THE EFFECT OF LYMPHOCYTES SECRETOME ON SKELETAL MUSCLE STEM CELLS REGENERATION WITH AGEING

S. AL-DABBAGH

PhD 2016

THE EFFECT OF LYMPHOCYTES SECRETOME ON SKELETAL MUSCLE STEM CELLS REGENERATION WITH AGEING

SARAH AL-DABBAGH

A Thesis Submitted in Partial Fulfilment of the requirements of Manchester Metropolitan University for the Degree of Doctor of Philosophy

School of Healthcare Science

Manchester Metropolitan University

2016

ABSTRACT

Older people experience skeletal muscle wasting, in part due to impaired proliferative capacity of quiescent skeletal muscle satellite cells and. The work presented in this thesis set out to examine the hypothesis that microenvironment of skeletal muscles can be influenced by immune cell secretions, which affect satellite cell proliferation, and that beneficial immune-muscle interactions in young people are blunted in elderly. The aim of this research was to investigate the role of ageing human lymphocytes on skeletal muscle cell behaviour. For this purpose, lymphocytes were isolated from whole blood of young (aged 18-25 years) and older (aged 78-85 years) healthy volunteers and older healthy volunteers. All the participants were healthy, with no history of muscle disease and not on immunosuppressant or corticosteroids treatment that affect immune function. Lymphocytes were cultured with, or without, anti-CD3/CD28 activators for 4 days to induce release of cytokines, interleukins and growth factors into the media. The secreted proteins were used to prepare conditioned media that were used to culture C2C12 myoblasts. Secretomes were analysed and fifteen secreted Th1/Th2 cytokines and IGF-I were quantified by multiplex immunoassay. The gene expression and protein concentrations of amphiregulin were determined from T-lymphocytes lysates by real-time PCR and ELISA respectively. The levels of CD25 and FoxP3 expression in lymphocytes were examined using flow cytometry. The expression of muscle transcription factors, MyoD and Myogenin were determined by real- time PCR. Activated Mek1/Erk1/2 and Akt/mTOR were measured by multiplex immunoassay. Our results demonstrate for the first time that a decrease in the levels of amphiregulin and CD25 coincides with the increase in FoxP3 with ageing, which may be involved in suppression of lymphocytes. Seven cytokines were differentially secreted by the young- compared with the old-activated lymphocytes. The secretome from young-activated lymphocytes had 30% ($P<0.005$) higher IGF-I concentrations compared with old and control treatments. The conditioned media from young -activated lymphocytes increased the rate of proliferation of myoblasts by ~3-fold ($P<0.005$) and caused an approximate 4-fold ($P<0.005$) increase in migration compared with non-activated lymphocyte control media. These responses were characterised the extended proliferation of young -treated myoblasts was also associated with a decrease in MyoD and Myogenin and an increase in mediators of proliferation Mek1/Erk1/2and a decrease in the key proteins for differentiation, Akt/mTOR. In contrast, myoblasts treated with conditioned media from old-activated lymphocytes exhibited a high degree of differentiation.

DEDICATION

I would like to dedicate this thesis to my mum and dad. Their belief in me throughout my life has helped me to become successful and has made me a better person.

Also to my husband, who has been a great support throughout the journey.

DECLERATION

All the data presented in this report are the results of my own efforts and have not previously been submitted in candidature for any other degree or diploma. In addition, this thesis contains no material previously published or written by another. I understand that any evidence of plagiarism and/or the use of unacknowledged third party data will be dealt with as a very serious matter.'

Signature: Sarah Al-Dabbagh Date:

ACKNOWLEDGMENTS

Many thanks to my Director of studies Dr. Nasser Al-Shanti; this thesis would never be completed without his help, support, patience and constant advice.

Special thanks to Dr. Jamie McPhee and Prof. Claire Stewart (John Moore University) for their help and valuable comments, proofreading and support throughout the study.

I thank my parents(Amir and Sammar Al-Dabbagh), for their continued love and support, my husband (Yousif Al-Aaraj) and my friend (Zena Al-Jadir) for their extreme patience, and understanding, encouragement, and being supportive and caring at all stages during my research.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1) Al-Shanti N[1], Durcan P, Al-Dabbagh S, Dimchev GA, Stewart CE (2014). Activated lymphocytes secretome inhibits differentiation and induces proliferation of C2C12 myoblasts. CellPhysiolBiochem**33**(1): 117-128.

2) Sarah Al-Dabbagh1, Jamie S. McPhee1, Christopher Murgatroyd1, Gillian Butler-Browne2, Claire E. Stewart 3 & Nasser Al-Shanti 1*(2015). The lymphocyte secretome from young adults enhances skeletal muscle proliferation and migration , but effects are attenuated in the secretome of older adults. Physiological Reports.

3) Saini, J., McPhee, J.S., Al-Dabbagh, S., Stewart, C.E. and Al-Shanti, N., 2016. Regenerative function of immune system: Modulation of muscle stem cells. Ageing Research Reviews, 27, pp.67-76.

PRESENTATIONS

A conference of Scandinavian Physiological society (Stockholm, Sweden August 2014). The effect of ageing of immune system on skeletal muscle proliferation and migration. Sarah Al-Dabbagh, Jamie McPhee & Nasser Al-Shanti.

SCIENCE & ENGINEERING RESEARCH SYMPOSIUM (MMU, September, 2015). The lymphocyte secretome from young adults enhances myoblast proliferation and migration, but effects are attenuated in the secretome of older adults". Sarah Al-Dabbagh1, Jamie S. McPhee1, Christopher Murgatroyd1, Gillian Butler- Browne2, Claire E. Stewart 3 & Nasser Al-Shanti 1.

TABLE OF CONTENTS

ABSTRACT .... **Error! Bookmark not defined.**

TABLE OF CONTENT...v

LIST OFFIGURES...x

LIST OF TABLES.. **Error! Bookmark not defined.**

CHAPTER 1 .... **Error! Bookmark not defined.**

INTRODUCTION .... **Error! Bookmark not defined.**

1.1. OVERVIEW .... **Error! Bookmark not defined.**

1.2. SKELETAL MUSCLE DEVELOPMENT AND REGENERATION .... **Error! Bookmark not defined.**

EMBRYONIC MYOGENESIS .... **Error! Bookmark not defined.**

POSTNATAL (ADULT) MYOGENESIS .... **Error! Bookmark not defined.**

1.3. SATELLITE CELLS RENEWAL AND ACTIVATION............ **Error! Bookmark not defined.**

1.4. MOLECULAR REGULATION OF SKELETAL MUSCLE REGENERATION: the myogenic regulatory factors (MRFs).. **Error! Bookmark not defined.**

1.5. SIGNALLING PATHWAYS THAT REGULATE REGENERATIVE MYOGENESIS....**Error! Bookmark not defined.**

1.5.1. IGF-1/MAPK-ERK1/2... **Error! Bookmark not defined.**

1.5.2. IGF/Akt/mTOR.. **Error! Bookmark not defined.**

1.6. MYOGENESIS AND AGEING (AGE- RELATED MUSCLE WASTING)...**Error! Bookmark not defined.**

AGED SATELLITE CELL POOL .... **Error! Bookmark not defined.**

1.7. PARABIOSIS-AN EXPERIMENTAL MODEL TO STUDY AGE -RELATED CHANGES IN SKELETAL MUSCLE MICROENVIROMENT............................... **Error! Bookmark not defined.**

1.8. IMMUNE SYSTEM .... **Error! Bookmark not defined.**

IN VIVO AND *IN VITRO* ACTIVATION OF IMMUNE SYSTEM............ **Error! Bookmark not defined.**

1.9 IMMUNE SYSTEM WITH AGEING .... **Error! Bookmark not defined.**

1.10. POSSIBLE INTERACTION BETWEEN SKELETAL MUSCLE CELLS AND IMMUNE ENVIRONMENT. .... **Error! Bookmark not defined.**

1.11. THERAPEUTIC POTENTIAL OF IMMUNE MUSCLE CELLS SECRETOME IN MUSCLE REGENERATION... **Error! Bookmark not defined.**

1.12 AIMS AND OBJECTIVES .... **Error! Bookmark not defined.**

CHAPTER 2 .... **Error! Bookmark not defined.**

2.1. MATERIALS, GENERAL EQUIPMENT AND SPECIALIZED SOFTWARE.................**Error! Bookmark not defined.**

2.1.1. CHEMICALS, SOLVENTS AND REAGENTS.................. **Error! Bookmark not defined.**

2.1.2. CELL CULTURE .... **Error! Bookmark not defined.**

2.1.3. CELL CULTURE REAGENTS .... **Error! Bookmark not defined.**

C2C12 .... **Error! Bookmark not defined.**

HUMAN LYMPHOCYTES CULTURE.. **Error! Bookmark not defined.**

2.1.4. PLASTICWARE.. **Error! Bookmark not defined.**

2.1.5. LIVE IMAGING.. **Error! Bookmark not defined.**

2.1.6. REAL-TIME PCR... **Error! Bookmark not defined.**

2.1.7. FLOW CYTOMETRY .... **Error! Bookmark not defined.**

2.2. METHODS AND PROTOCOLS .... **Error! Bookmark not defined.**

2.2.1. CELL CULTURE PREPARATION..................................... **Error! Bookmark not defined.**

CELL COUNTING BY TRYPAN BLUE EXCLUSION .... **Error! Bookmark not defined.**

2.4. BLOOD SAMPLE COLLECTION AND PROCESSING MANAGEMENT ....**Error! Bookmark not defined.**

2.4.3. CONDITIONED MEDIA (CM) PREPARATION AND C2C12 TREATMENT.........**Error! Bookmark not defined.**

2.6. CELL EXAMINATION BY FLOW CYTOMETRIC ANALYSIS.......**Error! Bookmark not defined.**

2.6.1. MEASUREMENT OF CD25 (IL2R-α) ON LYMPHOCYTES..........**Error! Bookmark not defined.**

2.6.2. MEASUREMENT OF CD14 MARKER ON HUMAN MONOCYTES...**Error! Bookmark not defined.**

2.6.3. MEASUREMENT OF FOXP3, A TREG CELL MARKER **Error! Bookmark not defined.**

2.6.4. PROPIDIUM IODIDE AND CELL CYCLE EXAMINATION.........**Error! Bookmark not defined.**

2.7. WOUND HEALING ASSAY WITH LIVE IMAGING EXAMINATION. .**Error! Bookmark not defined.**

2.8. MORPHOLOGICAL DIFFERENTIATION AND QUANTIFICATION OF THE PROLIFERATION AND DIFFERENTIATION PARAMETERS. **Error! Bookmark not defined.**

2.9. RNA EXTRACTION.. **Error! Bookmark not defined.**

2.10. REAL-TIME PCR.. **Error! Bookmark not defined.**

2.11. PROTEIN EXTRACTION AND ESTIMATION OF PROTEIN CONCENTRATION **Error! Bookmark not defined.**

EXTRACTION AND ESTIMATION OF PROTEIN CONCENTRATION **Error! Bookmark not defined.**

2.12. MULTIPLEX IMMUNOASSAY .... **Error! Bookmark not defined.**

2.13. MEASURMENT OF AMPHIREGULIN USING .... **Error! Bookmark not defined.**

2.15. STATISTICAL ANALYSIS... **Error! Bookmark not defined.**

CHAPTER 3 .... **Error! Bookmark not defined.**

3. OPTIMISATION OF LYMPHOCYTE CULTURE: HARVESTING OF CONDITIONED MEDIA TO IMPROVE C2C12 PROLIFERATION.......................... **Error! Bookmark not defined.**

3.1 INTRODUCTION .... **Error! Bookmark not defined.**

3.2. AIMS, HYPOTHESIS AND OBJECTIVES .... **Error! Bookmark not defined.**

3.3 METHODS .... **Error! Bookmark not defined.**

3.3.2. ADHERENT CULTURE TO DEPLETE MONOCYTES (MACROPHAGES) ....**Error! Bookmark not defined.**

3.3.3 OPTIMISATION OF ANTI-CD3 CONCENTRATION....... **Error! Bookmark not defined.**

3.3.4. OPTIMISATION OF ANTI-CD28 CONCENTRATION.... **Error! Bookmark not defined.**

3.3.5. MEASUREMENT OFCD25 (IL-2Á R) MARKER .... **Error! Bookmark not defined.**

3.3.6. HARVESTING LYMPHOCYTE SECRETOME .... **Error! Bookmark not defined.**

3.3.7. DETERMINATION OFTHE QUANTITY OF CYTOKINE SECRETION IN DAY 2 AND 4 ACTIVATED LYMPHOCYTES .... **Error! Bookmark not defined.**

3.3.8. CONDITIONED MEDIA (CM) PREPARATION AND VOLUME OPTIMIZATION .... **Error! Bookmark not defined.**

3.3.9. C2C12 CULTURESAND TREATMENT WITH CONDITIONED MEDIA ....**Error! Bookmark not defined.**

3.3.10. CELL CYCLE .... **Error! Bookmark not defined.**

3.11. STATISTICAL ANALYSES .... **Error! Bookmark not defined.**

3.4. RESULTS .... **Error! Bookmark not defined.**

3.4.1. ADHERENT CULTURE TO DEPLETE MONOCYTES (MACROPHAGES)...........**Error! Bookmark not defined.**

3.4.2. OPTIMISATION OF ANTI-CD3 CONCENTRATION...... **Error! Bookmark not defined.**

3.4.3. OPTIMIZATION OF LYMPHOCYTE INCUBATION TIME .... **Error! Bookmark not defined.**

3.4.4. OPTIMISATION OF ANTI-CD28 CONCENTRATION.... **Error! Bookmark not defined.**

3.4.5. OPTIMAL VOLUME OF SECRETOME TO MAKE CONDITIONED MEDIA (CM1) .... **Error! Bookmark not defined.**

3.4.6. CD25 (IL-2A R) MARKER ANALYSIS .... **Error! Bookmark not defined.**

3.4.7. QUANTITY OF CYTOKINE SECRETION IN DAY 2- AND 4 -ACTIVATED LYMPHOCYTES .... **Error! Bookmark not defined.**

3.4.8. C2C12 TREATMENT WITH CONDITIONED MEDIA .... **Error! Bookmark not defined.**

3.4.9. CELL CYCLE ANALYSIS... **Error! Bookmark not defined.**

3.5 DISCUSSION .... **Error! Bookmark not defined.**

3.5.1. OPTIMAL ANTI-CD3 CONCENTRATION FOR LYMPHOCYTE ACTIVATION **Error! Bookmark not defined.**

3.5.2. OPTIMIZATION OF ANTI-CD28 CONCENTRATION.... **Error! Bookmark not defined.**

3.5.3. OPTIMAL DURATION FOR LYMPHOCYTE ACTIVATION .... **Error! Bookmark not defined.**

3.5.4. CD25 MARKER (IL-2α R) MEASUREMENT ON LYMPHOCYTES FOLLOWING 2-& 4- ACTIVATION ..... **Error! Bookmark not defined.**

3.5.5. QUANTITATION OF CYTOKINE SECRETION IN DAY 2 AND 4- ACTIVATED LYMPHOCYTES ..... **Error! Bookmark not defined.**

3.5.6. OPTIMAL VOLUME OF SECRETOME TO MAKE CONDITIONED MEDIA .... **Error! Bookmark not defined.**

3.5.7. MYOBLAST CULTIVATION IN FORMULATED CONDITIONED MEDIA .... **Error! Bookmark not defined.**

3.5.7. CELL CYCLE ANALYSIS ..... **Error! Bookmark not defined.**

3.6. CONCLUSION ..... **Error! Bookmark not defined.**

CHAPTER 4 ..... **Error! Bookmark not defined.**

4. AGE- RELATED DIFFERENCES IN THE CHARACTERISTICS OF YOUNG AND OLD LYMPHOCYTES. ..... **Error! Bookmark not defined.**

4.1 INTRODUCTION ..... **Error! Bookmark not defined.**

4.2 AIMS, OBJECTIVES AND HYPOTHESIS ..... **Error! Bookmark not defined.**

4.3 METHODS ..... **Error! Bookmark not defined.**

4.3.1 HUMAN LYMPHOCYTES CULTURE ..... **Error! Bookmark not defined.**

4.4RESULTS ..... **Error! Bookmark not defined.**

4.4.1 GENE EXPRESSION ANALYSIS OF AMPHIREGULIN IN HUMAN YOUNG AND OLD T LYMPHOCYTES. ..... **Error! Bookmark not defined.**

4.4.2 GENE EXPRESSION ANALYSIS OF GDF-11 IN HUMAN YOUNG AND OLD T LYMPHOCYTES ..... **Error! Bookmark not defined.**

4.4. 3 QUANTITATION OF AMPHIREGULIN IN YOUNG AND OLD SECRETOMES.. **Error! Bookmark not defined.**

4.4.4 MEASUREMENT OF CD25 and FOXP3 EXPRESSION BY YOUNG AND OLD LYMPHOCYTES ..... **Error! Bookmark not defined.**

4.5 DISCUSSION ..... **Error! Bookmark not defined.**

CHAPTER 5 ..... **Error! Bookmark not defined.**

5. CHARACTERISATION OF YOUNG AND OLD SECRETOMES OBTAINED FROM LYMPHOCYTES ..... **Error! Bookmark not defined.**

5.1 INTRODUCTION ..... **Error! Bookmark not defined.**

5.2 AIM, OBJECTIVES AND HYPOTHESIS ..... **Error! Bookmark not defined.**

5.3 METHODS ..... **Error! Bookmark not defined.**

5.3.1 ISOLATION OF LYMPHOCYTES FROM FRESH BLOOD SAMPLES **Error! Bookmark not defined.**

5.3.2 IMMUNOASSAY ANALYSIS .... **Error! Bookmark not defined.**

5.3.3 STATISTICAL ANALYSIS .... **Error! Bookmark not defined.**

5.4 RESULTS .... **Error! Bookmark not defined.**

5.4.1 Th1/Th2 cytokines concentration in young and old secretomes .... **Error! Bookmark not defined.**

5.4.1 IGF in young and old secretomes .... **Error! Bookmark not defined.**

5.5 DISCUSSION .... **Error! Bookmark not defined.**

5.6 CONCLUSION .... **Error! Bookmark not defined.**

CHAPTER 6 .... **Error! Bookmark not defined.**

6. THE EFFECT OF YOUNG AND OLD ACTIVATED CONDITIONED MEDIA ON C2C12 PROLIFERATION AND MIGRATION .... **Error! Bookmark not defined.**

6.1 INTRODUCTION .... **Error! Bookmark not defined.**

6.2 AIMS, HYPOTHESIS AND OBJECTIVES .... **Error! Bookmark not defined.**

6.3 METHODS .... **Error! Bookmark not defined.**

6.3.1 ISOLATION OF LYMPHOCYTES FROM FRESH BLOOD SAMPLES **Error! Bookmark not defined.**

6.3.2 RECOVERY OF SECRETED CYTOKINES .... **Error! Bookmark not defined.**

6.3.3 CONDITIONED MEDIA (CM) PREPARATION AND C2C12 TREATMENT .... **Error! Bookmark not defined.**

6.3.4 MORPHOLOGICAL DIFFERENTIATION AND QUANTIFICATION OF THE PROLIFERATION AND DIFFERENTIATION PARAMETERS **Error! Bookmark not defined.**

6.3.5 CELL CYCLE ANALYSIS .... **Error! Bookmark not defined.**

6.3.6 MIGRATION STUDY .... **Error! Bookmark not defined.**

6.3.7 QUANTIFICATION OF DIFFRENTIATION PARAMETERS OF C2C12 CULTURED IN DM SUPPLEMENTED WITH YOUNG AND OLD HUMAN SERUM..... **Error! Bookmark not defined.**

6.3.8 MORPHOLOGCAL ASSESSMENT HUMAN SERA CELL CULTURE **Error! Bookmark not defined.**

6.3.9 STATISTICAL ANALYSIS .... **Error! Bookmark not defined.**

6.4 RESULTS .... **Error! Bookmark not defined.**

6.4.1 MOPHOLOGICAL DIFFERENCES IN THE C2C12 MYOBLASTS TREAED WITH CMs OF YOUNG AND OLD SECRETOME .... **Error! Bookmark not defined.**

6.4.2 DIFFERENTIATION OF C2C12 FOLLOWING YOUNG CM1 WITHDRAWAL..... **Error! Bookmark not defined.**

6.4.3 CELL CYCLE ANALYSIS .... **Error! Bookmark not defined.**

6.4.4. MIGRATION STUDY .... **Error! Bookmark not defined.**

6.4.5 DIFFRENTIATION PARAMETERS OF C2C12 CULTURED IN DM SUPPLIMENTED WITH YOUNG AND OLD HUMAN SERA................................ **Error! Bookmark not defined.**

6.5 DISSCUSSION... **Error! Bookmark not defined.**

6.5.1 MOPHOLOGICAL DIFFERENCES IN C2C12 MYOBLASTS TREAED WITH CM1 OF YOUNG AND OLD .... **Error! Bookmark not defined.**

6.5.2 DIFFERENTIATION OF C2C12 FOLLOWING YOUNG CM1 WITHDRAWAL.....**Error! Bookmark not defined.**

6.5.3 MIGRATION STUDY .... **Error! Bookmark not defined.**

6.5.4 CULTURING CELLS IN DM SUPPLEMENTED WITH YOUNG OR OLD HUMAN SERUM.. **Error! Bookmark not defined.**

6.6 CONCLUSION... **Error! Bookmark not defined.**

CHAPTER 7 .... **Error! Bookmark not defined.**

7. C2C12: CELLULAR AND MOLECULAR ADAPTATION OF C2C12 CELLSTREATED WITH YOUNG AND OLD CONDITIONED MEDIA................................ **Error! Bookmark not defined.**

7.1 INTRODUCTION .... **Error! Bookmark not defined.**

7.2 AIMS AND OBJECTIVES .... **Error! Bookmark not defined.**

7.3 METHODS .... **Error! Bookmark not defined.**

7.3.1 CULTURE OF C2C12 MYOBLASTS AND TREATMENT WITH CM1-4................**Error! Bookmark not defined.**

7.3.2 RNA ISOLATION AND REAL-TIME PCR .... **Error! Bookmark not defined.**

7.3.3 OPTIMAL TIME POINT TO ACTIVATE PHOSPHO-PROTEIN CASCADES.........**Error! Bookmark not defined.**

7.3.4DETERMINATION OF MYOBLAST LEVELS OF MAPK/ERK AND AKT/mTOR AFTER TREATMENT WITH YOUNG AND OLD CONDITIONED MEDIA.**Error! Bookmark not defined.**

7.3.5 MULTIPLEX/IMMUNOASSAY PROTOCOL................... **Error! Bookmark not defined.**

7.3.6 INVESTIGATING THE EFFECT OF IGF-1 ON C2C12 PROLIFERATION ....**Error! Bookmark not defined.**

7.4 STATISTICAL ANALYSIS .... **Error! Bookmark not defined.**

7.5 RESULTS .... **Error! Bookmark not defined.**

7.5.1 GENE EXPRESSION ANALYSIS OF MYOD AND MYOGENIN FOR YOUNG AND OLD CM1-4- TREATED MYOBLASTS .... **Error! Bookmark not defined.**

7.5.2 OPTIMAL DURATION TIME TO ACTIVATE PHOSPHO-PROTEINS CASCADES .... **Error! Bookmark not defined.**

7.5.3 MEK1/ERK 1/2 SIGNALLING PATHWAYS ARE INDUCED IN RESPONSE TO YOUNG CM1BUT EFFECTS ARE ATTENUATED IN RESPONSE TO OLD CM1 ....**Error! Bookmark not defined.**

7.5.4. AKT/mTOR SIGNALLING PATHWAY IS LOWER IN RESPONSE TO YOUNG CM1 COMPARED WITH OLD CM1 .... **Error! Bookmark not defined.**

7.5.5THE MAPK INHIBITOR UO126 ATTENUATES THE YOUNG CM1-INDUCED MYOBLAST PROLIFERATION. .... **Error! Bookmark not defined.**

7.5.6 PROTEOMICS STUDY .... **Error! Bookmark not defined.**

7.5.8 DETERMININGTHE AKT/mTOR SIGNALLING PATHWAY WITHIN C2C12CELLS TREATED WITH THE IGF-1R INHIBITOR OSI-906................. **Error! Bookmark not defined.**

7.6. DISCUSSION .... **Error! Bookmark not defined.**

7.8 CONCLUSION .... **Error! Bookmark not defined.**

CHAPTER 8 .... **Error! Bookmark not defined.**

8.1 GENERAL DISCUSSION .... **Error! Bookmark not defined.**

8.2 LIMITATIONS .... **Error! Bookmark not defined.**

8.3 FUTURE WORK .... **Error! Bookmark not defined.**

8.4 CONCLUSION .... **Error! Bookmark not defined.**

List of Figures:

Chapter 1

Figure 1.1: Aschematic representation of somatie.

Figure1.2: Skeletal muscle repair and regeneration.

Figure 1.3: The representative of reversible and irreversible cell cycle arrest.

Figure 1.4: Molecular regulation during embryonic and postnatal myogenesis.

Figure 1.5: A schematic representation of the GH-IGF in normal physiology

Figure 1.6: IGF-mediated signalling pathways.

Figure 1.7: Heterochronic-parabiosis between aged and young mice.

Figure 1.8: The interaction between innate and adaptive immunity.

Figure 1.9: Activated and quiescence states of T-cells.

Figure 1.10: The general cross-talk between immune cells and skeletal muscle cells during myogenesis following muscle injury.

Figure 1.11: Debris clearance after skeletal muscle injury.

Figure 1.12: The immunological and non-immunological functions of T-regs.

Figure 1.13: The effect of amphiregulin on skeletal muscle regeneration.

Chapter 2

Figure 2.1: Haemocytometer design and dimensions.

Figure 2.2: Engagement of T- cell receptors (TCR) CD3 and CD28 only.

Figure 2.3: Amicon ®Ultra-4 Centrifugal filter Units for secretome filtration.

Figure 2.4: A schematic overview of mechanism of flow cytometer.

Figure 2.5: DNA content through cell cycle phases.

Figure 2.6: Phases of separation using Trizol@ reagents.

Figure 2.7: Comparison of PCR products in real time PCR between SYBR® and Green and TaqMan®.

Figure 2.8: Ct value of RT-PCR.

Figure 2.9: The schematic presentation of multiplex immune assay steps.

Figure 2.10: Assay summary of amphiregulin human ELISA.

Chapter 3

Figure 3.1: Optimisation of lymphocytes culture and conditioned media.

Figure 3.2: Lymphocytes depleted of monocytes.

Figure 3.3: Anti-CD3 optimisation.

Figure 3.4: Optimisation for culture time of anti-CD3 activated vs non-activated at days 2, 4 and 6.

Figure: 3.5: Cell count for anti-CD3 activated vs non-activated at days 2, 4 and 6.

Figure 3.6: Optimisation of anti-CD28 concentrations for activated young lymphocytes following 4 days incubation.

Figure 3.7: Optimisation of secretome volume for CM preparation.

Figure 3.8: The expression of CD25 on lymphocytes at 0, 2, 4, 6 days of activation.

Figure 3.9: C2C12 treated with activated conditioned media (CM1)

Figure 3.10: The percentages of S phases of myoblasts treated with day 4 (CM1-4) and DM at 96 hours.

Figure 3.11: The percentages of S phase of myoblasts treated in CM1-4 and DM following 2, 4 and 6 days of activation.

Chapter 4

Figure 4.1: Amphiregulin gene expression in young and old activated lymphocytes.

Figure 4.2: GDF-11 gene expression in young and old activated lymphocytes.

Figure 4.3: Amphiregulin concentration in secretome of young and old activated lymphocytes.

Figure 4.4: %CD25 young and old activated lymphocytes.

Figure 4.5: % FoxP3 in young and old activated lymphocytes.

Figure 4.6: Flow cytometric analysis of PE-anti human FoxP3 and APC-anti human CD25 on lymphocytes.

Chapter 5

Figure 5.1: IGF-1 concentrations in young and old activated lymphocytes secretome.

Chapter 6

Figure 6.1: Representative images of C2C12 following 4 days incubation with young and old CM1-4.

Figure 6.2: Immunofluorescence analysis of skeletal muscle myosin heavy chain (MHC).

Figure 6.3: Differentiation parameters analysis of young and old secretome treated myoblasts.

Figure 6.4: Differentiation of C2C12 following young CM1 withdrawal.

Figure 6.5: S phases of myoblasts treated with conditioned media CM1-4 after 4 days.

Figure 6.6: Representative images for wound healing assay of C2C12 treated with CM1-4.

Figure 6.7: Cell count of wound healing assay for C2C12 treated with CM1-4.

Figure 6.8: Immunofluorescence analysis of skeletal muscle myosin heavy chain.

Chapter 7

Figure 7.1: RT-PCR analysis of MyoD mRNA gene expression following 4 days of incubation.

Figure 7.2: RT-PCR analysis of Myogenin mRNA gene expression following 4 days of incubation.

Figure 7.3: MFI of pAkt levels in C2C12 myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25, and 60 min.

Figure 7.4: MFI of mTOR levels in myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25 and 60 min.

Figure 7.5: MFI of IRS-1 levels in myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25, 60 min.

Figure 7.6: MFI for MEK1 in CM1 Young and Old treated C2C12.

Figure 7.7: MFI levels for ERK1/2 in young and old CM1, CM2, CM3 and CM4-treated C2C12.

Figure 7.8: MFI of pAKt levels in Young and Old CM1, CM2, CM3 and CM4-treated C2C12 myoblasts.

Figure 7.9: MFI of mTOR levels in Young and Old CM1-4 treated myoblasts.

Figure 7.10: Levels of activated Akt/mTOR and ERK1/2 in C2C12 cells treated with CM1 and 3, 5, 8 and 10µM of UO126.

Figure 7.11: Morphological assessment, cell count and cell cycle analysis for OSI-906-pre treated and un-treated cells cultured with CM1, DM and controls after 3 days.

Figure 7.12: The levels of phosphorylated Akt and mTOR for OSI-906-pre-treated and un-treated cells cultured with CM1, DM and controls.

ABBREVIATIONS

Areg: Amphiregulin

CCR2C-C: chemokine receptor type 2

CX3CR1CX3: chemokine receptor 1

DCs: Dendritic cells

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal- regulated kinase

FGF-2Fibroblast growth factor-2

GM-CSF: Granulocyte macrophage colony-stimulating factor

ECM: Extracellular matrix

GSK3β: Glycogen synthase kinase 3*Beta*

IL-1 β: Interlukin-1*Beta*

IL-10: Interlukin-10

IL-2: Interlukin-2

IL-2Rα: IL-2 receptor-alpha

IL-4: Interlukin-4

IL-6: Interlukin-6

INF-γ: Interferon –Gamma

IP-10: Interferon gamma-induced protein 10

iNOS: Inducible nitric oxide synthase

MAPK: Mitogen-Activated Protein Kinase

MIP-α: Macrophage inflammatory protein-α

Mpc: Muscle precursor cells

Myf5: Myogenic factors5

Mrf4: Myogenic regulatory factor 4

NFAT: Nuclear factor of activated lymphocytes

mTOR: Mammalian target of rapamycin

NFƘ-B: Nuclear factor kappa-Beta

PI3K: Phosphatidylinositol-3 kinase

RT: Room temperature

SDF: Stromal cell-derived factor

TCR: T cell receptor

TGF-β: Transforming growth factor- β

TLRs: Toll- like receptors

TNF-α: Tumour necrosis factor-α

Tregs: Regulatory T cells

VEGF: Vascular endothelial growth factor

LIST OF TABLES

Table 2.1: Serial dilution standards for BCA protein assay.

Table 2.2: Standard dilution preparation.

Table 5.1: Multiplex reading for anti-CD3/CD28 activated and non-activated lymphocyte secretome from young and old participants.

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

Skeletal muscle is a highly metabolic tissue, essential for stability and body movement, representing approximately 40-50% of the human body mass (Adibi, 1976). Muscle wasting is very common health problem, resulting from several life threatening diseases such as heart diseases and cancer cachexia. Ageing is also associated with reduced muscle mass, people aged between 40–70 lose on average 10–25% of lean body mass and people over age of 80 can lose up to 40% of mass (McPhee et al., 2013). These findings indicate that there is a diminished capacity of skeletal muscle cells to regenerate and grow with age. The diminished capacity of muscle repair and regeneration with ageing is suggested to result in part from reduction in skeletal muscle stem cell numbers (satellite cells) and function. Furthermore, in ageing, there is a marked decline in immune function. Following e.g. injury, immune cells contribute to the local environment that can influence the regenerative process of skeletal muscle cells. Muscle injury is accompanied by stimulation of precursor satellite cells by infiltration of immune cells, which in turn release growth factors and cytokines that affect muscle repair and regeneration (Tidball and Villalta, 2010). Therefore, the diminished ability of muscle regeneration in older age has been linked to the impaired immune response (Dumke and Lee, 2011).

1.2. SKELETAL MUSCLE DEVELOPMENT AND REGENERATION

EMBRYONIC MYOGENESIS

Development of somites in a vertebrate embryo results in the formation of anatomical cellular groups which later give rise to skeletal muscle (myotome), vertebrae (sclerotome) and dermis (dermatome). As shown in Figure 1.1, the somate can be divided into two distinct regions the hypaxial domain which forms limb muscles and the lateral trunk and the epaxial domain which forms dorsal muscles (Parker et al., 2003; Charge and Rudnicki, 2004; Buckingham, 2006). The hypaxial domain of the somite gives rise to precursor cells which migrate to the limb bud where key myogenic regulatory factors (MRFs) are located. These factors include the Mrf4 (myogenic

regulatory factor 4) and Myf5 (myogenic factor 5)(Buckingham, 2006) and their activity in proliferating, migrating and differentiating myoblasts can in fact be determined by neighbouring, thereby establishing myogenesis (Hawke and Garry 2001;Buckingham et al., 2003).

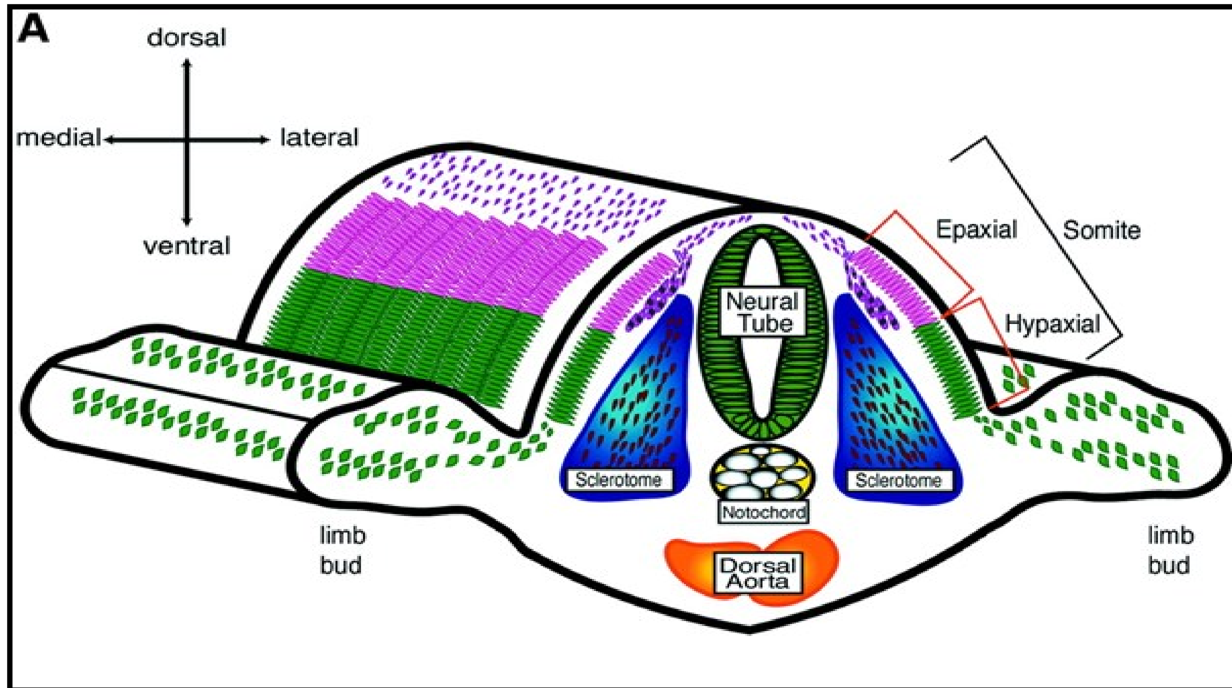


Figure 1.1: A schematic representation of somite: Somite is separated into epaxial and hypaxial regions with its surrounding structures including the notochord, neural tube and dorsal aorta provide signals inducing the migration of precursor cells. Precursor cells from the epaxial region of a somite contribute to the back musculature, while precursor cells originating from the hypaxial region migrate to the newly formed limb buds. Adapted from (Hawke and Garry 2001).

Once within the limb buds, precursor cells derived from the hypaxial domain begin to exclusively produce Pax thereby making the cells specific for myogenic lineage. Cell proliferation and differentiation of these embryonic precursor cells expressing Pax3+/Pax7+ (described in section 1.4) occurs whereas some cells leave the cell cycle becoming satellite cells (Yin et al., 2013).The first muscle fibres, known as primary fibres, form between E11 and E14 in the mouse limb. Between E 14 and E16, initially smaller in size, secondary myofibres form parallel to motor neural innervations are formed (Messina and Cossu, 2009; Sheard et al., 1991;

Gros et al., 2005). It has been proposed that these cell groups demonstrate intrinsic differences i.e. dissimilar genetic expression and in vitro behaviour (Biressi et al., 2007; Buckingham et al., 2003).

Once secondary fibres are formed and every fibre is surrounded by basal lamina, satellite cells attain their typical morphology of mononucleated cells present between basal lamina and plasma membrane of the fibre. These satellite cells undergo division at a slow rate during peri and post-natal development. Moreover, to enable hypertrophy postnatally, they merge with the adjacent fibres to offer new nuclei thereby amplifying the size of muscle fibre. They assume the dormant state at the end of post-natal growth; however, ensuing activation of these satellite cells play a crucial role in regeneration and muscle repair in case of any muscle damage.

POSTNATAL (ADULT) MYOGENESIS

Myofibres and innervating motor neurons constitute skeletal muscles which are vascularised to enable e.g. the provision of nutrients required for replenishment of energy stores and repair of muscles. Basal lamina or basement membrane refers to the endomysium (connective tissue) which surrounds the individual muscle fibres. A set of myofibresis collectively covered by different connective tissue is termed the perimysium. Finally, the epimysium is the outermost layer of the connective tissue which surrounds the whole bundle of myofibres. Actin and myosin myofibrils make up each myofibre and these myofibrils organize to form sarcomeres, functional units of the skeletal muscle (Yin et al., 2013; Borg and Caulfield, 1980).
Regeneration of skeletal muscle compromises of time-dependent and sequential phases of degeneration, inflammation, regeneration and remodelling-repair (Carosio et al., 2011). Muscle injury is followed by a degenerative phase and necrosis of the cells. Certain cytokines (e.g. IL-1, IL-6 and TNF-α) are discharged by damaged tissues which attract inflammatory immune cells to the site of damage (Madaro and Bouché, 2014). Immune cells have significant roles in clearing debris and activating satellite cells, a requirement of regeneration (Aurora and Olson, 2014). During the regenerative phase, the satellite cells are activated, proliferate and differentiate to repair the damage. Satellite cells are in the G0 phase (inactive, or dormant) of the cell cycle in an intact fibre, these cells become activated, leave the G0 phase to enter the G1 phase. When the satellite cells are activated, they move towards the damaged fibres and merge

with the necrotic myofibres. This is followed by differentiation of satellite cells for promoting repair of muscle fibres(Hawke, 2011; Turner and Badylak, 2012; Charge and Rudnicki, 2004). The satellite cells do not necessarily remain restricted to the site of muscle damage. Reports indicate that these cells can travel between myofibres and may go beyond to surpass the wall of basal lamina and connective tissue. In this way, they make sure that the regeneration is executed efficiently and the more extensive damage is repaired (Yin et al., 2013). Maturation of regenerated myofibres occurs during the final phase, which also involves remodelling of extracellular matrix (ECM) and finally the damaged muscles resume their function.

Even though the regenerative process requires active involvement of satellite cells, some of these cells undergo self-renewal and attain the inactive state so that there remains a sufficient supply of satellite cells to be used in any injury repair throughout the lifespan (Relaix and Zammit, 2012). It has been reported that muscle from aged mice was estimated to contain around 65% fewer functioning satellite cells than muscle from young mice (Cosgrove et al., 2014) and the overall number of satellite cells was also lower in aged mouse muscle compared to young mice muscle (Chakkalakal et al., 2012).

1.3. SATELLITE CELLS RENEWAL AND ACTIVATION

Following activation, satellite cells undergo asymmetric division, generating two daughter cells, one cells becoming committed myogenic precursor cell (mpc) or myoblast and the other, entering a quiescent state to maintain the satellite cell pool. In the asymmetric division phase, satellite cells express (Pax7+/Myf5-) and give rise to one satellite cell and one myogenic cell (Pax7+/Myf5+)(Halevy et al., 2004)(Figure 1.2). Thus, the self –renewing ability should in theory not end up with depletion of the satellite cell pool and therefore the ability of muscle to regenerate is maintained..

The satellite cells niche is important in regulating whether satellite cells undergo symmetric or asymmetric divisions. For example, if there is a need for a large number of cells, satellite stem cells may undergo asymmetric division to replenish the satellite cells pool and expand population following injury. Self-renewing satellite cell population (Pax7+/Myf5-) is governed by intrinsic and extrinsic factors such as Wnt7a and Notch (Conboy and Rando, 2002; Le Grand et al., 2009).

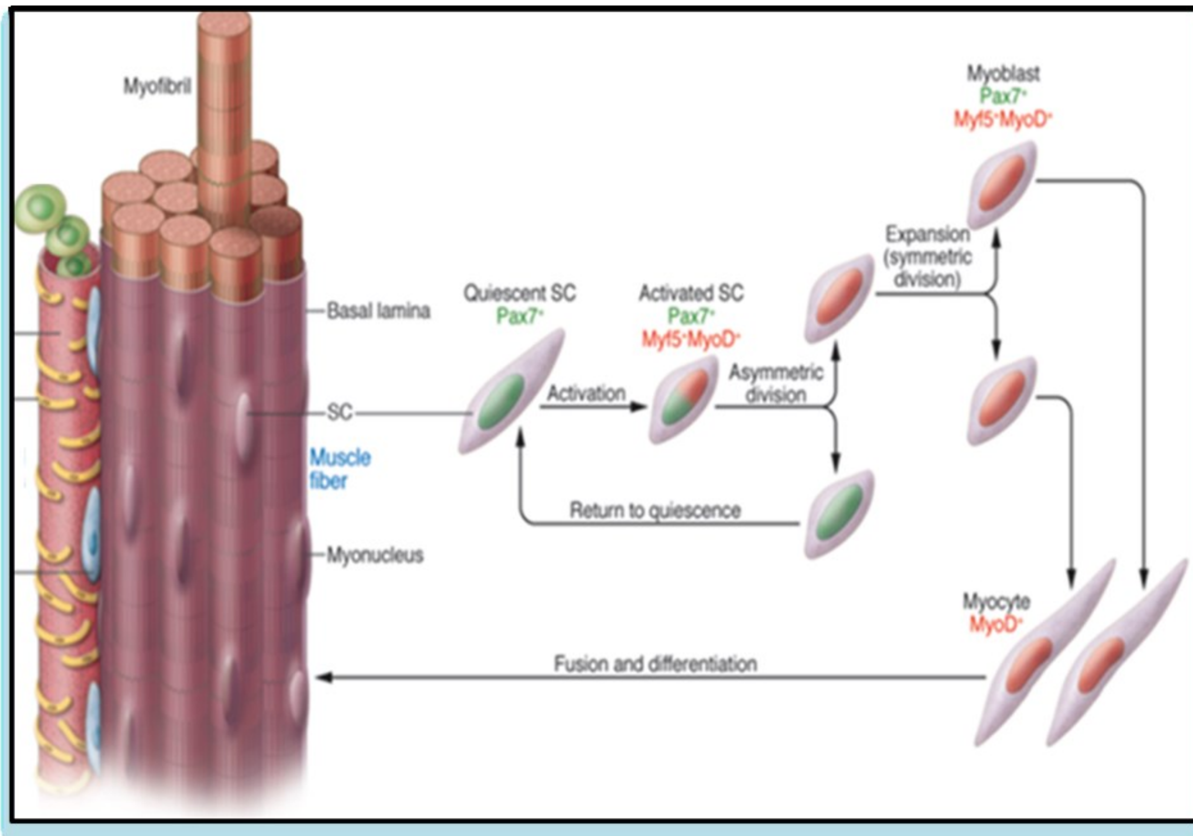


Figure 1.2: Skeletal muscle repair and regeneration. Satellite cells exist from quiescent phase and undergo asymmetric cell division. One of the cells that express (Pax7+/Myf5+) is activated and divided symmetrically to end up with myocytes formation. The other cell with high expression of (Pax 7/Myf5) returns to a quiescent phase to replenish non-activating satellite cells. When Myocytes express Myo D, they become differentiated to new myofibres and migrate to fuse at the site of injury. Adapted from (Tedesco et al., 2010).

It has been reported that Numb, is a crucial regulatory factor generating asymmetric satellite cell division. An activated daughter cell that inherits Numb will undergo myogenic development; this cycle progression is called irreversible G0 arrest (Dhawan and Rando, 2005). While a daughter cell that does not inherit Numb will undergo reversible G0 arrest in which the cell commits to self -renewal and returns to a quiescent state (Shen et al., 2002). The transition from a quiescent state to a proliferative state to is regulated by MyoD expression, which is also a key regulatory molecule for myogenic differentiation (de la Serna et al., 2001; Weintraub et al., 1989; Weintraub et al., 1991). In the presence of proliferative signals, the cells enter S phase and MyoD expression decreases. In contrast, in the presence of differentiation cues in G1, cells

exit cycle and initiate terminal differentiation that is accompanied by up regulation of MyoD that induces the expression of other key molecules such as p21, which are essential for differentiation to the myogenic lineage (Hawke et al., 2003; de la Serna et al., 2001), including retinoblastoma (Rb) ,cyclinD3 and myogenin (Kitzmann and Fernandez, 2001; Dhawan and Rando, 2005)(Figure1.3). In addition to these target genes, other genes become activated including; creatinine kinase, myosin heavy chain and acetyl choline receptors that assure the formation of functional mature myofibres (Hawke and Garry, 2001). To enable growth arrest, quiescence cues p27 and p130 are expressed and cells enter the quiescence state (G0), MyoD declines, target genes are inactive and consequently, cells return to a dormant arrest rather than develop to myogenic differentiation (Figure 1.3)(Dhawan and Rando, 2005).

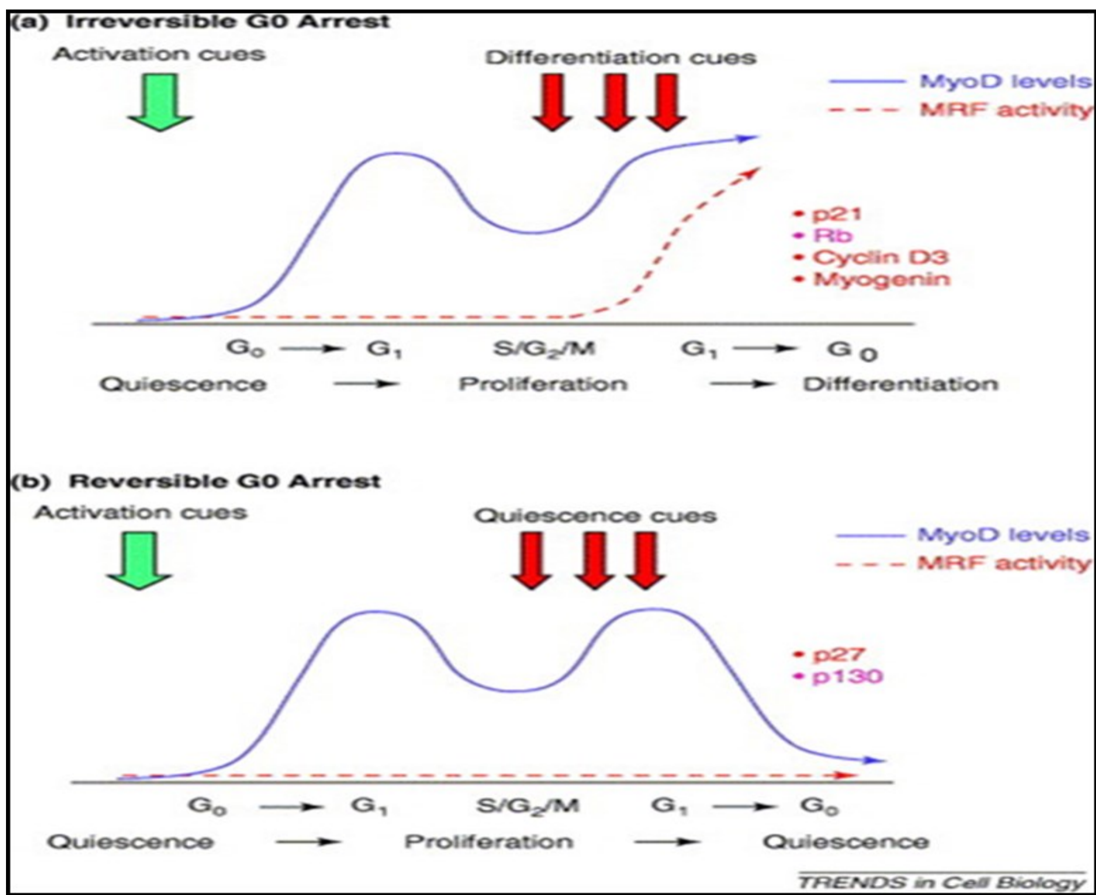


Figure 1.3: The representation of reversible and irreversible cell cycle arrest. Adapted from (Dhawan and Rando, 2005).

Replaced myofibre after damage will be functionally similar to the original myofibre in expression of MyoD, Myogenin and Myf4. These MRFs have key roles during fusion of myonuclei and generating mature myofibres (Charge and. Rudnicki, 2004).

Minor skeletal muscle injuries, occur routinely during daily activity and may induce a limited number of satellite cells to proliferate and differentiate, whereas major injury require an extended population of satellite cells to respond. Thus, satellite cell function is essential in maintaining muscle repair and regeneration throughout the life (Yablonka-Reuveni, 2011).

1.4. MOLECULAR REGULATION OF SKELETAL MUSCLE REGENERATION: the myogenic regulatory factors (MRFs)

The process of skeletal muscle regeneration, from satellite cell to mature muscle fibre parallels embryonic myogenesis, which is highly orchestrated by a series of genetic and myogenic regulatory factors. During embryonic myogenesis, paired box 3&7 (Pax3& Pax7) transcriptional factors are expressed by satellite cells and play key roles in myogenic specification and are able to compensate partially for each other during skeletal embryonic development (Buckingham et al., 2003; Gros et al., 2005). Pax3 is an important determinant for proliferation and migration of muscle precursors from the presomitic mesoderm (Sheard et al., 1991). Pax7 is essential in the initiation of satellite cell activation, proliferation and self-renewal to replenish the pool of quiescent cells (Turner and Badylak, 2012*)*. Additionally, Pax7 triggers the sequential activation of the basic helix-loop-helix (bHLH) family of MRFs. Following muscle precursor cells commitment to the skeletal muscle lineage, the process of embryonic myogenesis parallels the adult myogenesis, which is regulated by series of MRFs (Figure 1.4). MRFs involved in the proliferative and differentiating phases of muscle regeneration include Myf5, MyoD, Myogenin, Myf4, and MRF4. Myf5 is expressed early after injury in the regenerative process, Myf5 alone is intimately involved with the early proliferation of satellite cell myoblasts. Research has also revealed that Myf5-/- mice present with mild deficits in the regeneration of skeletal muscle, manifested through the fibrosis of the endomysium and excessive accumulation of adipocytes at the site of injury, compared to wild-type mice (Gayraud-Morel et al., 2007). MyoD is another MRF responsible for the early stages of skeletal muscle regeneration and performs key functions of the early proliferative phase similar in nature to Myf5, in a compensatory and redundant

manner. As minor deficits in muscle regeneration are observed in Myf5-/- animal models, similar deficits are also observed in MyoD-/- models (White et al., 2000). Although myogenesis still progresses when one of these two factors is deficient, it is not possible when both are devoid (Kassar-Duchossoy et al., 2004). Although several studies have been conducted on the MRFs responsible for early myogenesis and muscle regeneration (Olguin et al., 2007; Singh and Dilworth, 2013), the exact nature of the Myf5 MyoD molecular interaction still requires clarification to elucidate the synergistic action of these two MRFs. During normal muscle regeneration satellite cells will proliferate until a threshold is obtained, at this point the expression of MRFs involved with proliferation begin to decrease while differentiating MRFs expression increases. Myogenin is one of the MRFs identified as a requirement for the normal differentiation of myoblasts to myocytes and ultimately myofibres. As mentioned earlier Myf5-/- or MyoD-/- mice displayed only minor deficits in muscle regeneration, conversely MyoG-/- mice present with severe deficiencies, signifying myogenin is unquestionably essential for unflawed muscle development (Venuti et al., 1995). Additionally, myogenin does not possess the characteristics of redundancy or compensation during embryonic myogenesis with any of the other MRFs as exist between Myf5 and MyoD. Research has demonstrated that a flawed type of adult muscle regeneration is still possible without myogenin as MyoG-/- mice are still able to regenerate muscle, but are two-thirds smaller than wild-type littermates (Knapp et al., 2006).This study suggests that mechanisms independent of myogenin can partially compensate for deficiency during adult regeneration. During normal skeletal muscle cell regeneration, the final MRF to be expressed is muscle regulatory factor 4 (MRF4). It can be appreciated that the three MRFs described above each have a unique timing and magnitude of expression, which suggests the configuration of myogenic regulatory factor expression has significant impact on the regenerative process. Research has also shown MyoD expressing cells to have impacts on the expression of MRF4 in the late stages of regeneration. MyoD-/- mice also exhibit inhibited MRF4 expression compared to wild-type mice, whether this action is direct or indirect still remains to be explored. Ultimately, the expression of MRFs through the stages of muscular restoration (Figure1.4) occurs once satellite cells become activated; the expression of MRFs is a complex and essential process needed to ensure proper muscular regeneration.

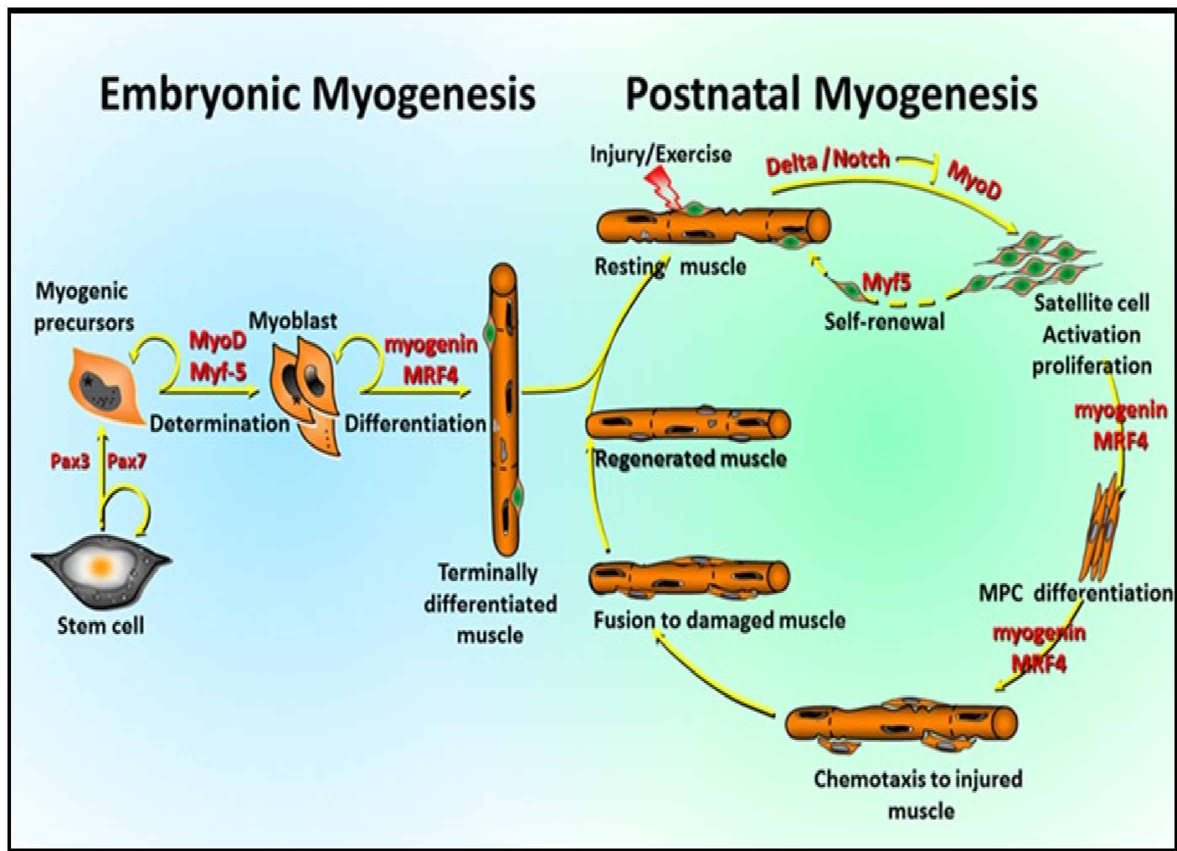


Figure1.4: Molecular regulation during embryonic and postnatal myogenesis. Upon response to injury/damage, resident satellite cells are activated, migrate to the damaged area and proliferate to generate a progeny of fusion competent myoblasts. Some of the satellite cells will self-renew and return to quiescent stage to contribute to future regeneration events. The generated progeny of myoblasts will fuse with each other or add new myonuclei to the damaged myofibre, thus promoting its repair. Figurer is adapted from (Al-Shanti and Stewart, 2009).

1.5. SIGNALLING PATHWAYS THAT REGULATE REGENERATIVE MYOGENESIS

In adult vertebrate, skeletal muscle hypertrophy occurs in response to weight-bearing exercise and because of increased protein synthesis (Glass, 2010). Enhanced protein synthesis enables new contractile proteins (myosin and actin) to be added to pre-existing muscle fibres, leading to generation of greater overall muscle force.

Hypertrophy can be stimulated by insulin -like growth factors (IGF), which are potent growth factors that produced, by the liver (Sjögren et al., 1999; Kasprzak and Adamek, 2012). IGFs

bind to surface receptors which are expressed on most skeletal muscle tissues in human and rodents (Figure 1.5)(Kasprzak and Adamek, 2012). IGF-I is known to induce proliferation and differentiation of myoblasts, increase muscle myofibre size and the number of fused myonucleus in differentiated myofibres (Yin et al., 2013). In addition to promoting muscle regeneration, IGF-1 acts as inhibitor for skeletal muscle atrophy Glass, 2010; Fuentes et al., 2011; Sukho, 2003). These findings have been confirmed *in vivo* and *in vitro* (Musarò et al., 1999; Rommel et al., 2001). Activated satellite cells express insulin-like growth factor binding proteins (IGFBPs), which are secreted proteins that regulate the function, transport and half-life of IGF-I. The mechanism of IGF-I effect on satellite cells proliferation and differentiation is mediated by IGF-1 receptors (IGF-IR). Binding IGF-I to IGF-IR recruits the insulin receptor substrate (IRS-1).Activation of IRS-1 triggers Raf /Ras /mitogen-activated protein kinases(MAPK)/extracellular signal regulated kinase (ERK) andphosphatidylinositol-3 kinase (PI3k)/Akt pathways (Figure1.5)(Moelling et al., 2002).The cross- talk between these signalling pathways is essential for IGF-1 to induce skeletal muscle hypertrophy (Chambard et al., 2007; David J Glass, 2003)

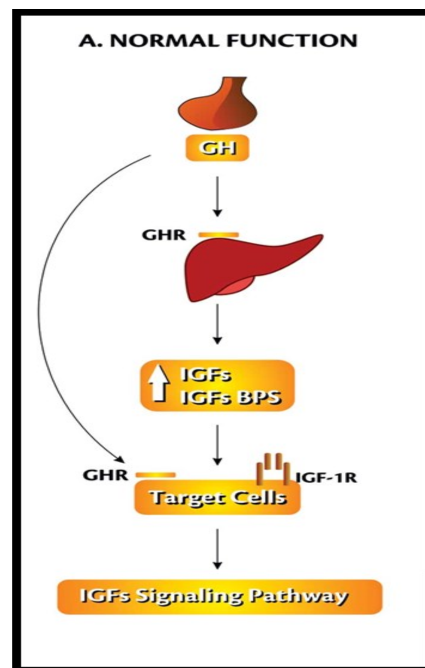


Figure1.5: Schematic representation of the GH-IGF in normal physiology: During postnatal life, liver is the main organ of IGF production and this growth factor is produced mainly upon effect of growth hormone (GH) secreted from pituitary gland. Binding of GH to growth hormone receptor (GHR) on liver cells promote IGF production which in turn bind to their receptors on skeletal muscle cells to initiate signalling cascade. Adapted from (Kasprzak and Adamek, 2012).

1.5.1. IGF-1/MAPK-ERK1/2

ERK1 and ERK2 isoforms are highly conserved serine/threonine kinases that are crucial for muscle cells proliferation (Jones et al., 2001). Activation of both isoforms leads to autophosphorylation of threonine and tyrosine, which in turn, leads to phosphorylation of several substrates that regulate transcription and translation of genes involved in cell cycle events (Sukho, 2003) (Figure 1.6). In mammals, it has been shown that inhibition of ERK1/2 leads to diminished cell cycle progression (Chambard et al., 2007). Blocking ERK1/2 in Raf-expressing myoblasts promotes myoblast fusion and differentiation (Li and Johnson, 2006). However, ERK1/2 is a positive regulator for skeletal muscle regeneration; it is required for terminal differentiation and preventing apoptosis (Li and Johnson, 2006; Fuentes et al., 2011; Saini et al., 2008).

1.5.2. IGF/Akt/mTOR

The Akt/mTOR signalling pathway is important in regulating myogenesis particularly in protein synthesis and skeletal muscle differentiation. Activated PI3k is essential for IGF function to induce hypertrophy. Activation of IRS-1 leads toPI3k phosphorylation (Fuentes et al., 2011), which in turn phosphorylates phosphoinositide-dependent protein kinase (PDK1) that translocates to the cell surface membrane (Glass, 2003) (Figure 1.6). Once activated, Akt1phosphorylates several substrates, including proteins that block apoptosis, promote protein synthesis and cell proliferation (Matsui et al., 2003; Vivanco and Sawyers, 2002). It was demonstrated that inhibition of PI3k prevented IGF-I-mediated hypertrophy *in vitro* (Rommel et al., 2001). The findings that Akt is also essential to promote IGF-I- induced hypertrophy have been reported (Rommel et al., 2001; Takahashi et al., 2002) and suggest that Akt is subsequently activated upon PI3k phosphorylation and both members function downstream for IGF. Phosphorylated Akt activates mammalian target of rapamycin (mTOR). IGF-I activation will stimulate downstream signalling pathways activating mTOR and p70S6k (Figure 1.6). mTOR also can be activated by amino acids and efficiently initiates downstream signalling to activate 70S6k. Thus, mTOR is vital in maintaining regular function of several factors that lead to protein synthesis. Both phosphorylated Akt and mTOR are elevated during skeletal muscle hypertrophy.

Rapamycin is a chemical that acts as an mTOR inhibitor (Pallafacchina et al., 2002). Also it was found to block activation of p70S6k, inhibit hypertrophy and protein synthesis. *In vitro* studied have shown that rapamycin blocks partially IGF-1 induced skeletal muscle hypertrophy, suggesting activation of other alternative pathways stimulating Akt induced hypertrophy (Rommel et al., 2001; Pallafacchina et al., 2002). Treatment with rapamycin blocks activation downstream of p70S6k but did not affect Akt and mTOR activity (Bodine et al., 2001), indicating that Akt subsequently activates mTOR and downstream p70S6k during hypertrophy (Figure1.6).

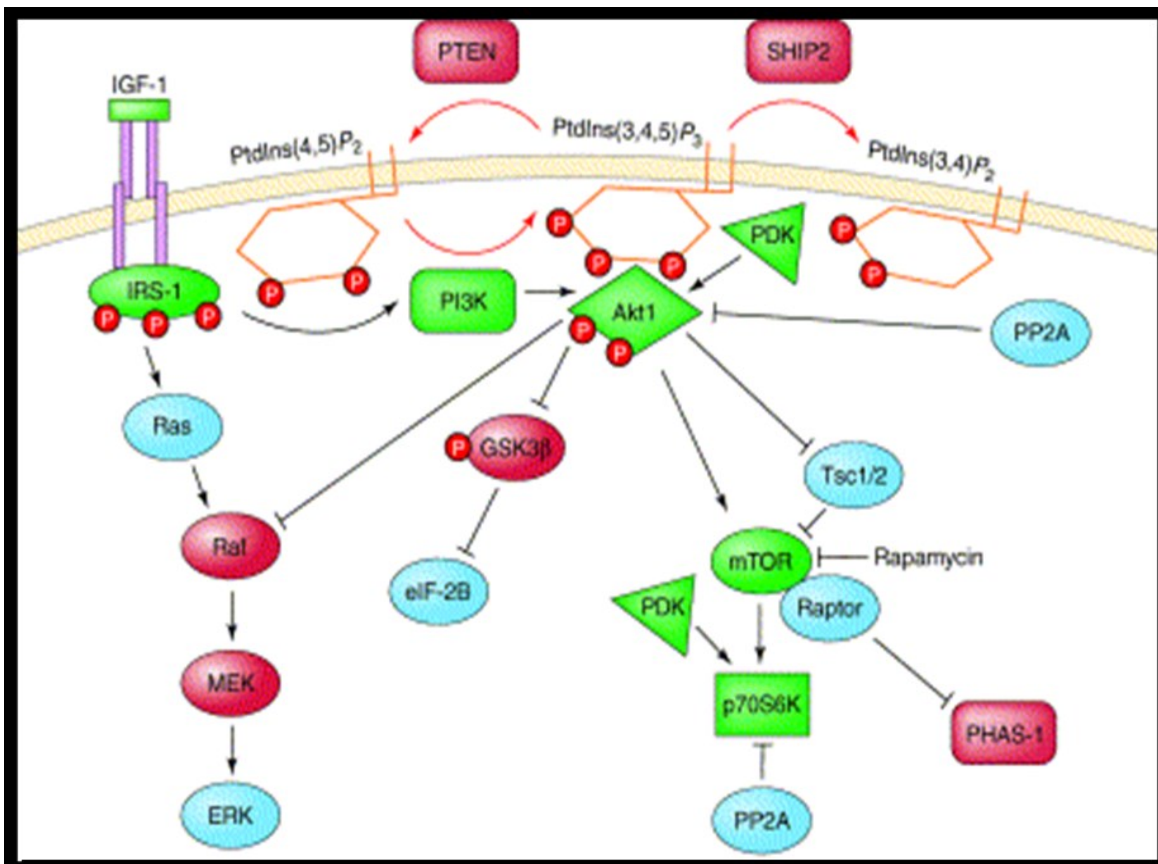


Figure 1.6: IGF-1-mediated signalling pathways: The schematic representation of signalling pathway for IGF-1/MAPK-ERK1/2 and IGF/ Akt/ mTOR cascades. Signalling proteins that have negative impacts on hypertrophy are shown in red boxes, and proteins whose activation induce hypertrophy are shown in green boxes. Proteins that have not been assayed for their effects on hypertrophy were shown in blue boxes. Adapted from (Glass, 2003).

1.6. MYOGENESIS AND AGEING (AGE- RELATED MUSCLE WASTING)

Ageing is associated with a decline in muscle mass, strength and a reduced ability to regenerate in a condition known as sarcopenia. Moreover, in ageing there is an increase in skeletal muscle fibrosis in concert with reduced quality of muscular tissue leading to impaired muscle regeneration, increased frailty and weakness.

The impairment in neuromuscular function is considered to be the major contributing factor to age-related muscle wasting so it is important to understand the causes. Causes may include deficits intrinsic to old muscle cells or their satellite cells (as described above) or may also be due to changes in the 'environment' that are heavily influenced by non-muscle cell factors, such as inflammatory molecules, growth factors and hormones that may all change in ageing. To shed some light on this, a study reported that autografts of muscle from old rats (2 years) regenerate efficiently when transplanted into the limb of a young rat (4 months) (Carlson and Faulkner, 1996; Gutmann and Carlson, 1976). Another cross-age transplantation study was conducted on young (4 months) and old (24 months) rats found that the mass and maximum force of old muscles grafted into young hosts were not significantly different from those of young muscles also transplanted into young hosts (Carlson and Faulkner, 1989).To gain further insights, a series of muscle transplantation experiments were conducted in which muscle was transplanted between geriatric (27–29 months old) and young (3 months old) mice. When young muscle was transplanted into the old host there was a delayed regenerative response that was related to a reduction of inflammatory cells in geriatric hosts (Shavlakadze et al., 2010). These results suggest the muscle itself is not necessarily impaired in the older rodents and therefore points towards the environment as a potential cause of muscle wasting in old age. However, the picture is more complex, because as discussed below, there are several reports of reduced satellite cell number and activation in older age which may or may not be related to the environment.

AGED SATELLITE CELL POOL

The ageing of skeletal muscle and associated reduction in satellite cell (SC) turnover substantially diminish the self-renewal process of SCs and hence muscle fibre repair mechanisms, causing reduced ability for muscular regeneration. One experiment found that stimulating SC proliferation 24 hours after eccentric knee extensor exercise leads 51% increase in SC numbers in elderly participants, however, a significantly greater increase of 141% was observed in the young participants (Dreyer et al., 2006); contributing further data for the age related decline of SC quantity. Ageing also instigates a change in muscle fibre phenotype associated with type II fibre atrophy (Verdijk et al., 2014), which then influences the associated loss of type II muscle fibre SCs in humans. Furthermore, studies have also shown that the satellite cells nuclei are reduced by roughly 50% in old (aged 71 years) compared to young people (aged 29 years) (Sajko et al., 2004). There is evidence that the reduction of SC concentration precedes the deficiencies observed during myogenesis in elderly people (Shefer et al., 2006).

A recent study reported that *p16* depression, a master regulator for cellular senescence, impairs satellite cells activation, proliferation and self-renewal of old mice (Bentzinger and Rudnicki, 2014). Furthermore, Bernet et al (2014) showed that increased P38 MAPK signalling pathway in response to elevated level of fibroblast growth factor -2 (FGF-2) in the aged niche impairs asymmetric self- renewal of aged satellite cells (Bernett et al., 2014).

Even though there is valuable evidence that the deficiencies observed in old myogenesis are a consequence of decreased satellite cell concentrations (Day et al., 2010), a decline in aged extrinsic (niche) and intrinsic regulatory signals (Notch Signalling, Wnt signalling, Niche EFG2, p38 MAPK, JAK-STAT and TEWAK signalling) which regulate satellite cell functions also involved in impaired muscle regeneration. Thus, age related changes in the intrinsic characteristic of satellite cells, as well as the extrinsic microenvironment are associated with impaired muscle regeneration.

1.7. PARABIOSIS-AN EXPERIMENTAL MODEL TO STUDY AGE -RELATED CHANGES IN SKELETAL MUSCLE MICROENVIROMENT

In the last few decades, the parabiosis model in which two animals share the same blood stream, re-emerged and has yielded very interesting results in endocrinology, tumour biology and immunology. This model enabled scientists to aim to identify the circulatory modulators involving growth factors, hormones and cytokines that influence tissue health and function. Following successful results, scientists developed the heterochronic-parabiosis (young-aged) model (Figure 1.7) to study whether exposure to young circulatory mediators could rejuvenate old tissue function.

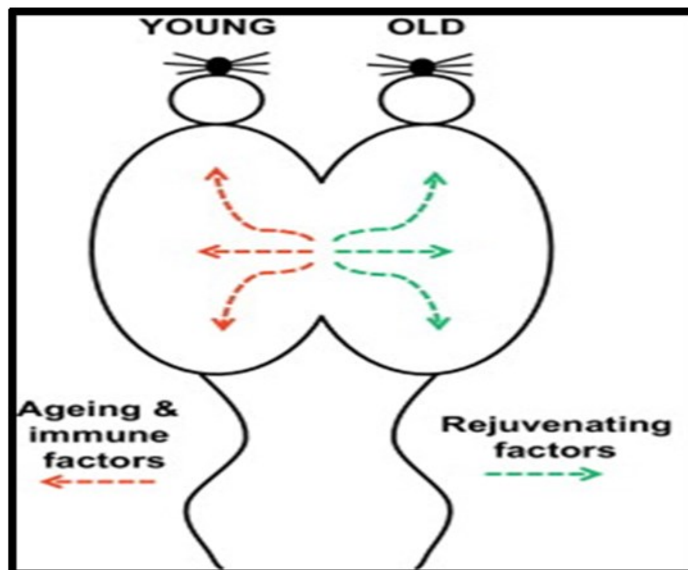


Figure 1.7: Heterochronic-parabiosis between aged and young mice. The cross connection between young and aged mice, allows continuous circulation of growth factors at physiological level. Adapted from **http://www.sciencedirect.com**.

This model has been applied to improve the understanding of stem cells in skeletal muscle, brain and spinal cord.

Conboy and colleagues found that exposure of old microenvironment to young circulatory factors through heterochronic-parabiosis rejuvenated the function of stem cells in spinal cord (Ruckh et al., 2012) and liver in mice (Conboy et al., 2005). Similar findings were obtained in brain (Villeda et al., 2011) and heart (Loffredo et al., 2013) in aged mice. Moreover, heterochronic-parabiosis was able to restore the Notch signalling pathway and the proliferative capacity of senescent muscle stem when exposed to factors present in young serum in mice (Conboy et al., 2005). Furthermore, the experimental results of the parabiosis model in mice

suggested that blood factors from a young circulation can significantly affect the function of aging tissues, as indicated by the rejuvenation and function of endogenous, old skeletal muscle satellite cells after injury using dry ice (Conboy et al., 2005). In addition, exposing a young mouse to an old systemic environment inhibited skeletal muscle regeneration (Brack et al., 2007).

These studies showed that there are key regulatory molecules provided by young hosts, which are able to reverse age related impairment in skeletal tissues. In attempt to identify candidate circulatory proteins in young blood, Sinha and colleagues reported that supplementation of systemic GDF-11 levels, which normally reduce with age, by heterochronic-parabiosis reversed age –related skeletal muscle and stem cells dysfunction in mice (Sinha et al., 2014).

However, blood contains thousands of proteins which may induce the rejuvenation process and therefore it is perhaps naive to think that one factor alone will underpin this process. It is also noteworthy that immune cells can cross between animals in parabiosis models and due to their role in tissue regeneration they could offer a more sophisticated regulatory mechanism for directing regeneration where it is needed, rather than relying on generally circulating molecules. Therefore, a better understanding of these cells and their secreted mediators may enhance the search for the candidate proteins which induce muscle rejuvenation.

1.8. IMMUNE SYSTEM

The immune system provides a defence mechanism against foreign antigens. It compromises of two classes: Innate and adaptive immunity (Figure1.8). Innate immunity includes chemical and physical barriers such as skin and mucous membranes as well as phagocytes and dendritic cells (DCs). Adaptive immunity is triggered by innate immunity via releasing cytokines (e.g. INF-γ) and cell-cell interaction between DCs and lymphocytes (Münz et al., 2005). The degree of specificity arises from the presence of major histocompatibility molecules (MHCI) on CD8 +T cells and (MHCII) on CD4+ T cells. CD4+T and CD8+ T cells activated after exposure to Antigen presenting cells (APCs) then differentiate to effector T cells such as cytotoxic T cells and helper T cells (Th). Th cells play important roles in controlling T and B-lymphocytes (Figure 1.8). Th cells produce IL-2 to activate CD8. In addition, Th cells release co-stimulatory

cytokines to activate B-lymphocytes and differentiate them to anti-body –releasing cells (Murray, 1998).

During normal physiological conditions, immune cells circulate within the blood and the lymphatic system, with considerable accumulations in lymphoid organs, but are also dispersed amongst most tissues; peripheral tissues also contain a population of resident immune cells primarily consisting of macrophages and DCs. However, during pathophysiological conditions supplementary leukocytes and lymphocytes can rapidly infiltrate tissues. These leukocytes become activated and release soluble cytokines that can influence the function of the surrounding environment.

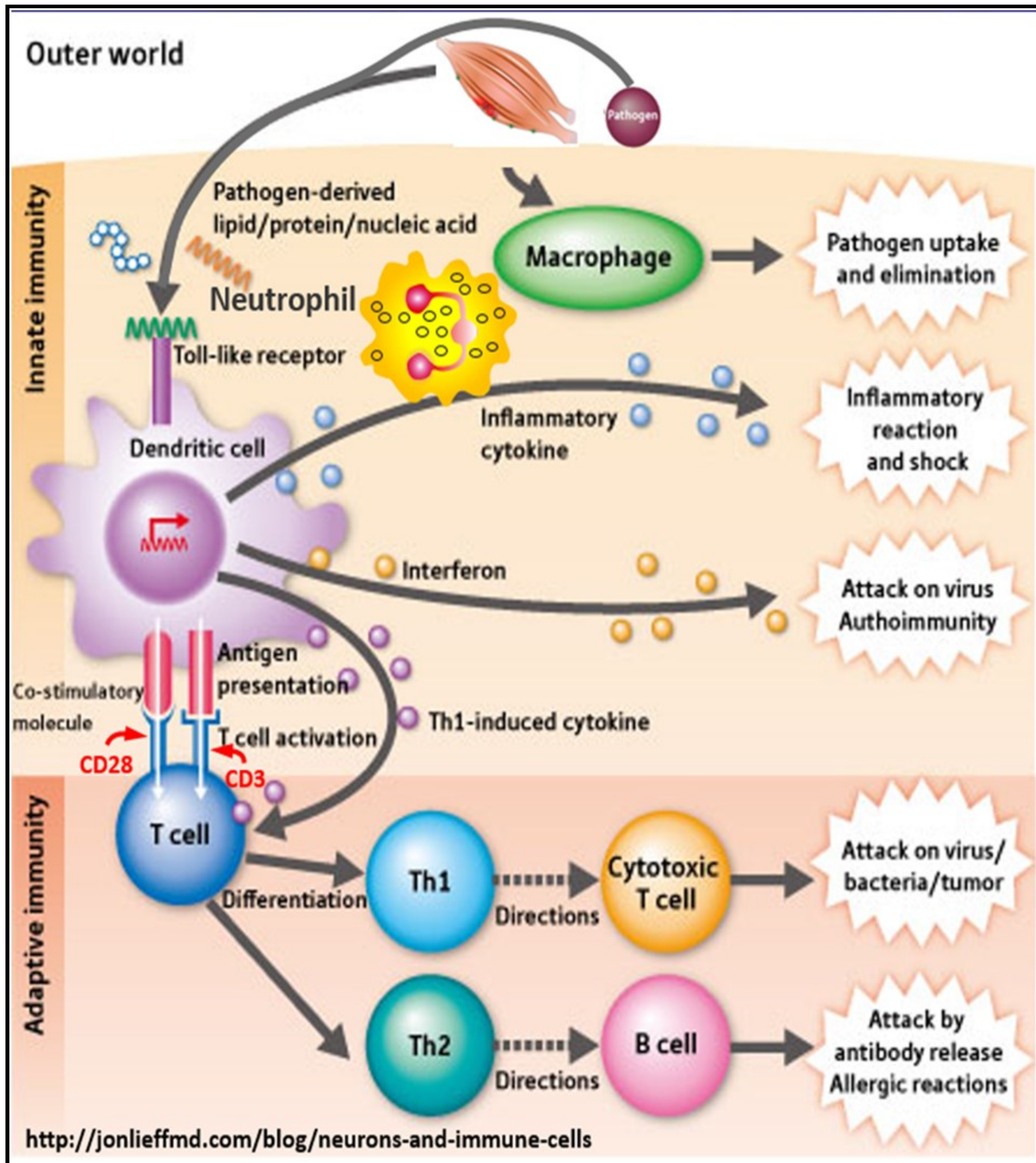


Figure 1.8: The interaction between innate and adaptive immunity. Upon exposure to pathogen, or in response to muscle injury, the interaction between dendritic cells and T cells occurs by MHC molecules. Consequently, T cell become activated and differentiated to cytotoxic T cells or T-helper T (Th1 and Th2) cells. Th1 cells support cytotoxic T cells while Th2 support antibody releasing B- cells. Adapted from http://jonlieffmd.com.

***IN VIVO* AND *IN VITRO* ACTIVATION OF IMMUNE SYSTEM**

T cell activation plays an important role in the regulation of immune responses including initiation of multiple signalling cascades originating from cells surface receptors (TCR) engaged by Major histocompatibility complex (MHC) on APC.TCR is a heterodimer composed of six different chains which are important for ligand recognition. CD3 molecules including, CD3-Gamma, CD3-Delta, CD3 Epsilon and CD3-Zeta, which are responsible for recognition of specific antigen by T lymphocytes. Engagement of TCR/CD3 by MHC leads to phosphorylation of tyrosine containing molecules including ERK (Extracellular signal- regulated kinase), JNK (c-Jun N terminal Kinase), NF-Kappa β (Nuclear factor- Kappa β) and NFAT (Nuclear factor of activated lymphocytes) resulting in T cell activation and IL-2 production (Figure1.9). T cell activation is regulated by CD28 receptor which provides essential co-stimulatory signal resulting in T cell expansion (Schwartz, 1990). Under normal condition, T lymphocytes, engagement of TCR-CD3 complex, results in co- stimulation by CD80-CD28 and recruitment of LAT (Linker of activated T-Cells) which leads to augmentation of IL-2 production and increase T cell proliferation (Jerome, 2008). Partial activation of TCR and/or the absence of co-stimulatory signals generate insufficient signals to trigger phosphorylation of ERK, JNK, nuclear factor kappa –Beta (NFK-β) and nuclear factor of activated T cells (NFAT)(Villarino et al., 2007; Sprent and Surh, 2011) (Figure 9). Therefore, IL-2 production is reduced. Anergy, unresponsive state, and quiescent states also arise from the presence of inhibitory molecules. TOB (Transducer of erbB2**)** is an inhibitory regulator of T cells proliferation and IL-2 production, it is selectively expressed in un-stimulated T lymphocytes and anergic T cells (Cooper et al., 2004). Inhibition of IL-2 by TOB is mediated by SMAD proteins. The latter mediate signals triggered by TGF-β through its receptors; TGF-B RII and TGF-BRI which in turn phosphorylate the downstreamSMAD2, SMAD3 and SMAD4 and translocate to the nucleus. The SMAD complex binds to the IL-2 promoter repressing IL-2 production (Tzachanis and Boussiotis, 2009)(Figure 1.9).

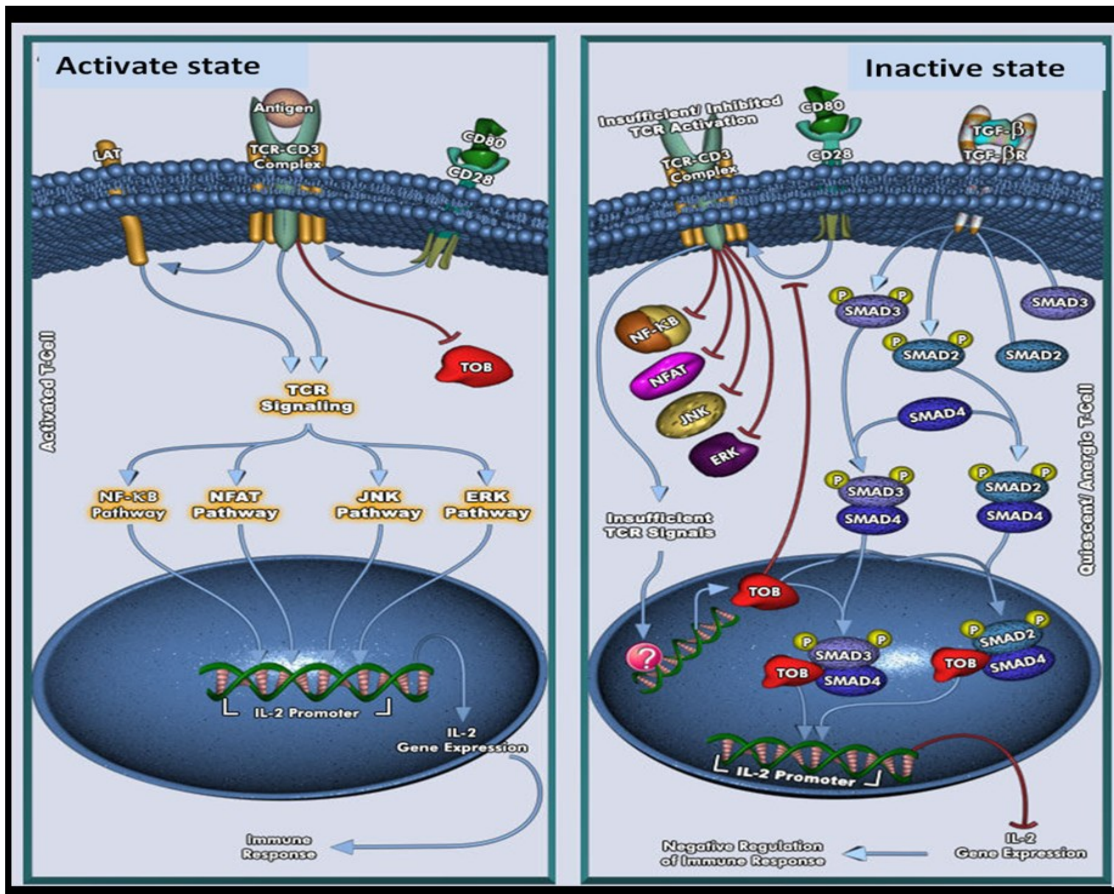


Figure 1.9: Activated and quiescence states of T cells. Activation of TCR/CD3 receptors and co-stimulatory CD28 molecules trigger sufficient signalling pathways that lead to activation of IL_2 gene promoter and then IL-2 gene transcription (left panel). Partial activation of TCR/CD3 and the absence of CD28 a co-stimulatory signal generate weak activation signal and inhibit promoter of IL-2 gene resulting hindered in IL-2 transcription (right panel). Negative regulatory molecules; TOB and SMAD complex repress IL-2 gene promoter and suppress IL-2 transcription (right panel). Adapted fromhttp://www.ebioscience.com

1.9 IMMUNE SYSTEM WITH AGEING

With ageing, many biological functions become impaired, including immune system. Age related decline of immune cells reduces the capability to respond to a challenge (Weng, 2006). Innate immune response is adversely affected with senescence (Salam et al., 2013). It has been observed that macrophages from old individuals express lower MHC molecule and Toll like receptors (TLRs) compared with young, which could negatively affect on their antigen

presentations resulting in lower cytokines productions in such as IL-6 and tumour necrosis factor-α (TNF-α) (Renshaw et al., 2002). Other components of the innate system that are adversely affected with increasing age are DCs. DCs reside in all tissue types and are identified by their elevated phagocytic and antigens presentation capabilities. DCs prime naïve T cells by their excreted cytokines which determine the differentiation and development of T cells. Aged DCs produce fewer secreted cytokines such as IL-12, suggesting age-related changes in function of T lymphocytes could be a consequence of impaired DC activity with ageing. Like DCs and macrophages, neutrophils also are functionally affected with ageing. After an infection, neutrophils are the first immune cells migrate to the site of infection. While the numbers of infiltrating neutrophils is not impacted with age, they display biological dysfunction in aspects of chemotaxis and nitric oxide production (Minet-Quinard et al., 2010).

The aged related decline in adaptive response has been well documented. Researchers showed that with increasing age, the number of T cells and clonal expansion TCR signalling are negatively affected thus, contributing to significant alteration in immune response to infections and tumours. For instance, upon stimulation, the interaction between T cell receptors (TCR) and APC cells is decreased which subsequently leads to low proliferative rate of T CD4 and CD8 cells and the reduction in CD4 T cells releasing cytokines. Together these events contribute to poor differentiation to Th1 and Th2. For example, IL-2 production from aged naïve TCD4 cells in mice is significantly lower compared to young counterparts. This subsequently leads to reduced numbers of CD4, Th1 and Th2 subsets, thus negatively influencing immune responses (Haynes et al., 1999; Salam et al., 2013; Buckingham, 2006). Moreover, It was observed that age related T CD4 dysfunction reflected on the responsiveness of CD8 TCR repertoire towards viral infection (Haynes and Maue, 2009) and to antibody production by B lymphocytes (Weng, 2006). Apart from T CD4 and CD8 changes, regulatory T cells (Tregs) are also functionally affected with ageing. It is well documented that the number of suppressor regulatory T cells (Treg) increases with age(Haynes and Maue, 2009; Weng, 2006; Nishioka et al., 2006). The age related defects in naïve T CD4 cells result from chronological ageing of naïve cell populations rather that from the chronological ageing of people. In response to antigen, naïve T cells are activated and migrate from the pool to encounter antigen, where as some remain locally within secondary lymphoid organs and then die off. In aged individuals, the equilibrium is disturbed because of reduced thymic output, suggesting that the pool contains aged subsets of lymphocytes which in turn lead to reduced conversion of naïve cells to memory cells and accumulation of age

related defects in CD4 cells. Taking all these events together, age related changes in the components of the immune system could contribute to immune response impairment in aged people.

In the last few years, there has been growing attention to the contribution of cellular and humoral components of the immune system in the regeneration of damaged tissues, including skeletal muscle, heart, and the nervous system. Recent discoveries in immune-mediated regeneration have been made in skeletal muscle, a well-studied model for adult mammalian regeneration that employs activation of satellite cells, the resident progenitors of the muscle. While the evaluation of macrophages in skeletal muscle regeneration has long been emphasized (Arnold et al., 2007), both eosinophils and regulatory T cells (Tregs) have now been shown to be necessary mediators of satellite cell activation and subsequently myotubes formation following muscle damage (Heredia et al., 2013; Wynn et al., 2013). In addition, discrete spatial and temporal regulations of the immune response to injury or disease determine the systemic milieu and consequently the future fate of the tissue.

1.10. POSSIBLE INTERACTION BETWEEN SKELETAL MUSCLE CELLS AND IMMUNE ENVIRONMENT.

After acute muscle injury, a sequence of events is initiated by populations of immune cells leading to inflammation followed by muscle regeneration (Figure 1.10).

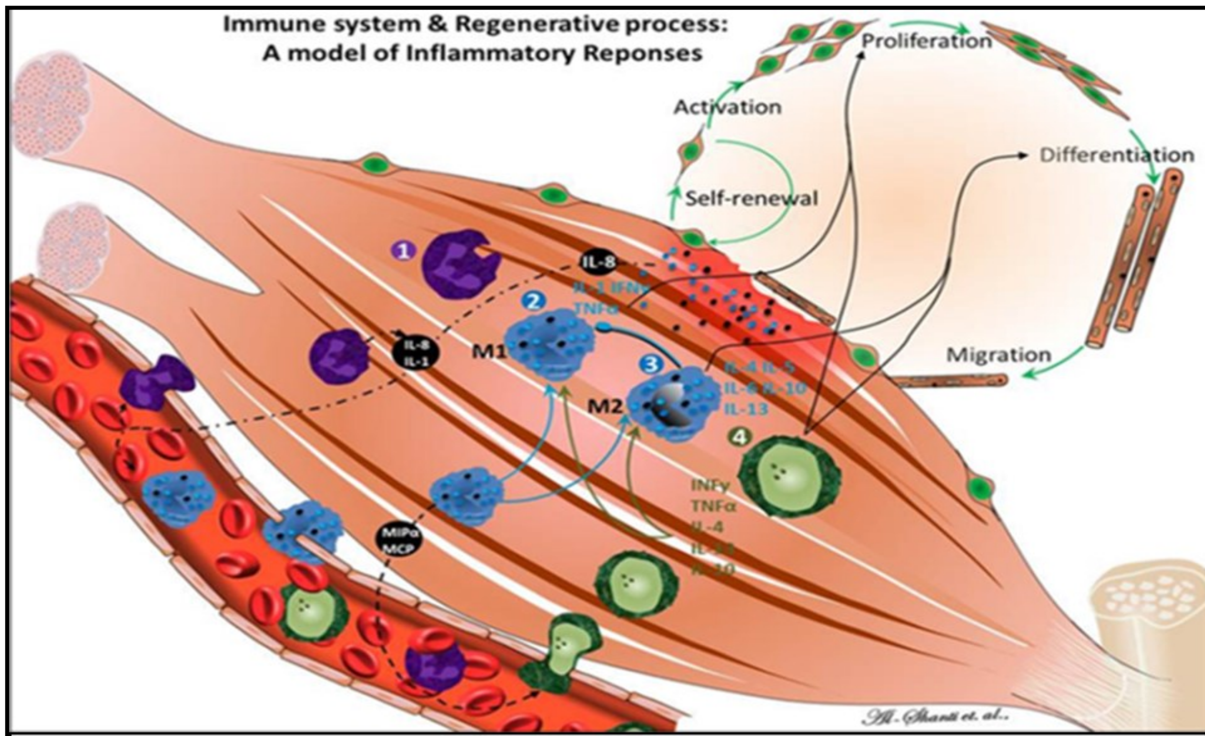


Figure 1.1010: Cross-talk between immune cells and satellite cells during myogenesis following muscle injury. After acute muscle injury, Th1 cytokines induce neutrophils and subsequently macrophages during the M1 phase. During M1, cytokines are released that promote muscle damage. M1 undergoes a transition to M2, which inhibits M1 function and releases anti-inflammatory cytokines that induce muscle repair and regeneration. T–cells release interleukins that promote muscle regeneration (this diagram was designed by Al-Shanti et al.).

Neutrophils are the first immune cells to migrate to the site of muscle injury with, numbers peaking between 6 - 24 hours post- injury. Macrophages are the second responders to the muscle injury and their numbers peaks 48 hours post injury then begin to decrease sharply. Declining macrophage invasion is followed by elevated numbers of lymphocytes that remain at high level for approximately 10 days (Pedersen and Toft, 2000; Shephard, 1998). The inflammatory response following muscle injury is accompanied by macrophages and neutrophils derived free radicals which target cellular debris for phagocytosis and subsequently lead a clean microenvironment for muscle repair and regeneration (Malm et al., 2000). The persistent presence of lymphocytes may provoke myogenesis and repair of skeletal muscle. However, with ageing, the immune function becomes impaired (Haynes and Maue, 2009; Nishioka et al., 2006) and the ability of skeletal muscle for repair and regeneration declines, leading to decreased

muscle mass, quality and function. Therefore, it could be suggested that impaired immune function with ageing could influence the microenvironment of skeletal muscle leading to inefficient muscle regeneration and repair (Dumke and Lees, 2011).

Cytokines and interleukins released from immune cells during inflammatory responses contribute to muscle adaptation during satellite cell proliferation and differentiation stages by affecting transcriptional activities and viability of skeletal muscle cells (Tidball and Villalta, 2010). Therefore, a better understanding of the cross talk between immune cells and skeletal muscle cells could help to provide therapeutic strategies for the reduction and treatment of muscle wasting with old age. To date, characterisation of immune specific factors capable of influencing skeletal muscle cells remain elusive.

1.10.1 CROSS TALK BETWEEN SKELETAL MUSCLE AND INNATE IMMUNE SYSTEM (MACROPHAGES).

Upon muscle injury, the innate immune response is initiated and driven by Th1 cytokines particularly, interferon -ɣ (INF-ɣ), IL-6 and TNF-α. These cytokines recruit immune cells for muscle repair and regeneration. Macrophages and monocytes within peripheral tissues have many important functions within this microenvironment, including phagocytosis, enzyme secretion, as well as growth factor and cytokine/chemokine production. These modulators aid in the recruitment of supplementary immune cells and provide a clean microenvironment for muscle repair and regeneration (Malm et al., 2000). As previously stated, neutrophils are the first population to migrate to the damaged tissue. Infiltrating neutrophils have important functions involving phagocytosis and TNF-α secretion. Neutrophil-secreted TNF-α triggers macrophage activation to a phenotype named M1. Influx of M1 macrophages promptly increases over the first 24 hours after muscle injury takes place, eventually reaching peak concentrations after 3 days (Saclier et al., 2013) after which numbers decline. The invading M1 phenotypes cells are responsible for driving inflammatory responses and stimulating muscle damage (Aurora and Olson, 2014). After the initial infiltration and decline of M1 macrophages, a secondary population of M2 macrophages enters the muscle reaching peak concentrations between 4 and 6 days, before reducing in number over several days or weeks. M2 macrophages are responsible for muscle differentiation and regeneration (Tidball and Villalta, 2010). M1

macrophages originating in the blood as monocytes are distinguishable by their expression of the glycoprotein lymphocyte antigen 6C (Ly6C) as well as receptors for the CX3C chemokine receptor1 (CX3CR1) and C-C chemokine receptor type 2 (CCR2)(Geissmann et al., 2003)

Ly6C+ monocytes differentiate into M1 macrophages in tissue and are acknowledged as inflammatory cells as they ultimately produce pro-inflammatory cytokines (Jetten et al., 2014). CCR2 (aka MCP-1) and its ligand CCL2 are potentially involved with the recruitment of Ly6C+ to the site of injury supporting the pro-inflammatory response. Conversely,Ly6C- cells differentiate into M2 macrophages and perform anti-inflammatory tasks, such as enhancing the reparative process. CX3CR1 and CCR2 chemokine receptors recruit the Ly6C- cells in a similar way to CCR2 recruiting LyC6+ cells. The CCL2/CCR2 axis is one of the most extensively described and is involved with the inflammatory response after skeletal muscle injury takes place. Research with CCR2-/- mice has discovered that monocyte recruitment to the site of injury is significantly impaired, while neutrophil and other lymphocytes remain unaffected. Studies with CCR2-/- mice not only display impaired muscle regeneration as a result of inadequate macrophage recruitment, arrested angiogenesis along with increased fibrosis and excess adipocyte accumulation at the injury site are also observed (Martinez et al., 2010). Noteworthy, another study involving CCR2-/- mice revealed that bone marrow transplants coming from wild-type mice result in a substantial increase in the regenerative capacity of skeletal muscle. This finding proposes that CCR2 released by proliferating myocytes and resident immune cells unmistakably recruit bone marrow derived monocytes (Sun et al., 2009). However, the same results are not observed in studies involving CCL2-/- mice. Minor deficiencies in regenerative capacity are perceived in comparison to CCR2-/- mice, which may indicate alternative chemokine (C-C motif) ligands can bind with the CCR2 receptor and support the recruitment of monocytes and ultimately improve regenerative capacity (Lu, Huang, Ransohoff, et al., 2011), yet CCL2 is still required for completely successful muscle regeneration. The cytokine IL-6 is also implicated in the initial infiltration of monocytes and macrophages during the inflammatory response shortly after muscle damage. Studies involving IL-6-/- mice have revealed a significant decrease in the early infiltration of monocytes and macrophage to the injury site. This results in the manifestation of diminished myofibres mass, along with intensified fibrosis of the muscle (Zhang et al., 2013). In wild-type mice, a large proportion of IL-6 production is an outcome of early monocyte and macrophage infiltration, which can trigger the expression of CCL2 and CCL3. Thus promoting the maximum infiltration

of monocyte and macrophage concentrations seen in the first 24 hours after injury. Granulocyte colony-stimulating factor (G-CSF) is required for typical myoblast proliferation and myofiber development throughout the muscle regeneration process(Hara et al., 2011). Analysis shows that macrophages release G-CSF when stimulated by IL-6, revealing another important role for IL-6 function during the early stages of inflammation and regeneration. Taking these results into consideration it can be recognized that the release of cytokines/chemokines can alter satellite cell activation, proliferation and/or differentiation to mature myotubes and muscle fibres. When examining differentiation of M2 phenotype macrophages a considerably more complex development takes place (Mantovani et al., 2004). M2a macrophages emerge from the exposure to adaptive immune response cytokines Interleukin 4 (IL-4) and IL-13, which stimulate the complex phases of tissue restoration and injury healing. M2b macrophage regulates the activation of Toll-like receptor immune complexes, leading to the release of anti-inflammatory chemokines such as IL-10 and the inflammatory cytokines TNF-α, and IFN-γ. The complex interactions between these cytokines and the healing muscle tissue are needed to regulate the timing of the immune response. The release of IL-10 by M2b macrophages also supports the recruitment of M2c macrophages. The M2c macrophages release the cytokines that are essential for the cessation of M1 macrophage infiltration and activity. Additionally, M2c macrophages release supplementary IL-10, which stimulates the proliferation of myoblasts leading to the promotion of muscle growth and regeneration (Deng et al., 2012). Through increasing knowledge of the exact mechanisms involved with the transitions between the different phenotypes, an insight into the manipulation of the process may provide unique therapies to increase regenerative capacity of skeletal muscle. It has been illustrated that shortly after injury M1 macrophage infiltration to the damage site occurs rapidly. The M2 macrophages that follow the M1 macrophages exhibit qualities of the M2c phenotype. This is logical as they mitigate the activation and actions of M1 macrophages (Arnold et al., 2007). During the early repair of damaged muscle tissue, the M1 macrophages are responsible for phagocytosis of necrotic muscle fibres as well as cytokine secretion and antigen presentation. Furthermore, TNF-α stimulates the M1 macrophages to produce inducible nitric oxide synthase (iNOS). This leads to cytotoxic concentrations of nitric oxide (NO) being produced, which are needed to destroy intracellular pathogens but as a consequence simultaneously promotes further muscle damage (Villalta et al., 2009). M2a macrophages are observed at their highest concentrations in the end stages of muscle repair due to their functions in the repair sequence. While sub-phenotypes of

M2b macrophages are observed throughout the repair process as having fluctuating levels during the alternatively activated immune response, since their production of IL-10 is needed to promote anti-inflammatory actions during muscle regeneration (Bosurgi et al., 2012). IL-10-/- animal models have disclosed that the transition of macrophages from the M1 to M2 phenotype is significantly impaired in the absence of IL-10, resulting in a corresponding impairment of muscle regeneration. Additionally, myoblast cell cultures supplemented with IL-10 and M2 macrophages enhance proliferation (Deng et al., 2012). Therefore, IL-10 performs important roles involved with mediating the conversion of M1 to M2 macrophages after muscle damage occurs, encouraging the proliferation of myoblasts and maturation of myofibers. Although there has been progress in the study of these mechanisms and persuasive evidence for the manifestation of diverse macrophage phenotypes in muscle repair, a robust knowledge of their precise roles is still elusive. Interestingly, research has presented evidence that the inflammation caused by macrophages and their associated pro-inflammatory cytokines have beneficial effects on myoblast proliferation while inhibiting differentiation (Tidball, 2005). As myoblasts switch from proliferation to differentiation, a shift from M1 macrophages to M2 macrophages and anti-inflammatory cytokines occurs concurrently (Figure 1.11).

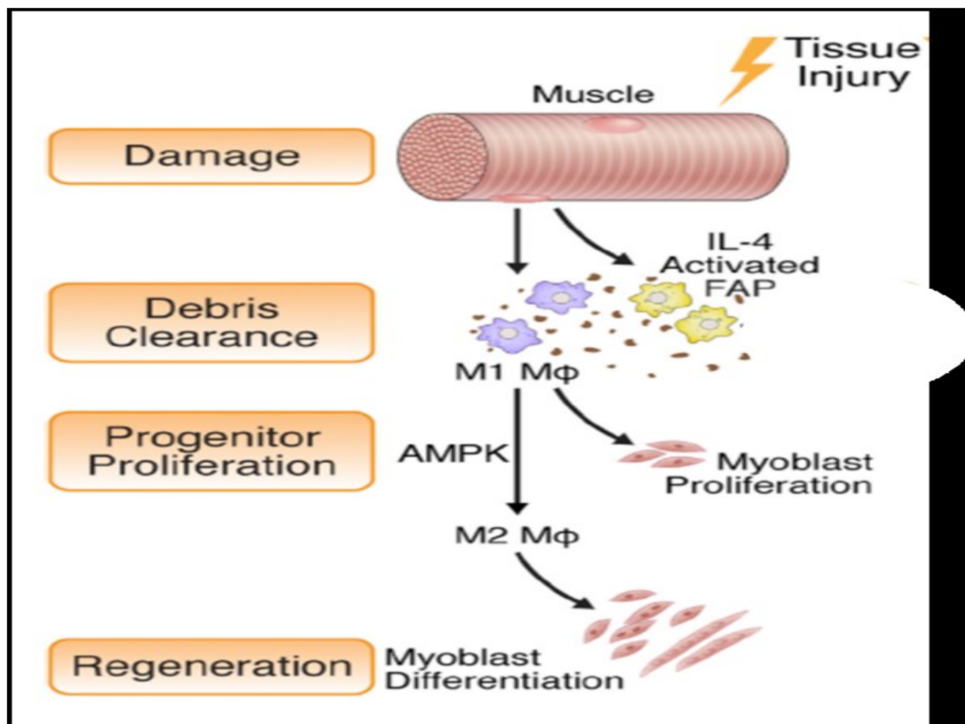


Figure 1.11 : Debris Clearance after skeletal muscle injury. Following muscle injury, debris clearance orchestrated by the immune system is a key activator of subsequent skeletal regenerative steps, including satellite cells activation, proliferation and differentiation. Debris removal is orchestrated by macrophages M1 and fibro/adipocyte progenitors (FAPs.). FAPs function activity depends on eosinophil derived IL-4; the absence of on eosinophil derived IL-4, FAPs convert to fat causing muscular dysfunction. Phagocytic M1 (MØ) promotes satellite cells activation and proliferation shifts to M2 via AMP-activated protein kinase mediated signalling (AMPK) to provide an environment that stimulates myoblasts differentiation. Adapted from (Aurora and Olson, 2014).

The cytokine transference diminishes the pro-inflammatory response and supports the differentiation of muscle fibres (Deng et al., 2012). The positive effects exerted by the anti-inflammatory cytokines produced by M2 macrophages on the differentiation of myoblasts have also been shown *in vitro* (Tidball, 2005; Arnold et al., 2007). M2 macrophages also produce IGF-1, a hormone known for this growth promoting skeletal muscle hypertrophy through the up regulation of MRFs. CCR2-/- mice not only display reductions in the adequate infiltration of monocytes and macrophages, a significant reduction in IGF-1 production and impaired muscle regeneration are correspondingly observed (Lu, Huang, Saederup, et al., 2011). This fascinating discovery suggests that macrophages provide growth factors that aid in the repair of muscle tissue damage by encouraging IGF-1 stimulated satellite cell proliferation. Overall, the production of these preliminary mediators via resident immune cells recruit the infiltration of circulating immune cells, thus allowing for the elimination of necrotic tissue along with the augmentation of myoblast proliferation and differentiation.

1.10.2. CROSS TALK BETWEEN SKELETAL MUSCLE AND ADAPTIVE IMMUNE SYSTEM (LYMPHOCYTES)

In the last decade, there has been a remarkable increase in the number of descriptive studies concerning the interactions between innate immune responses and muscle regeneration. However, the role of the adaptive immune system (T lymphocytes) in muscle regeneration relatively less well developed. Just as macrophages and cells involved with innate immunity are detected during acute muscle injury repair, adaptive immune cells such as T cells are also present during the regeneration process (Cheng et al., 2008). When observing the infiltration of innate

immune cells into the site of muscle injury a precise timeline is noted, T-cell infiltration also adheres to this timeline, with increasing concentrations reported approximately 3 days subsequent to cardiotoxin injection and remaining elevated for at least 10 days (Cheng et al., 2008). Recent studies investigating how T-cells regulate muscular regeneration indicate that T-cell cytokine secretions promote the proliferative and migratory actions of satellite cells, isolated from young muscle (Dumke and Lees, 2011). This finding adds evidence for the T-cell regulation of muscle repair. However, while the T-cell stimulus is observed as a positive influence on satellite cell proliferation/migration during acute injury repair, in animals that have dystrophic myopathies, T-cell suppression results in improved muscle regeneration with decreased fibrosis (Farini et al., 2007). Additionally, it is acknowledged that non-dystrophic T-cell deficient mice exhibit impaired muscle growth and increased fibrosis (Morrison et al., 2005). This is indicative of T-cell actions being distinct dependant on whether the response is to acute or chronic inflammation. Innate immunity's regulatory control of muscular regeneration is through cytokine and growth factor secretions; similarly, T-cells also release various signalling molecules, warranting further investigation. Furthermore, distinguishing between T-cell sub-phenotypes present during regenerations also important.

1.10.3 REGULATORY T (TREG) CELLS

Regulatory T (Treg) cells, determined as the $FoxP3^{+}CD4^{+}$ sub-phenotype are involved in immune response regulation (Josefowicz et al., 2012) inhibiting neutrophils and controlling the shift from M1 pro-inflammatory macrophages to M2 anti-inflammatory macrophages by IL-10 production(Figure 1.12) (Burzyn et al.,2013).

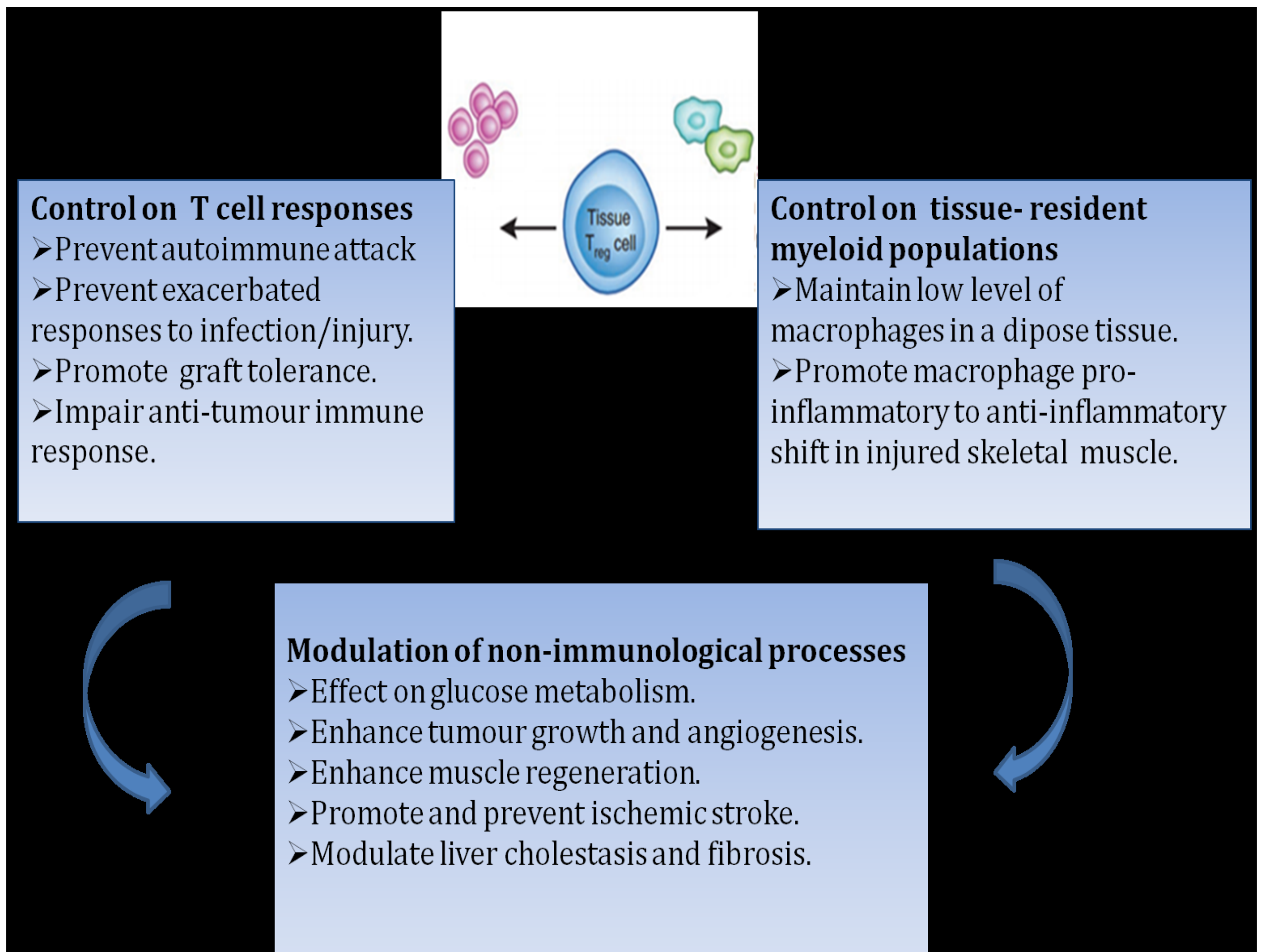


Figure1.12: The immunological and non-immunological functions of Tregs. The function of Tregs in tissues can be divided in to three groups: Control T cell responses, control myeloid populations and modulating the non-immunological processes Adapted from (Burzyn et al.,2013).

Tregs cells also control non-immunological process. For example, they promote angiogenesis by producing vascular endothelial growth factors (VEGF) (Zhou et al., 2015), regulate ischemic stroke development (Kleinschnitz et al., 2013), liver cholestasis and fibrosis (Katz et al., 2011). Recently, Tregs were detected in large quantities during acute muscle injury repair and regeneration (Burzyn et al.,2013). These cells produce regenerative factors such as IL-10, CCR1, PGDF and amphiregulin (Areg) that are known to accelerate skeletal muscle repair and regeneration(Figure 1.12) (Burzyn et al., 2013).Genetic ablation of T reg has shown that muscle Tregs directly enhance satellite cell activation and differentiation by secreting Areg (Burzyn, ,

et al., 2013) (Figure 1.13). Areg is a member of EGF family and a pro -regenerative that capable to initiate its effect on muscle regeneration through EGF receptor (EGFR). EGFR is expressed by different cell types involving satellite cells and a myoblast cell line (Burzyn, et al., 2013). Once amphiregulin interacts with EGFR on skeletal muscle cells, signalling of both Ras/Raf/MAPK/ERK and PI3K/Akt/mTOR are activated (Rundhaug and Fischer, 2010) (Figure 1.13).

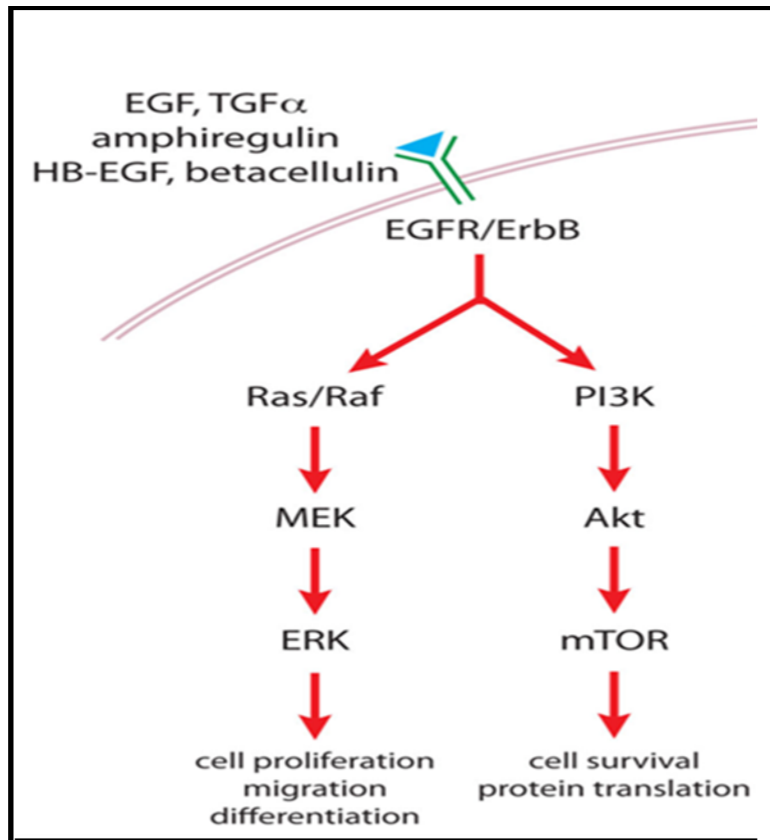


Figure 1.13: The effect of amphiregulin on skeletal muscle regeneration. Adapted from (Rundhaug and Fischer, 2010)

Researchers examining the role Foxp3+CD4+ Treg cells (Muscle Treg) play during muscle repair have provided some elucidation on T cell/satellite cell interactions. Using mouse models with muscular injury induced via cardiotoxin, it was discovered that the Treg cells number increases within the injured muscle as the innate immune cells shifts from a pro to anti-inflammatory phenotype (Burzyn et al., 2013). The authors of this study also found that elimination of Treg cells during the reparative process resulted in reduced regeneration. In consideration of these results, it can be appreciated that T cells add another level of complexity

to the regulation of muscle regeneration. Genetic ablation of Tregs has shown that muscle Tregs directly enhance satellite cell activation and differentiation by secreting amphiregulin (Burzyn et al., 2013).

Age has a significant impact on multiple satellite cells signalling pathways during myogenesis, aged satellite cell response to T-cells are no exception. Research conducted by Dumke and Lees (Dumke and Lees, 2011) has demonstrated that T-cell cytokine secretions have only a negligible progressive impact on satellite cell proliferation and migration, along with suppressed differentiation in aged satellite cells. This finding reinforces the concept that there are inherent variances in the response to environmental signals, dependant on age, occurring among satellite cells isolated from the skeletal muscle. Therefore, the authors speculate that satellite cell reactions to T-cell signalling may further age-related reductions in skeletal muscle regeneration. Various studies have revealed growth factors and cytokines which influence satellite cell functions are secreted from T cells (e.g. FGF2, IFN-γ, TGF-β, TNF-α, and IL-4) (Blotnick et al., 1994; De Rosa et al., 2004; Levings et al., 2002). The challenge for future studies will be to determine the specifics of the T-cell released factors responsible for altering satellite cell function during muscular regeneration throughout aging. Allowing for the advancement of directed therapeutic mediation in aged and/or diseased muscle. One proposed avenue of research that may provide further clarification of the mechanisms involved with T-cell/satellite cell interactions is to investigate what effect the secretome of activated lymphocytes has on cultured satellite cells. Experiments with C2C12 murine myoblast cell line have demonstrated that the secretome from human anti-CD3 activated T-lymphocytes inhibits differentiation while promoting proliferation (Al-Shanti et al., 2014). Importantly, the discovery that immune cell secretome can be used to manipulate myoblast functions allows for novel studies using human cell lines, while concurrently acquiring further intelligence of the constituent parts of the immune secretome.

1.11. THERAPEUTIC POTENTIAL OF IMMUNE MUSCLE CELLS SECRETOME IN MUSCLE REGENERATION.

Although the investigation of the immune cell secretome on skeletal muscle satellite cells is still in the early stages, studies have been performed using the secretome to enhance wound healing. Interesting results have been demonstrated when the secretome collected from young, healthy,

human activated T-lymphocyte cultures is applied to punch biopsy wounds on mice. Experiments show advanced wound healing in mice treated with the secretome directly (Mildner et al., 2013). Additionally, the authors found that when the secretome was applied to human skin cells in *vitro*, signalling molecules involved with increased proliferation and migration were activated. Similarly, promising evidence for the use of the secretome in treating myocarditis has been exhibited. When experimental autoimmune myocarditis (EAM) has been induced in animal models, it can be attenuated with large doses of mononuclear cell secretome (Konrad Hoetzenecker et al., 2013). Recently, we showed that the secretome from young human activated lymphocytes markedly induced proliferation and blocked the differentiation of C2C12 cells (Al-Shanti et al., 2011). In view of these results it can be appreciated that the immunomodulating impacts of immune cell secretome has the potential for diverse applications, including skeletal muscle regeneration. Therefore, a better understanding of the cross talk between immune cells and skeletal muscle cells could help to provide therapeutic strategies for the reduction and treatment of muscle wasting with old age. To date, characterisation of immune specific factors capable of influencing skeletal muscle cells remain elusive. In the present work, it was hypothesised that as we age, the ability of our immune and skeletal muscle systems to interact, and therefore to function optimally, are compromised.

1.12 AIMS AND OBJECTIVES

The overall aim of the work presented in this thesis was to develop a better understanding of the role of immune cells (principally lymphocytes) in muscle satellite cell proliferation, migration and differentiation, and to determine whether differences exist between immune cells isolated from young and from older donors.

Thus, the objectives are:

1. To optimise the lymphocytes cell culture conditions, including determination of effective activation time, anti-CD3 and CD28 concentrations and cell counts.
2. To determine effective concentrations of the lymphocytes' secretome collected from young adults when used to enrich the cell-culture medium of C2C12 (p5) cells.
3. To characterise young and old lymphocytes isolated from fresh blood samples of healthy donors by analysing CD25 receptors, amphiregulin expression and FoxP3 receptors.
4. To identify candidate regulatory factors secreted from young and old lymphocytes that have the potential to modify C2C12 activity.
5. To explore the impact of harvested secretomes from young and old activated lymphocytes on C2C12 proliferation and migration, compared to the controls of the study.
6. To determine the biochemical (MAPK/ERK1/2 and Akt/mTOR signalling pathways) and molecular (MyoD and myogenin) adaptations of C2C12 cells treated as above.

CHAPTER 2

2.1. MATERIALS, GENERAL EQUIPMENT AND SPECIALIZED SOFTWARE

2.1.1. CHEMICALS, SOLVENTS AND REAGENTS

All general chemicals and solvents (analytical (AnalaR)) or molecular biology/tissue culture grade) were purchased from Sigma (Poole, UK) and BDH (Poole, UK), unless otherwise specified. All inhibitors were purchased from Selleckchem (USA).

2.1.2. CELL CULTURE

All cell culture experiments were performed under a Class II microbiological safety cabinet (Labcaire SC-R Recirculating Class II, North Somerset, UK). All cell incubations were performed in Triple Red Laboratory Technology Nuaire DH Autoflow CO2 Air Jacketed Incubator (Buckinghamshire, UK). Liquid, media and supernatants were discarded using an IBS Integra Biosciences Vacusafe Comfort (Chur, Switzerland). All solutions used for cell culture were prepared with distilled water from Elgastat option 4 water purifier (Elga Ltd., High Wycombe,UK). Cell microscopy and photography were performed using Leica Microsystems (Wetzlar, Germany) CMS GMbH light microscope (Leica DMI6000B) with digital photography (Leica CTR 6000), using Leica Application Suite software (Wetzlar, Germany). For fluorescent microscopy, images were taken using Leica DMI 6000B imaging system with FITC-fluorescence L5 filter cube (excitation wavelength=480/40nm).

2.1.3. CELL CULTURE REAGENTS

C2C12

Sterile Dulbecco's Modified Eagle's Medium (DMEM) w/ 4.5g glucose per Litre, w/ L-Glutamine (584mg/L) was purchased from LonzaBioWhittaker (Wokingham, UK). Heat-

inactivated (hi) foetal bovine serum (FBS) was purchased from Gibco (Paisley, UK) and hi horse serum (HS) - from Southern Group Laboratory (Corby, UK). Sterile penicillin-streptomycin solution and 10x trypsin solution were purchased from BioWhittaker (Wokingham, UK). L-glutamine was purchased from BDH (Poole, UK) and non-sterile gelatine type A from porcine skin was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Phosphate buffered saline (PBS) tablets were from Oxoid Ltd. (Basingstoke, UK).All solutions were prepared in non-sterile distilled water and were sterilised by autoclaving at 121°Cin a bench-top autoclave from Prestige Medical (Birmingham, UK) before usage in cell culture.

HUMAN LYMPHOCYTES CULTURE

RPMI-1640 was purchased from (Lonza, Belguim), Ficoll–Paque PLUS (GE healthcare Life Science, UK), recombinant interleukin- 2 (rhIL-2, 10µg, R & D System) and L-glutamine was purchased from BDH (Poole, UK). Human anti-CD3 was purchased from (CliagAG, Switzerland) and anti-CD28 from (BD Pharmingen TM, San Diego). Sterile AB human serum purchased from Invitrogen (Brown Deer, USA).

2.1.4. PLASTICWARE

Tissue culture flasks (T75 and T175), 12 and 6 well Nunclon™Δ surface multi-well dishes were purchased from Nunc Life Sciences, Thermo Fisher Scientific (Rockslide, Denmark). 96 well plates for protein assays were also purchased from Nunc Life Sciences, Thermo Fisher Scientific (Rosklide, Denmark). MicroAmp® Optical 96-Well Reaction Plates for Applied BiosystemsStepOnePlus Real-time PCR system were purchased from Applied Biosystems (Warrington, UK). Cryogenic vials, 15m and 50ml sterile centrifuge tubes, cell scrapers and pipette tips for tissue culture, biochemistry and flow cytometry were purchased from Fisher Scientific (Loughborough, UK) and 0.25, 0.5, 1.5 ml RNase Free Microfuge tubes for RNA work were purchased from Applied Biosystems (Ambion-The RNA Company, Cheshire, UK). 0.5, 1.5 and 2 ml tubes were also purchased from Eppendorf (Hamburg, Germany). Syringes were from Terumo (Leuven, Belgium) and syringe filters (0.22 µM) from Corning (Lowell, MA, USA).

2.1.5. LIVE IMAGING

Live imaging microscopy was performed using a Leica DMI6000B microscope equipped with an autoflow incubator, CO_2 controller, heating unit and temperature controller, thus allowing maintenance of 5% CO_2, 37°C humidified atmosphere. Videos were generated and exported using the Leica Application Suite software (Wetzlar, Germany).

2.1.6. REAL-TIME PCR

RNA concentrations and purity were assessed using a Biotech Photometer (WPA UV1101, Biochrom, Cambridge, UK). Applied Biosystems StepOnePlus Real-time PCR system. Results were analyzed using a StepOne Software v2.2.2 (Applied Biosystems, Life Technologies). Reagents for real time PCR were from Applied Biosystems (Carlsbad, CA, USA).

2.1.7. FLOW CYTOMETRY

Flow Cytometry was performed on a BD FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) with Cell Quest Pro Software (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using ModfitTM software (Verity Software House, Topsham, ME, USA). Reagents for Flow cytometry were purchased from BD (BD Biosciences, San Jose, CA, USA).

2.2. METHODS AND PROTOCOLS

2.2.1. CELL CULTURE PREPARATION

Cell culture experiments were performed with the C2C12 cell line, which was purchased from the American Tissue Culture Collection (ATCC; Rockville, MD, USA).

The cell cultures in the study were prepared following thawing the frozen stock. The cells were induced to grow to reach the desired population. Then proliferating cells were transferred to different conditioned media which were designed for the experiments. Unused cell cultures were

preserved by cryopreservation for long- term storage. These procedures used in the preparation of cell cultures for the study are described below.

2.2.1.1. STIMULATING PROLIFERATION OF MOUSE C2C12 SKELETAL MYOBLASTS.

C2C12, murine skeletal myoblasts, are derived from satellite cells, whose behaviour corresponds to that of a progenitor lineage. These cells are a subclone of C2 myoblasts (Yaffe and Saxel, 1977) which spontaneously differentiate in culture after serum removal (Blau et al., 1983). The C2C12 cells were purchased from ATCC (Rockville, MD, USA) and maintained by growing cells at a density of $2x10^6$ cell / flask in 0.2% pre-gelatinised T75 flasks in a humidified atmosphere of 5% CO_2 at 37°C. The cells were grown in GM Dulbecco's Modified Eagles Media (DMEM), supplemented with 10% foetal bovine saline defined (hi FBS), 1% L-glutamate and 1% of 10000U (Penicillin/Streptomycin). Myoblasts undergo myogenic differentiation upon replacement of GM with differentiation media (DM), supplemented with 2% horse serum (hi HS), 1% Pen-strep and 200mM L-glutamine.

2.2.1.2. CELL PASSAGING

Passaging of cells was performed by aspirating culture media and washing cells with PBS to remove any remaining serum. Cells weretrypsinised with 0.05% Trypsin/0.02%Ethylenediaminetetraacetic acid (EDTA) for 3-5 minutes at 37°C to detach cells adhering to the substrate and re-suspending the cells in pre-warmed GM (in a ratio of least 1:6 of trypsin:GM). The purpose of suspending cells in media is to deactivate the action of the trypsin (EDTA). Cells were counted using a haemocytometer and the numbers obtained were adjusted for dilution and stock volumes. The cells were grown in GM in a flask or a multi-well dish, pre-coated with 0.2% gelatine. Working volumes were normally 10ml of media for T75 flasks, 5 ml in T25 flasks, 2ml of media/well for a 6-well plate and 1ml of media/well for a 12-well plate.

2.2.1.3 CELL CRYOPRESERVATION AND RESURRECTION

CELL CULTURE PROLIFERATION AND MAINTENANCE OF C2C12 SKELETAL MYOBLASTS (GM)

For the preparation of cell stocks, confluent cell monolayers were trypsinised and cells counted with a Neubauer haemocytometer. Cell numbers were diluted in GM to 1×106 cells/ml. 10% dimethyl sulphoxide (DMSO; BDH, Poole, UK) was added to the cell suspension in a drop-wise manner and cells were transferred to 1ml cryovials. DMSO partially solubilises and stabilises the cell membrane, making it less prone to puncture. This is mainly because draws water out of the cells, dehydrating them and protecting intracellular components from ice crystal damage.

Consequently, cryovials were placed into freezing chambers purchased from Nalgene (Rochester, NY, USA) containing isopropyl alcohol and the chamber was transferred for overnight storage at -80°C. This procedure allows gradual freezing of the cells at a rate of -1°C/.min, which allows the reduction in metabolism of the cells and allows enhanced survival upon resuscitation. Following overnight incubation at -80°C, cryovials were transferred to liquid nitrogen for long-term storage. The growth of ice crystals is reduced in liquid nitrogen for long-term storage.

For restoring cell stocks from liquid nitrogen, cryovialswere placed at room temperature in a cell culture safety cabinet until thawed and the cell suspension quickly transferred to a 0.2% gelatine pre-coated flask, containing warm GM.

2.2.1.4. RECONSTITUTION OF INHIBITORS FOR C2C12 TREATMENT

All inhibitors including the Mek inhibitor(UO126) and IGF-R inhibitor (Linsitinib-OSI906) were purchased from Selleckchem. Inhibitors were diluted in DMSO according to manufacturer's instructions and stored at -20°C. Both inhibitors were kept inmMconcentration stocks which were diluted to working concentrations in appropriate DMEM media before treatment.

2.3CELL COUNTING BY TRYPAN BLUE EXCLUSION

CELL COUNTING BY TRYPAN BLUE EXCLUSION

For cell counting, the cell suspension was syringed to destroy clumps forming and prepared in a 1:1 dilution in trypan blue stain (Bio Whittaker, Wokingham, UK) and cells were counted with a Neubauer haemocytometer (Assistent, Sondheim, Germany). Viable cells exclude the trypan blue dye and only the dead cells absorb the stain.

PRINCIPLE

Cell counting was performed using a Neubauer haemocytometer consisting of a thick glass with a grid of lines etched in the middle. The grid isan arrangement of different sizes of squares which makes it possible to count the number of cells in a known volume of solution. The counting chamber coverslipshould restfirmlyon a counting chamber at a distance of 0.1mm above the base of the slide(Figure 2.1). When cell suspensions fill the chamber, they can be observed under the microscope and the cells are counted in a chosen number of squares in fields of view. From these counts, the cell number per ml of suspension can be calculated. Percentage of dead cells vs.Percentage of viable cells were estimated using trypan blue, which will only penetrate membranes of dead/non-viable cells. Viable cells exclude the stain and stay small while dead cells become, larger and dark blue

PROCEDURE

Prior to use, the haemocytometer was cleaned with 70% ethanol**.** Edges were moistened and the coverslip positioned over the grooves until Newton's rings were observed.This indicated that the coverslip was accurately placedand fixedover the counting area. Equal volumes of cell suspension and 0.4% trypanblue were mixed and pipetted into the chamber by capillary action. Cells were counted in quadruplicate with each counting area of the hemocytometer, representing a total volume of $0.1mm^3$. Since $1cm^3$ is equivalent to 1 ml, the cell concentration per ml was calculated using the following equations

Cells per ml = Average cell count × dilution factor × 1×104 cells/ml

E.g.: average count is 50 cells × dilution factor of 2 × 1×10^4= 1×10^6 cells/ml

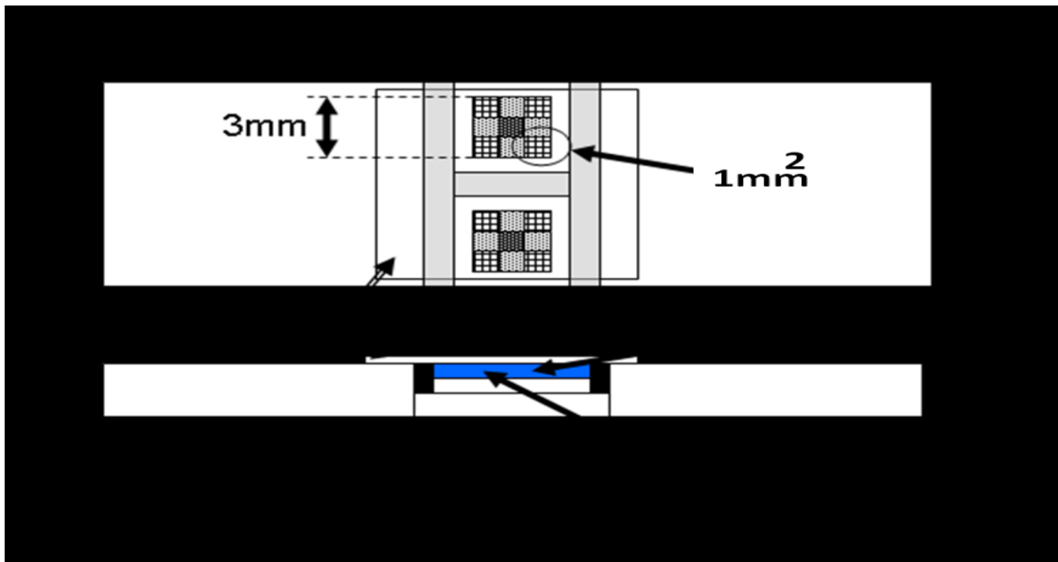


Figure 2.1: Haemocytometer design and dimensions. Adapted from https://www.phe-culturecollections.org.uk

2.4. BLOOD SAMPLE COLLECTION AND PROCESSING MANAGEMENT

Blood samples were collected from healthy young and old participants predominantly males, who were recruited to take part in this study. All recruited participants in this study were healthy on a normal diet, not on immunosuppressant or corticosteroids and immunosuppressant treatment that affect immune function. During the recruitment process, potential participants were provided with the details of this study. Information of this study was provided in verbal and written formats in order for potential participants to make an informed decision on whether or not to participate. Participants were encouraged to understand this information and make a considered decision on their own, away from the researchers. A copy of the signed consent form was provided to the participants for their information. Participants had the right to withdraw from the experiment at any time. They also completed a medical questionnaire in which all recorded details were completely anonymous (free from any potential identification). This was achieved by allocating each participant with a non-identifiable participant number (PN). The participants were asked to give blood by a phlebotomist. The needle was inserted in an antecubital vein to obtain at least 10 ml blood in EDTA tubes. After obtaining the blood, the needle was removed and gauze applied at the site of venepuncture. The participants were then free to leave the laboratory. All raw samples and data were stored anonymously under a

participant number (PN) rather than by name. Data storage complied with the Data Protection Act (1998). Participants had the right to withdraw from the study at any time after the experimental procedure commenced, all that participant's samples and data stored in whatever format would have been destroyed.

2.4.1. *IN VIRTO* ACTIVATION OF HUMAN LYMPHOCYTES FROM FRESH BLOOD SAMPLES

Lymphocytes were isolated from fresh blood samples by Ficoll–PaquePLUS depending on the gradient density. Diluted blood with PBS (ratio 1:1) was layered carefully over 15 ml Ficoll – Paque PLUS. The blood - Ficoll-Paque PLUS layers were centrifuged at 500xg for 30 minutes at 18°C. Following that, the layer of lymphocytes was carefully removed and washed twice with RPMI-1640 media).Prior to lymphocytes culture, monocytes were depleted from the cell preparation by pre-plating for 15 minutes at 37°C (Karanfilov et al., 1999). To mimic in vivo activation, isolated lymphocytes were cultured at $1x10^6$ cell/ml on pre-coated 96 well flat-bottomed micro well plates with anti-human anti-CD3 (OKT3 ,10ng/ml)(Figure 2.2)(Garland et al., 2002; Al-Shanti et al., 2014a)overnight at 4°C. Lymphocytes were developed in RPMI-1640 containing 10% (v/v) AB human serum, anti-CD28 (Figure 15), 1% Penicillin/Streptomycin and 50U/ml of human recombinant interleukin- 2 (rhIL-2, 10µg, R & D System).In negative controls cells were incubated in the absence of anti- CD3/CD28 and supplemented with IL-2 in a complete RPMI-1640 media. Cells were cultured at different incubation times to identify the optimal stimulation. For cell counting, lymphocytes were re-suspended in a 1:1 dilution in trypan blue stain before being counted with a Neubauer haemocytometer.

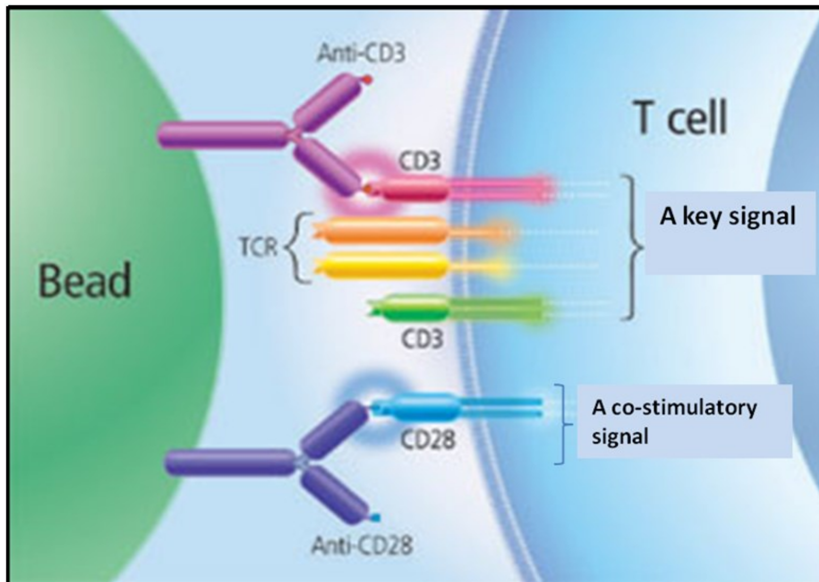


Figure 2.2: Engagement of T cell receptor (TCR) CD3 and CD28 by anti-CD3 and anti-CD28 of T lymphocytes activation *in vitro*. Adapted from http://www.the-scientist.com

2.4.2. SECRETOME RECOVERY

A volume of 4 ml of lymphocytes secretomes from each experimental culture time was centrifuged in an Ultracel-3 Membrane tube (Figure 2.3) for 30 minutes at 5400xg to prepare conditioned media. Alternatively, secretomes were aliquoted and kept at -80°C for multiplex immune assay analysis.

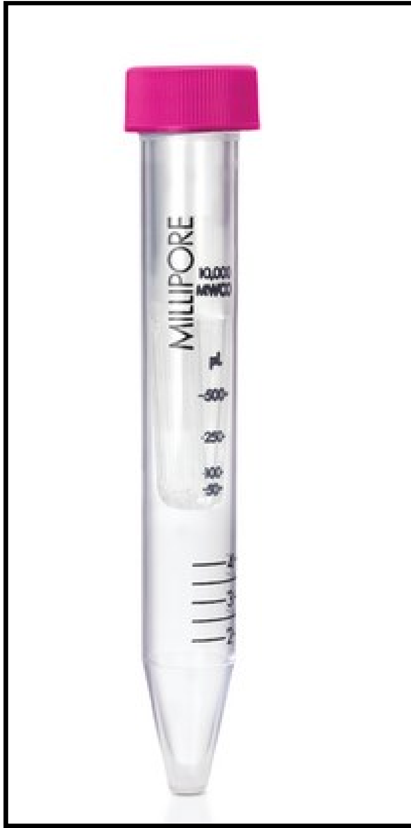


Figure 2.3: Amicon® Ultra-4 Centrifugal Filter Units for secretome filtration

2.4.3. CONDITIONED MEDIA (CM) PREPARATION AND C2C12 TREATMENT.

A cell-free culture supernatant was used for the conditioned media and prepared by sterile filtration of the media in which the lymphocytes were cultured. The conditioned media was added to the usual C2C12 culture media at the desired concentration. The remaining concentrate of the secretome (200µl) was mixed with DM (DMEM) to form CM. Four types of CM were prepared. CM1 contained concentrate filtered obtained from activated lymphocyte culture. CM2 contained concentrate obtained from non-activated lymphocyte cultured with IL-2 but without anti-CD3 and CD28. CM3, was a control containing boiled activated lymphocyte secretome - the secretome was boiled for 10 minutes in a water bath to destroy all cytokines. CM4 contained concentrate from RPMI-1640 supplemented only with IL-2 but without lymphocytes. This control was included to exclude any effects of the 10% of human AB, which is added to RPMI-

1460, a lymphocyte culture medium. To treat C2C12 with these CMs, cells were cultured a day before in pre –gelatinised 6-well plates at a concentration of $50x10^3$cells/ml of completed growth media (GM). Cells were incubated overnight in a humidified atmosphere with CO2 at 37^0C. After 24 hours, cells were washed twice with PBS prior to replacing GM with conditioned media (CM1 Young and Old, CM2 Young and Old, CM3 Boiled, and CM4 and incubated for particular period of time.

2.5 HUMAN SERUM SEPARATION FROM FRESH BLOOD SAMPLES

Blood samples were collected from young and old participants using a serum separated tube (SST) and left for an hour to clot at room temperature. The clot was removed by centrifuging at 1000-2000×g for 10 minutes. Following centrifugation, the liquid component (serum) was transferred into a clean polypropylene tube and stored at -80°C for DM preparation. Serum is the blood extract which contains all the proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens and hormones. To destroy heat-labile complement proteins, serum samples were heated in a water bath for 30 minutes at 56°C. Serum samples were aliquoted and kept at -80°C till further analysis.

2.6. CELL EXAMINATION BY FLOW CYTOMETRIC ANALYSIS.

PRINCIPLE

Flow cytometry is a widely used analytical tool in biomedical research and clinical diagnostics. It is a powerful tool to study the expression levels of both surface-expressed (such as CD4, CD69, CD25etc.) and intracellular (such as Foxp3) proteins on a single-cell basis and the data can be obtained relatively quickly.Using this technique, multiple molecules can be determined simultaneously on the same cells, which can help to identify different cell populations within a given sample, and differential expression levels can accurately be measured and compared across various cell populations. In addition to the advantages of using flow cytometry in terms of its speed, high precision, and accuracy, the constant hardware updates and improved capability for software-based data analysis make flow cytometry one of the most advanced technologies for cellular quantitative analysis.

Flow cytometry is a useful technique in counting and examining the cell characteristics. It consists of three main systems: fluidics, optics and electronic systems, as it shown in (Figure 2.4).The cells are suspended in a fluid sheath and injected into the fluidics system. The latter transports the stream of liquid to laser beam .Laser light is scattered by the passing particles and any passing fluorescence. Scattered light is collected by detectors. Forward scatter (FSc) is collected by the detectors orientated to the laser beam. The scattered light is an indicator for relative particle size, whilst side scatter (SSc) is picked up by detectors aimed perpendicular to the laser source, and it is an indication of the internal complexity of the cells. Fluorescent detectors may also give information on fluorescent chemicals found in the cells.

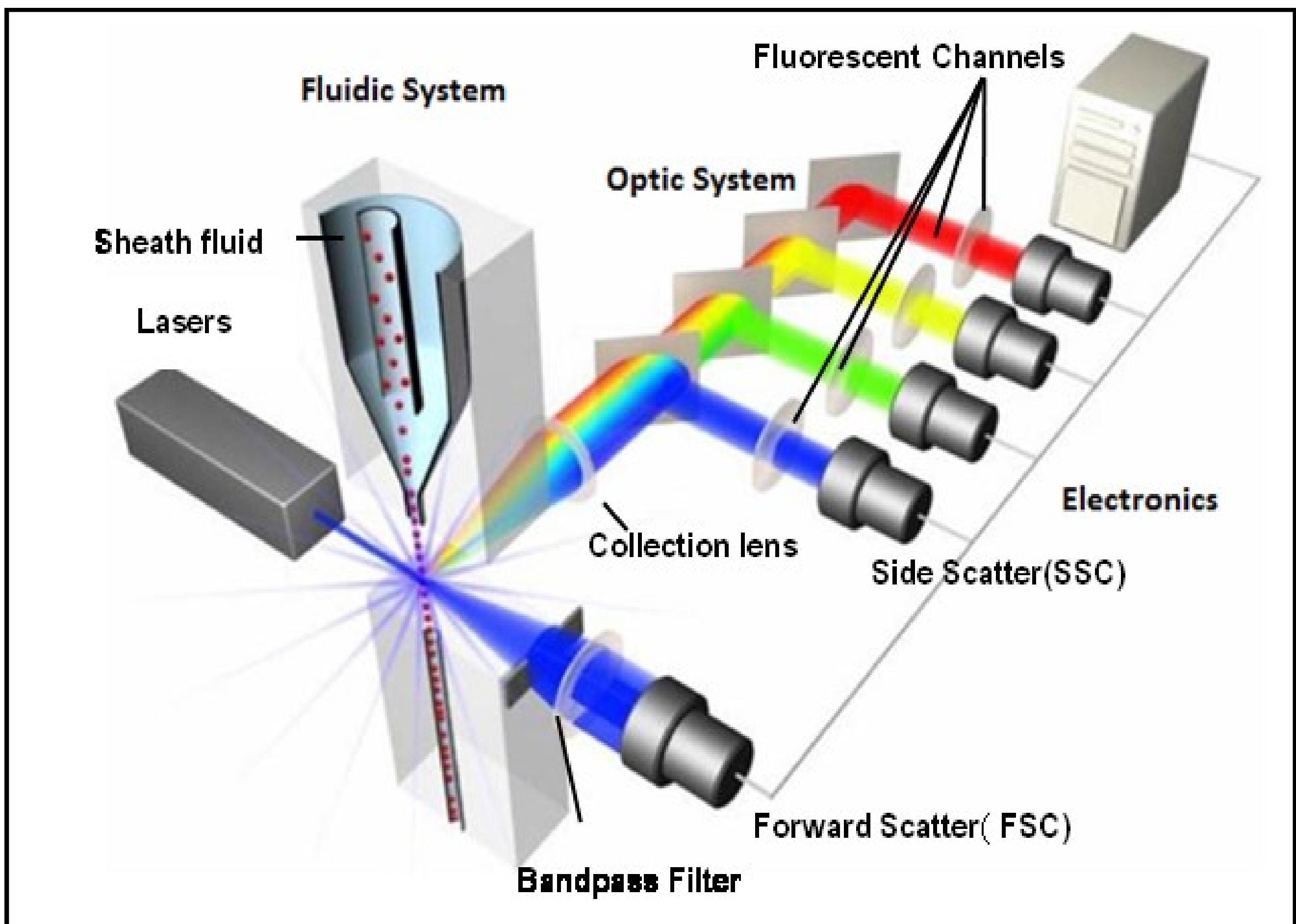


Figure 2.4: A Schematic overview of the mechanisms of Flow Cytometer. Adapted from: www.labome.com

2.6.1. MEASUREMENT OF CD25 (IL2R-α) ON LYMPHOCYTES

CD25 (IL2R α) is a ligand-binding alpha subunit of interleukin- 2 receptor alpha (IL-2Rα). It is a marker for lymphocyte activation. The expression of CD25 on anti-CD3/CD28- activated and non- activated were measured. Cells were homogenised and re-suspended in 500µl of stain buffer (FBS) ready made-up (BD Pharmingen™) and stained with 10µl of human anti-CD25-APC conjugated (Sigma-Aldrich). After 30 minutes of incubation in the dark at room temperature (RT), the cells were centrifuged at 300xg for 10 min. The supernatant was discarded; single-cell suspensions were prepared in 500µl of sheath fluid solution for each tube and transferred to the FACS tubes prior to measurement by a FACS Calibur flow cytometer. An isotype -matched control antibody was to establish the threshold for negative staining.

2.6.2. MEASUREMENT OF CD14 MARKER ON HUMAN MONOCYTES

CD14 is the major lipopolysaccharide (LPS)-binding protein. It is synthesised and highly expressed by monocytes (Jersmann, 2005). A cell suspension of $1x10^6$ cell/ ml was centrifuged at 300xg for 10 minutes. Following centrifugation, the supernatant was discarded and pellet was washed with PBS, then re-centrifuged for 10 minutes at 3000xg. The pellet formed following centrifugation was homogenised by using a 21-gauge blunt-ended needle. Then the cells were incubated in the dark at RT with a volume of 500µl of stain buffer and 20µl of APC Mouse anti-human CD14 (BD Pharmingen™). After 30 minutes of incubation at RT, the supernatant was discarded; single-cell suspensions were prepared in 500µl of sheath fluid solution for each tube for flow cytometric analysis**.** Isotype was applied as a negative control to establish the thresholds for negative staining.

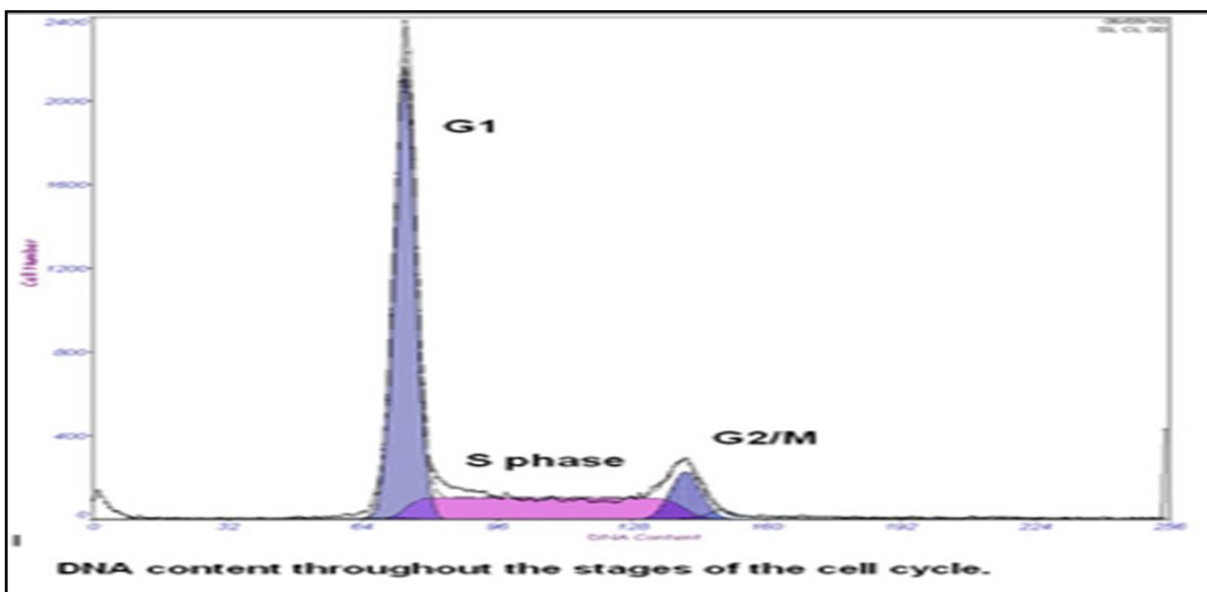
2.6.3. MEASUREMENT OF FOXP3, A TREG CELL MARKER

Activated lymphocytes by anti-CD3/CD28 were harvested following 4 days of activation. Cells were homogenised and re-suspended in 200µl of stain buffer (FBS) ready made-up (BD Pharmingen™) and stained with 20µl of PE-human FoxP3 antibody (BD Pharmingen™). Following 30 minutes 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 500µl of sheath fluid solution for each tube and transferred to the FACS tubes prior to measurement by a FACS Calibur flow cytometer. Isotype -matched control antibody was used to establish the thresholds for negative staining.

2.6.4. PROPIDIUM IODIDE AND CELL CYCLE EXAMINATION

Flow cytometry is a useful technique that is used to measure cell cycle progression. To perform this, propidium iodide (PI) is used. PI is a fluorescent intercalating agent that binds to the nucleic acid.

Cells life cycle involves different phases. In theG1 phase the cell division has not initiated but the centrioles start replicating. In the S phase, DNA starts replicating so more DNA contents will form. In the G2 /M phase, the final preparation for mitosis in which DNA is fully synthesised and the contents become twice of the G1 phase (Figure 2.5). In flow cytometry analysis, the intensity of PI binding to DNA will be higher. Therefore, if cells have been induced to proliferate, more DNA content will form, and the cells in S phase will be accumulated thus, the percentage of fluorescence intensity will be increased



DNA content throughout the stages of the cell cycle.

Figure 2.5: **DNA content through cell cycle phases.** Adapted from http://www.grc.nia.nih.gov/

METHOD

To assess the proliferation and differentiation of CMs- treated C2C12myoblasts, cells were washed with PBS twice and trypsinised. Then the cells were collected in 15ml tubes and centrifuged at 300xg for 10 minutes. The PBS supernatant was aspirated and cells werewashed twice with PBS and centrifuged at 300xg for 10 minutes. After centrifugation, the PBS supernatant was removed and cells were fixed with 3ml of 75% EtOH, which was added in a drop wise manner to the cell pellet while vortexing (ethanol helps PI to intercalate into double-strand DNA).Samples were stored overnight at -20°C. Following fixation with 75% EtOH, cells centrifuged for 10 minutes at 300xg. Following centrifugation, the EtOH supernatant was removed, and cells were re-suspended and centrifuged twice with 1 ml PBS. Cells were then mixed and transferred to 3ml BD Falcon flow cytometry tubes (BD Biosciences, San Jose, CA, USA). The volume of 10ul of PI (50 µg/ml) was added to the cell suspension. Stained samples were incubated in the dark at RT for 30 minutes then samples were stored at 4°C before flow cytometric analysis was performed.

2.7. WOUND HEALING ASSAY WITH LIVE IMAGING EXAMINATION.

In order to investigate the migration potential of C2C12 cells in response to specific factors, a wound healing assay was established. In the wound healing assay, a scratch/wound was created in a monolayer of confluent cells, usually using a pipette tip. Live observation of wound closure progress over the time was undertaken by using live imaging microscopy that can provide useful information not only on the migration of cells but also on morphology and migrating distance in response to particular factors.

WOUND HEALING METHOD

C2C12 were grown in 12-well plates in GM until 80% confluent. Cell monolayers were washed twice with PBS and incubated for 20 hours in DMEM, supplemented with 0.1%HS to attain a quiescent state. After removing the medium, cells were pre-incubated with MitomycinC (10ug/ml) (Rogel et al., 2011; Gamell et al., 2008) for 3hrs (Gamell et al., 2008) to arrest the

proliferation phase (Frommer, 2008). C2C12 monolayers were washed three times with quiescence medium and scratched using a 200µl sterile pipette tip. The cells were washed 3 times to remove dead cells and debris. They were cultured in the various experimental CMs for 18 hrs at 37˚C and 5% CO2. Images were obtained at 0 and after 18 hours of migration using a Leica DMI6000B microscope, ensuring the same X and Y coordinates were imaged each time. The number of cells migration towards the central area, from which the cells were initially evacuated following the scratch, was quantified using ImageJ in each treatment

2.8. MORPHOLOGICAL DIFFERENTIATION AND QUANTIFICATION OF THE PROLIFERATION AND DIFFERENTIATION PARAMETERS.

Two fluorescent stains were used to evaluate morphological parameters of CMs- treated C2C12 :Texas Red®-X Phalloidin is a fluorescently- labelled stain which binds specifically to F- actin in skeletal muscle cells and anti-Myosin Heavy Chain (MyHC) Alexa Fluor® 488 that binds to heavy chains of myosin.

Cells after CMs treatment were fixed in 3.7% formaldehyde solution and incubated at RT for 5 min. Fixed cells were washed 3 times with PBS before permealisation with 0.1% Triton-X-100 for 5 min. Following Triton removal, cells were stained with either Texas Red®-X Phalloidin (200 unit/ml, Sigma Aldrich, Pool, UK) or anti-Myosin Heavy Chain (MyHC) Alexa Fluor® 488 (eBioscience, UK) and DAPI nuclear counterstain solution (5mg/ml, IHCWORLD). After 30 min of incubation, the treated cells were observed using a fluorescent microscope. The differentiation parameters were assessed using ImageJ software(Schneider, andEliceiri, 2012) to evaluate the morphometric parameters for myotube development. The differentiation parameters involved were : fusion index (FI) which was calculated by dividing the total number of infused nuclei per a myotube (>2 nuclei) to the total number of nuclei counted over the field , total myotubes area (MA), calculated by counting the total area of myotubes in a field over the entire image(Ren et al., 2008; Ricotti et al., 2011). Aspect ratio (AR) which was calculated by dividing the length of myotubes to their width (Grubišić, et al., 2014). Ten microscopic fields were scanned for each parameter. Images were obtained using a Leica DMI 6000B imaging system and a FITC-fluorescence L5 filter cube.

2.9. RNA EXTRACTION

Highly purified RNA is required for real-time Reverse Transcription Polymerase Chain Reaction (real time RT-PCR). RNA was isolated from cells using Guanidiniumthiocyanate-phenol-chloroform extraction, with Trizol® reagent (Invitrogen, Life Technologies, Paisley, UK). Trizol® contains phenol and Guanidiniumthiocyanate, which have cell lysing and protein denaturing actions, therefore it prevents the activity of RNAse and DNAse enzymes. Following appropriate treatment, 330µl of Trizol® was added to each well of a 6-well plate and 500 µl for each $1x10^6$ lymphocytes. Then the cells were left for 5 minutes at RT and stored at -20C° until further processing. Cell monolayers were collected by scraper to disturb the cells, while the lymphocyte suspension was homogenised by repeated pipetting prior to storage. For RNA isolation, chloroform and Trizol® in a 1:5 ratio were added to the lysate, the solution was mixed, then incubated for 10min at RT and centrifuged for 16min at 12,000xg. Chloroform, along with the phenol contained in the Trizol® reagent, causes the proteins to denature and precipitate and separates the RNA into an aqueous supernatant. Following addition of chloroform and centrifugation, separation of the solution into three phases is visible (aqueous phase, interphase and organic phase), as shown in Figure 2.6. The majority of the RNA is present in the aqueous phase, while DNA and proteins are in the interphase and organic phases respectively. After separation of the solution into three phases, the aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added. Isopropanol precipitates and recovers the RNA from the aqueous phase, which becomes visible as a pellet after 10min of centrifugation at 12,000xg. The RNA pellet was washed with 75% EtOH, centrifuged at 8,000xg for 10min and air dried. Tris-EDTA (TE) buffer (Applied Biosystems, Ambion, Cheshire, UK) was added to reconstitute the RNA.

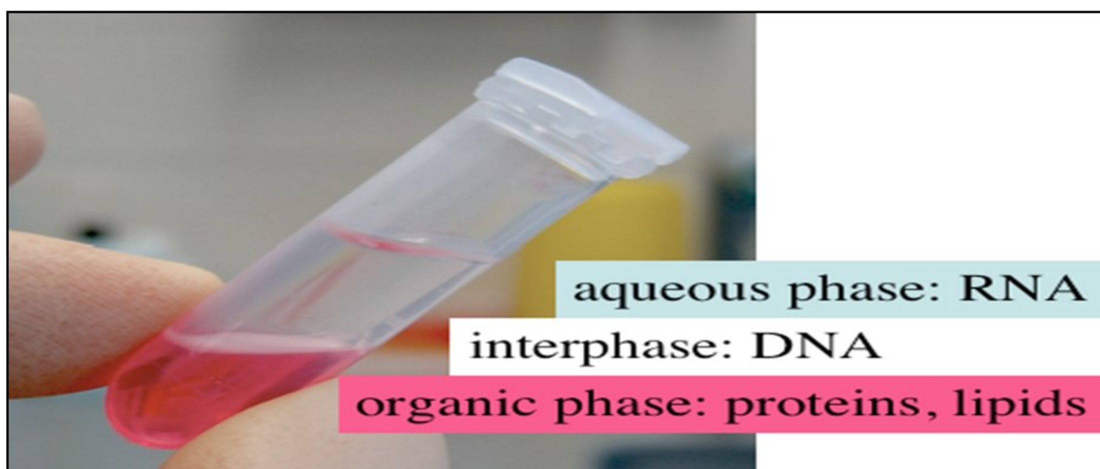


Figure 2.6: Phases separation using Trizol®reagent. The formation of three phases after chloroform addition to the Trizol® lysate and centrifugation. RNA is isolated from the aqueous phase. Adapted from www.openwetware.org

After reconstitution in TE buffer, RNA concentration and purity were assessed by UV spectroscopy, using a Biotech Photometer (NanoDrop, Wilmington, DE, USA). The absorbance of the diluted RNA sample was measured at 260 and 280 nm. A ratio of 260/280 is indication for high purity of RNA. It is known that A260 reading of 1.0 is equivalent of 40µg/ml of RNA. A RNA samples of high purity were stored at −80 °C for the use of real-time PCR.

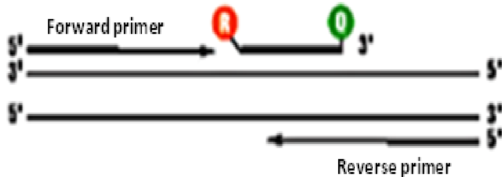
2.10. REAL-TIME PCR

PRINCIPLE

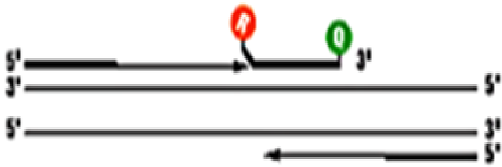
Gene expression can be altered in response to various conditions or different stages of cell development and is a key step in how many cellular processes, including differentiation, are accomplished. Real-time PCR (RT-PCR) is a commonly used method for investigating gene expression. It quantifies the amount of mRNA molecules produced by a specific gene after it has been transcribed. The method depends on reverse transcription of the previously isolated mRNA (section 2.9) into complementary DNA (cDNA) and amplifying the cDNA to be detected and measured as the reaction progresses (hence, the method is named real-time PCR). The detection of amplified product is performed by labelling the DNA with molecules emitting fluorescence, which is proportionally increased with the amplification of the cDNA molecules with each cycle of the reaction. Two commonly used fluorescent tags in real-time PCR are SYBR® Green dye and TaqMan® probes. SYBR® Green dye is the cheaper alternative - it binds to all double-stranded DNA and is able to detect non-specifically all amplified double-stranded DNA molecules. In contrast, TaqMan® probes are more expensive, but can be synthesised for each unique target sequence and therefore emit fluorescence only when the specific PCR product is amplified. Comparison of both chemistries is presented in Figure 2.7.

TAQMAN PROBE-BASED ASSAY CHEMISTRY

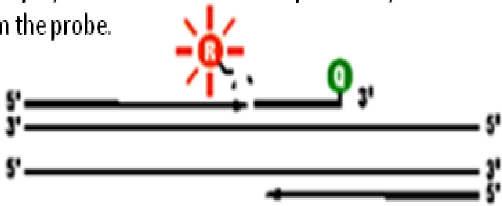
1. Polymerization: A fluorescent reporter IR dye and quencher are attached to the 5 and 3 ends of a TaqMan probe respectively.



2. Standard displacement :When the probe is intact, the reporter dye emission is quenched.



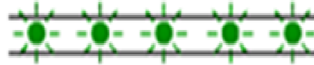
3.Cleavage: During each extension cycle, the DNA polymerase cleaves the reported dye from the probe.



4. Polymerization completed: once reported separated from the quencher, the reporter dye emits its characteristics fluorescence.

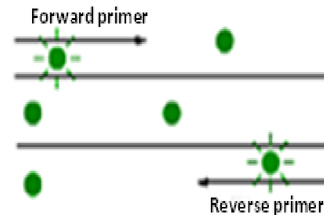
SYBR GREEN ASSAY CHEMISTRY

1. Reaction setup: The SYBR Green I Dye fluoresces when bound to double–standard DNA .



2. Denaturation : When the DNA is denatured, the SYBR Green I Dye is released and the fluorescence is dramatically reduced.

3. Polymerization: During extension, primers anneal and PCR product is generated.



4. Polymerization completed: When polymerization is complete, the SYBR Green I Dye binds to the double-strand product, resulting in a net increase in fluorescence detected by the 7900HT.

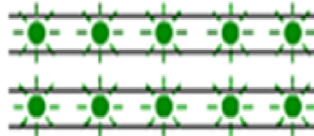


Figure 2.7: **Comparison of PCR products in real-time PCR between SYBR® Green and TaqMan®.**TaqMan® detection is more specific, as it uses a specially designed probe for each target sequence, which emits fluorescence only when the target sequence has been replicated. SYBR® Green dye is less specific, as it binds to any double stranded DNA present in the reaction.

The increase in fluorescence with each cycle is detected. This enables estimation of the rate of amplification and the abundance of the cDNA molecules generated. The higher the gene expression, i.e. the starting concentration of cDNA, the fewer number of amplification cycles it will take for the levels of fluorescence to obtain a certain threshold (represented as the Ct value). Thus, the Ct value is inversely proportional to the gene expression level (i.e. the higher the Ct level the lower the amount of target nucleic acid in the sample). Comparison of Ct values between different samples is therefore indicative of how gene expression between these samples differs. Representation of an example of 3 samples with different gene expression levels and how this is represented by their Ct values is shown in Figure 2.8.

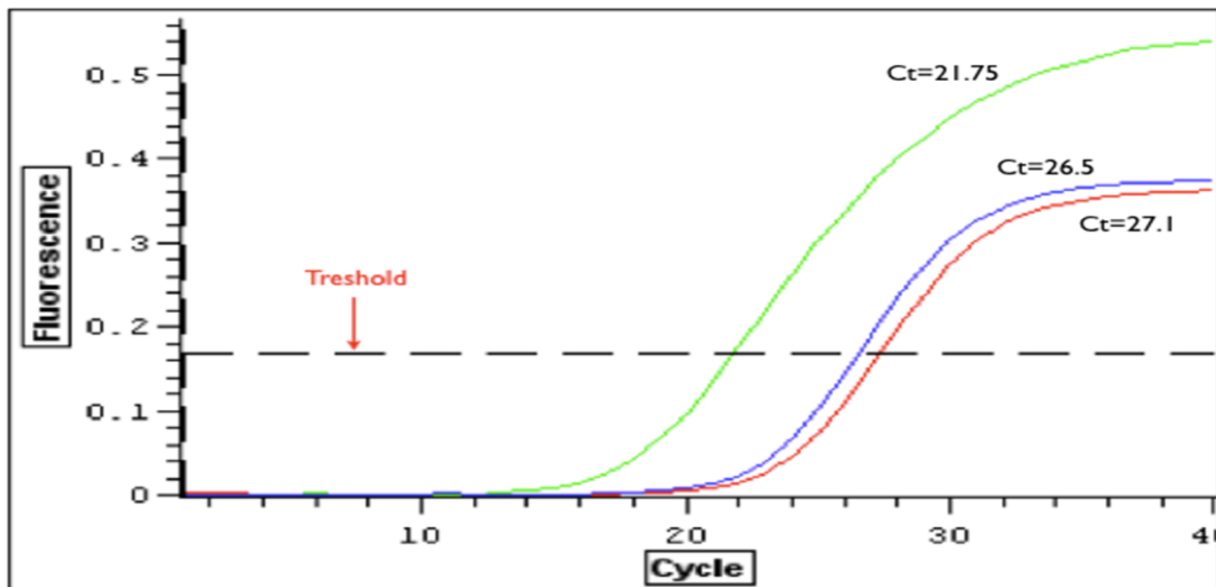


Figure 2.8: Ct value of real time PCR. Real-time PCR analysis demonstrates the number of cycles required for 3 different samples to reach a specific fluorescence threshold (represented by their Ct value). The lower the Ct value or the number of cycles required to reach the fluorescence threshold, the higher the expression of the gene. Thus, the sample represented by the green curve is the one with highest gene expression (Ct=21.75), followed by the blue one (Ct=26.5) and the red sample is the one with the lowest gene expression (Ct=27.1). Adapted from www.appliedbiosystems.com.

TAQMAN® METHOD

For TaqMan® detection, TaqMan® RNA-to-CT™ 1-Step Kit was used (Applied Biosystems, San Jose, CA, USA). The kit contains both AmpliTaq Gold® DNA polymerase, required for DNA amplification and ArrayScript™ UP reverse transcriptase, the enzyme required for the reverse transcription of mRNA into cDNA. The final volume per reaction was 20µl, composed of 8.5µl of RNA (50ng/µl for C2C12 and 80ng/ µl for lymphocytes), 10µl master mix containing the DNA polymerase enzyme, 1µl of primer mix and 0.5µl RT enzyme. Applied BiosystemsStepOnePlus real-time PCR system with StepOne Software v2.2.2 (Applied Biosystems, Life Technologies) was used. The following program cycles were used:

Stage	Step	Temperature	Time
Holding	Reverse Transcription	48ºC	15 min
Holding	Activation of TAQ polymerase	95ºC	10 min
Cycling (40 cycles)	Denaturation	95ºC	15 sec
	Annealing/Extension	60ºC	1min

The following primer sets for C2C12 were ordered from Applied Biosystems and were used with the TaqMan® detection method: GAPDH (mm99999915_m1), MyoD (mm00440387_m1), Myogenin(mm00446194_m1) and for lymphocytes GAPDH(Hs00950669-m1),Amphiregulin(hs00950669-m1) and GDF11 (Hs00195156-m1).

ANALYSIS

Relative quantification of gene expression was determined automatically by Opticon Monitor version 3.1.32 (MJ Geneworks Inc., Bio-Rad Laboratories, Inc., Hercules, CA, USA) and StepOne Software v2.2 (Applied Biosystems, Life Technologies). The relative quantification is based on analysing changes in gene expression relative to a reference sample (control). One sample from the experiments was set as a calibrator/control and fold change with respect to this

calibrator was determined for all samples of the experiment. All samples were normalised to a housekeeping gene (GAPDH), which expression is known to remain consistent in C2C12 cells and lymphocytes, regardless of the treatment and activation respectively.

2.11. PROTEIN EXTRACTION AND ESTIMATION OF PROTEIN CONCENTRATION

PROTEIN EXTRACTION FROM C2C12 MONOLAYERS

Following appropriate treatments, samples for protein assay were prepared by adding 200µl of ice-cold cell signalling lysis buffer (Merck, MA, USA) per well (for 12-well plates). Following removal of cells from the substrata using a scraper, lysed cells were collected and centrifuged for 2min at 8,000xg at 4°C to form a pellet and discard insoluble debris; samples were stored at -20°C for protein estimation

EXTRACTION AND ESTIMATION OF PROTEIN CONCENTRATION

PROTEIN EXTRACTION FROM C2C12 MONOLAYER

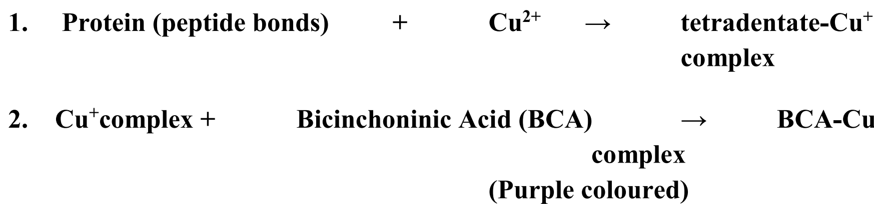
Following appropriate treatments, samples for protein assay were prepared by adding 200µL of ice cold cell signalling lysis buffer(Merck MA, USA) per well (for 12well plate). Following removal of cells from the substrata, using a scraper, lysed cells were collected and centrifuged for 2min at 8,000xg at 4°C to pellet and discard insoluble debris; samples were stored at -20ºC for protein estimation.

ESTIMATION OF PROTEIN CONCENTRATION

PRINCIPLE OF Pierce™ BCA PROTEIN ASSAY

The bicinchoninic acid protein (BCA) assay depends on BCA is sensitive reagent for total protein determination. The principle of this protein assay depends on formation of a Cu^{1+}protein

complex. BCA in this method acts as a detector reagent for Cu^{1+} which is formed when CU^{2+} is reduced by protein in an alkaline environment. The purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{1+}). Thus, the amount of Cu^{2+} reduction is proportional to the amount of BCA-Cu^{+} complex colour. The macromolecular structure of the protein, the number of peptide bonds and the presence of four particular amino acids (Cysteine, Cystine, Tryptophan and Tyrosine) are reported to be responsible for colour formation with BCA. The reaction is summarised in the following equations:



1. Protein (peptide bonds) + Cu^{2+} → tetradentate-Cu^{+} complex

2. Cu^{+}complex + Bicinchoninic Acid (BCA) → BCA-Cu^{+} complex (Purple coloured)

↓

Read absorbance at 540nm-590nm

METHOD

For the protein determination, BSA protein standards were prepared in lysis buffer (at concentrations of 2000, 1500, 1000, 750, 500, 250, 125 and 25.0µg/ml as illustrated in the table below.

For the protein determination, BSA protein standards were prepared in lysis buffer (at concentrations of 2000, 1500, 1000, 750, 500, 250, 125, 25,0µg/ml. As illustrated in table below

Table 2.1: Serial dilution standards for BCA protein assay.

Vial	Volume of lysis buffer(µl)	Volume and Adapted of BSA	Final BSA concentration (µg/ml)
A	0	300 from stock	2000
B	125	375 from stock	1500
C	325	325 from stock	1000
D	175	175 of vial B dilution	750
E	325	325f of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0

The protein assay was performed using BCA Protein Assay purchased from Pierce (Rockford, IL, USA). BCA reagents A & B were mixed in a ratio of 1:50. The volume of 200µl of the mixed solution was added in a 96 well plate with 10µl of the protein samples or standards. The 96-well plate was incubated at 37^{0}C and absorbance was read at 562nm using a Bio-Tek ELISA plate reader (Winooski, VT, USA). Standard curves were generated automatically by plotting the average blank-corrected 630nM measurement of the loaded BSA standards against pre-programmed known concentrations in mg.ml^{-1} and sample concentrations were calculated from the standard curve. The average 562 nm absorbance measurement of the blank standard replicates was subtracted from the 562nm absorbance reading of all individual standard and unknown sample replicates.

2.12. MULTIPLEX IMMUNOASSAY

Multiplex bead immunoassay was performed to quantify cytokines existing in harvested young and old secretomes obtained from lymphocytes (Th1/Th2) and IGF-1. In addition, this technique was applied to quantify the potential candidates of activated proteins in cell extracts of C2C12 following treatment with CM. The results were read using the Luminex 200 system (Bio-Plex) running the xPONENT analytical software.

PRINCIPLE

In multiplex bead immune assays, beads (microspheres) are internally labelled with two fluorescent dyes, one labelling the capture antibody and the second labelling the detection antibody. A biotinylated detection antibody is introduced after the beads capture an analyte from the sample. Thus, the ratio of signals emitted by the two-labelled antibodies reveals the analyte concentration. The reaction mixture is then incubated with the reporter molecule streptavidin-PE conjugate to complete the reaction on the surface of each microsphere. Following incubation, the ratio of the fluorescent signal emitted from each discrete antibody couplet in the array is scanned by two lasers. The first laser (green colour) excites the reporter streptavidin-PE dye of the assay. The second laser (red colour) is used to excite the dyes inside the beads (Figure 2.9). The ratio of the fluorescent signal emitted from each discrete antibody couplet in the array is measured and identified using high-speed digital signal processors.

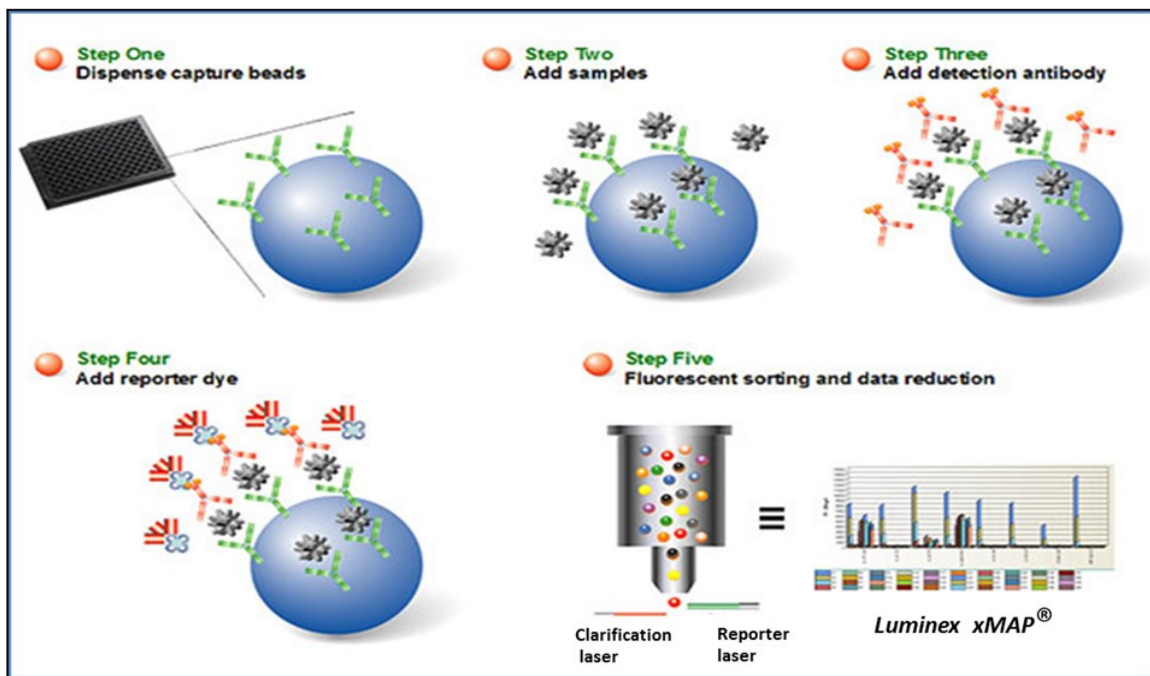


Figure 2.9: The schematic representation of multiplex immune assay steps. Adapted from (http://www.bio-rad.com/en-ae/applications-technologies/bio-plex-multiplex-immunoassays).

2.12.1 MEK-1/ERK1/2 AND AKT/MTOR SIGNALLING MGNETIC BEADS ASSAY

METHOD

Proteins of treated cells were extracted and estimated as described in 2.10. Samples were diluted according to the manufacturer's protocol to attain the final concentration of 10µg/well. A volume of 25 µl of beads suspension was added to each well. Twenty-five µl of assay buffer was added to blank wells and 25ul of lysates to the controls and samples well. The controls of MAPK/SPAK and Akt/mTOR are shown in the table below. The plates were incubated for 18 hours on shakers at 800rpm at 4°C. Lysates were removed by a vacuum manifold (EMD Millipore, USA) and incubated with a Biotin-labelled detection antibody in the dark at RT. Following removal of the detection antibody, 25ul of Streptavidin-Phycoerythrin (SAPE) was added to each well and incubated for 15 min. with shaking at RT with the dark. SAPE was removed and 25ul of amplification buffer was added and incubated for 15 min. on a shaker at 4°C. Amplification buffer was aspirated from all the wells which were then loaded with 150ul of assay buffer prior to reading the plates on a Luminex 200 TM.

2.12.2. TH1/TH2 MULTIPLEX IMMUNOASSAY

The human cytokines Affymetrix magnetic dead assay (AffymetrixeBioscience, USA) was performed according to manufacturer's instructions. Briefly, an aliquot of the supernatant collected from lymphocyte cultures (described above) was thawed. Fifty µl of each sample and 50 µl of standards and controls were added to the wells. The plate was incubated overnight on a shaker at 4°C. Following washing twice with wash buffer, 25 µl of detection antibody was added to each well and incubated at RT in the dark for 30 min. Following washing, 50 µl of Streptavidin-PE was added to each well. The plate was sealed and kept at RT in the dark for 30 min. The samples were aspirated and beads re-suspended in 120 µl of reading buffer for 5 min. The plate was read using a Luminex 200TM.

2.12.3. HUMAN IGF-1 IMMUNOASSAY

The assay was performed according to the manufacturer's protocol using a human magnetic bead panel (EMD Millipore, MA, USA). The plate was washed with assay buffer then 25 µl of standards and controls were added to the appropriate wells and 25 µl of sample was added to each well. A volume of 25 µl of bead suspension was added to all wells which were then incubated overnight on a plate shaker (500-700rpm) at 4°C. The wells were then washed three

times with wash buffer. A volume of 50 µl of detection antibody was added to each well and the plate was incubated for 1 hour at RT. Streptavidin-Phycoerythrin was added to each well and incubated for 30 min at RT. All wells were washed three times and 100µl of sheath fluid were added prior to reading on a Luminex 200 TM plate reader.

2.13. MEASURMENT OF AMPHIREGULIN USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Amphiregulin human enzyme-linked immunosorbent assay (ELISA) of 96-well plate (Abcam, UK) was applied for the quantitative measurement of Amphiregulin in young and old secretomes. Briefly, standards were prepared according to Table 2.2. A volume of 100 µl of samples and standards were added to their appropriate wells and incubated overnight with gentle shaking at 4°C. Following incubation, the plate was washed with 1X wash solution and 100 µl of 1X biotinylated Amphiregulin detection antibody was added to all wells. The plate was incubated for 1 hour at RT with gentle shaking. The plate was re-washed four times with 1X wash solution and 100ul of 1X HRP-Streptavidin solution was added to all wells. The plate was sealed and incubated for 45 minutes at RT with gentle shaking. The solution was discarded from all wells and washed four times with 1X wash solution. A volume of 100ul of TMB one-step substrate reagent was added to each well. The plate was sealed and kept at RT for 30 minutes in the dark with gentle shaking. The plate was read at 450nm by a Bio-Tek ELISA plate reader (Winooski, VT, USA) immediately after adding 50µl of stop solution to each well. The protocol is summarised below in Figure 2.10.

Table 2.2: Standard dilution preparation.

Standard #	Volume to Dilute (µL)	Diluent (µL)	Total Volume (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	40	460	500	50,000	4,000
2	200	400	600	4,000	1,333
3	200	400	600	1,333	444.4
4	200	400	600	444.4	148.1
5	200	400	600	148.1	49.38
6	200	400	600	49.38	16.46
7	0	400	400	0	0

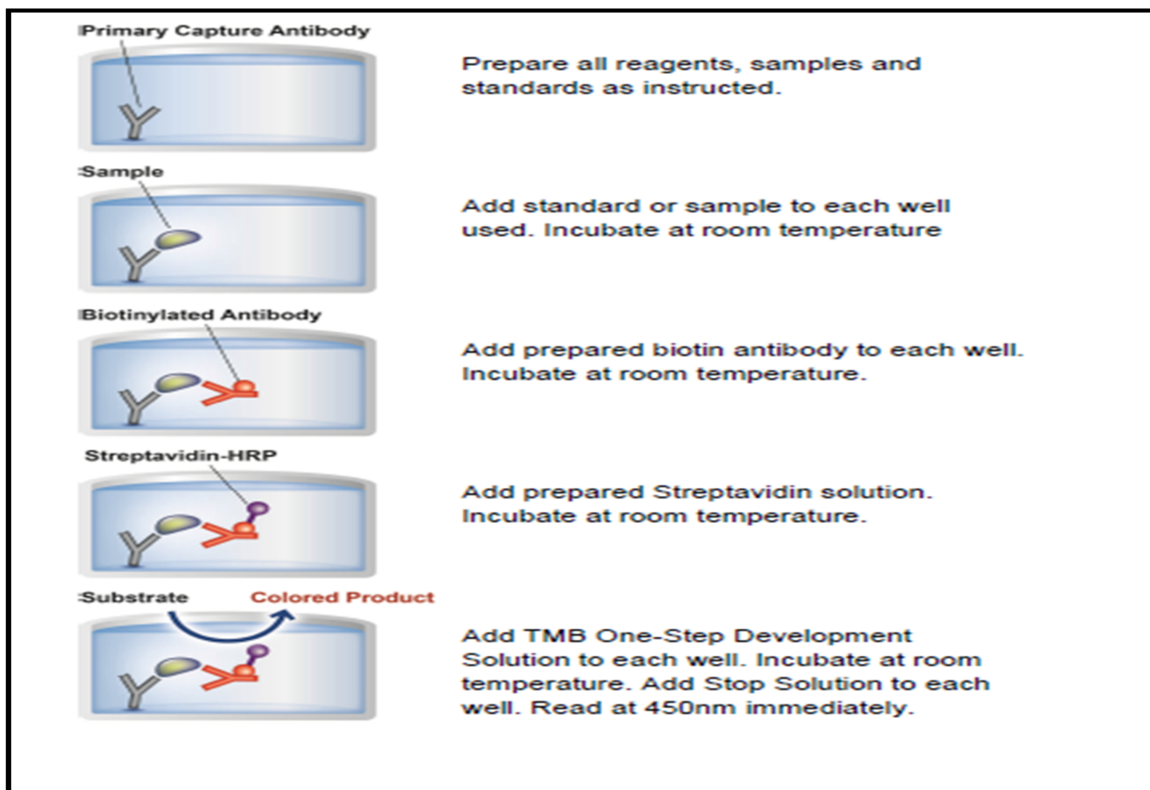


Figure 2.10: Assay summary of Amphiregulin human ELISA. Adapted from www.abcam.com.

2.14 PROTEOMICS STUDY

Harvested secretomes obtained from young and old participants were transferred into clean polypropylene tubes. Secretome samples were kept at -80°C and sent to Liverpool John Moores University for proteomics analysis. Prior to start the procedure, the protein concentrations were measured and albumin was depleted by centrifugation according to manufacturer's protocol. Briefly, Albumin Depletion Resinwas placed in a collection tube and centrifuged at 12000xg for 1 minute to remove access liquid. A volume of 200µl of binding /wash buffer was added to the spin column prior to centrifugation at 12000xg for 1 minute. Following removal of access fluid was discarded; a spin column was placed into a new collection tube. Twenty -five µl of albumin containing sample was added to resin and incubated for 2 min at RT to ensure maximal albumin binding. Samples were centrifuged at 12000xg for 1 min and spin column was transferred to a new collection tube. Resin was washed three times by 50µl of Binding buffer and centrifuged at 12000xg for 1 minute. For mass spectrometry analysis, aliquots of 50µg of protein (Albumin-depleted) were precipitated in 5 volumes of acetone for 1 h at −20 °C. Pellets were re-suspended in 0.1% (w/v) Rapigest SF (Waters; Milford, MA, USA) in 50 mM ammonium bicarbonate and incubated at 80°C for 15 min DTT was added to a final concentration of 1 mM and incubated at 60°C for 15 min followed by incubation in dark at 4°C in the presence of 5mM of iodoacetamide. A protein was digested overnight at 37°C by adding sequencing grade trypsin (Promega; Madison, WI, USA) at ratio of 1:50. The reaction was ended by the addition of 2µl concentrated TFA and centrifuged at 13000xg for 5 min. Label-free liquid chromatography-mass spectrometry (LC-MS) analysis was performed using a quadrupole-high capacity ion-trap (HCT Ultra ETD II; Bruker Daltonics, Bremen, Germany) coupled online via an electrospray ionisation source to a nano-flow HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA, USA). Tryptic digests (0.8 µg/µl) were diluted with aqueous 0.1% formic acid (FA) at ratio 1:10 and 5 µl loaded via a Zorbax 300SB C_{18} 5µm, 5 × 300 µm pre-column (Agilent Technologies Ltd.). Peptides separation was performed using a Zorbax 300SB C18 3.5 µm, 15 cm × 75 µm analytical reverse phase column (Agilent Technologies Ltd.) at a flow rate of 300 nL min−1 using a non-linear gradient rising to 40% acetonitrile 0.1% FA over 160 min.. Mass spectra for LC-MS profiling were recorded between 200 m/z and 2,500 m/z using Standard Enhanced mode [8,100 (m/z)/s]. From triplicate analysis of a pooled standard including each HCR and LCR sample, equivalent data-dependent tandem mass spectrometry (MS/MS) spectra were collected. MS/MS spectra of collision-induced dissociation fragment ions were recorded for the 5 most abundant precursors from each survey scan (350 m/z to 1,600 m/z). MS/MS spectra were

exported in Mascot generic format and searched against the Swiss-Prot database (2011.6) restricted to “Rattus” (7617 sequences) using a locally implemented Mascot server (version 2.2.03). The enzyme specificity was trypsin allowing one missed cleavage, carbamidomethyl modification of cysteine (fixed), deamidation of asparagine and glutamine (variable), oxidation of methionine (variable) and an *m/z* error of ± 0.5 Da.

2.15. STATISTICAL ANALYSIS

Statistical analyses and significance of data were determined using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical significance for the difference between more than two groups was determined using One way ANOVA and Bonferroni multiple comparisons test. Statistical significance was set at $P < 0.05$. All experiments were performed at least 3 times in triplicate.

CHAPTER 3

3. OPTIMISATION OF LYMPHOCYTE CULTURE: HARVESTING OF CONDITIONED MEDIA TO IMPROVE C2C12 PROLIFERATION

3.1 INTRODUCTION

Immune cells are required for skeletal muscle repair and regeneration (Tidball and Villalta, 2010). Following injury, immune cells infiltrate to damaged areas and release numerous cytokines, chemoattractants/interleukins and growth factors. The infiltrating immune cells and their secretome function to clear the injury site to provide a clean environment for muscle regeneration (Aurora and Olson, 2014). Thus, alterations in the systemic microenvironment, constituting immune cells and their secretome, directly affect satellite cells and myogenesis. For instance, recent evidence suggested that the high levels of fibroblast growth factor (FGF) in the aged muscle tissue niche impair asymmetric self-renewal and myogenic differentiation of aged mouse satellite cells (Bernet et al., 2014). Interlukin-6 (IL-6) released by neutrophils and lymphocytes, as well as from myofibres in response to exercise, contributes to satellite cell activation and myofibre regeneration, but high levels of IL-6 have been linked to an age- related muscle wasting (Haddad et al., 2005).T-lymphocytes are the main contributing factor to the local milieu of satellite cells in injured/damaged muscle. In rodent models, following muscle injury, infiltrating T cells remain elevated for 10 days post-injury (Bondesen et al., 2006; Cheng et al., 2008).It is well established that the concentration of T lymphocytes increases in blood after exercise (Pedersen and Toft, 2000; Grounds, 1998), however, very little is known about the specific function in skeletal muscle satellite cell proliferation and differentiation. Stimulated lymphocytes release numerous growth factors and cytokines (this includes, but is not limited to, transforming growth factor-β (TGF-β), IL-10, IL-7 and IL-4), which may be involved in the early inflammatory stages that trigger muscle regeneration (Cannon and St Pierre, 1998; Tidball, 1995). A study conducted on the *csid/mdx* model (*mdx*: dystrophic mouse, *scid*: immune deficient mouse) to examine the cross talk between T lymphocytes and skeletal muscle found that these mice exhibited reduced fibrosis and degeneration (Farini et al., 2007). Therefore, it is crucial to develop and optimise an *in vitro* system to activateC2C12, as occurs in vivo during regeneration, in the presence of the immune-influenced environment. Hence further studies need to be conducted to ascertain the optimal activation time of lymphocytes and preparation of optimal conditioned media from harvested secretomes. In this chapter the optimisation

conditions were applied to young lymphocytes only. Some results of this chapter have been published in one journal paper (Al-shanti et al.,2014)

3.2. AIMS, HYPOTHESIS AND OBJECTIVES

The aim of the word presented in this chapter was to optimise human lymphocytes culture by isolating and activating them *in vitro* and collecting the secreted proteins/cytokines (secretome). The secretome was used to prepare conditioned media for skeletal muscle cell cultures and the effects on proliferation and differentiation recorded. **This was an important step needed to address the overall hypothesis** that activated lymphocytes contain cytokine and growth factors able to induce muscle cell proliferation and differentiation. Therefore, the **objectives** were, firstly, to optimise the lymphocytes cell culture (to determine optimal activation time, anti-CD3 concentration, anti-C28 concentration and secretome volume). Secondly, to use the lymphocyte secretome to enrich the cell-culture medium of C2C12 (p5) cells and to examine the effects on C2C12 proliferation and differentiation. Methods are outlined in figure 3.1.

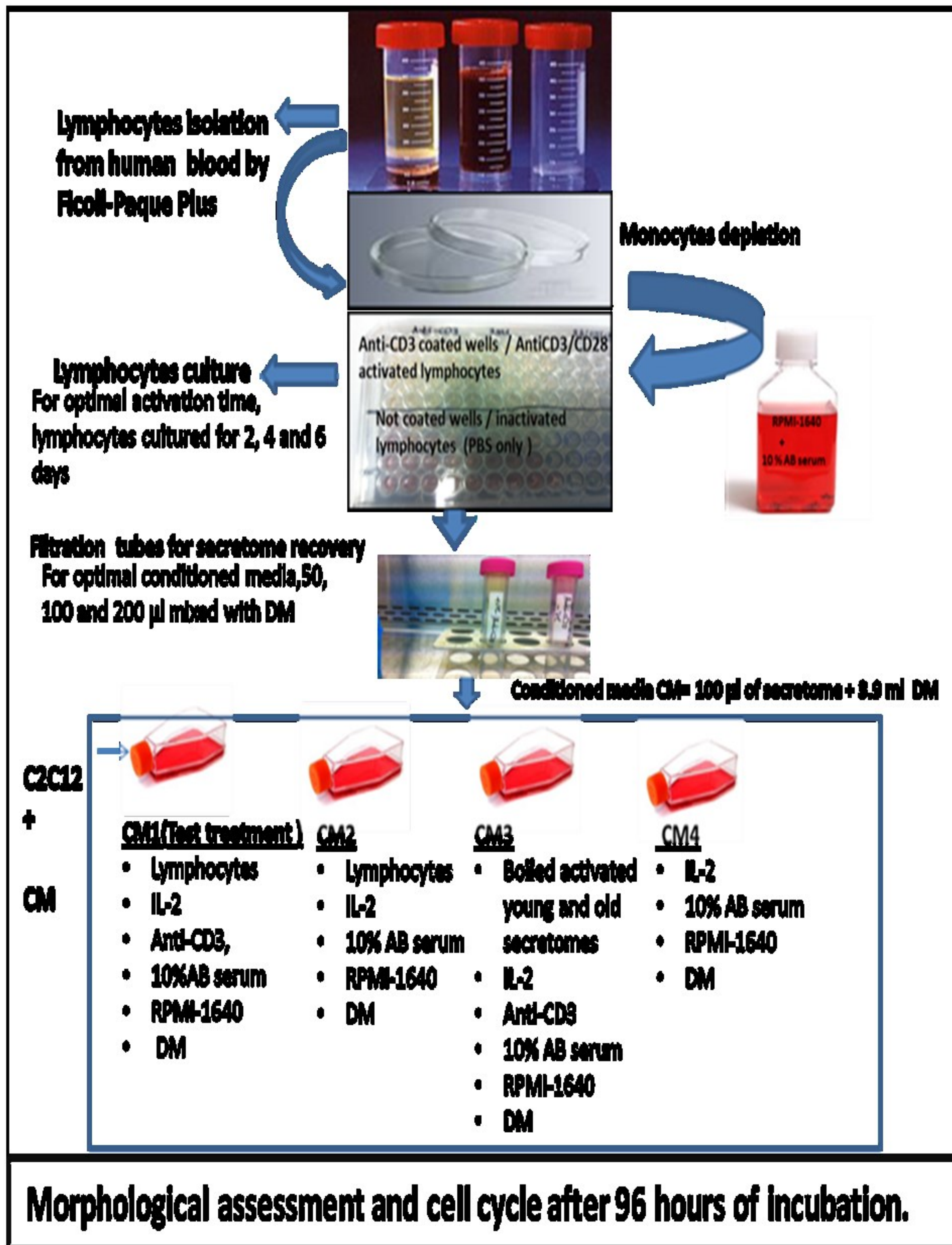


Figure 3.1: **Optimisation of lymphocytes culture and conditioned media.**

3.3 METHODS

3.3.1. ISOLATION OF HUMAN LYMPHOCYTES FROM VENOUS BLOOD

Ethical approval for the study was granted at Manchester Metropolitan University. Fresh venous human blood was collected from healthy volunteers (n=20, 3 female and 17 male, aged 18-25 years old) after their informed written consent. Lymphocytes were isolated by Ficoll–PaquePLUS and incubated in supplemented RPMI-1640 (as described in 2.2.3)in the presence or absence of anti-CD28 at 37°C, 5% CO_2 for 2, 4 and 6 days. The harvested secretomes were kept at -80°Cfor multiplex analysis to quantify cytokine concentration at different activation culture times.

3.3.2. ADHERENT CULTURE TO DEPLETE MONOCYTES (MACROPHAGES)

In order to purify isolated lymphocytes from fresh blood samples (as described in 3.3.1.), cells were suspended in supplemented RPMI-1640 and pre-plated in a tissue culture dish for 15 minutes at 37°C, 5% CO_2. This procedure eliminates adherent cells (monocytes)(Karanfilov et al., 1999). The non-adherent cells were then removed, and prepared for CD14 marker measurement as described in 2.6.2.

3.3.3 OPTIMISATION OF ANTI-CD3 CONCENTRATION

To achieve the optimal anti-CD3 concentration for lymphocyte activation, lymphocytes were cultured in pre-coated 96- well plates with four different concentrations of anti-CD3 (0.12, 0.25, 0.5 and 1µg/ml). These lymphocytes were counted using a haemocytometer (as described in 2.3.6) to determine the best proliferation rate of lymphocytes after anti-CD3 activation.The negative control was non-activated lymphocytes.

3.3.4. OPTIMISATION OF ANTI-CD28 CONCENTRATION

A co-stimulatory signal to activate the CD28 receptor is essential to induce the T-cell receptor (TCR) and stimulate the IL-2 and IL-2 receptors, leading to full activation of lymphocytes. To achieve the optimal anti-CD28 concentration for lymphocyte activation, anti -CD28 was added at different concentrations to the cell suspension (2.5, 5 and 7.5 µg/ml) prior to culturing in pre-coated anti-CD3 plates. Following 4 days of incubation, lymphocytes were counted using a haemocytometer to determine the best proliferation rate of lymphocytes upon anti-CD3/CD28

activation. Non-activated lymphocytes (cultured in the absence of ant-CD3/anti-CD28) were set as controls for this experiment. The secretomes of anti-CD3/CD28 -activated young lymphocytes and non-activated were kept at -80°C for ELISA multiplex analysis.

3.3.5. MEASUREMENT OFCD25 (IL-2Á R) MARKER

CD25 (IL2R α) is a ligand-binding alpha subunit of interleukin- 2 receptor alpha (IL-2Rα). It is a marker for lymphocytes activation. The expression of CD25 of anti-CD3/CD28-activated young lymphocytes at 0, 2, 4 and 6 days of incubation were measured as described in 2.6.1.

3.3.6. HARVESTING LYMPHOCYTE SECRETOME

The secretome includes all of the proteins secreted from the activated/cultured lymphocyte cells. The lymphocytes secrete the proteins into the surrounding culture media and the media is processed so that the protein concentrate can be harvested from the supernatant of the culture media. The secretome contains numerous enzymes, growth factors, cytokines and interleukins or other immune soluble mediators. Most cytokines secreted from lymphocytes are of low molecular weight, ranging between 8-25 kDa (Paul and Seder, 1994). In this protocol, the secreted cytokines from lymphocytes following activation with anti-CD3/CD28 were purified by using an Amicon®Ultra-4 centrifugal filter device (Millipore, Watford, UK). This device is used to filter and purify proteins found in tissue culture, which weigh more than 3kDa. Following 2, 4 and 6 days of lymphocyte culture (experiment to establish the optimal culture period for the lymphocytes), the supernatant (secretome) was collected and filtered, as the manufacture recommended. Then, 4 ml of lymphocytes secretome from each experimental culture time was centrifuged in an Ultracel -3 Membrane tube for 30 minutes at 5400xg to be applied later in conditioned media preparations.

3.3.7. DETERMINATION OFTHE QUANTITY OF CYTOKINE SECRETION IN DAY 2 AND 4 ACTIVATED LYMPHOCYTES

Harvested secretomes from day 2 -and 4- activated and non-activated lymphocytes were analysed using Multiplex ELISA-based analysis. Eight cytokines EGF, IFNα-2, IFN-γ,IL-1, IL-3, IL-6, IP-10 and TNF-β were measured by multiplex in a single plate of 96 -wells as described in 2.12.2

3.3.8. CONDITIONED MEDIA (CM) PREPARATION AND VOLUME OPTIMIZATION

Conditioned media was prepared by sterile filtration of the media in which the lymphocytes were cultured. The harvested secretome from activated and non-activated lymphocytes was added to the usual C2C12 culture media at the concentrate of the secretome was mixed with 3.8 ml of DM to obtain conditioned media. Three volumes of concentrated secretome obtained from activated lymphocytes were mixed with3.8 ml DM: 50, 100 and 200µl to make up the optimal conditioned media. Four conditioned media were set up. **CM1 young** contained the secretome obtained from activated anti-CD3/CD8-lymphocytes (positive control treatment).**CM2 young** contained the secretome obtained from non -activated lymphocytes (negative control). **CM3 boiled young** contained boiled secretome obtained from young lymphocytes. **CM4** contained the RPMI-1640 and IL-2 without lymphocytes (negative control). This control was included to determine the effect of 10% human serum).

3.3.9. C2C12 CULTURESAND TREATMENT WITH CONDITIONED MEDIA

C2C12, murine skeletal myoblasts were maintained by growing cells at a density of $2x10^6$ cell / flask in 0.2% pre-gelatinised T75 flasks in a humidified 5 % CO2 at 37°C. The cells were grown in GM. In order to examine the effect of designed CMs on C2C12 regeneration,C2C12 cells were cultured in pre –gelatinised 6-well plate at concentration of $50x10^3$ cells/ ml of completed growth media (GM). Cells were incubated overnight in a humidified atmosphere CO_2 at 37^{0}C. After 24 hours, cells were washed twice with PBS prior to replacing GM with conditioned media (CM1, CM2, CM3, and CM4) and DM.

Morphological studies were performed at 24, 48, 72 and 96 hours of CM incubation using a Leica DMI6000B microscope. Cell counts using a haemocytometer and cell-cycle analysis were performed at 96 hours

3.3.10. CELL CYCLE

After 96 hours of CM incubation, cells were collected and prepared for cell cycle analysis. Sample preparation and the propidium iodide method for FACS are described in 2.6.4.

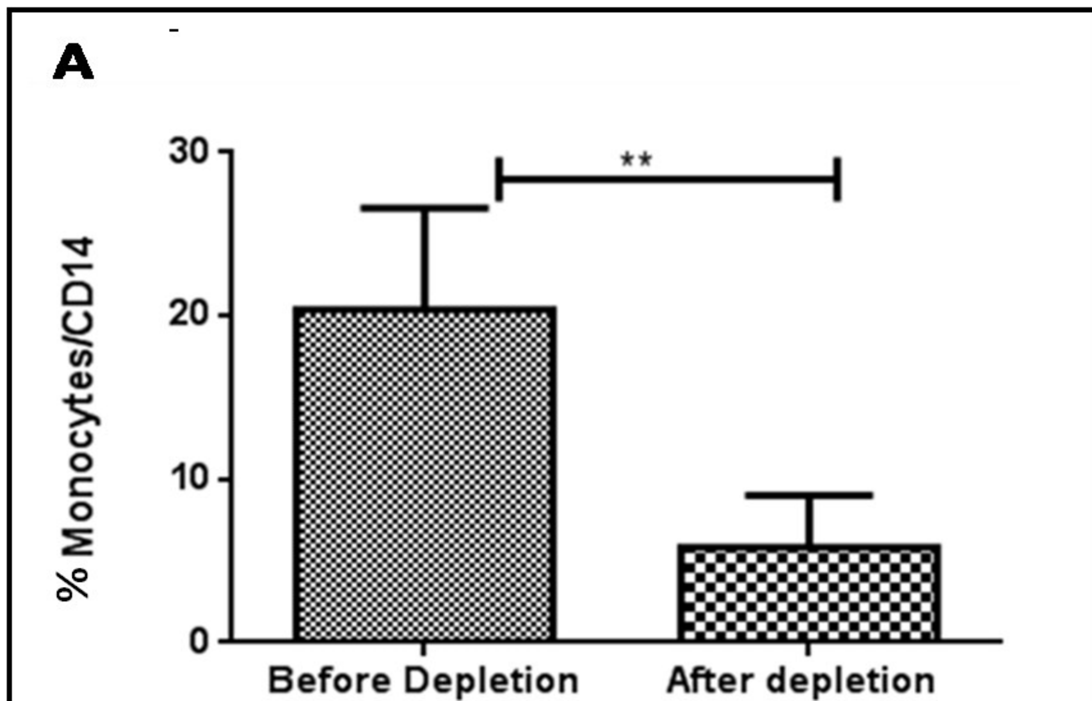
3.11. STATISTICAL ANALYSES

Results were analysed using GraphPad Prism version 6 software. Statistical analysis between more than two mean of treatment was determined using one- way ANOVA and Bonferroni correction for multiple comparisons. Two- way ANOVA followed by Bonferroni post-test analysis was used to determine the comparison among groups of treated cell. All results were presented as ± standard deviation of the mean (±SD). Results were statistically significant at the level of 5% ($P<0.05$) and indicated on figures *, whereas $p<0.01$ was marked **, $p<0.001$ was marked *** and $p<0.0001$ marked as ****. The percentages of Coefficient variation (%CV) were determined for repeated independent experiments by dividing standard deviation by the mean for each group of treated cells .

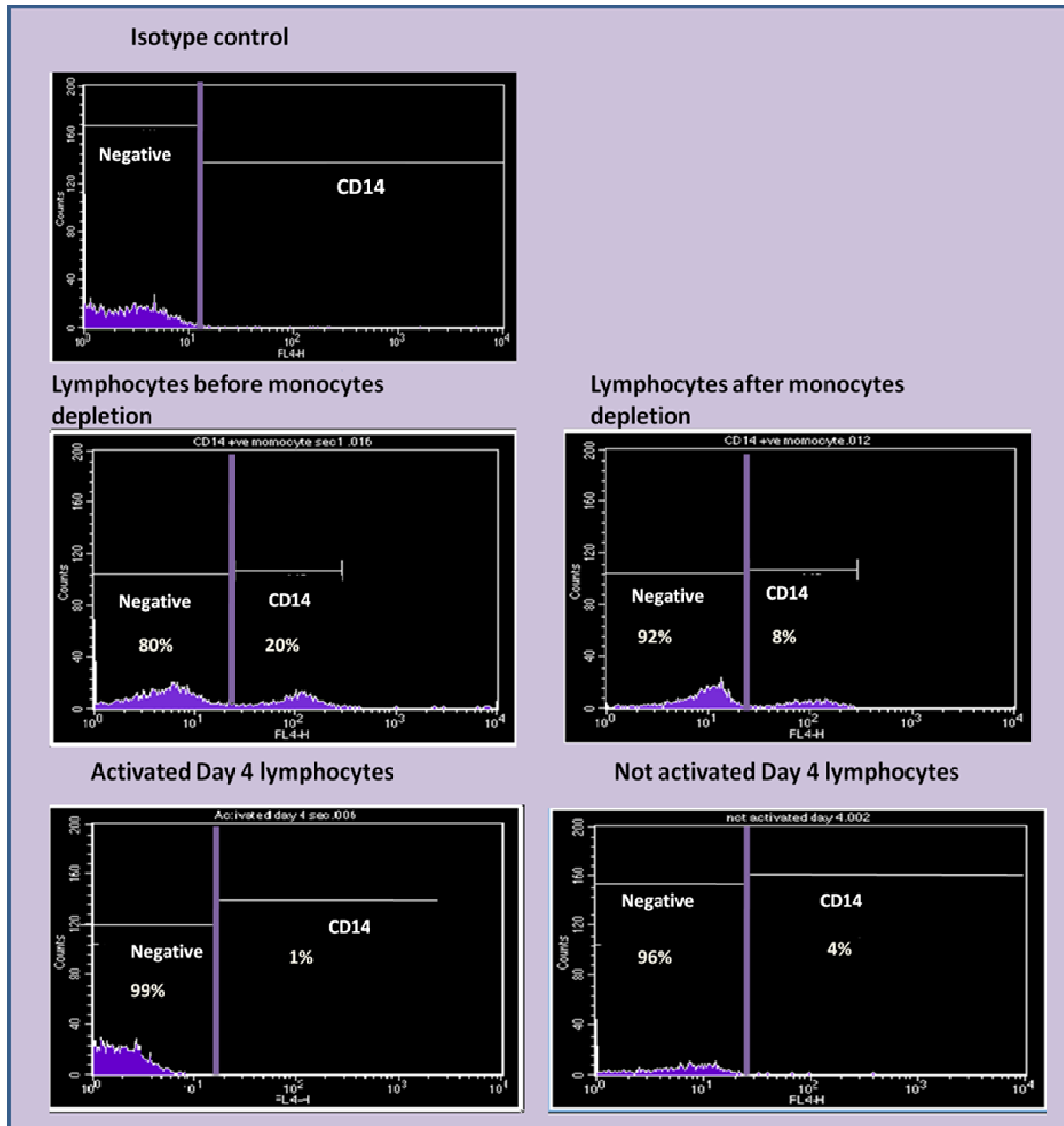
3.4. RESULTS

3.4.1. ADHERENT CULTURE TO DEPLETE MONOCYTES (MACROPHAGES)

Monocytes expressing CD14 were measured before and after depletion culture. A small percentage of CD14 was found before depletion culture (20±5%).This percentage was reduced significantly to (8±3%) following 15 minutes of depletion culture (Figure 21A and B). A minor percentage of lymphocytes was found 5±2. No monocytes expressing CD14 were found after 4 days of incubation (Figure 3.2A and B).



B



C

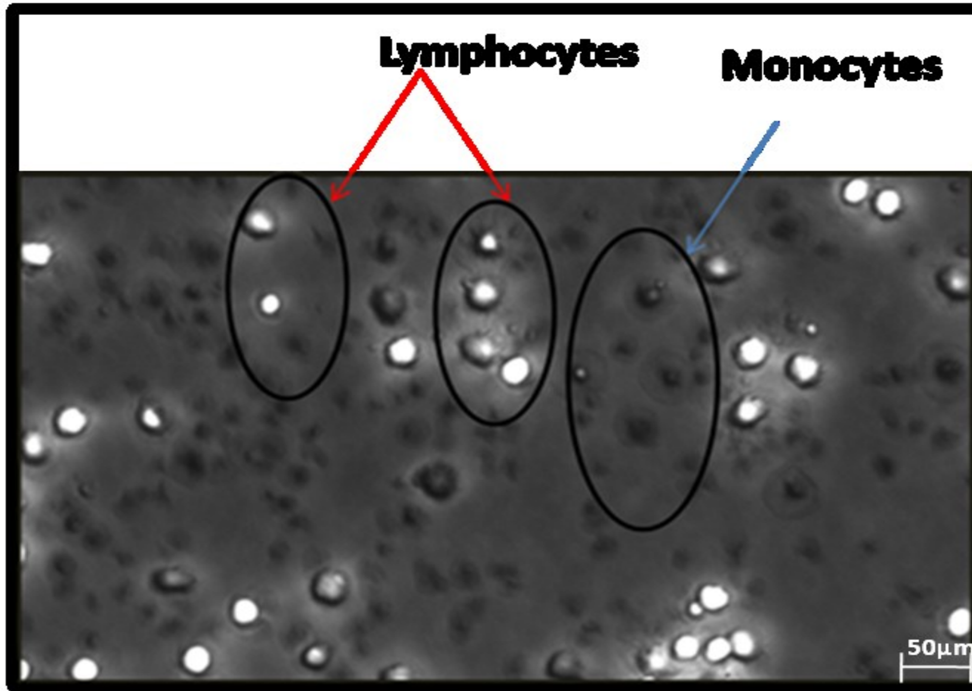


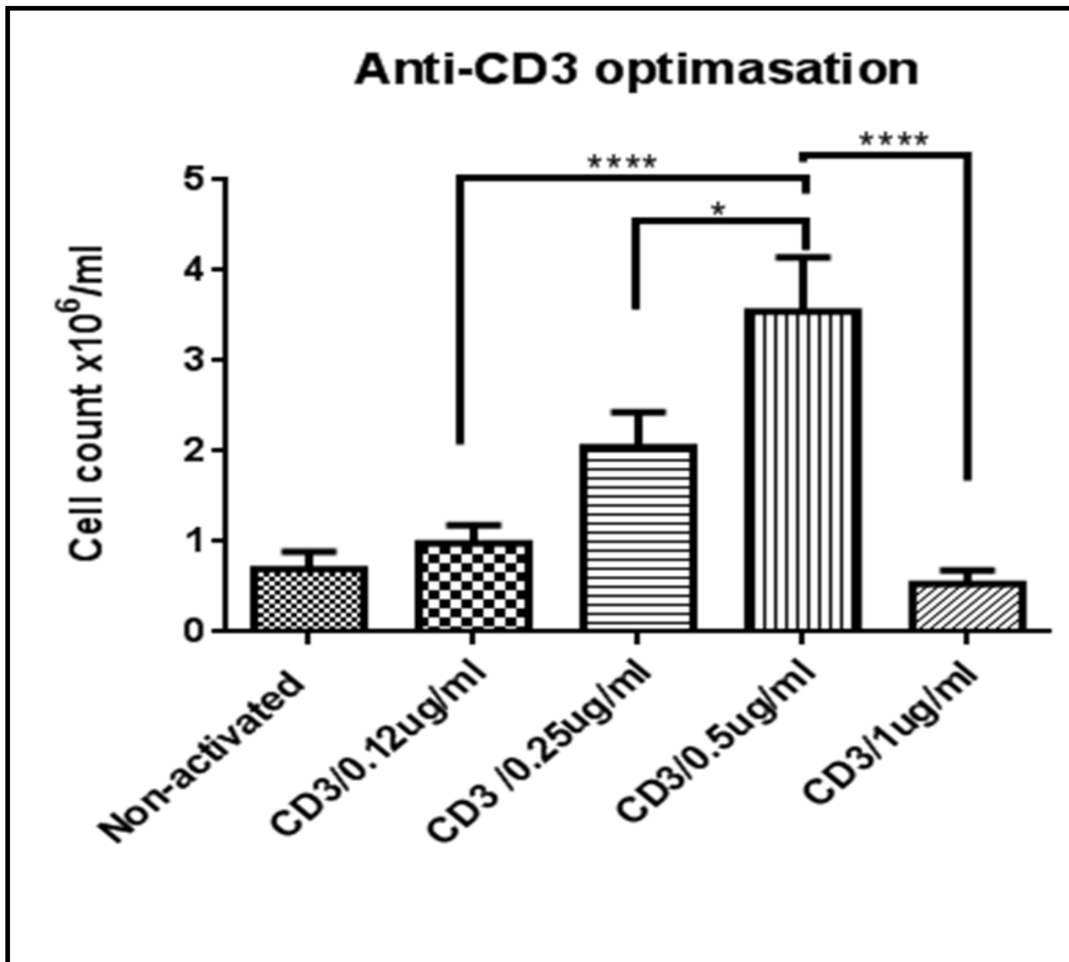
Figure 3.2: Lymphocytes depleted of monocytes. **A)** The comparison between CD14 expression before and after depletion culture at 0 time. Data represent the means ±SD (n=6 blood samples). Significant decrease was observed in CD14/Monocytes expression following 15 minutes of depletion culture ($p<0.05$). Results were presented as ±SD (n=5) of the mean. **B)** Histograms for CD14 receptors. Upper panel represents isotype control; middle panel represents lymphocytes before (left) and after (right) depletion at 0 time. Bottom panel; activated lymphocytes after depletion (left) and non- activated lymphocytes (right). **C)** A contrast image representing the adherent monocytes following 15 minutes incubation of isolation lymphocyte suspension from blood. Image obtained at 10x magnification. Scale Bar=50µm

3.4.2. OPTIMISATION OF ANTI-CD3 CONCENTRATION

In this study, four concentrations of anti-CD3 were prepared to identify the optimal activation of lymphocytes. The best activation was achieved at a concentration of0.5 µg/ml. This finding was confirmed by cell counting and morphological assessments. The proliferation rate of activated lymphocytes at 0.5µg/ml was 2-to-3 fold higher than the base line. Lymphocyte counts increased from $1x10^6$ to $3.5±0.6 x10^6$ cells /ml. The concentration of 0.12µg/ml and 0.25µg/ml

of anti-CD3 resulted in lower cell counts compared with 0.5ug /ml. Cell counts for 0.12 and 0.25µg/ml were 0.9± $0.1x10^6$ and 2.12±0.4 $x10^6$cells/ml respectively (Figure 3.3A). The highest concentration of 1 µg/ml showed cytotoxic effects on lymphocytes and inhibited their proliferation. Cells counts decreased from 0time to 96 hours dramatically ($1x10^6$ to 0.7±0.2 $x10^5$ cells/ml) (Figure 3.3A). Phase contrast images were taken as shown in Figure 3.3B.

A



B

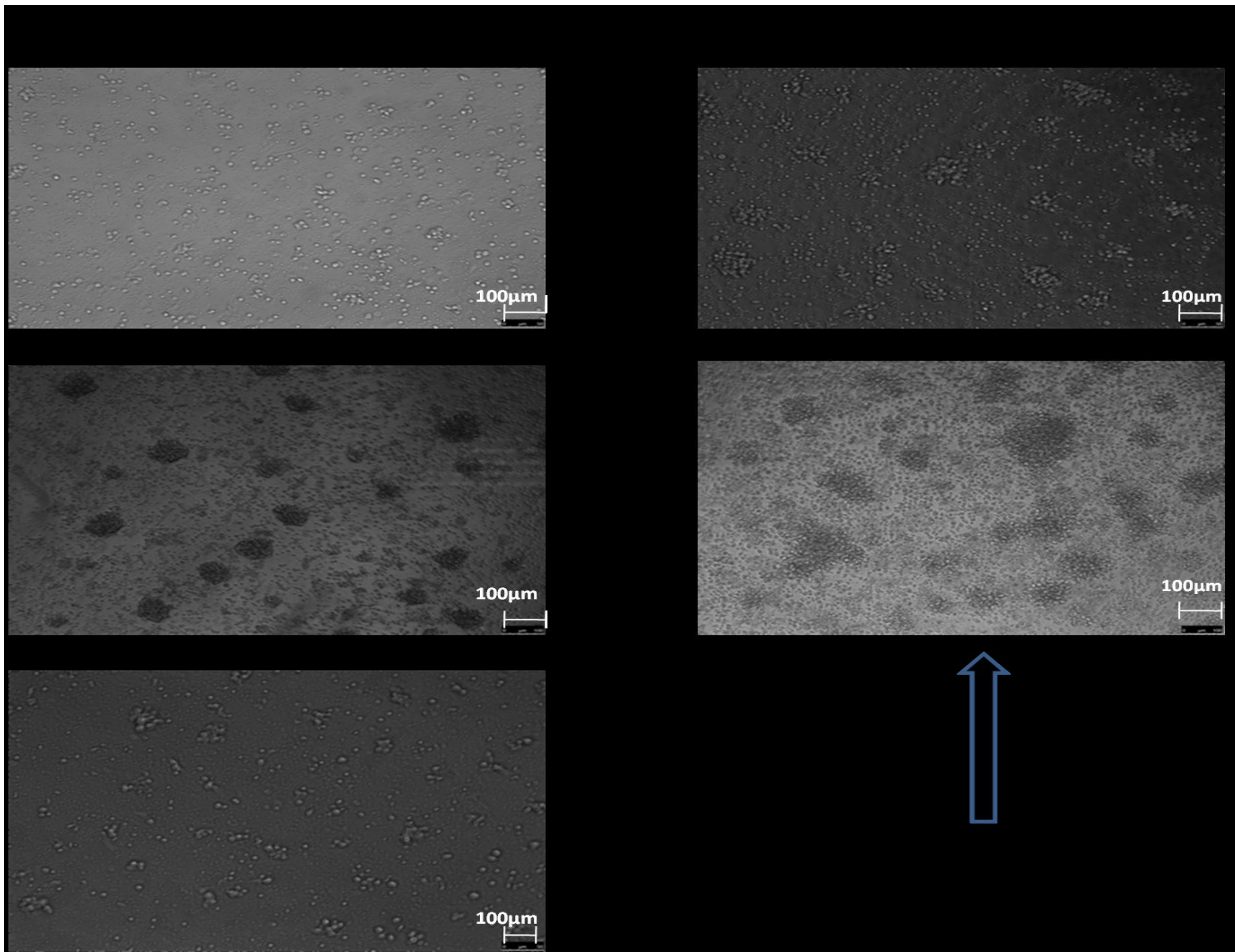


Figure 3.3: Optimisation of anti-CD3 concentration. **A**) Cell count of lymphocytes after 4 days of activation)**.** The proliferation rate of activated lymphocytes at 0.5µg/ml was the highest compared to the anti-CD3-activated lymphocytes at 0.12 and 0.25 µg/ml. The results represent mean of ±SD (n=5). **B)** Phase contrast images of lymphocytes at different concentrations of anti-CD3 after 4 days of incubation. Images obtained at 10x Magnification. The largest lymphocyte colonies were found after incubation with 0.5 µg/ml anti-CD3. Variations in the background brightness resulted from taking images from different sites of the well plate.

3.4.3. OPTIMIZATION OF LYMPHOCYTE INCUBATION TIME

Activated and non-activated lymphocytes were cultured for 2, 4 and 6 days to determine the optimal activation time. Lymphocytes cultured for 4 days exhibited higher cell counts and formed bigger colony-forming units than cells cultured for 2 and 6 days (2.7±0.11 x 10^6 cell/ml

vs $1.58 \pm 0.10 \times 10^6$ cell/ml and $1.6 \pm 0.15 \times 10^6$cell/ml respectively, $p<0.0001$). Activated cells at day 2 were not significantly higher compared to non- activated cells (1.58 ± 0.10 cells/ml $\times 10^6$ *vs* $1.24 \pm 0.09 \times 10^6$ cells/ml, $p>0.05$)(Figure 3.4 A, day 4, left panel).The counts of activated cells at day 4 and day 6 were significantly different compared to their non- activated cells ($2.7 \pm 0.11 \times 10^6$cells/ml and $1.6 \pm 0.15 \times 10^6$cells/ml vs $1.1 \pm 0.12 \times 10^6$ and $0.5 \pm 0.08 \times 10^6$ cells/ml;$p>0.0001$, respectively)(Figure 3.5)

Anti-CD3 activated lymphocytes **Non-activated lymphocytes**

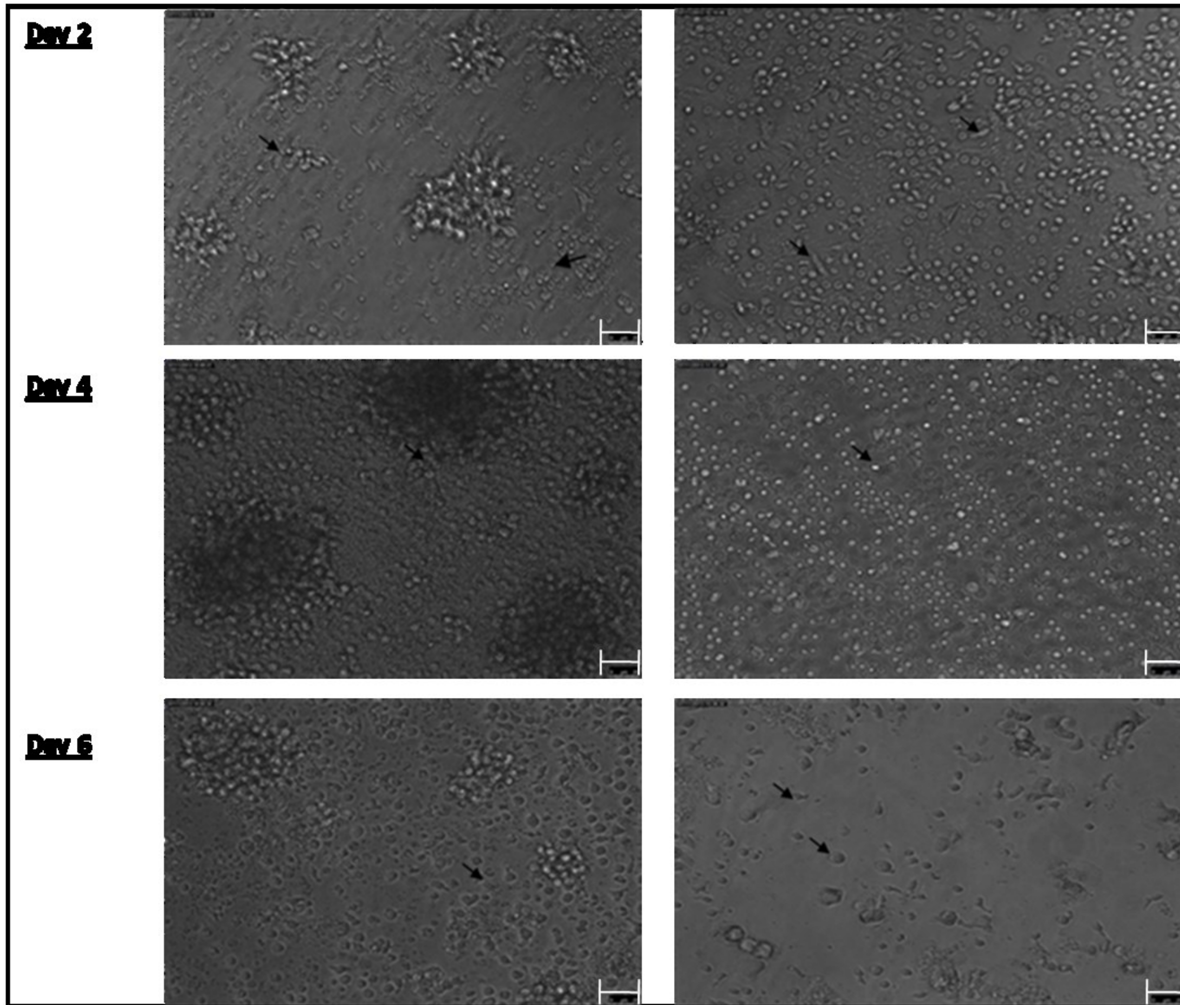


Figure 3.4: **Optimisation of culture time for Anti-CD3 activated vs non-activated lymphocytes at days 2, 4 and 6.** Activated cells formed colony units (left panel) whereas non-activated did not (right panel). At day 4, the number and size of colony forming of activated lymphocytes was higher compared to those in days 2 and 6 (upper and lower images, left panel). Homogeneity of immune cells at day 2, 4 and 6 varies. At day 4 (left panel) the dominant population of immune cells was characterised by having a circular shape (see arrow), whereas immune cells at days 2 and 6 were heterogeneous in size and shape (see arrow). Non-activated immune cells density in day 6 was lower compared with day 2 and 4 non-activated cells (right panel). Lymphocytes images were obtained at 10 x 1.6 Mag. Scale bar: 100μm.

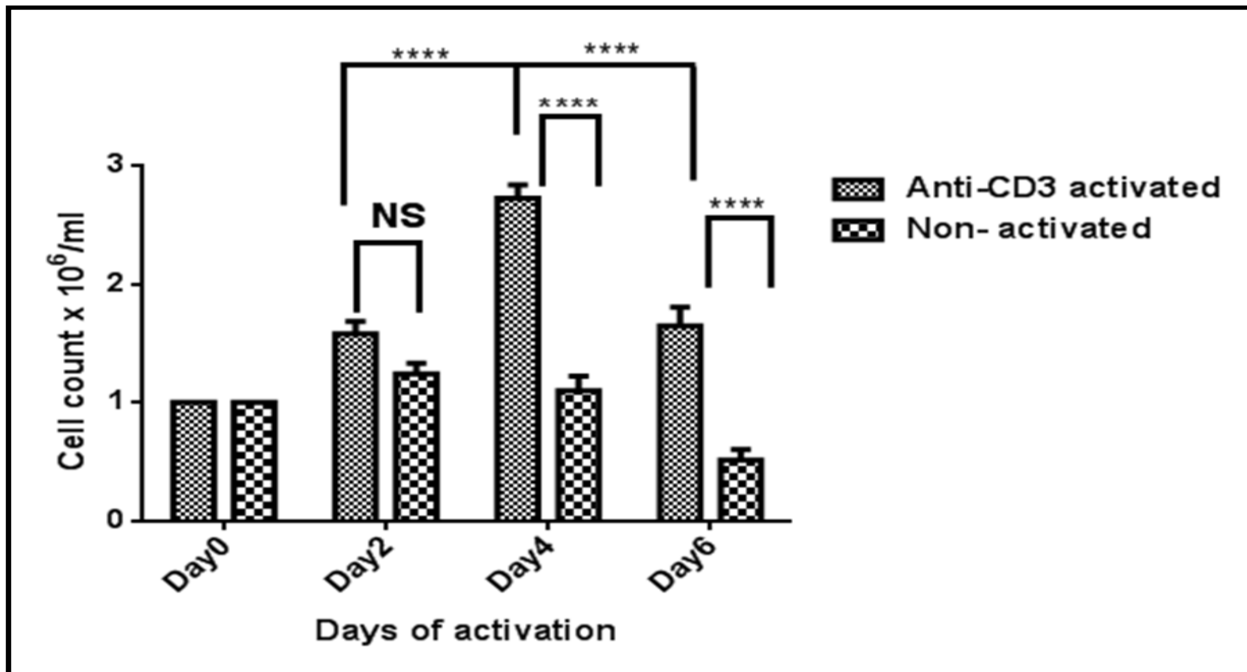


Figure 3.5: Cell count of anti-CD3 activated vs non-activated lymphocytes at days 2, 4 and 6. Activated cells at day 4 had the highest proliferative rate compared to days 2 and 6, $p>0.0001$.The number of lymphocytes decreased at day 6. Data represent means ±SD (n=10) undertaken in duplicates.

3.4.4. OPTIMISATION OF ANTI-CD28 CONCENTRATION

Lymphocytes were activated with 0.5ug/ml of anti-CD3 and a range of anti-CD28 concentrations (2.5, 5, and 7 µg/ml). Following 4 days of activation, the proliferation rate was assessed to determine the optimal concentration for anti-CD28 with anti-CD3 by cell counting. The findings show that the cell count of activated lymphocytes atanti-CD3/anti-CD28;5µg/ml) was significantly higher ($5x10^6$µg/ml) than anti-CD3/CD28;2.5µg/ml); ($3.5x10^6$cells/ml;$p>0.05$),anti-CD3/CD28(7µg/ml)($2.5x10^6$cells/ml; $p>0.001$) and the cell count for negative control (CD3+/-CD28)($3.12x10^6$cells/ml; $p>0.001$)(Figure 3.6).

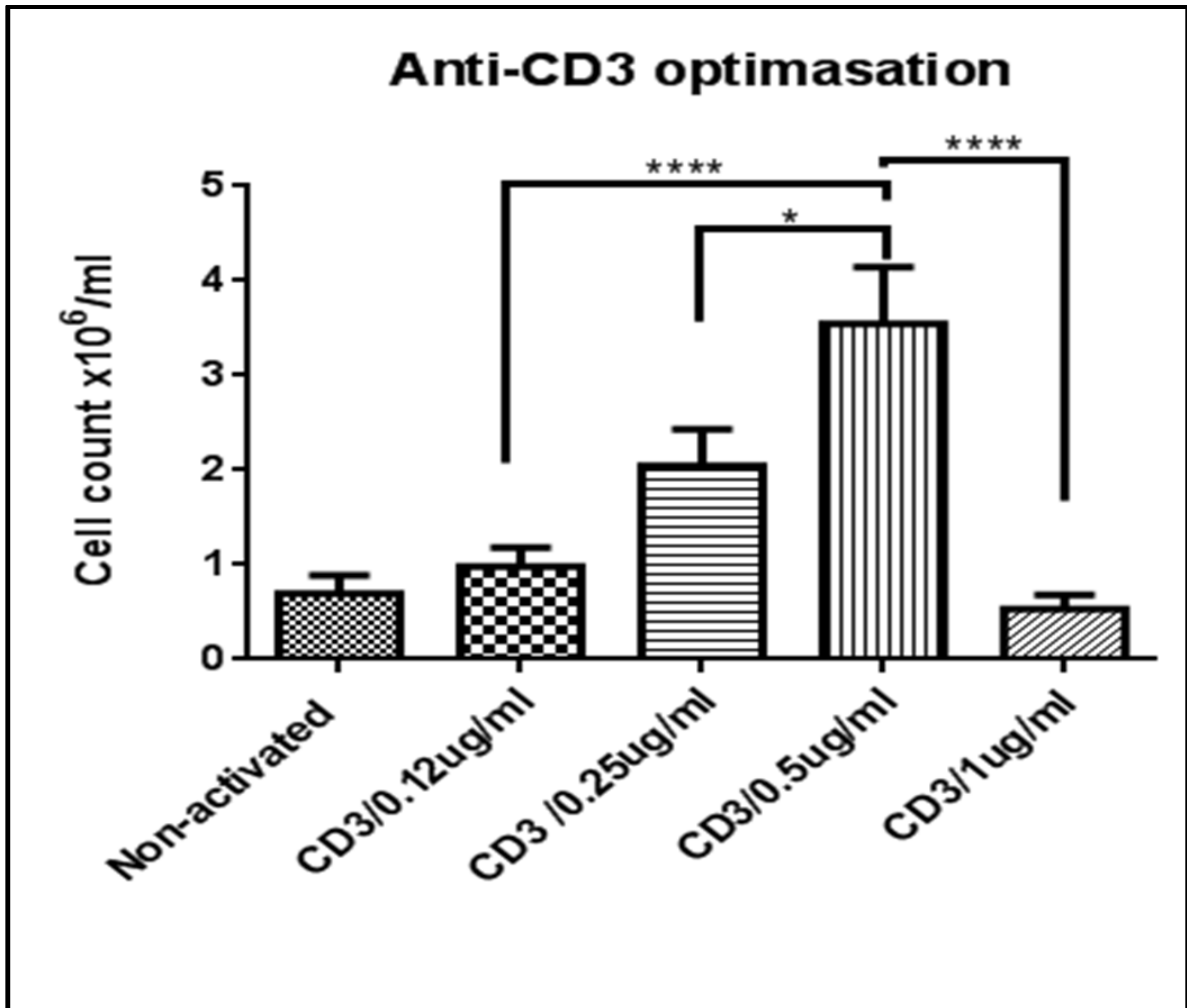


Figure 3.6: **Optimisation of anti- CD28 concentrations for activated young lymphocytes following 4 days' incubation.** Lymphocytes were activated at different concentrations of anti-CD28 and cultured in (0.5µg/ml) anti-CD3 pre-coated wells for 4 days. The data represent means ±SD (n=4) in triplicate**.** % CV for non –activated; 15%, CD3 0.12µg/ml; 7%, CD3/0.25µg/ml16%, CD3/0.5µg/ml;10% and CD3/1µg/ml; 35%.

3.4.5. OPTIMAL VOLUME OF SECRETOME TO MAKE CONDITIONED MEDIA (CM1)

C2C12 cells were cultivated in three conditioned media of anti-CD3- activated cells (CM1) for 96 hours. In order to identify the optimal concentration of secretome to add to the C2C12 DM, three volumes of secretome (50µl, 100µl and 200µl) were mixed with 4ml DM to make the

conditioned media. C2C12 cells grown in conditioned media including 50µl secretome exhibited differentiation and myotube formation (Figure 3.7A), whereasC2C12 cells grown in conditioned media composed of 100 µl of secretome and DM exhibited proliferation (no myotube formation)(Figure 3.7 B). C2C12 cultivated in conditioned media with 200 µl of secretome did not grow in a healthy manner (Figure 3.7 C).

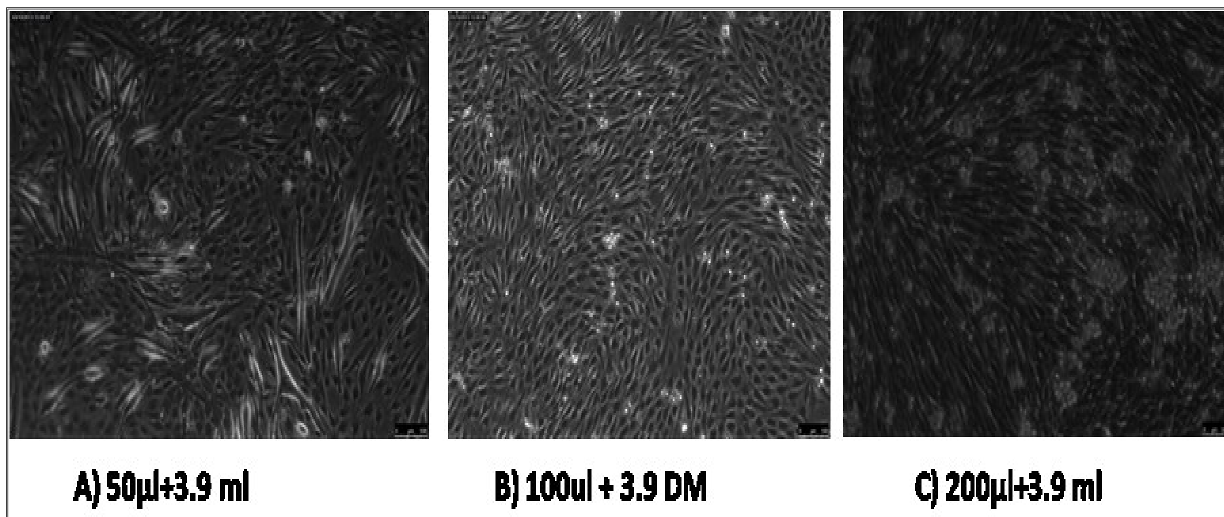


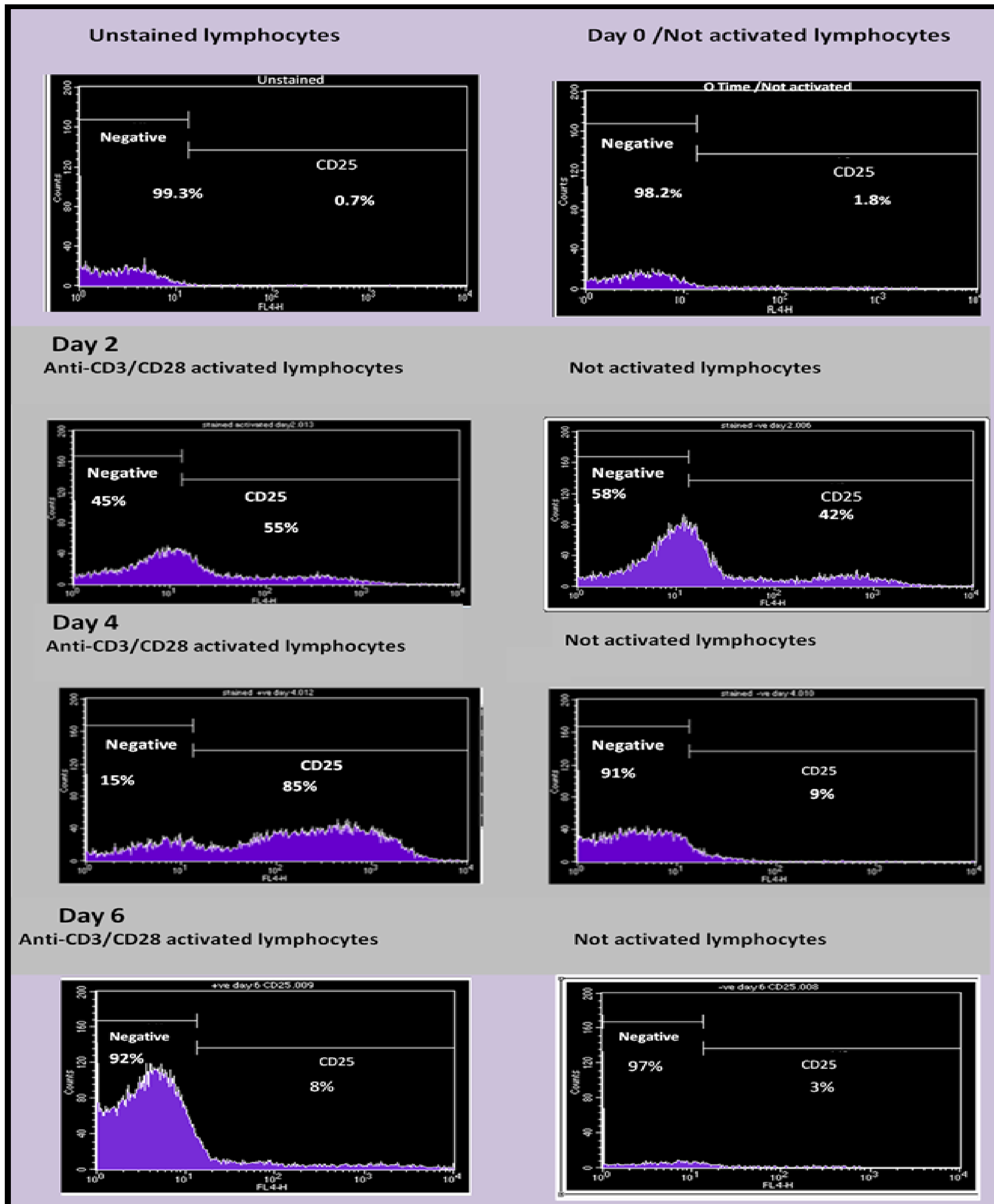
Figure 3.7: C2C12 cultivated at 50x1000cell/ml in CM1for 96 hours. Myoblasts treated in CM(A) exhibited differentiation and myotube formation, whereas (B) C2C12maintained proliferation. C2C12 grown in (C) did not exhibit healthy growth. The data represent at least 4 experiments. Scale presents 75µm.

3.4.6. CD25 (IL-2A R) MARKER ANALYSIS

IL-2Rα analysis was used for lymphocyte detection as a first step to evaluate the effect of lymphocytes on skeletal muscle generation. CD25 analysis was conducted on activated and non-activated lymphocytes after 2, 4 and 6 days incubation. CD25 is an early activation marker for T lymphocytes and essential for activation and proliferation of T cells (Chao et al., 2002; Smith and Cantrell, 1985).The results showed that CD25 expression on day 4 activated lymphocytes

was significantly higher compared to day 2 and 6 activated cells (87±8.4 % vs 49.5±2.5%; ($p<0.05$) and 18.7±10%;($p<0.0001$)(Figure 3.8 A and B).

A)



B)

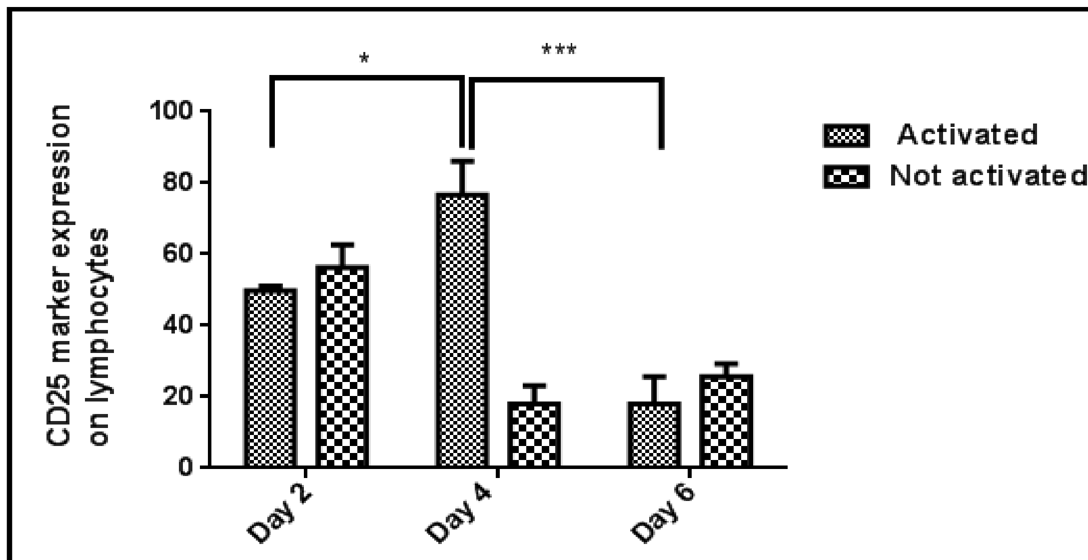


Figure 3.8: The expression of CD25 lymphocytes at 0, 2, 4 and 6 days of activation. A) At 0 time resting lymphocytes express only 1.8% anti-CD25 (upper panel). From day 2 of activation (second panel) to day 4 of activation (third panel), the expression of the IL-2α receptor on lymphocytes increased from (55%) to (85%). At day 6, the expression of CD25 marker decreased significantly to 8% (lower panel). **B)** The graph represents the mean value of CD marker expression on activated and non activated lymphocytes at day 2, 4 and 6. The data represent means ±SD (n=5) in triplicates.

3.4.7. QUANTITY OF CYTOKINE SECRETION IN DAY 2- AND 4 -ACTIVATED LYMPHOCYTES

In order to gain some insight into the quantity of secreted cytokines from activated/non-activated lymphocytes at day 2 and 4 incubation, eight cytokines in the secretome were screened. Briefly, the level of cytokines' expressed by day 2 was lower than for cytokines in day 4 -activated lymphocytes. IL-3 was significantly higher expressed by day 4activated lymphocytes (469ng/ml) than non-activated; (10.3ng/ml);($p<0.001$). Similarly, tumour necrosis factor-alpha (TNF-α) and interferon –gamma (IFN-γ) were detected at higher concentrations in activated lymphocytes compared with non- activated. TNF-α was detected at a concentration of

248ng/ml vs 30ng/ml in activated vs non- activated secretomes. IFN –γ was quantified at 2235 ng/ml in activated lymphocyte secretomes vs 1533 ng/ml in non-activated secretomes.

3.4.8. C2C12 TREATMENT WITH CONDITIONED MEDIA

C2C12 myoblasts were treated with CM1 or CM2 of lymphocytes following 2, 4 or 6 days of C2C12 culture. CM3-4 and DM were set as control treatments in this study. A morphological examination of CM1-4 treated myoblasts was at 96 hours. The CM1 (anti-CD3/CD28 activated lymphocytes) treated myoblasts at day 2 exhibited myotube formation and were not significantly different to other controls CM2-4 and DM at 72 hours, whereas day 4 CM1- treated myoblasts showed accelerated cell proliferation and did not exhibit multinucleated myotube formation at the same time point (Figure 3.9).Myoblasts cultivated in day 6 CM1 also showed an extended proliferation, but there was also myotube formation and more dead cells were detected.

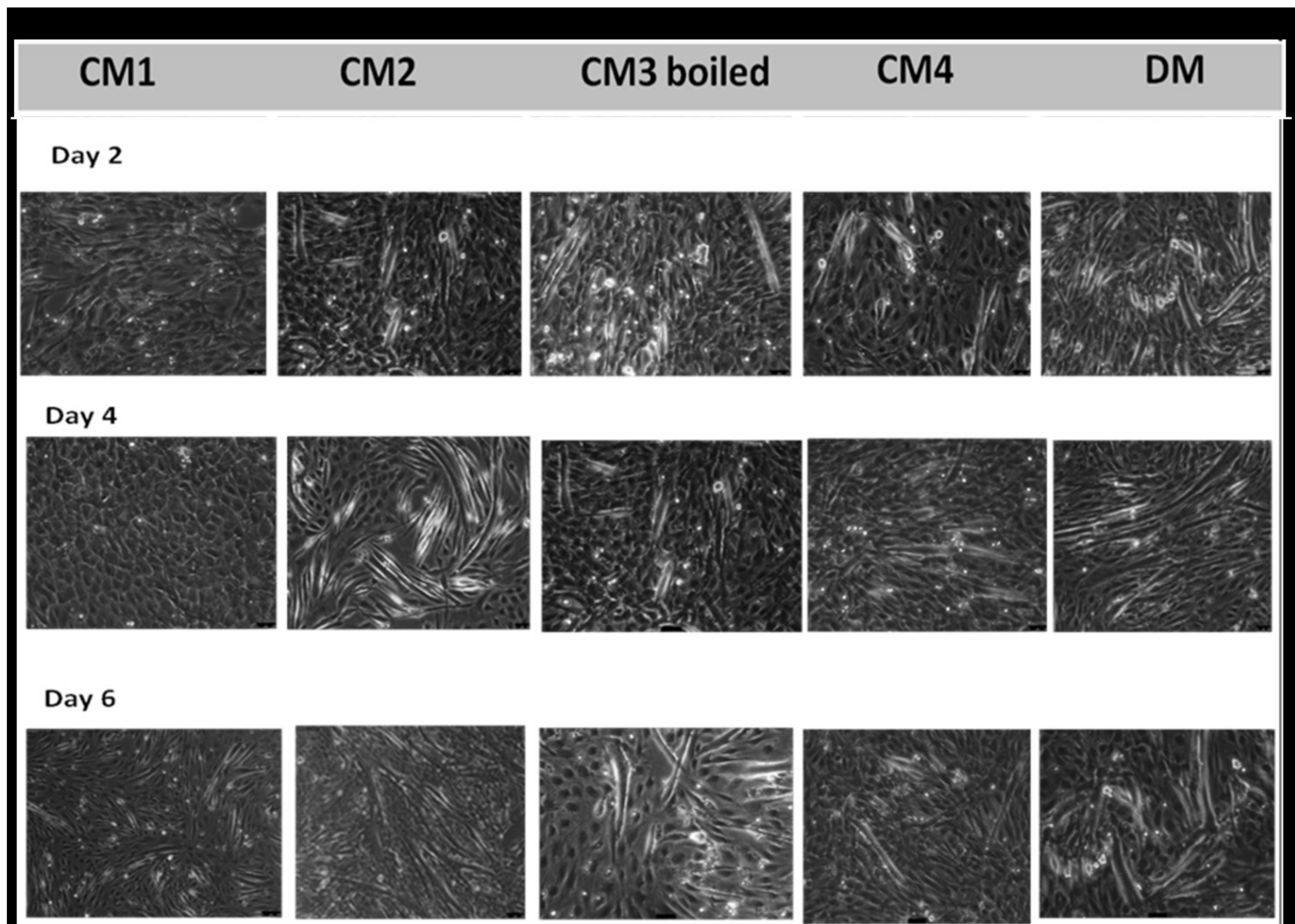


Figure 3.9: C2C12 treated with conditioned media (CMs). Myoblasts cultivated with CM1 (first panel), CM2 (second panel), CM3 boiled (third panel) CM4 (forth panel) or DM only for 96 hours. Images obtained at 10x1.6 Mag**.**

3.4.9. CELL CYCLE ANALYSIS

Cell cycle analysis was measured by flow cytometry, which is a widely used procedure to analyse cell proliferation and differentiation through assessing the S phase of dividing cells. This analysis was used to assess cell cycle progression of myoblast treated in conditioned media. The results showed that % S phase of day2 CM1- treated cells (22%±5) was not significantly different compared with CM2-4 (15%±5, 25%±5 and 20%±5, respectively) and S% phase of DM was 15%±2 ($p>0.05$). Similar to the C2C12 myoblast count, %S phase of day 4 CM1-treated cells was significantly higher than the various control conditions used in this study. Percentages of S phase of CM1 was 60±5% ($p<0.001$) compared with CM2-4. Percentages of S

phase were 15±5, 25±5 and 20±5% respectively, and in DM was 10±5% (Figure 32). Day 6 CM1 treated myoblasts (28% ±2) showed higher proliferation rates than day 2 CM1, but compared to controls, the proliferation rate was not significantly different compared with CM2-4; %S phase;20%±5,20%±5,25%±5 and DM 10%±5 ($p>0.05$)(Figure 3.11).

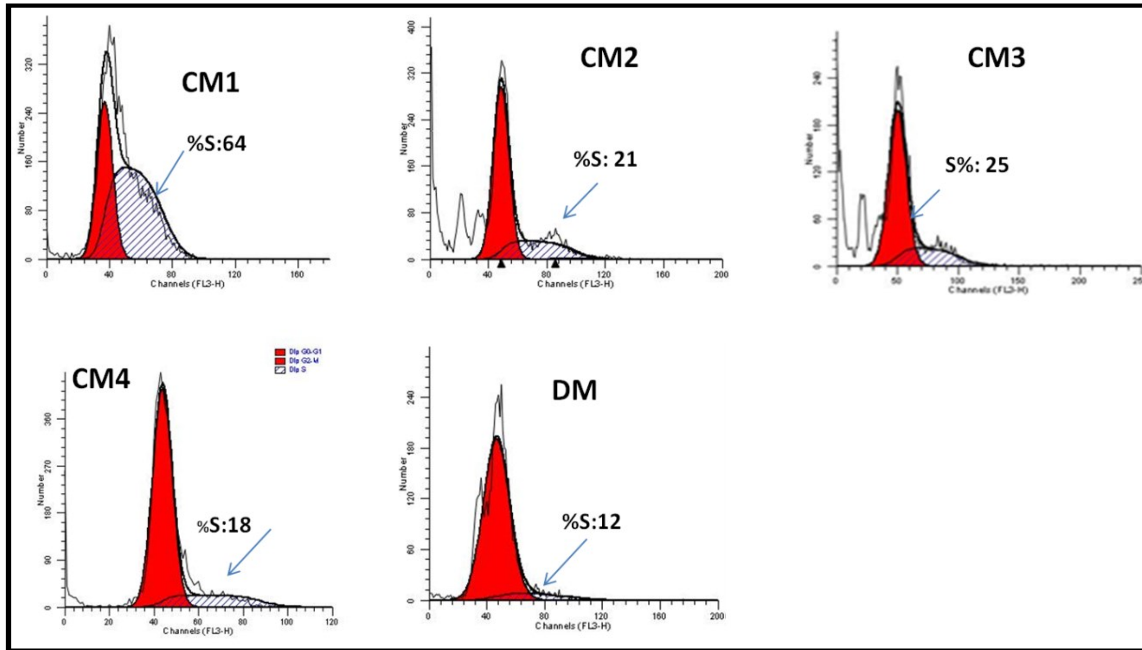


Figure 3.10: % S phase of myoblasts treated with day 4 conditioned media (CM1-4) and DM at 96 hours.

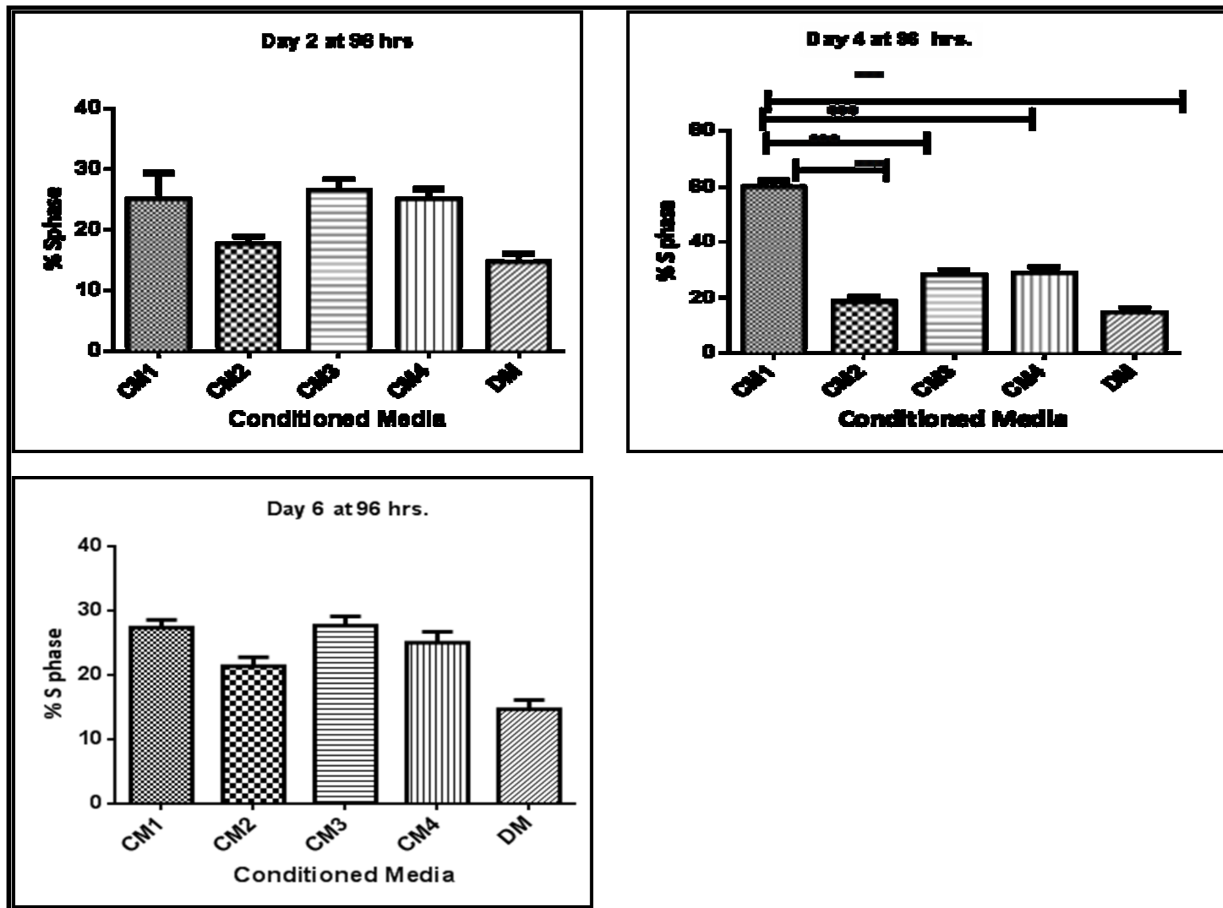


Figure 3.11: The percentage of S phase of myoblasts treated in conditioned media CM1- 4 and DM following 2, 4 and 6 days of activation. Data represent means ±SD (n=5) in duplicates.

3.5 DISCUSSION

3.5.1. OPTIMAL ANTI-CD3 CONCENTRATION FOR LYMPHOCYTE ACTIVATION

According to the cell count study for cultured lymphocytes, the concentration of 0.5µg/ml anti-CD3 induced the greatest lymphocyte proliferation (Al-Shanti et al., 2014). Lymphocyte

numbers increased from time point 0 to 96 hours from $1x10^6$to$5x10^6$cells/ml. Lymphocyte count at anti-CD3 concentrations of 0.25 and 0.12 µg/ml was significantly lower than for 0.5µg/ml ($p<0.001$). Also, at this concentration of 0.5µg/ml lymphocytes grew in colony forming units that were larger than the colonies shown at the lower anti-CD3 concentrations (0.25µg/ml and 0.12 µg/ml)(Holmes, 2008; Zheng et al., 2008)(Figure 3.3 A and B).

3.5.2. OPTIMIZATION OF ANTI-CD28 CONCENTRATION

The results clearly showed that 5µg/ml of co-stimulatory anti-CD28 was the optimal concentration to obtain the highest proliferation rate of lymphocytes in the presence of 0.5µg/ml of anti-CD3.The cell count of activated lymphocytes at concentrations of 2.5µg /ml and7µg/ml was significantly lower compared to 5µg/ml.

Anti-CD3 is a central signalling pathway for lymphocytes proliferation but addition of anti-CD28 provides co- stimulation for the expanding cells to release (Li and Kurlander, 2010; Schwartz, 1990) through activation of multiple signalling pathways including ERK and JNK and nuclear factor-kappa-B(NF-kappa-B)(Jerome, 2008). These findings were supported in that our study found the cell count of anti-CD3 at day 4 was significantly lower ($p>0.0001$) than anti-CD3 and anti-CD28 activated lymphocytes at the same period of activation.

3.5.3. OPTIMAL DURATION FOR LYMPHOCYTE ACTIVATION

Lymphocytes were activated by anti-CD3 and IL-2 (Tsoukas et al., 1985) at different incubation periods for 2, 4 and 6 days at 37°C in a humidified atmosphere. The optimal duration time for anti-CD3/IL-2 activated lymphocytes was found at day 4. This was confirmed by: 1) the size of colony forming units of lymphocytes at day 4 was larger than at days 2 and 6; 2), the cell count of lymphocytes at day 4 was significantly higher compared with days 2 and 6 ($p<0.05$)(Curti et al., 1996). 3) at day 4, the immune cells proliferated into large homogenous groups characterised by round regular shapes and small spherical nuclei (see arrows in Figure 3.4). 4) The lymphocyte culture in 2 and 6 days exhibited heterogeneous populations of immune cells that could represent other innate immune cell groups (see arrows in Figure 3.4). 5) The cell count and size-forming unit of lymphocytes started to reduce at day 6, and higher number of dead cells were detected in non-activated lymphocytes culture (Figure3.4 and 3.5).

3.5.4. CD25 MARKER (IL-2α R) MEASUREMENT ON LYMPHOCYTES FOLLOWING 2-& 4- ACTIVATION

To investigate the presence of activated lymphocytes, the CD25 marker (IL-2αR) was measured in lymphocyte cultures from days 2 and 4. CD25 is a specific marker for T cell activation and proliferation (Tsoukas et al., 1985; Brocardo et al., 2001). The data demonstrated that the expression of CD25 was higher in day 4(87±4) than day 2 (49±2), while at day 6 activation, the expression decreased significantly to (18±10, $p>0.0001$) (Figure 3.8 A and B).This finding was expected because most of the cells died. The peak expression of the IL-2 receptor following day 4 activation could indicate that IL-2 receptors expressed on lymphocytes were already engaged by the exogenous IL-2 that was added in the lymphocytes culture medium. Therefore, the number of un-bound IL-2 receptors to conjugate with anti-CD25 was very limited. Additionally, CD25 is the middle T- lymphocyte activation marker (Rea et al., 1999) and the way of activating lymphocytes by using anti-CD3/CD28/IL-2 is effectively expansion of lymphocyte proliferation and enhanced endogenous IL-2 production leading to increased IL-2 R expression (Schwartz, 1990; Jerome, 2008). Another possibility is that CD25 was expressed on immunoregulatory T cells (Treg) which represent 5-10% of CD4+Tcells (Piccirillo and Shevach, 2004).

3.5.5. QUANTITATION OF CYTOKINE SECRETION IN DAY 2 AND 4-ACTIVATED LYMPHOCYTES

Our data suggest that factors secreted by lymphocytes can extend the period of proliferation of myoblasts. It is therefore of interest to identify which secreted factors promote this regulatory role. Based on findings of the multiplex assay carried out on the lymphocyte secretomes, it seems that the cytokines and growth factors released by day 2 activated T lymphocytes were not sufficient to exhibit their mitogenic effects on CM1 treated myoblasts. The results indicated that the concentration of cytokines released from day2-activated lymphocytes was lower than for day 4. The cytokines which were expressed at high concentrations after 4 days of lymphocyte activation were IL-1, IL-3, TNF-β and IFN-y.

In addition, the study results have shown that IL-1 concentration was higher in the secretome of day 4 activated lymphocytes compared with non-activated lymphocytes. Moreover, the IL-1 concentration in day 4 activated lymphocytes was 1-2 fold higher compared to day 2-activated

lymphocytes. It is well established that IL-1 is a key stimulator of IL-2, 3, 6 and TNF (Tidball, 1995). IL-3, 6 and TNF are secreted by CD4+ Th2, while IL-3 and TNF-α are produced from CD4+Th1 cells (Mosmann and Sad, 1996). TNF-α is known to have a mitogenic effect on skeletal muscle myoblasts (Li, 2003). This finding also was supported by Al-Shanti. et al., (2008) who suggested that the combined effect of IL-6 and TNF-α induced satellite cell proliferation (Al-Shanti et al., 2008). Surprisingly, IFN-γ was also detected at similar concentrations in the secretome of day 4 activated and non-activated lymphocytes. IFN-γ secreted by activated lymphocytes has been shown to promote skeletal muscle regeneration and to stimulate wound healing (De Rosa et al., 2004; Krug et al., 1996). However, another finding demonstrated that INF-γ could negatively affect skeletal muscle proliferation. It was shown that blocking the receptor of IFN-γ decreased proliferation rate of the C2C12 myoblasts (Foster et al., 2003).

3.5.6. OPTIMAL VOLUME OF SECRETOME TO MAKE CONDITIONED MEDIA

Since the ultimate aim of this study was to extract the secretome of activated lymphocytes and use it to enrich the growth medium of C2C12 cells, it was necessary to determine the optimum volume of secretome. Three different volumes of secretome (50µl, 100µl and 200µl) were assessed to determine the response of C2C12 cells treated in CM1. The lower volume of day 4 activated lymphocytes (50µl) did not stimulate C2C12 proliferation (Figure 3.7 A), possibly because the existing cytokines were not at sufficiently high concentrations to exhibit their proliferative effects on skeletal muscle myoblasts. The conditioned media supplemented with the highest volume of secretome impaired satellite cells growth and caused C2C12 cell death (see arrows in Figure 3.7C). This finding raises the possibility that high concentrations of cytokines negatively affect cell growth and development, although this would have to be confirmed in follow-up studies. Several studies showed a strong relationship between muscular atrophy (muscle wasting) and high concentrations of cytokines. For example, cachexia, cancer and sarcopenia are strongly related to high concentrations of TNF-α and IFN-γ (Yeh and Schuster, 1999; Lutz and Quinn, 2012). The conditioned media supplemented with 100 µl of secretome was the optimal volume because cultivated C2C12 in this volume exhibited higher proliferation rates (Figure 3.7 A). The C2C12 cell count was significantly higher after enrichment of CM1 with 100µl of secretome compared with other secretome volumes ($p<0.05$).

3.5.7. MYOBLAST CULTIVATION IN FORMULATED CONDITIONED MEDIA

The results showed that CM controls did not inhibit differentiation of myoblasts to multinucleated myotubes. All CM controls were not significantly different from one another. In the main experiment, C2C12 cells were cultured in CM1 (composed of secretome of anti-CD3/CD28 activated lymphocytes at day 2, 4 and 6). The results showed that the conditioned media from day2 activated lymphocytes exhibited differentiation to myotube formation over a normal timescale (Figure 3.9 upper panel). Cell counts were not significantly different compared with CM2-4 control conditions (Figure 3.9, upper panel).The C2C12 cells cultured with CM1 enriched with the secretome of day 4 activated lymphocytes showed the highest proliferation rates and no myotubes formation (Figure 3.9, middle panel). The cell count of CM1 enriched with the day 4-activated lymphocytes was significantly higher compared with CM controls, CM2-4 and DM. On the other hand, myoblasts treated with CM2 (day 4 non-activated lymphocytes) displayed fusion of mononucleated cells to form multinucleated myotubes in the normal way that was not significantly different from control conditions CM3-4. However, C2C12 cultured in CM1 (secretome of day 6 activated lymphocytes) exhibited proliferation but was not significant compared to controls (Figure 3.9; lower panel). Moreover, more dead myoblasts were detected at 96 hours in day 6 CM1 culture.

3.5.7. CELL CYCLE ANALYSIS

Cell cycle progression is a commonly used method to characterise the development of satellite cells (Andres and Walsh, 1996). Data from this study was supported by S phase assessment of each CM. Consistent with other findings that suggest the CM1 enriched with the secretome of 4-day activated lymphocytes is the optimal condition, the S phase of myoblasts treated with CM1 day 4 increased significantly compared with other CM2-4 controls (Figures 3.10 and 3.11). In contrast, S phase of day 2 and day 6 were not significantly increased compared to the controls (Figure 3.11).

The day 6 incubation time of activated lymphocytes was excluded from the optimisation study due to the low lymphocyte count following their culture, low %S phase of myoblasts, low

myoblast count and high numbers of dead cells after 96 hours of incubation in conditioned media. Based on the results obtained from this study, the secretome of day 4 activated lymphocytes had an inhibitory influence on the differentiation of CM1-treated myoblasts, and instead extended the period of proliferation. Extending the proliferative capacity of muscle precursor cells is highly relevant and the results obtained by Mildner et al., (2013) demonstrated that the secretome from human mononuclear cells accelerate proliferation and wound healing of skin post injury (Mildner et al., 2013). Another study demonstrated that the anti-inflammatory mediators harvested from the secretome of mononuclear cells effectively attenuated the development of autoimmune myocarditis (Hoetzenecker et al., 2013). Taken together, these results clearly identify a primary role for the immune cells in promoting the proliferation of precursor muscle cells and the regeneration of tissue. Details of the possible muscle-immune interactions are considered below.

Activated T-cells produce cytokines and growth factors that have mitogenic effects on skeletal muscle regeneration (Dumke and Lees, 2011). These secreted proteins include fibroblast growth factor-2 (FGF-2)(Srully Blotnick et al., 1994), (IFN-γ) (De Rosa et al., 2004; Mann et al., 2011), TGF-β (Megan K Levings et al., 2002), TNF-α (Mann et al., 2011); (Schaerli et al., 2004) and (IL-4)(Fritsch et al., 2005; De Rosa et al., 2004). Interestingly, TNF-β and IFN were detected at high concentration in the secretome of day 4 activated lymphocytes by multiplex assay. It is known that mitogens activate cell proliferation and prevent differentiation (Sharon A. Coolican et al., 1997; Delmas et al., 2001). Another explanation could be the effect TGF-β which is strongly associated with T cells infiltration. Reduced expression of T- cells was associated with a decreased level of TGF-β in patients with Duchenne muscular dystrophy (DMD). The third possibility relates to possible effects of insulin like growth factor (IGF), which promotes satellite cell proliferation (Machida and Booth, 2004). It is possible that IGF is one of a number of proteins secreted by T-cells. A previous study showed that the synergistic effect of IGF with IL-6 extended the period of proliferation of C2C12 myoblasts in a similar way to that observed in the present study following enrichment of the growth medium with the lymphocyte secretome (Al-Shanti et al., 2014). However, it is well established that IGF promotes fusion of mononucleated myoblasts and stimulates hypertrophy via increasing the number of myonuclei and the size of myotubes *in vivo* (Yin et al., 2013).

3.6. CONCLUSION

The results show that culturing activated CD3/CD28- lymphocytes for 4 days is the optimal period of activation evidenced by the highest cells count, CD25 expression and cytokine secretion. In addition, It is suggested that the cytokines released by day 4 activated lymphocytes were able to induce proliferation of C2C12 following 4 days' incubation with CM1 compared to CM2, CM3 boiled and CM4. As a consequence of the results obtained in this chapter, it was decided to apply the same optimised experimental design to study the effect of secretome obtained from elderly participants on C2C12 regeneration.

CHAPTER 4

4. AGE- RELATED DIFFERENCES IN THE CHARACTERISTICS OF YOUNG AND OLD LYMPHOCYTES.

4.1 INTRODUCTION

Ageing related changes to the immune system increases vulnerability of older people to infectious disease and tumours (Vasto et al., 2009). The reasons for the age-related declines in immune function are not well understood. However, genetics and environmental risk factors as well as thymic dystrophy are thought to contribute (Salam et al., 2013). A decline in thymus activity reduces the immune system's homeostatic response to antigens and leads to a corresponding reduction in T-cell production and more generally, an overall decrease in circulating T-cells (Qi et al., 2014). This is not the only age-dependent change to immune cells, as the remaining T-cells also undergo alterations to their gene expression which affects their function.*In vivo*, T-cells are activated either by antigens or chemokines and respond by releasing cytokines, chemokines and growth factors to combat antigens or to orchestrate tissue repair. The activation of T-cells can be mimicked in vitro by anti-CD3 and/or anti-CD28 (Schwartz, 1990). *In vivo* and *vitro* studies have shown that naive T CD4 cells from aged mice exhibit remarkable age-related changes to proliferation (Swain et al., 2005; Haynes et al., 1999). These changes resulted in part from alterations in signalling pathways in T cells including tyrosine phosphorylation, NFAT and NFκB (Haynes et al., 2004; Grossmann et al., 1991; Grossmann et al., 1995).The defects in proliferation of naive T CD4cells following stimulation generate fewer effector populations that are smaller, less differentiated and that released lower levels of Th1 and Th2 cytokines (Swain et al., 2005).

The findings from the previous chapter demonstrated that T-cells collected from human volunteers and cultured in vitro with anti-CD3/CD28 showed their maximal activation after 4 days of culture, as evidenced by the highest CD25 expression. The secreted proteins harvested from activated T-cells from young adults were shown to increase the rate of proliferation of skeletal muscle C2C12 cells. However, it remains unclear which molecular mechanisms underlie cellular activation of young and old lymphocytes, and subsequently influence the behaviour of myoblasts contributing to muscle regeneration. It is well known that Tregs, expressing FoxP3 (+) CD25 (+), are important for muscle repair via amphiregulin expression in addition to their potential role in suppressing immune responses (Burzyn et al., 2013)

Furthermore, recent reports found that circulating Growth Differentiation Factor-11 (GDF11) levels improved the functional and strength of muscle in aged mice aged 22 months (Sinha etal., 2014). Thus, the purpose of this study was to identify Tregs, FoxP3, Amphiregulin and GDF11 expression in T-cells collected from young and older human volunteers.

4.2 AIMS, OBJECTIVES AND HYPOTHESIS

The aim of this study was to measure the expression of GDF11 and amphiregulin by Tregs FoxP3 cells collected from young and older adults and then cultured in vivo and activated (or not) by anti-CD3/anti-CD28.**The objectives** were, firstly, to assess CD25 and FoxP3 receptors on young and old T –lymphocytes. Secondly, to measure the expression of amphiregulin and GDF-11 in young and old T-lymphocytes which are related to muscle repair. **The hypothesis** was that Treg FoxP3, CD25 markers and expression of amphiregulin and GDF-11 would be higher in young individuals compared to old individuals.

4.3 METHODS

4.3.1 HUMAN LYMPHOCYTES CULTURE

Lymphocytes were isolated from fresh blood samples by Ficoll-Paque PLUS (as described in 2.4.1). Lymphocytes were activated by CD3/CD28 and incubated in the presence of IL-2 in completed RPMI-1640 media for 4 days in a humidified 5% CO_2 atmosphere at 37°C. In negative controls, the cells were incubated without anti- CD3/CD28 and supplemented with IL-2 in a completed RPMI-1640 media. It was assumed that the optimal culture conditions which were established using young lymphocytes will be optimal for culturing old lymphocytes. In all the experiments, un-pooled secretomes were applied for conditioned media preparations.

4.3.2 RNA ISOLATION AND REAL TIME PCR

RNA extraction and TaqMan® Real time PCR methods were performed as described in sections 2.9 and 2.10 respectively. The following predesigned primer sets were obtained from Applied Biosystem and were used in TaqMan® detection method: GAPDH (Hs00950669-m1), a house keeping gene, Amphiregulin (Hs00950669) and GDF-11(Hs00195156-m1). GAPDH was used as a reference gene for lymphocytes (Duran et al., 2005; Lai et al., 2003; Carolan et al., 2014; Rao et al., 2006) and performed in parallel with target genes. mRNA was isolated from lymphocytes obtained from young participants (5 male, aged 18-25) years and old participants (5 male, aged 75-86) .

4.3.3 MEASUREMENT OF CD25 AND FOXP3 EXPRESSED BY YOUNG AND OLD LYMPHOCYTES

Detection of transcription factor FoxP3 (Treg marker) and CD25 (activation marker) were performed on activated young and old lymphocytes. After 4 days of activation, cells were harvested and stained with CD25 (as described in 2.6.1). To detect FoxoP3 Tregs, samples were centrifuged at 300xg for 10 minutes. The supernatant was removed and cells were fixed with 4% of Paraformaldehyde solution for 10 minutes in the dark at RT. Following incubation, cells were washed with 2 ml of Stain Buffer and centrifuged at 300xg for 10 minutes. Wash buffer was removed and cells were incubated with premeabilization buffer (1X BD Prem , San Diego, CA, USA) for 30 minutes in the dark at RT. To wash cells, 2 ml of stain Buffer was added and samples centrifuged at 300xg for 10 minutes. The supernatant was discarded and cells were stained with anti-human FoxP3 (as described in 2.6.3). CD25 and FOXP3 receptors expression

were assessed on lymphocytes obtained from young participants (5 male, aged 18-25) years and old participants (5 male, aged 78-86).

4.3.4 QUANTITATIVE MEASUREMENT OF AMPHIREGULIN IN YOUNG AND OLD SECRETOMES

Amphiregulin human ELISA of 96-well plate (abcam) was applied for the quantitative measurement of amphiregulin in young and old secretomes (as described in 2.13). Protein estimation for all samples was determined prior to quantitative measurement of Amphiregulin concentration (as described in 2.10). Amphiregulin concentrations were measured from 6 young secretome obtained from young individuals (6 male aged 18-25 years and old secretome from old individuals (5 male and one female, aged 78-86 years).

4.5 STATISTICAL ANALYSIS

One-way ANOVA was used to compare effects between all the experimental conditions of activated and non-activated lymphocytes followed by Bonferroni post test analysis. Results were presented as mean ± standard deviation (±SD). Results were statistically significant at the level of 5% ($P<0.05$) and indicated on figures or in tables using*, whereas $p<0.01$ was marked **, $p<0.001$ was marked *** and $p<0.0001$ marked as ****. The percentages of CV were determined for repeated independent experiments to compare the degree of variation from one experiment to another. % CV(s) were calculated by dividing standard deviation by the mean for each group of treated cells .

4.4RESULTS

4.4.1 GENE EXPRESSION ANALYSIS OF AMPHIREGULIN IN HUMAN YOUNG AND OLD T LYMPHOCYTES.

As it is shown in Figure (4.1), the fold difference of Amphiregulin expression in secretomes of Young activated lymphocytes compared with non-activated was significantly higher compared to Old activated vs non-activated lymphocytes (2.14±0.7 *vs* 0.54±0.08 respectively; $p<0.0001$).

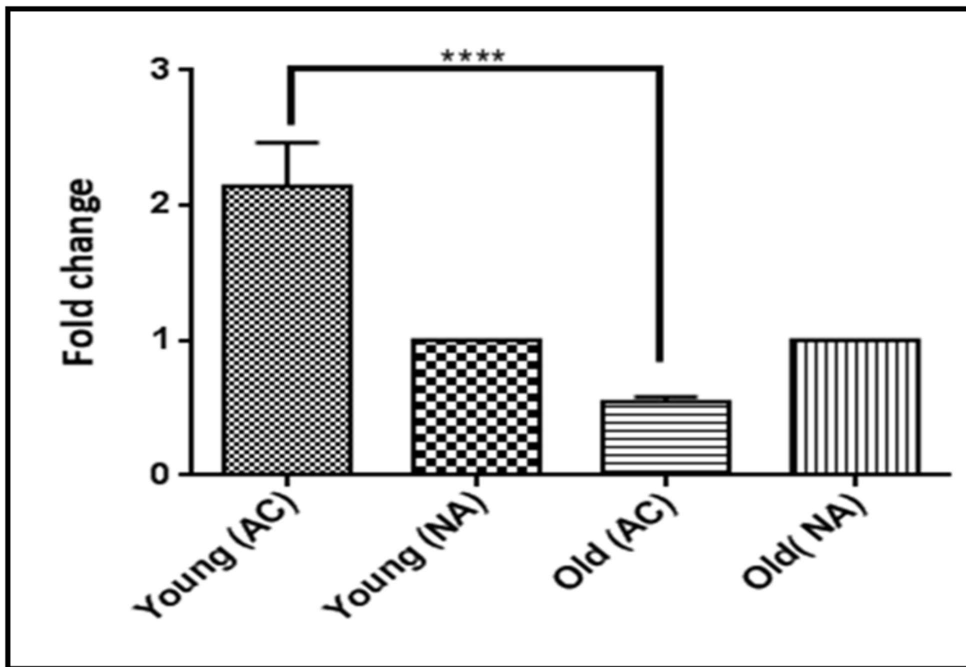


Figure 4.1: Amphiregulin gene expression in young and old activated lymphocytes after 4 days of incubation. All values are represented as a fold change to young not activated lymphocytes. Young activated lymphocytes significantly express 4 folds higher compared to Old activated lymphocytes ($p<0.0001$). The controls for this experiment were not significantly different. Results represent SD± means of (n= 5young aged 18-25 years) and (n=5old aged 75-86 years) in triplicates. AC: Activated, NA: Non-activated. %CVfor Young (AC); 34%, Young (NA); 0%, Old (AC);16% and Old(NA);7%.

4.4.2 GENE EXPRESSION ANALYSIS OF GDF-11 IN HUMAN YOUNG AND OLD T LYMPHOCYTES

The results indicated that there were no significant differences in GDF-11 gene expression in Young and Old activated lymphocytes compared with their levels seen in non-activated lymphocytes (0.8±0.4 *vs* 0.8±0.2; p>0.05 respectively)(Figure 4.2).

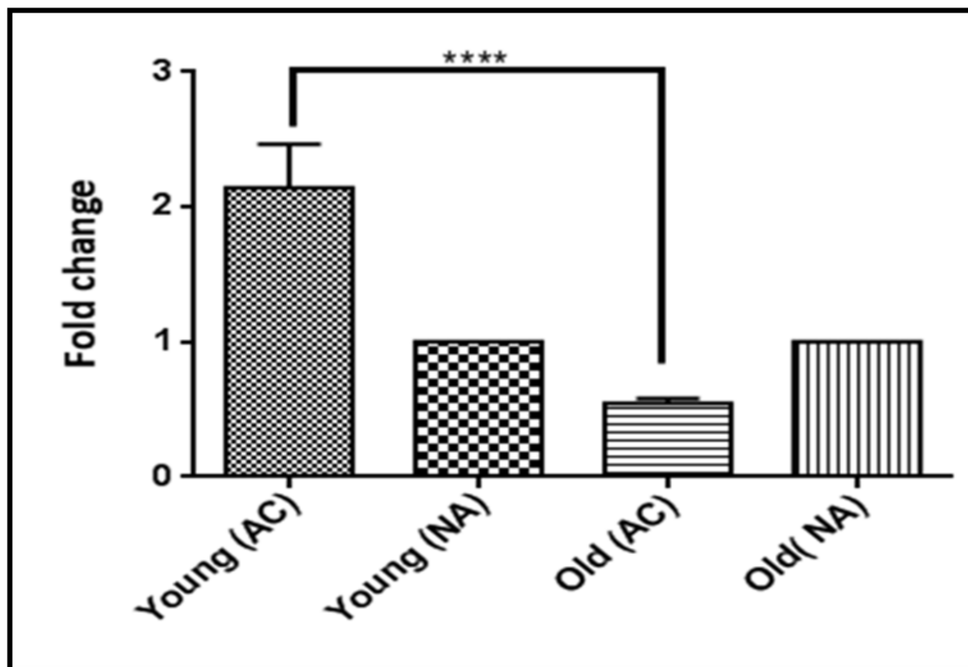


Figure 4.2: GDF-11 gene expression in young and old activated lymphocytes. Fold changes of GDF-11 for activated Young and Old lymphocytes were not significantly different (p>0.05). Results represent SD± of (n= 5 young aged 18-25 years) and (n=5 old, aged 75-86years) in triplicates. AC: Activated, NA: Non-activated. %CV for Young (AC); 52%, Young (NC); 0%, Old (AC); 23% and Old (NC);14%.

4.4. 3 QUANTITATION OF AMPHIREGULIN IN YOUNG AND OLD SECRETOMES

Quantitative analysis for Amphiregulin in Young and Old secretomes showed that the concentrations of amphiregulin in Young activated secretomes were significantly higher compared to Young non-activated and Old activated secretomes (904±323pg/ml *vs* 266±237 pg/ml; p<0.05 and 367±208pg/ml; p<0.001 respectively). Slight difference was observed between Old activated and non-activated secretomes but the difference did not reach statistical significance (367±244pg/ml and 183±208pg/ml; p>0.05)(Figure 4.3).

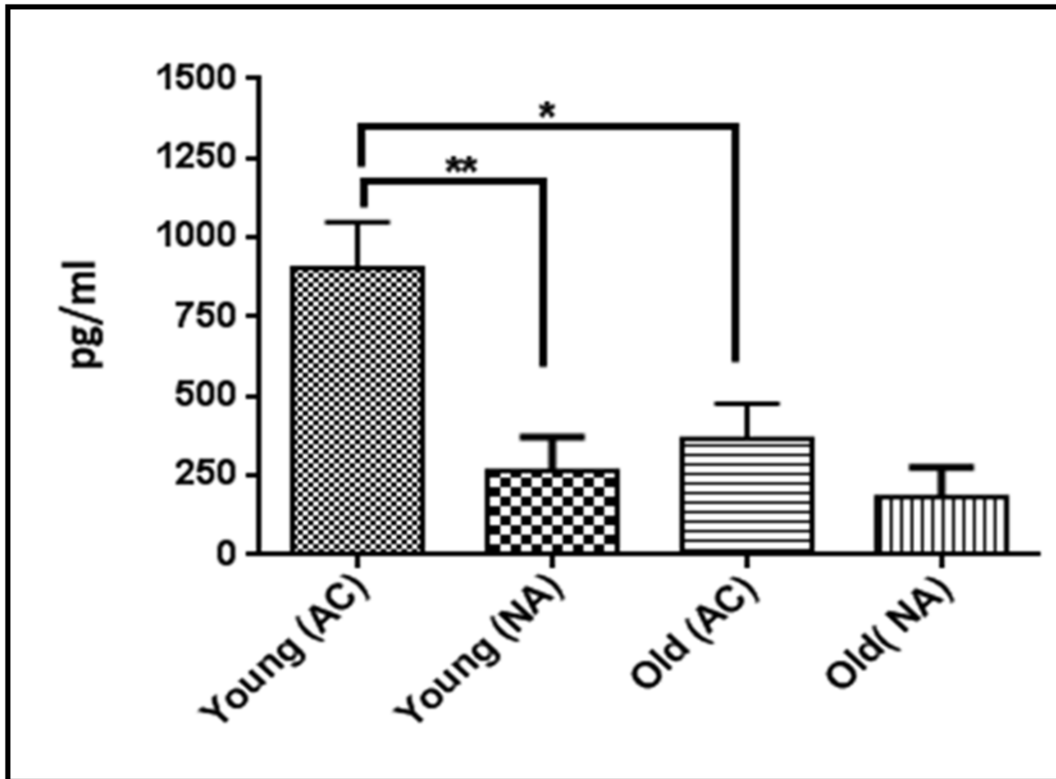


Figure 4.3: Amphiregulin concentration in secretomes of young and old lymphocytes. The concentration of Amphiregulin in young secretomes was significantly higher ($p<0.05$) compared to old secretomes. Data represent means ±SD (n=6samples of each young aged (18-25 years) and old (78-86 years) in triplicates. Ac: Activated, NA: Non-activated. %CV for Young (AC) ; 36%, Young (NA); 89%, Old (AC)67% and Old (NA); 13%.

4.4.4 MEASUREMENT OF CD25 and FOXP3 EXPRESSION BY YOUNG AND OLD LYMPHOCYTES

The results of CD25 analysis in Figure (4.4 B) indicated that %CD25 expression was approximately 3 folds higher on Young activated compared to Old activated lymphocytes (67.6±10.3% *vs* 24.6±5.5% respectively; $p<0.001$). In addition, a significant difference was observed between Young activated and non-activated lymphocytes in % CD25 expression (67.6±10% vs 9.7±5%; $p<0.01$). Similarly, the percentage of CD25 was significantly higher in Old activated compared to non activated (24.6±5.5% *vs* 4.1±3%; $p<0.05$)(Figure 4.4 and 4.6). Data analysis for FoxP3 percentage revealed that Old activated lymphocytes expressed approximately 2 folds higher than Young activated lymphocytes (35±13% vs 15±5%; $p<0.05$).

Also, significant difference was observed between Old activated and non-activated in percentages of FoxP3 expression (35±13% vs 10.5±1.3%; $p<0.05$) (Figure 4.5 and 4.6).

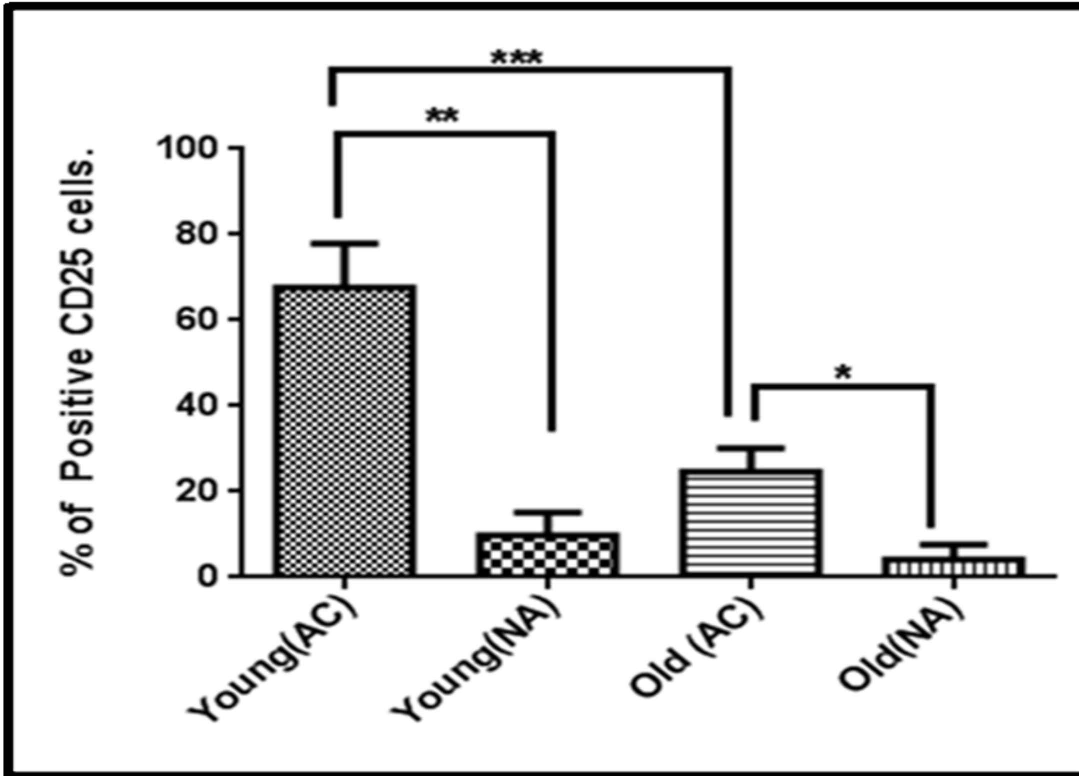


Figure 4.4: % of CD25 Young and Old lymphocytes. CD25 expression was significantly higher on Young compared to Old cells ($p>0.001$). Data represent means ±SD (n=5) of each young (22±3years) and old (80±5 years) in duplicates**.** Ac: Activated and NA: Non-activated.

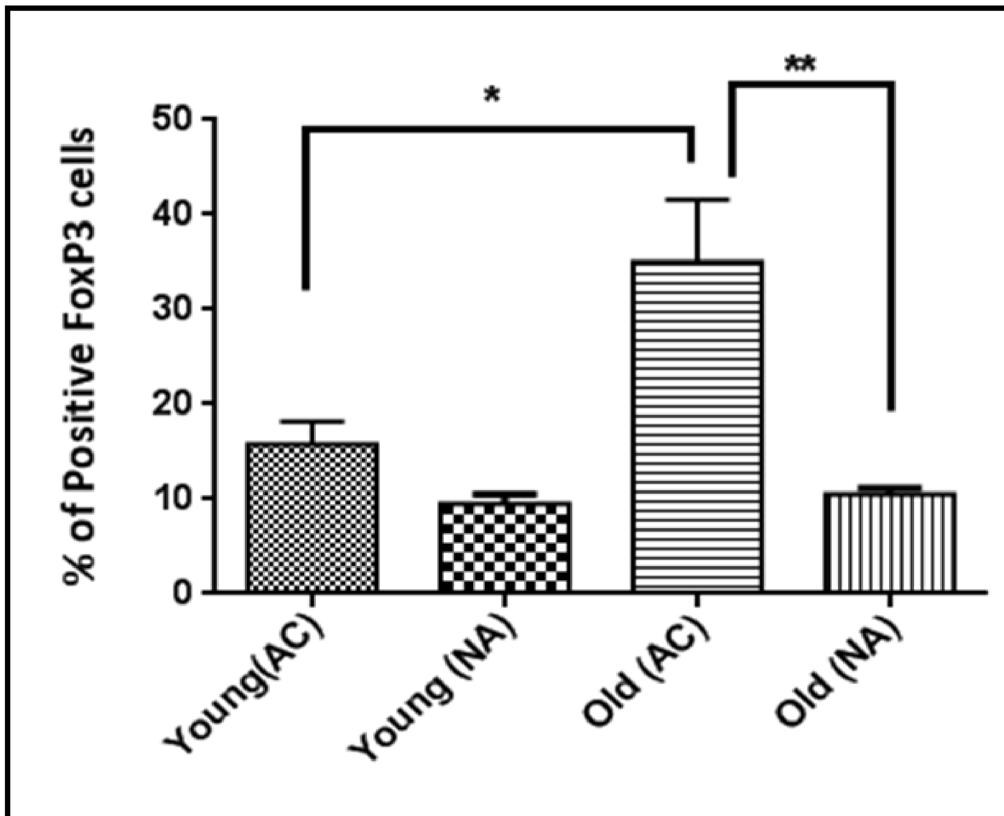


Figure 4.5:% FoxP3 in young and old lymphocytes. % FoxP3 was significantly higher on Old compared to young lymphocytes ($p<0.05$). Data represent means ±SD (n=5) of each young (18-25 years) and old (75-86 years) in duplicates.

.

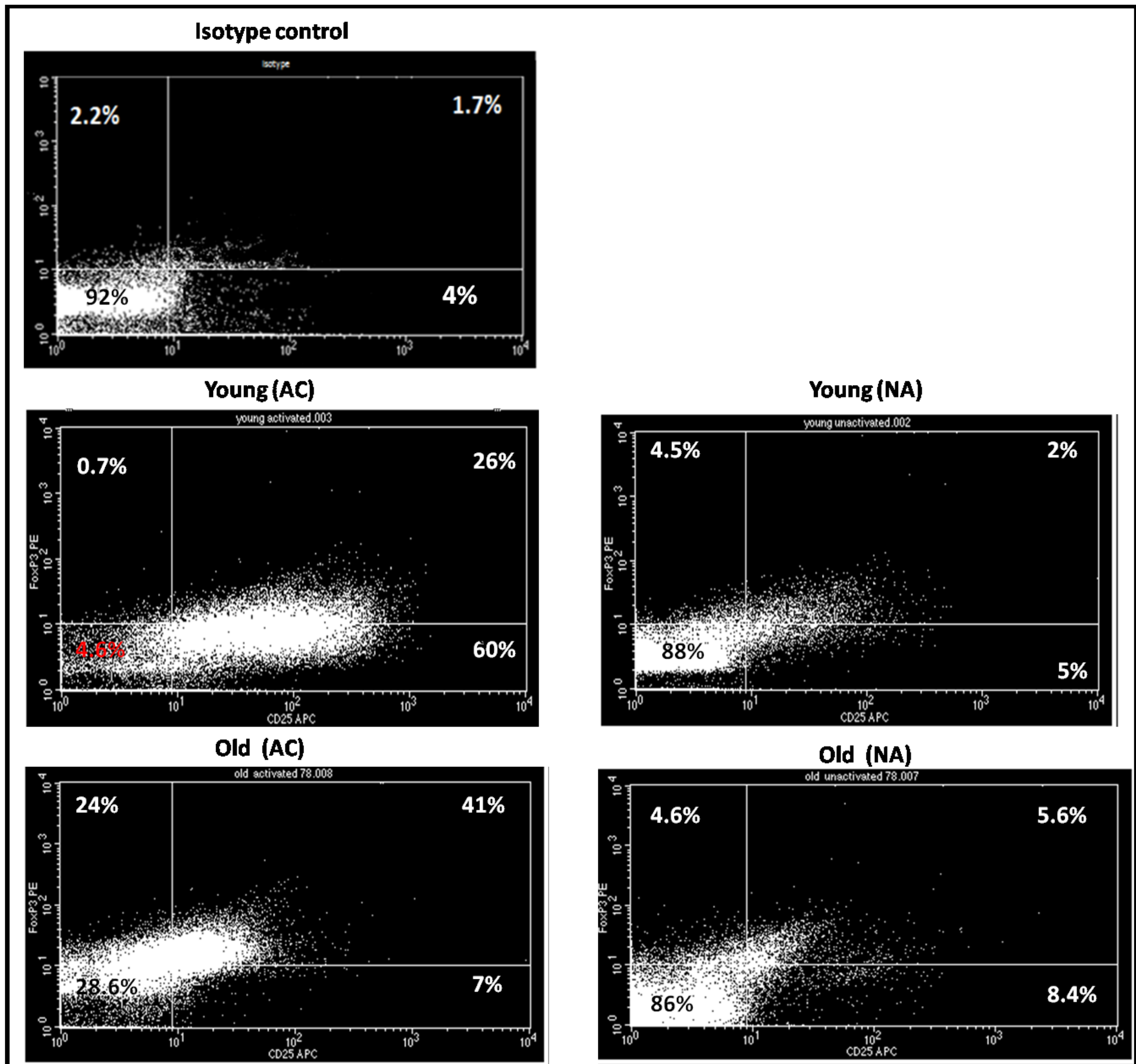


Figure 4.6: Flow cytometric analysis of PE-anti human FoxP3 and APC- anti human CD25 on lymphocytes.%FoxP3 expression was significantly higher in Old activated lymphocytes (lower panel- left) compared to Young activated lymphocytes (middle panel -left). %CD25 expression was significantly higher on Young activated (middle panel -left) compared to Old activated lymphocytes (lower panel -left). Each dot plot includes 4 quadrants which are: Upper right (+FoxP3), Lower right (-FoxP3), Upper left (+CD25+FoxP3) and Lower left is +CD25).

4.5 DISCUSSION

The results from the present study indicate that CD25/FoxP3 Tregs and Amphiregulin growth factor are subject to change with ageing. No difference was observed in the gene expression of GDF11. A recent report has shown that muscular Treg Foxp3 cells potentiate muscle repair and regeneration via amphiregulin growth factor (Burzyn et al., 2013). The results presented here provide the first evidence, to our knowledge, that Amphiregulin expressed by Young activated lymphocytes was higher compared to old activated lymphocytes. Our results were confirmed by measuring gene expression and quantification of secreted amphiregulin in young and old secretomes. Also, the current results demonstrated that reduction % of Treg FoxP3 in young activated lymphocytes tended to correspond to increase amphiregulin production.

Parabiosis studies showed that elevated levels of circulatory GDF-11 may enhance skeletal muscle repair, reverse impairment of skeletal muscle dysfunction in aged mice (Sinha et al., 2014), promote neurogenesis (Katsimpardi et al., 2014) and reverse cardiac hypertrophy in aged mice (Loffredo et al., 2013). Since GDF-11 is closely related to myostatin, which is known as a negative regulatory factor for skeletal muscle regeneration (Rı´os et al., 2002; Trendelenburg et al., 2009; Sartori et al., 2009), it seems surprising that GDF-11 positively enhanced myogenesis, in contrast to the demonstrated negative effects induced by myostatin. A recent manuscript reported that GDF-11 is similar to the myostatin, increases with ageing contributing to decreases in satellite cell proliferation and attenuates skeletal muscle regeneration in aged mice (Egerman et al., 2015). The finding of this latter study is more reliable and specific compared to the previous studies mentioned above, as the techniques applied for this work were more specific and reproducible. However, our data did not reveal any preferential influence of ageing on GDF-11 expression. One possible reason for this finding is that our work was restricted to the cytokines and growth factors secreted from lymphocytes.

However, the finding of increased Treg FoxP3 with ageing is comparable to previous studies conducted on humans which demonstrated that there is a measurable increase in the percentage of Treg FoxP3 cells in old compared to young subjects (Lages et al., 2008; Gregg et al., 2005; Gottenberg et al., 2005; Ruby and Weinberg, 2009). Others showed that the percentage of Treg FoxP3 cells following antigen stimulation was higher in skin biopsies from old compared to young individuals (Agius et al., 2009). In addition, further results from mice found expansion of absolute number of Treg FoxP3 in mice with advancing age (Chiu et al., 2007; Sharma et al.,

2006). Thus the age-related variations in immune competence may partially be a reflection of changing function in Tregs (Dejaco et al., 2006).

Muscular Tregs are different from other counterparts existing in lymphoid and non-lymphoid tissues (Burzyn et al., 2013). They display a distinct transcriptome, gene expression profile and TCR repertoire for an antigenic selection. The substantial difference in these characteristics could add an advantageous effect of muscular Tregs on skeletal muscle repair and satellite cell expansion. *In vivo* and *in vitro* studies have shown the direct effect of FoxP3 Treg on myogenesis, enhancing satellite cell proliferation and promoting effective skeletal muscle regeneration in mice (Castiglioni et al., 2015).

The most recent studies have shown that depletion of muscular Tregs altered the physiological features of muscle repair and attenuated lymphoid cells shifting from the pro-inflammatory to the anti-inflammatory phenotype in injured muscle with Ctx in mice. It is well known that myeloid cells are crucial for efficient muscle repair and that any change in the proportion of lymphoid cells can negatively affect the series of events that orchestrate muscle repair and regeneration (Tidball & Villalta, 2010). Recently, a novel study highlighted the role of Treg FoxP3 in recruiting proper muscle repair. The study demonstrated that skeletal muscle in aged mice (aged 6 months) failed to undergo proper muscle repair as a result of reducing infiltration of Treg cells in injured muscle skeletal muscle regeneration compared to young mice (2 months) (Kuswanto et al., 2016). However, in the current study, the findings of an increase in the percentage of Treg FoxP3 was not comparable to the findings of muscular Treg in the other studies mentioned. The reason could be that the measurement of % FoxP3 was conducted on *in vitro* stimulated lymphocytes from fresh blood samples of young and aged participants, not on muscle–infiltrating Treg cells.

In addition to the age-related changes in FoxP3, our data showed that there is a decline in CD25 expression, which is known as a key marker of IL-2 production upon T cell receptor (TCR) stimulation (Chao et al., 2002). The reduced expression of CD25 is consistently observed in the aged, naive CD4 T cells as a result of reduce thymic output (Salam et al., 2013; Weng, 2006). This is reflected in our results which showed that the young lymphocytes responded differently compared to old to the exogenous addition of IL-2 *in vitro*. This age- related defect in CD25 expression leads to poorly differentiated of Th1 and Th2 subsets (Goronzy et al., 2007; Arnold et al., 2011; Haynes et al., 1999). For example, it has been shown for CD4 + T cells that Th1

cell numbers decrease first, followed later by Th2 cells (Chiu et al., 2002). Th2 subset is a source of amphiregulin production (Paul and Zhu, 2010; Licona-Limón et al., 2013; Qi et al., 2009; Zaiss et al., 2006). Thus, suppression of CD25 expression in aged people (Salam et al., 2013) could lead to poor production of amphiregulin, which is known as inducer factor for Treg FoxP3 (Zaiss et al., 2006). Other factors were also shown to induce amphiregulin biological function. These include IL-1β and TNF-α (Streicher et al., 2007; Woodworth et al., 1995), IL-3and IL-4 (Qi et al., 2012). Taken all together, our data suggested that accumulating Treg FoxP3 cells, suppressor T- cells, in old adults attenuate the activation of old T- cells (Saito et al., 2010; Fessler et al., 2013) resulting in lower CD25 expression. This may explain, at least in part, the age-related impairment of the regulation of the immune response.

4.6. CONCLUSION

The percentage of Treg FoxP3 increases with ageing. The concentration of Amphiregulin growth factor is inversely associated with the percentage of Treg FoxP3 cell population. Defects of immune cells with ageing contribute to the changes of cytokines and growth factors production.

CHAPTER 5

5. CHARACTERISATION OF YOUNG AND OLD SECRETOMES OBTAINED FROM LYMPHOCYTES

5.1 INTRODUCTION

A reduction in thymus output with ageing is partly compensated by homeostatic proliferation of existing naive CD4 T cells in addition to the shifting in other T cells compartments (Linton and Dorshkind, 2004; Swain et al., 2005). The long- term maintenance of naive T cells through homeostatic mechanisms generates defects and functional characteristics of T cells. For example, it was shown that naive CD4T cells show less proliferative capacity and IL-2 production in response to antigens compared to naive CD4T cells that have not undergone homeostatic expansion (Swain et al., 2005). In addition, aged T- cell populations express lower levels of CD25 expression and T-cell receptor TCR repertoire (Swain et al. 2005; Salam, et al. 2013). *In vivo* and *i*n *vitro* studies showed that in aged mice, naive T cells express lower levels of CD25 receptors and release less IL-2 following stimulation with antigen or anti-CD3/CD28 (Swain et al. 2005). These defects in the immune response attenuate the quality of TCR signalling cascades, reducing the clonal expansion and cytokine production from naive T cells (Haynes et al., 2004). Furthermore, memory T cells from old mice exhibit major defects in initial signalling pathways, cytokine production and proliferative capacity (Haynes et al., 1999). All these observations suggest that the accumulation of impaired memory T cells in old mice could play a role in the changes of cytokines production. Alteration in cytokines production from aged naive T cells are accompanied by shifting of T cell subsets (Nagelkerken et al., 1991) resulting in an accumulation of Treg FoxP3 cells and memory cells at the expense of naive T cells (Seddiki et al., 2006, Hwang et al., 2009).

However, the possible effect of ageing on T cell subsets of human cytokines is very limited and controversial with studies indicating that the production of TNF-α. INF-y and IL-4 may be elevated, unchanged or reduced (Fagiolo et al., 1993; Sindermann et al., 1993).

In the previous chapter, the results clearly showed that aged lymphocytes express higher levels of FoxP3 and lower levels of CD25 compared to young lymphocytes. These observed differences may play a role in the production of cytokines, interleukins and growth factors. Thus, in this part of the study, the production of cytokines (Th1and Th2) and growth factor (IGF-1) from young and old individuals was examined for possible differences in T cell populations of young and old people. To achieve this, the concentrations of 15 Th1/Th2 cytokines and IGF-1 were analysed in the secretomes of6 of each young and old individuals, activated and non-

activated lymphocytes after 4 days of *in vitro* incubation.

5.2 AIM, OBJECTIVES AND HYPOTHESIS

The aim was to determine whether activated young and old lymphocytes show differences in cytokines and IGF-1 production. The objectives were to quantify the cytokines and IGF-1 from young and old lymphocytes. The hypothesis was that there are differences in cytokines and IGF-1 production from young and old lymphocytes.

5.3 METHODS

5.3.1 ISOLATION OF LYMPHOCYTES FROM FRESH BLOOD SAMPLES

Lymphocytes were isolated from fresh blood samples obtained from healthy young and old participants. Lymphocytes were isolated using Ficoll–Paque PLUS (as mentioned in 2.4.1). Prior to counting and culturing the lymphocytes, macrophages were depleted (as mentioned in 2.4.1). To mimic *in vivo* activation, isolated lymphocytes from both aged groups were cultured as described in 2.4.1. Proteins estimation (2.11) for secretomes obtained from young and old subjects were performed prior to immunoassay analysis. It was assumed that the optimal culture conditions which were established using young lymphocytes will be optimal for culturing old lymphocytes. In all the experiments, un-pooled secretomes were applied for conditioned media preparations.

5.3.2 IMMUNOASSAY ANALYSIS

Harvested secretomes from young and old participants that were used to prepare conditioned media (CM1 and CM2) were screened by multiplex analysis. Fifteen cytokines were quantified from young (n=5, aged 25-30 years) and old (n=5, aged 75-81) and IGF-1 were quantified from young (n=6, aged 25-30 years) and old (n=6, aged 75±8 years) participants (as described in 2.12.2 and 2.12.3) respectively.

5.3.3 STATISTICAL ANALYSIS

Two- way ANOVA was used to assess the interaction effect of age and activation status (activated or non-activated lymphocytes) on the cytokines followed by Bonferroni post-test analysis. Results are presented as mean and standard deviation (±SD) of the mean. Analyses were carried out using GraphPad and significance accepted as $p<0.05$. The percentages of CV were determined for repeated independent experiments by dividing standard deviation by the mean for each group of treated cells .

5.4 RESULTS

5.4.1 Th1/Th2 cytokines concentration in young and old secretomes

Table 5.1 shows the concentrations of 15 Th1/Th2 cytokines that were detected in the secretomes of young and old, activated and non-activated lymphocytes. Seven cytokines were significantly different in the secretome from Old activated lymphocytes compared with the Young activated lymphocytes. Old activated lymphocyte secretome had lower concentrations of TNF-α, IL-1β, INF-γ and IL-12 compared to Young (all $p<0.05$), and higher concentrations of IL-4, IL-5 and IL-8 (all $p<0.05$). Furthermore, cytokine concentrations in Old activated lymphocyte secretome were significantly lower than the non-activated lymphocyte secretome ($p<0.05$). Similarly, all cytokines in old activated lymphocyte secretome were significantly higher than in old non-activated lymphocyte secretome. No significant difference was observed between activated young and old secretomes in the concentrations of IL-18, MIP-α (macrophage inflammatory protein-α), SDF (stromal cell-derived factor), IP-10 (interferon gamma-induced protein 10) and GM-CSF (granulocyte macrophage-colony stimulating factor).

Table 5.1: Multiplex reading foranti-CD3/CD28 activated and non-activated lymphocyte secretome from young and old participants.

Cytokines	Young activated pg/ml	Young non-activated pg/ml	Old activated pg/ml	Old non- activated pg/ml	Young vs Old (P value)
IL-1β	694±118	53±18	181±61	51±9	×†
IL-4	256±76	25.24±12	1187±300	22.3±13	×†‡¥
IL-12	24±10	1.±1	10±3	2±1	×†
INF-γ	21985±259	150±123	19391±5962	312±57	†‡¥
TNF-α	7949±581	18±4	5524±406	33±3	×†‡
IL-5	829±140	6±4	1820±388	39±20	×†‡¥
IL-6	7609±1749	7442±385	3613±554	1580±117	¥
IL-8	2481±89	2427±166	22652±1000	2520±68	×‡¥
IL-2	1610±1496	466±377	1378±600	257±67	ns¥
IL-13	4565±2369	111±60	6170±58	192±75	†‡¥
IL-18	1129±205	116±1	1101±12	94±18	†‡¥
MIP-a	4932±355	230±43	5751±1671	387±96	†‡¥
SDF	9943±4976	1009±4571	9592±9080	8015±1944	‡¥
IP-10	3419±1259	885±600	2903±1150	409±236	ns¥
GM-CSF	32841±15172	82±85	22652±10234	82±10	†¥

All data were statistically analysed by one-way ANOVA and two-way ANOVA, p values of young vs old only were shown in the table.×is young activated vs old activated. †indicates young activated vs young non-activated. ‡ indicates old activated vs old non-activated. ¥ indicates the interaction effect of age and activation status on the cytokines. Results represent the mean ±SD for 105 young and 5 old) subjects. Samples were tested in triplicate.

5.4.1 IGF in young and old secretomes

The concentrations of IGF-I was measured directly from the secretome (i.e. the secretome was used directly, not after dilution into the conditioned media). The young lymphocytes, activated *in vitro* using anti-CD3/CD28, secreted approximately 30% more (1100 ±84 ng/ml; p<0.005)

IGF-I compared to Old CM1 (845 ±68 ng/ml; $p<0.005$) and compared to the non-activated Young lymphocytes (both $p<0.05$). There was no significant difference between the Old activated and non-activated lymphocytes in IGF-I concentrations. Data are shown in Figure 5.1.

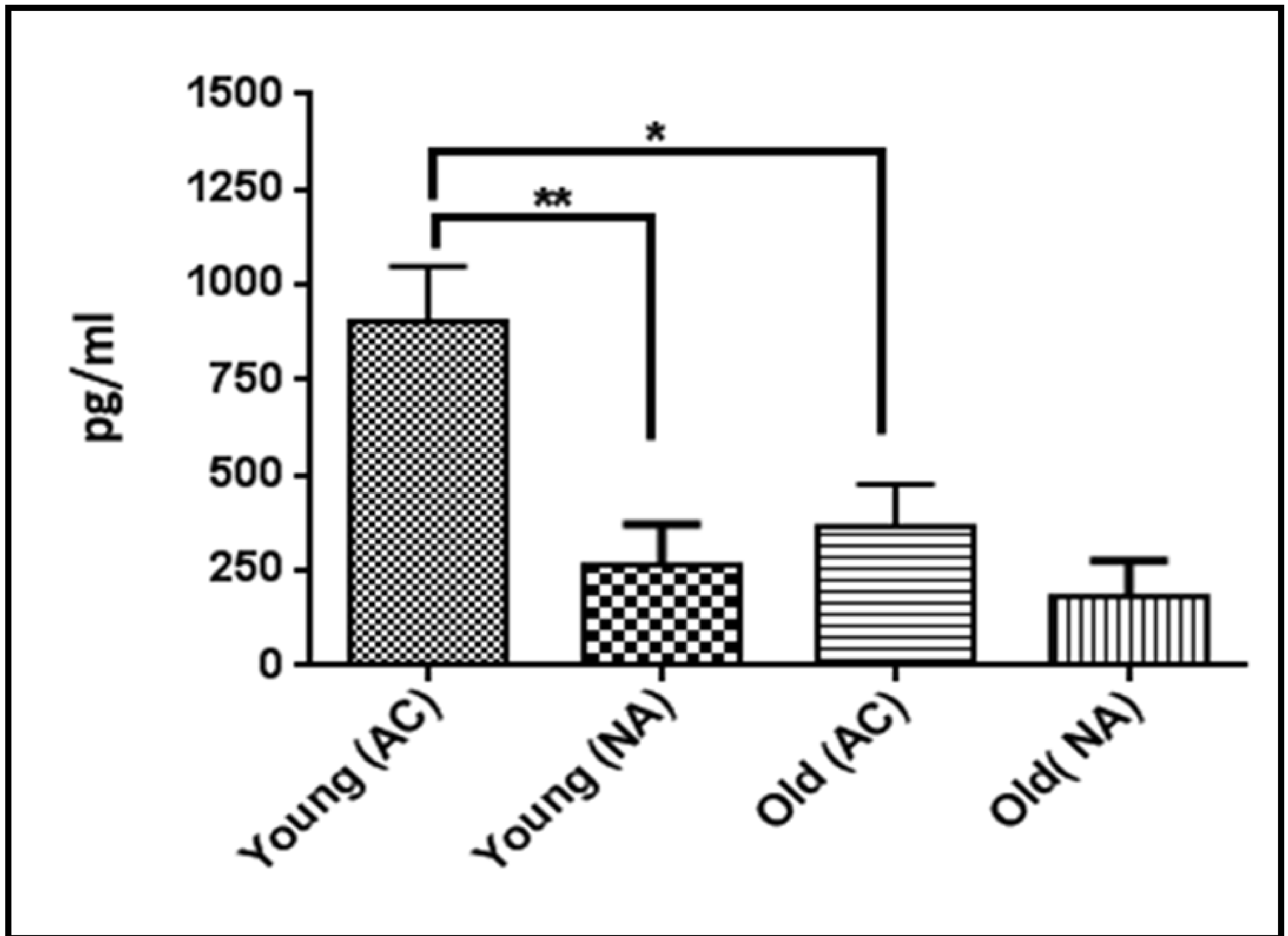


Figure 5.1: IGF-1 concentrations in young and old secretomes of activated and non-activated lymphocytes. The concentration of IGF-1 in young positive secretomes was significantly higher compared to old activated secretomes ($p<0.01$). Results represent the mean ±SD for 12 (6 young and 6 old) subjects. Samples were tested in triplicate .%CV for Young Activated;7%, Young Non-activated ;7%, Old Activated ;10% and Old non-activated 17%.

5.5 DISCUSSION

Age associated thymic involution has been proposed as aprinciple factor that influences the hormones and cytokines production(Salam et al., 2013).

Our results showed that IGF -1 concentration in the old secretome was significantly lower compared to young activated secretome. IGF-I is secreted by skeletal muscles and plays a key role in their growth, differentiation and repair. Two previous reports showed that IGF-I is also secreted by activated T-lymphocytes (Leng et al., 2009; Merchav et al., 1988; Johnson et al., 1992). Our results showed 30% (1100ng/ml± 84) higher IGF-I levels secreted by young activated lymphocytes compared to old (845ng/ml±68) when measured directly from the secretomes.

The multiplex cytokine results showed that IL-1β, TNF-α, IL-12, and IFN-γ were significantly lower in old activated lymphocytes compared to young, while IL-4, IL-5 and IL-8 were higher in old compared to young. Previous work demonstrated that TNF-α and IL-6 were decreased in old participants compared to young men following *in vitro* LPS stimulation (Bruunsgaard et al., 1999). The finding of this study support our study that showed decreased levels of inflammatory cytokines IL-6 and TNF-α in old subjects. It was suggested that TNF-α promotes skeletal muscle wasting by inhibiting the IGF signalling pathway (Grounds, 2002). These results are similar to our findings which showed lower concentrations of TNF-α, IL-6 and IGF in old activated compared to young.

Our data showed that IL-2, IL-12 and IFN-y were lower in old secretomes compared to young. Several studies have reported that the combination of these cytokines is essential for T lymphocyte recruitment and activation. IL-12 is an inducer for Th1 responses (Van der Pouw Kraan et al., 1997) augments IFN-γ production and induces IL-2R alpha-chain (CD25 expression) resulting in the expansion of Th1 clones (Kohno et al., 1997; Leong et al., 2014). In addition IL-12 is a critical factor required for optimal IL-2-dependant proliferation, stimulation and clonal expansion of T lymphocytes (Valenzuela et al., 2002). This effect is mediated through up-regulation of CD25 expression. Moreover, both cytokines act synergistically to induce IL-12αR expression, promoting cross-regulation of receptor expression (Valenzuela et al., 2002).

Production of IL-2 is indicative marker for TCR stimulation of naive CD4 cells. TCR stimulation leads to proliferation and differentiation to Th1 and Th2. It is well known that IL-2 reduces with ageing, resulting in poor regeneration of either Th1 or Th2 response and altered

cytokines production. However, our results showed lower production of IL-2 from old lymphocytes compared to young was not significant, this could be due to the exogenous addition of IL-2 that may have restored the capacity of T cells to respond to IL-2.

The results of the current study show an age –related increase of IL-4. Our data was confirmed by prior studies which showed increased IL-4 production with age (Sindermann et al., 1993; Arnalich et al., 1994; Nijhuis et al., 1994; Born et al., 1995). IL-4 is as anti-inflammatory cytokine released from Th2 cells. It has pleiotropic effects on the secretion of other cytokines. For example, IL-4 was found to attenuate the secretion of IL-6, TNF-α and IL-1-β. Hence, the reduction of IL-1-β, TNF-α and IL-6 in old secretomes could result from the inhibitory effect of IL-4 (Nolan et al., 2005; Te Velde et al., 1990; Zhou et al., 1994).

Age-related increase in IL-8 production was also observed. This finding is in agreement with a study that found elevated expression of IL-8 by stimulated lymphocytes of the elderly (Rink et al., 1998). Consistently, another study demonstrated detection of elevated level of pro-inflammatory IL-8 in plasma of aged individuals (Podkowka et al., 2001).

Altered production in IL-18, MIP-a, SDF, IP-10 and GM-CSF were also observed in secretomes of activated young and old secretomes. Albeit the changes in expression of these cytokines were not statistically significant, but they may exert some effects in combination with each or other cytokines, although this cannot be confirmed from the present studies.

IL-18 was higher in young compared to old secretomes. This factor is known as an inducer for INF-y (Dinarello, 2006), however, in the current study, the elevated expression of INF-y in activated young secretomes is unlikely to be induced by IL-18 since its concentration is slightly different in old compared to young secretomes.

Similarly, our data showed higher concentration of GM-CSF in activated young compared to activated old secretomes (Kim et al., 2011). Decline in GM-CSF levels with ageing affects the transmembrane signalling and function of immune cells, which in turn increase the susceptibility to infections (Seres et al., 1993).

Low concentrations of SDF were detected in old secretomes compared to young. This candidate is involved in mediating migration of mesenchymal stromal cells *in vivo and in vitro* (Marquez-Curtis and Janowska-Wieczorek, 2013); its influence is accelerated in combination with TNF-α (Fu et al., 2009).

MIP-α is a chemokine which is associated with the development of several diseases such as rheumatoid arthritis and coronary heart disease. Our results showed higher levels of MIP-α in activated old secretome compared to activated young. Elevated expression of MIP-α in the supernatant of old lymphocytes caused an enhanced leukocyte chemotaxis response compared to old (Chen et al., 2003), suggesting that the age-related change in T-cell chemokine expression influences the functional consequence of immune cells.

In our case, these factors acting separately or combined may have resulted in a comprise in the efficiency of the immune system and ultimately affect the capability of lymphocytes to produce cytokines as well as growth factors.

5.6 CONCLUSION

In conclusion, significantly lower concentrations of TNF-α, IL-1β, INF-y and IL-12 were detected in the secretome from old activated lymphocytes compared to young activated lymphocytes, and higher concentrations of IL-4, IL-5 and IL-8. Old activated secretome had lower concentrations of SDF, IL-18 and GM-CSF compared to young activated secretome and lower concentrations of MIP-α.

CHAPTER 6

6. THE EFFECT OF YOUNG AND OLD ACTIVATED CONDITIONED MEDIA ON C2C12 PROLIFERATION AND MIGRATION

6.1 INTRODUCTION

The progressive loss of skeletal muscle mass and strength characteristic of old age is associated with a failure to appropriately activate the satellite cells (Goldspink et al., 1994; Corsetti et al., 2008), impaired proliferative capacity to produce enough myoblasts essential for muscle regeneration (Conboy et al., 2003) and also slowed rates of migration(Price et al., 2007). The failure to activate and proliferate satellite cells in older age is not necessarily related only to intrinsic deficiencies, but the microenvironment is also implicated (Dumke and Lees, 2011; Barberi et al., 2013). For example, skeletal muscle grafted from a young rat and transplanted into an old host showed impaired regeneration, but conversely muscle tissue grafted from an old rat showed extensive regeneration after transplantation into a young host(Carlson and Faulkner, 1989). In a Heterochronic Parabiosis model, skeletal muscles of older mice regenerated after the circulation was shared with younger mice (Conboy et al., 2005). In addition, young satellite cells cultured with serum from old mice showed impaired proliferation, while old satellite cells cultured with young plasma proliferated normally (Conboy et al., 2005). These findings suggest that circulating factors, inherent in aging, negatively impact satellite cell activation and muscle regeneration. In Chapter 3, the results show that the proteins secreted by young activated lymphocytes elicit marked regulatory effects on skeletal muscle proliferation and differentiation; enhancing the former and postponing the latter (Al-Shanti et al., 2014). In addition, the results of the previous chapters show that activated lymphocytes from young donors show differences from old donors in CD25, amphiregulin and FoxP3 expression, and also in their cytokines and IGF-1secretion. Therefore, in this chapter, the secretome from freshly isolated peripheral blood mononuclear cells (PBMC) derived from young and older men was examined for their effects on the rates of proliferation, differentiation, and migration of myoblasts.

In this study, lymphocytes were isolated from the fresh blood of young (n=20, aged 21±3 years)and old (n=18, aged 81±3 years) men. After 4 days of activation and prior to C2C12 treatment with young and old secretome, lymphocytes from both groups were counted and the protein concentration for young and old secretomes were estimated. The un-pooled harvested young and old secretomes were used to prepare conditioned media(CM1-CM3) for C2C12 experimentation. The C2C12 cells were cultured with differentiated media (DM) containing

serum obtained from young volunteers (n=7, aged 18-25 years) and old volunteers (n=7, aged 78-85 years). Conventional DM supplemented with horse serum was set as control for C2C12 differentiation. The morphological changes, differentiation parameters and migration for young and old CM- treated myoblasts were determined. All the results in this chapter were published in *Physiological Reports* (Al-Dabbagh et al., 2015).

6.2 AIMS, HYPOTHESIS AND OBJECTIVES

The aims of this part of the research were to establish whether age –related changes in cytokines and growth factors released by lymphocytes affect C2C12 proliferation, differentiation and/or migration. **The objectives** in this chapter were firstly; to compare the effect of young and old conditioned media, which was prepared from activated young and old secretomes, on C2C12 proliferation and migration. Secondly, to compare the ability of myoblasts to terminally differentiate in the presence of 2% of either young or old human serum and the conventional DM.

The hypothesis was that conditioned media prepared from lymphocytes isolated from young adults would improve proliferation and migration of C2C12 cells, while the effects of lymphocytes from older adults would attenuate proliferation and migration. Similarly, the age of human serum donors would have a different effect on the differentiation of C2C12 compared to horse serum.

6.3 METHODS

6.3.1 ISOLATION OF LYMPHOCYTES FROM FRESH BLOOD SAMPLES

Lymphocytes were isolated from fresh blood samples obtained from healthy volunteers (n=20, 3 female and 17 male, aged 18-25years) and healthy old participants (n=18, 2 female and 16 male, aged 78-85 years). Lymphocytes were isolated using Ficoll–Paque PLUS (as mentioned in 2.4.1). Prior to counting and culturing the lymphocytes, macrophages were depleted (as mentioned in 3.3.2). Cells were cultured at 37°C, 5% CO_2 for 4 days (Al-Shanti et al., 2014) in supplemented RPMI- 1640 in the absence or presence of 5µg/ml anti-CD28. Following 4 days of activation, lymphocytes obtained from young and old participants were counted by haemocytometer. In all the experiments, un-pooled secretomes were applied for conditioned media preparations.

6.3.2 RECOVERY OF SECRETED CYTOKINES

The secretome was harvested for each treatment group separately (activated and non-activated; young and elderly) filtered and purified using an Ultracel-3 membrane tube. The method is detailed in 3.3.6. The harvested secretomes were assessed for protein concentration (as described in 2.10) and equal concentrations were used in all the following experiments. In all experiments un-pooled secretomes were applied for conditioned media preparations.

6.3.3 CONDITIONED MEDIA (CM) PREPARATION AND C2C12 TREATMENT

Using the methods described in section 3.3.6, four different CMs were prepared from young and old secretomes. These were as follows: CM1 young and old (test treatment), CM2 young and old (negative control); CM3 boiled young and old and CM4 (negative controls). C2C12 myoblasts were maintained by growing cells in (GM) as described in 2.2.1.1 and treated with CMs as described in 2.4.3.

6.3.4 MORPHOLOGICAL DIFFERENTIATION AND QUANTIFICATION OF THE PROLIFERATION AND DIFFERENTIATION PARAMETERS.

After 4 days of CMs treatment, C2C12 cultures were stained and incubated with Texas Red®-X Phalloidin, Anti-Myosin Heavy Chain (MyHC) Alexa Fluor® 488 and DAPI nuclear counterstain solution as described in 2.8. The parameters of differentiation were assessed using ImageJ software (Schneider, Rasband, Eliceiri, et al., 2012) to evaluate the morphometric parameters for myotube development as described in 2.8. The differentiation parameters included: fusion index% (**FI %),** the total myotube area% (**MA %**), (Ricotti et al., 2011; Ren et al., 2008) and the aspect ratio (**AR**) (Grubiši et al., 2014).Ten random microscopic fields were scanned for each parameter at 10x magnification.

6.3.5 CELL CYCLE ANALYSIS

After 4 days of CMs treatment, C2C12 cells were collected for analysis by flow cytometry. The method was discussed in section 2.6.4.

6.3.6 MIGRATION STUDY

Migration study was undertaken as described in section 2.7.

6.3.7 QUANTIFICATION OF DIFFRENTIATION PARAMETERS OF C2C12 CULTURED IN DM SUPPLEMENTED WITH YOUNG AND OLD HUMAN SERUM

Human sera were separated from fresh blood samples from young volunteers (n=7, male, aged20-28 years) and old volunteers (n=7, male, aged 78±81 years) as described in 2.5. Two types of DM were prepared ;Young human sera DM (DMEM), supplemented with 1% L-glutamate and 1% 10000U (Penicillin/Streptomycin) and 2% heat inactivated (hi) young human serum; and old human serum DM (DMEM) media supplemented with 1% L-glutamate and 1% of 10000U (Penicillin/Streptomycin) and 2% hi old human serum. In all experiments were devised using unpooled sera to prepare DM.

6.3.8 MORPHOLOGCAL ASSESSMENT HUMAN SERA CELL CULTURE

Cells were grown in pre-gelatinised 12 well- plate in GM until confluency of 70-80% was attained. Following 24 hours, cells were washed with PBS and young and old human sera DM was added to cell monolayers and incubated for 5 days in a humidified incubator at 37 °C and 5% CO_2. Conventional DM was also set as a control. Media was refreshed at day 3. Cells were stained with (MyHC) Alexa Fluor® 488 and DAPI nuclear counterstain as described in 2.2.4. The morphological differentiation parameters of myotubes including FI % (%Fusion Index), Aspect Ratio (AR) and Myotube Area% (MA %) were calculated using image J as described in 2.8.

6.3.9 STATISTICAL ANALYSIS

One-way ANOVA was used to compare effects between all the experimental conditions followed by Bonferroni post test analysis. Results were presented as mean ± standard deviation (±SD). Analyses were carried out using GraphPad and significance accepted as $p<0.05$ and marked with * where $p<0.001$ marked with **, and $p<0.0001$ denotes with ***. The percentages of CV were determined for repeated independent experiments by dividing standard deviation by the mean for each group of treated cells .

6.4 RESULTS

6.4.1 MOPHOLOGICAL DIFFERENCES IN THE C2C12 MYOBLASTS TREAED WITH CMs OF YOUNG AND OLD SECRETOME

To evaluate the degree of differentiation and proliferation, immunofluorescence of a Texas Red®-X Phalloidin (Figure 6.1) and a high affinity Alexa Fluor-488 MyHC and DAPI nuclear counterstain were performed (Figure 6.2).

Analysis of differentiation parameters FI%, MA% and AR, (Figure 6.3) showed that Young CM1-treated myoblasts exhibited reduced differentiation and extended proliferation, while in Old CM1-treated myoblasts the majority of cells exhibited myotube formation, thus confirming data above. This was evident as lower FI% in Young compared to Old CM1 (5±0.7% vs 14.7±1.6%; respectively. $p<0.001$.) and few myotubes as shown by very low %MA in Young compared to Old (2.4±0.9% vs 12±0.8%; respectively, $p<0.001$). The AR was significantly higher for myotubes in Old CM1 compared with Young CM1 (13.3±1.3, 2.8±8.7, respectively; $p<0.05$).

There was no significant difference in % FI, %MA and AR for the control conditions of Young and Old CM2 and CM3 and CM4 (24±3.6, 34±8 vs 29±4, 32±2.5 vs 41±2, respectively; $p>0.05$),(15.6±6, 18±2 vs 25±2, 25±3 vs 15±3, respectively; $p>0.05$) and (17±4, 15±1 vs 16±3, 15±3 vs 14±3; respectively; $p>0.05$).

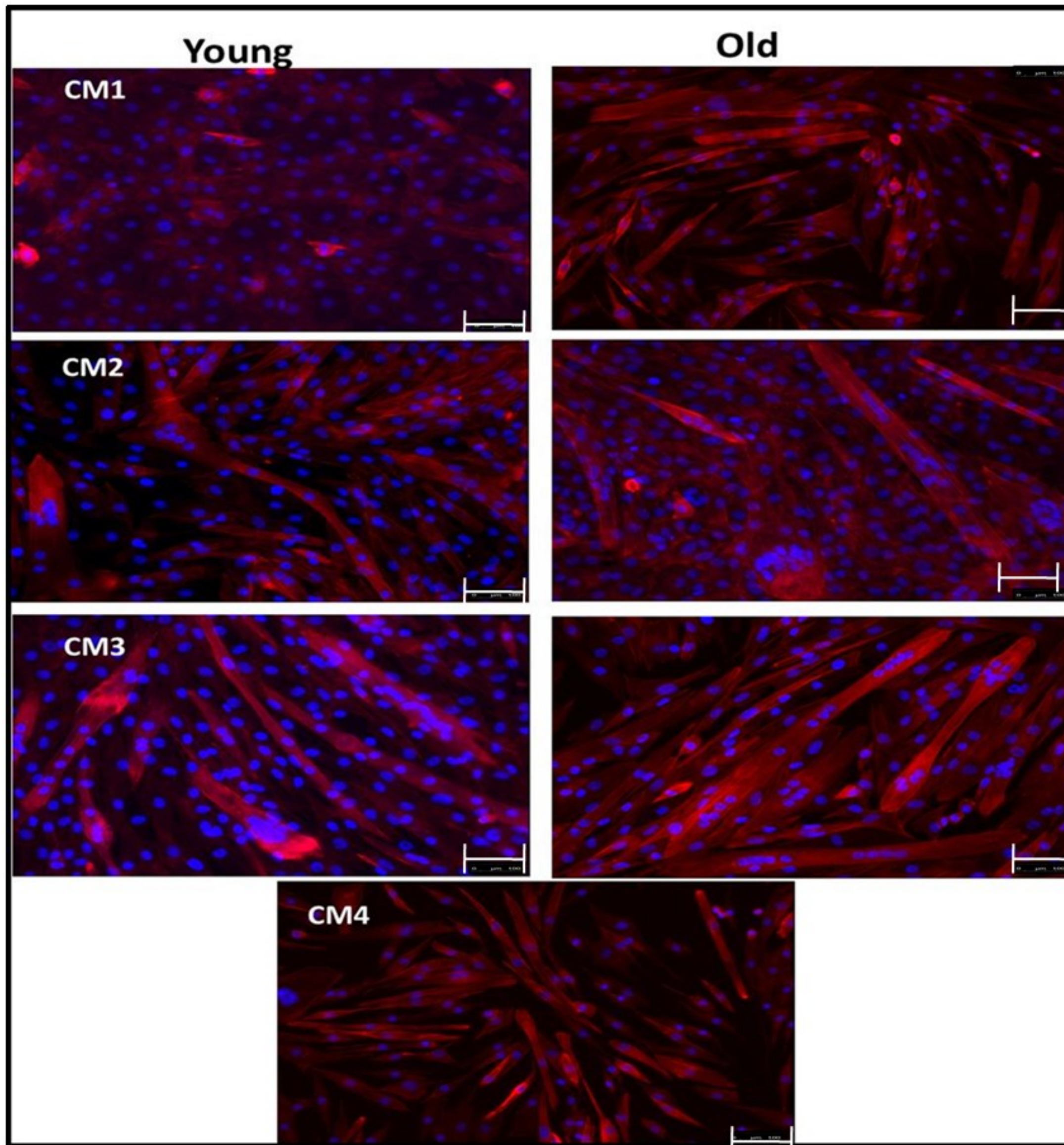


Figure 6.1: Representative images of C2C12 cells following 4 days incubation with CMs. C2C12 cell cultures stained with Texas Red®-X Phalloidin and DAPI (blue) nuclear counterstain. Young CM1-treated myoblasts exhibited an extended proliferation (upper left panel), while the majority of Old CM1-treated myoblasts exhibited differentiation to myotubes formation (upper right panel). All negative controls develop myoblasts fusion into myotubes following 4 days of incubation. Images were obtained by a Leica microscope at x10 Mag. Scale bar=75µm.

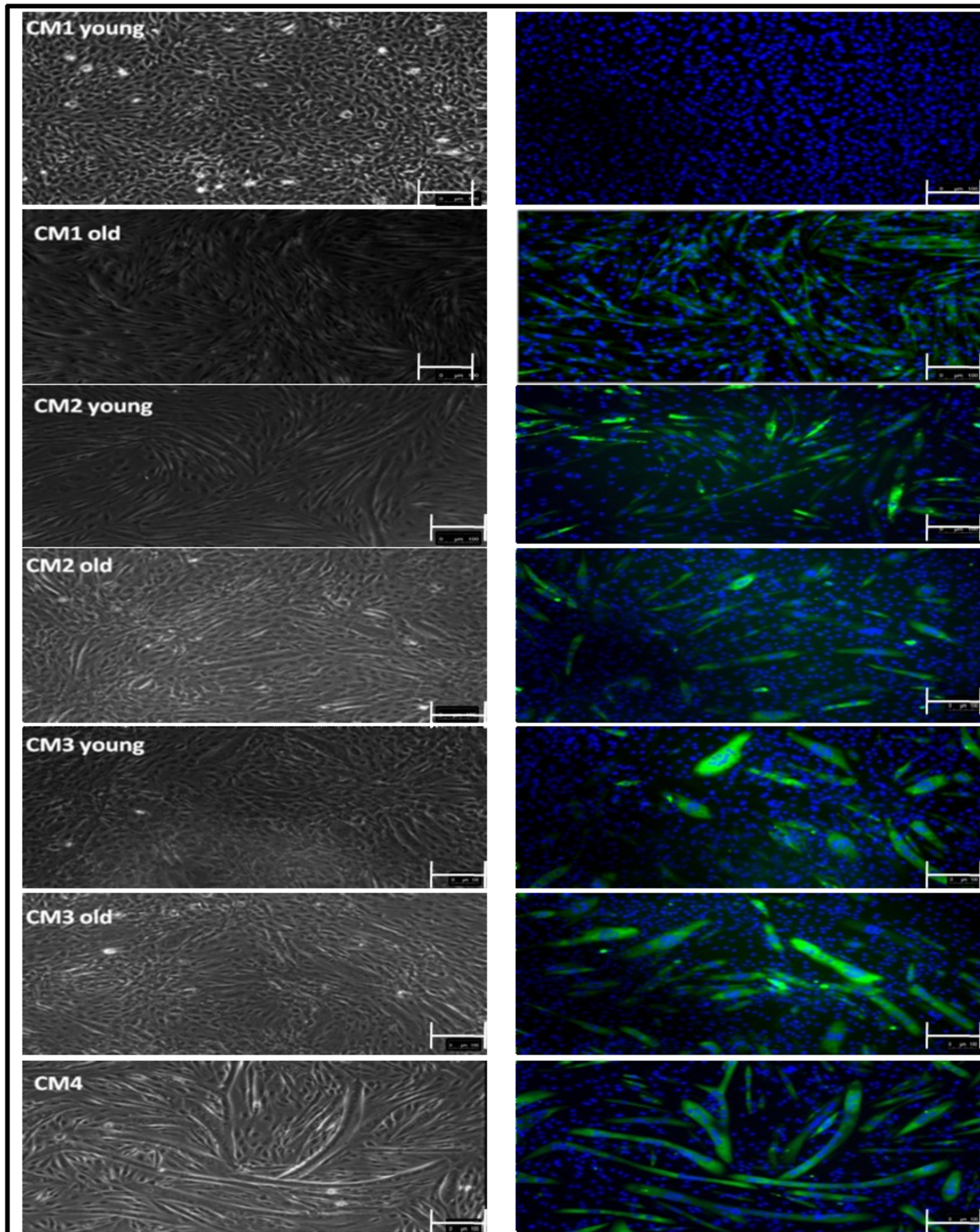


Figure 6.2: Immunofluorescence analysis of skeletal myosin heavy chain (MHC). Representative images of C2C12 after 4 days of CMs treatment (right panel) and C2C12 stained immunofluorescence of a high-affinity MHC (marker of differentiation) heavy-chain Alexa Fluor-488-MyHC (green) and DAPI (blue) nuclear counterstain (left panel). CM2, CM3 and CM4 develop myoblasts fusion into myotubes following 4 days of incubation. Images were taken from random fields by Leica DMI6000B microscope at x10Mag. Scale bar: 75µm**.**

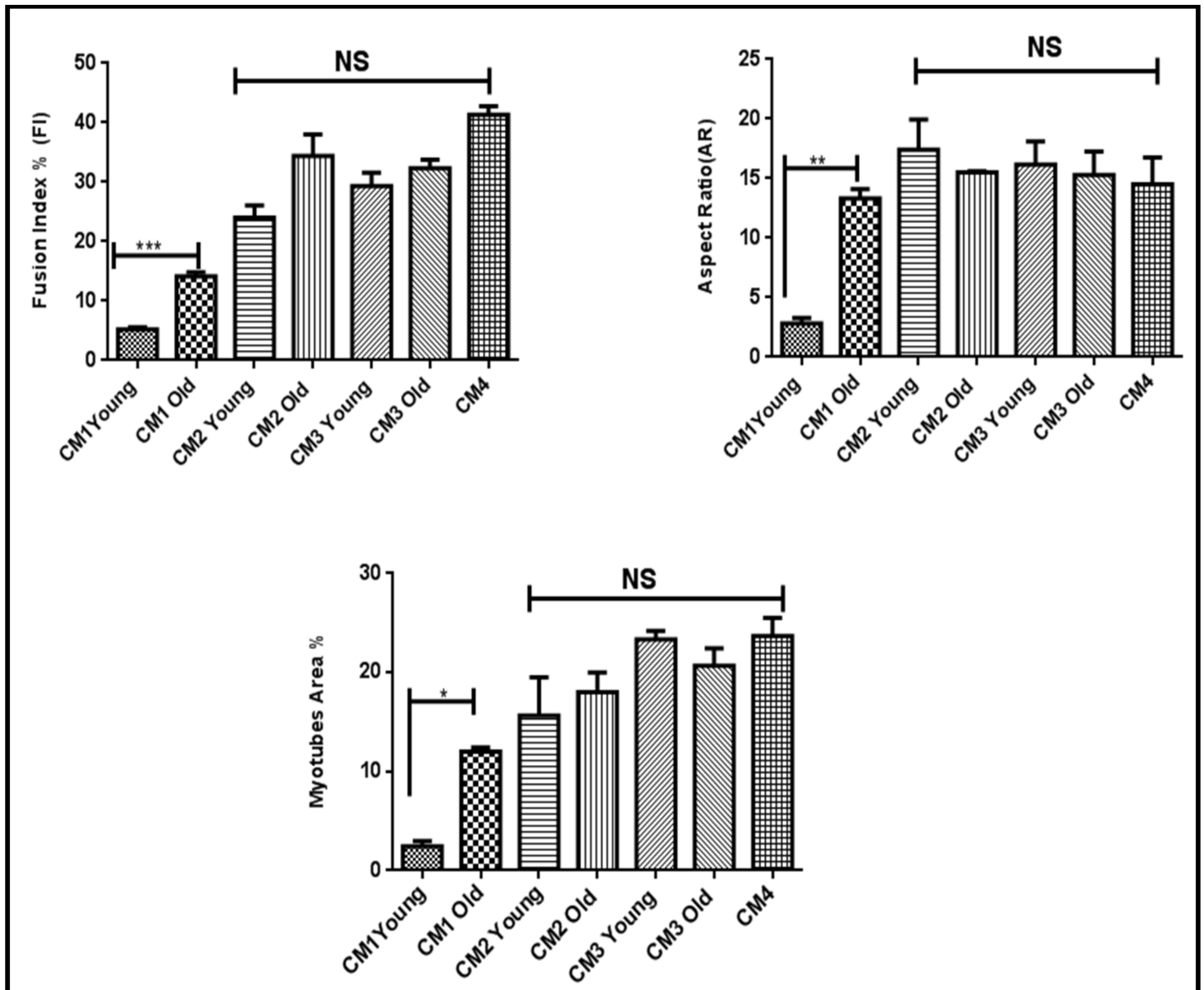


Figure 6.3: Differentiation parameters analysis of young and old secretome treated-myoblasts. %FI: Young CM1-treated myoblasts exhibited less differentiation and an extended proliferation thus lower FI%, lower fused nuclei and extended mononuclear cells compared to Old CM1-treated myoblasts that exhibited differentiation to myotubes formation and significantly higher fused nuclei per myotubes (>2 nuclei),($p<0.001$). AR: The aspect ratio (AR) of myotubes in cells treated with old CM was higher compared to cells cultured in young CM, ($p<0.05$). MA%: The total area of myotubes over the entire image in cells cultured with young CM1 was significantly lower compared to cells treated with Old CM1, ($p<0.0001$). Ten random microscopic fields were scanned for each parameter at 10x magnification. The data represent the means ±SD (n=20) from young and (n=17) from old in duplicates. CM1: Activated conditioned media, CM2: Non-activated conditioned media,CM3: Boiled activated conditioned media and CM4 : no lymphocyte conditioned media.

6.4.2 DIFFERENTIATION OF C2C12 FOLLOWING YOUNG CM1 WITHDRAWAL

Differentiation is a key step during myogenesis. Young CM1 was withdrawn following two days of culture and a standard differentiation media was applied for the subsequent five days. The myoblasts differentiated by aligning and fusing to form myotubes (Figure 6.4).

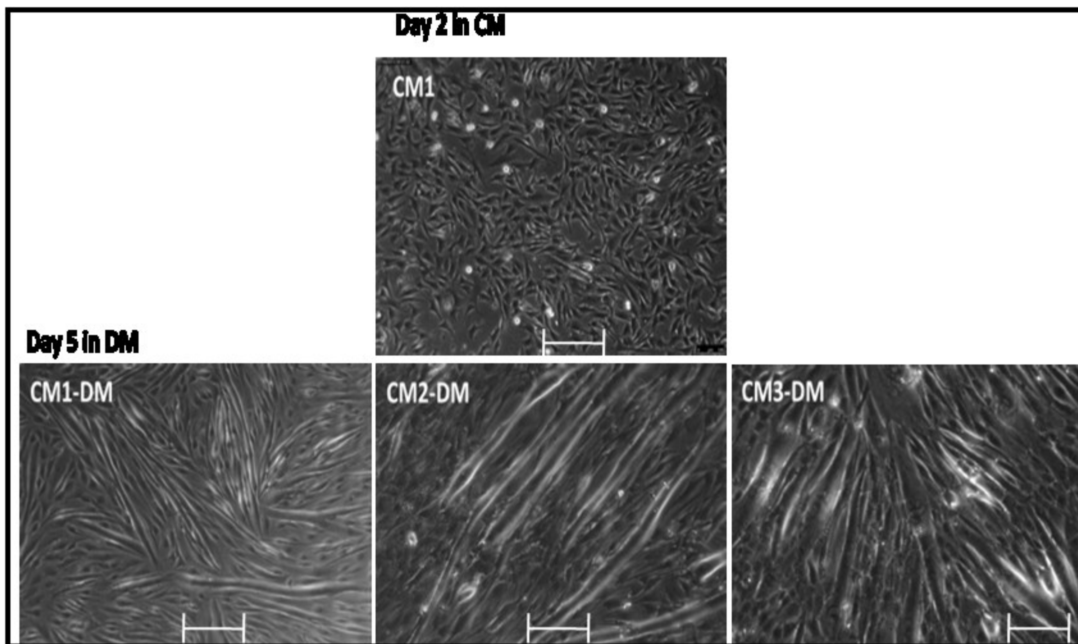


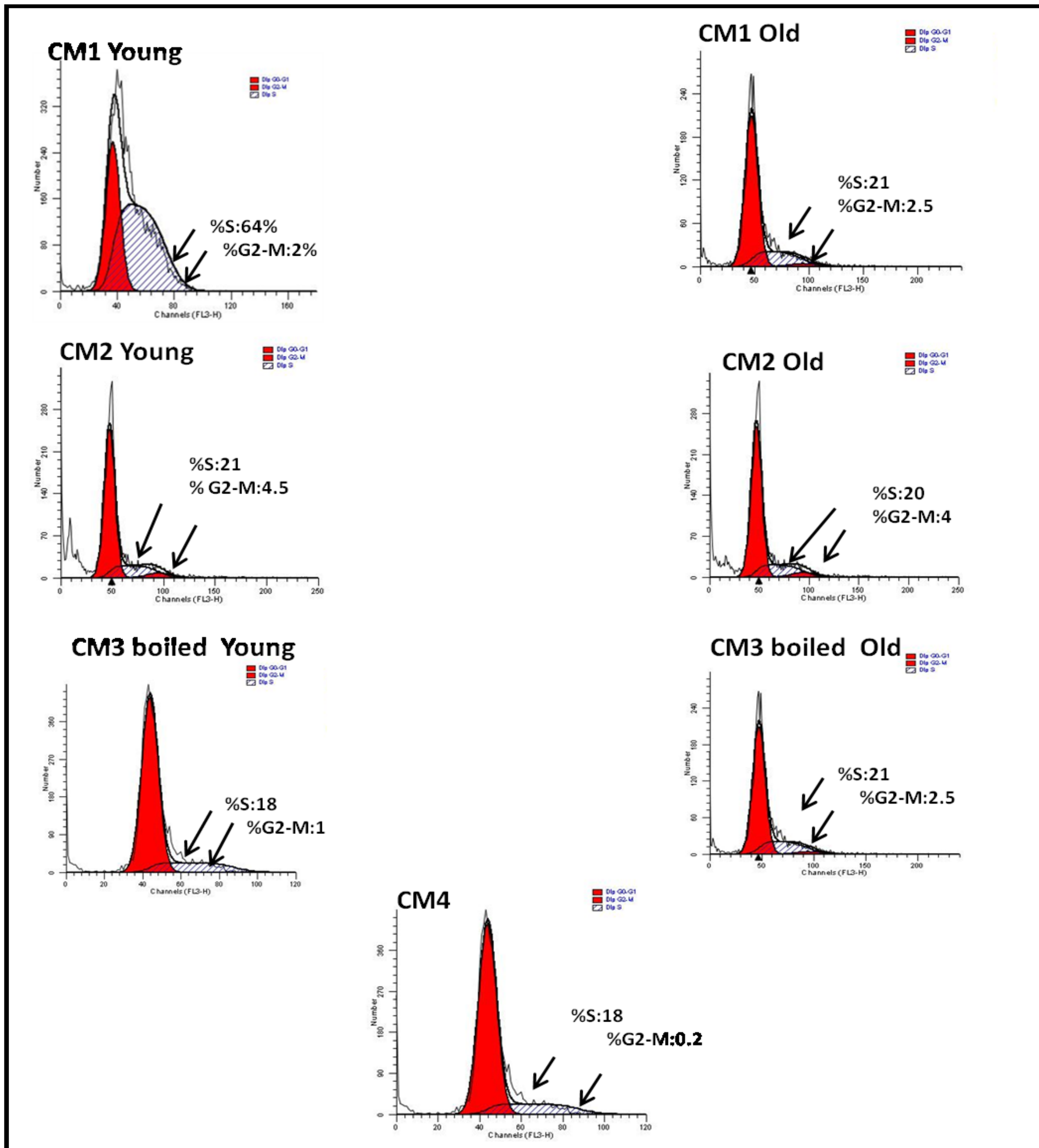
Figure 6.4: Differentiation of C2C12 following young CM1 withdrawal. Shifting CM to DM after 2 days incubation of myoblasts cultured in young CM1. The proliferative myoblasts form myotubes following 3 days of incubation with DM. Scale bar: 100µm.

6.4.3 CELL CYCLE ANALYSIS

Analysis of myoblast cell cycle progression showed that %S-phase of Young CM1-treated cells was significantly higher than Old CM1-treated cells (59.8±5.7% vs 23±1.3%, respectively, $p<0.001$) and higher than all other control conditions CM2, CM3, CM4 (60±5 vs 24.6±8, 26.7±5, 26±4, 20±7 and 23.7±3 respectively; $p>0.01$)(Figure 6.5 A and B). There were no significant

differences among Young and Old CM2, CM3 or CM4 (24.6±8 vs 26.7±5 vs 26 ±4, vs 20±7 vs 23.7±3 respectively; p >0.05)(Figure 6.5 A and B).

A)



B)

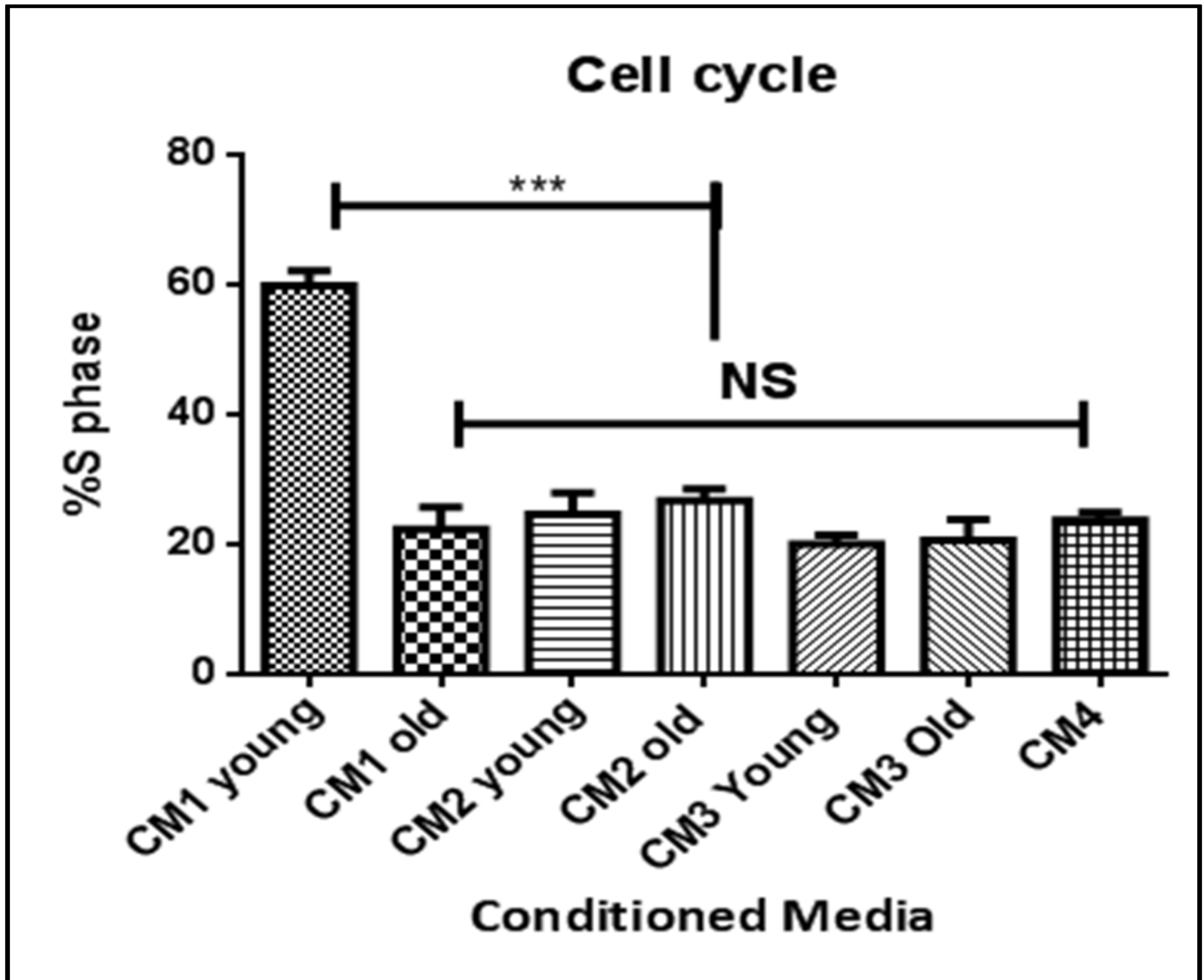


Figure 6.5:%S phases of myoblasts treated in conditioned media CM1-4 after 4 days. A) Representative photographs of C2C12 cell cycle analysis. B) The graph represents % S phases of C2C12 treated in CMs. Young CM1 treated myoblast showed higher %S phase compared to Old CM1 treated myoblasts, P<0.001. All controls of our study were not significantly different, p>0.05. The graph represents four independent experiments. The results represent means ±SD (n=20) from young and (n=17) from old.: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media and CM4: no lymphocytes conditioned media. % CV: for CM1 young ;9%, CM1 old; 40%, CM2 young ;34%, CM2 old; 19%, CM3 young; 23%, CM3old ;28% and CM4 ;14%. CM1

6.4.4. MIGRATION STUDY

Using a scratch assay, migration was assessed 18 hrs after the cells were removed from a small section in the middle of the well (artificial 'injury' model). Young CM1 induced significant myoblast migration that was almost 4-fold higher than the migration that occurred in the Old CM1 condition (73.3±6.02 cells vs 20.7±2.5 cells, respectively $P<0.001$; Figure 6.7). There were no significant differences between the Young and Old CM2, CM3 and CM4 (24.6±6 vs 26 ±5 vs 20±7 vs 23 ±3 respectively; $p>0.05$; Figure 6.7).

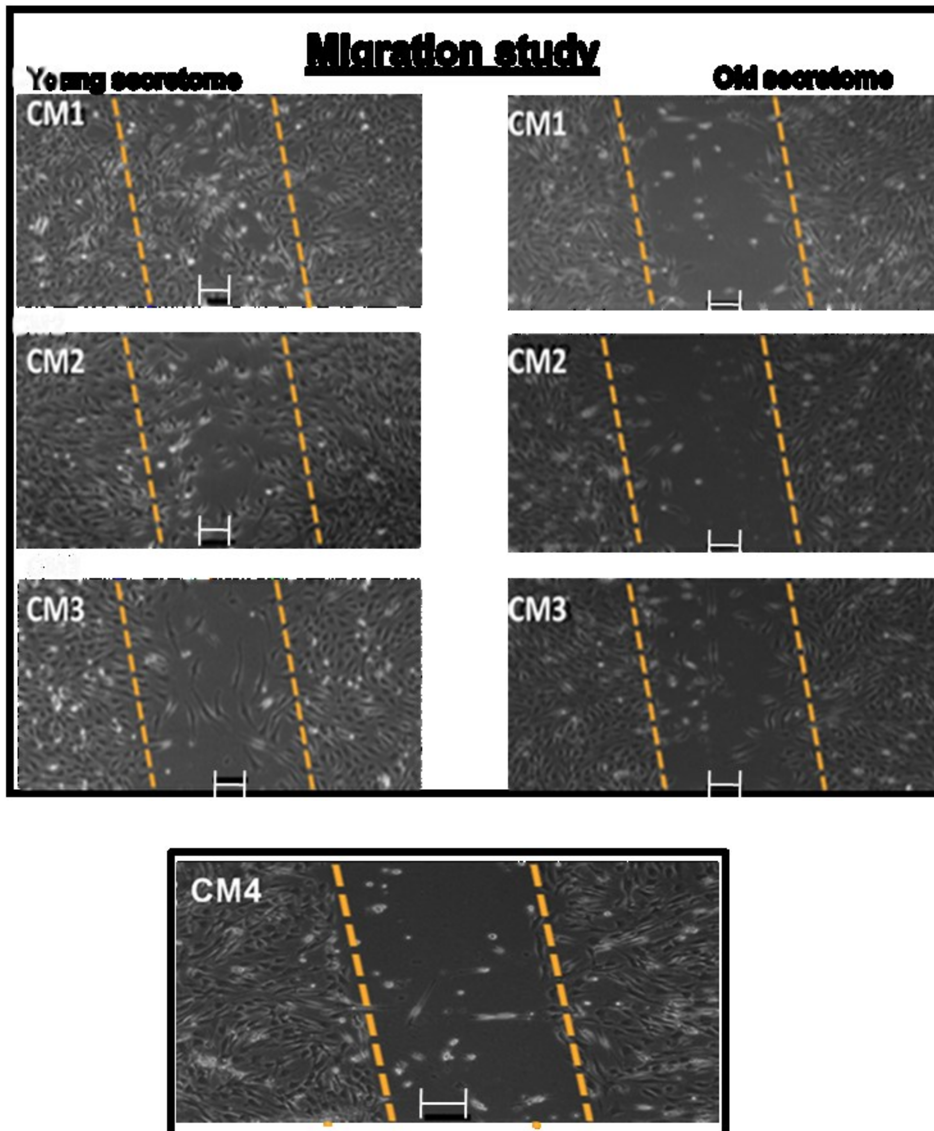


Figure 6.6: Representative images of wound healing assay for C2C12 treated with young and old secretomes. A phase contract images to compare the effect of young and old secretomes on C2C12 myoblast migration to the wound area after 18 hours. Scale bar=100µm.

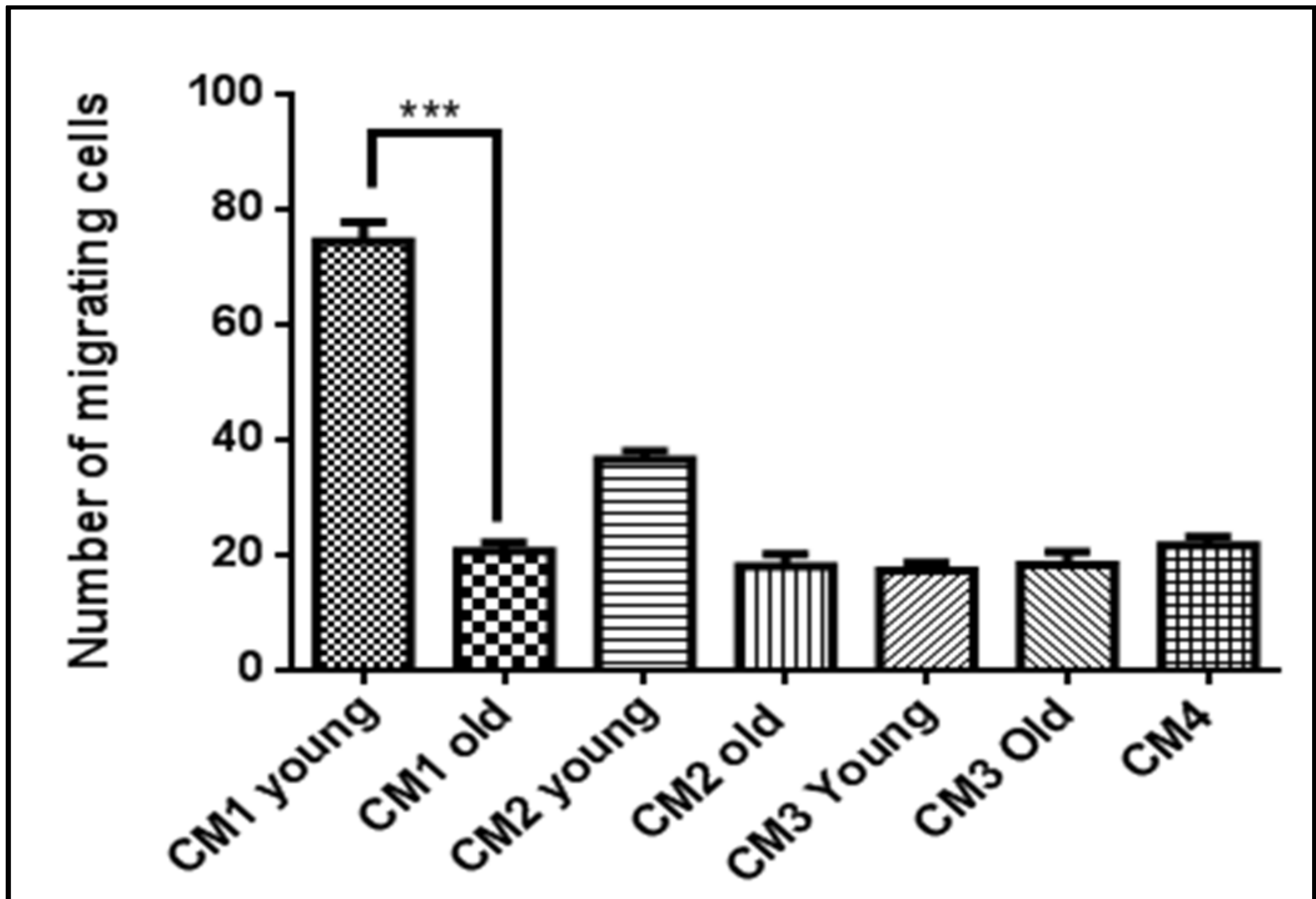


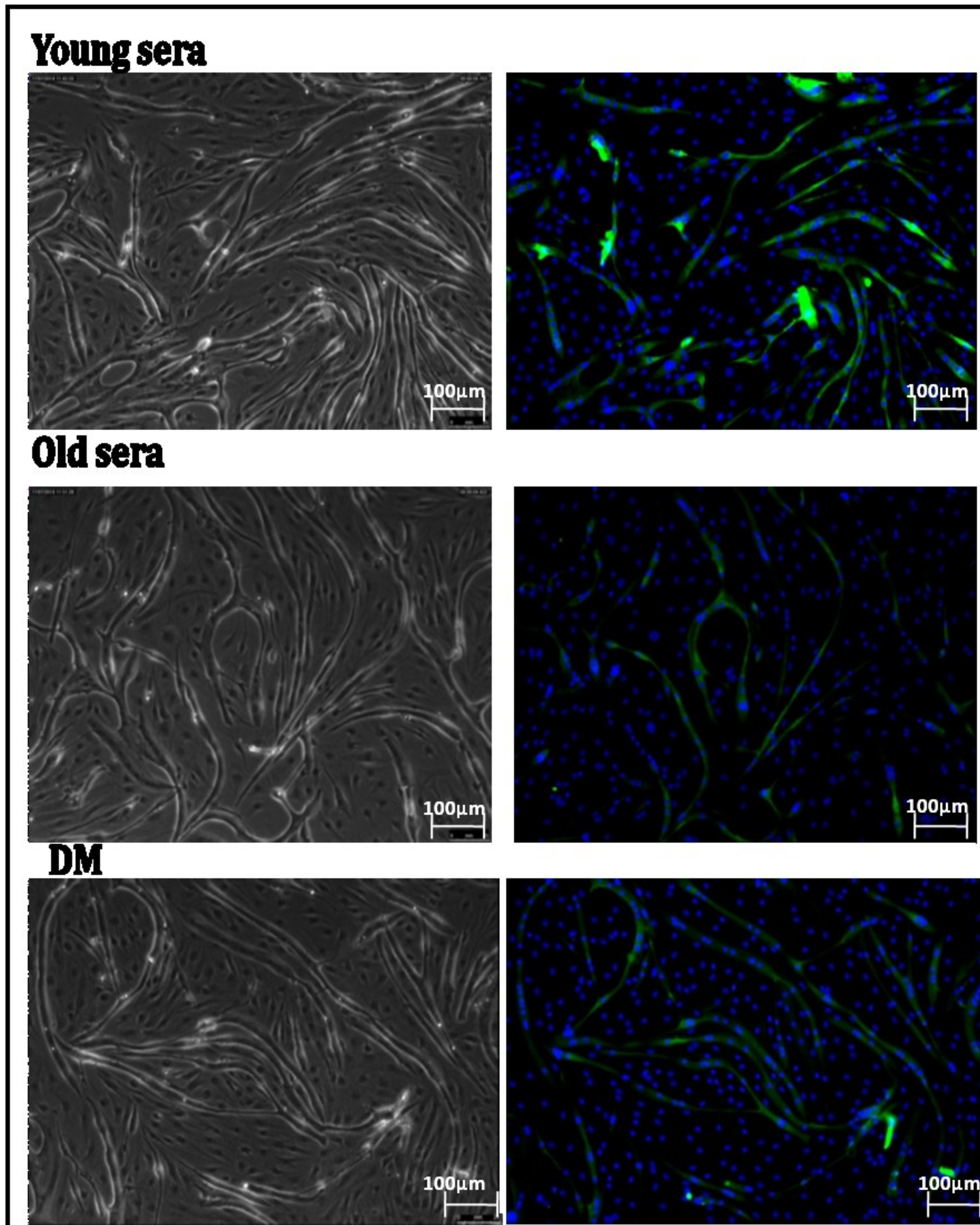
Figure 6.7: Cell count analysis for wound healing assays of C2C12 treated with young and old secretomes. A quantitative analysis of the invaded area was obtained was 4 folds higher in the presence of young secretome vs old, $p<0.0001$. All CMs (CM2-4) were not significantly different, $p>0.05$. The results represent the means of ±SD (n=5) of young and (n=5) of old in triplicates CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media and CM4: no lymphocytes conditioned media. . %CV for CM1 young ; 6%, CM1 old; 9%, CM2 young ;12%, CM2 old 20%, CM3 young ;15%, CM3 old ;25% and CM4 ; 15%.

6.4.5 DIFFRENTIATION PARAMETERS OF C2C12 CULTURED IN DM SUPPLIMENTED WITH YOUNG AND OLD HUMAN SERA

The evaluation of differentiation degree was performed. FI%, AR and MA% were calculated for myoblasts stained with a high affinity Alexa Fluor-488 MyHC and DAPI nuclear counterstain. The results showed that following 5 days incubation, Young DM, old DM and

conventional DM treated myoblasts exhibited extended differentiation; higher C2C12 expressed MHC protein (Figure 6.8 A). This was evident as AR was not significantly different among Young DM, Old DM and conventional DM treatments (16.3±1.1, 13.7±2 and 15 ±1, $p > 0.05$, Figure 6.8 B). MA% of Old DM was slightly lower compared to Young DM and conventional DM but statistically not significant (17.4 ±3% *vs* 24.5±1.9% and 25.5±2%, $p > 0.05$, Figure 6.8 B). However, as it is shown in figure 4.6, FI% of Old DM treated cells was significantly lower compared to young and conventional DM (15±2% vs 28±2%, $p < 0.05$ and vs 37±2% respectively, $p > 0.001$, Figure 6.8 B).

A)



B)

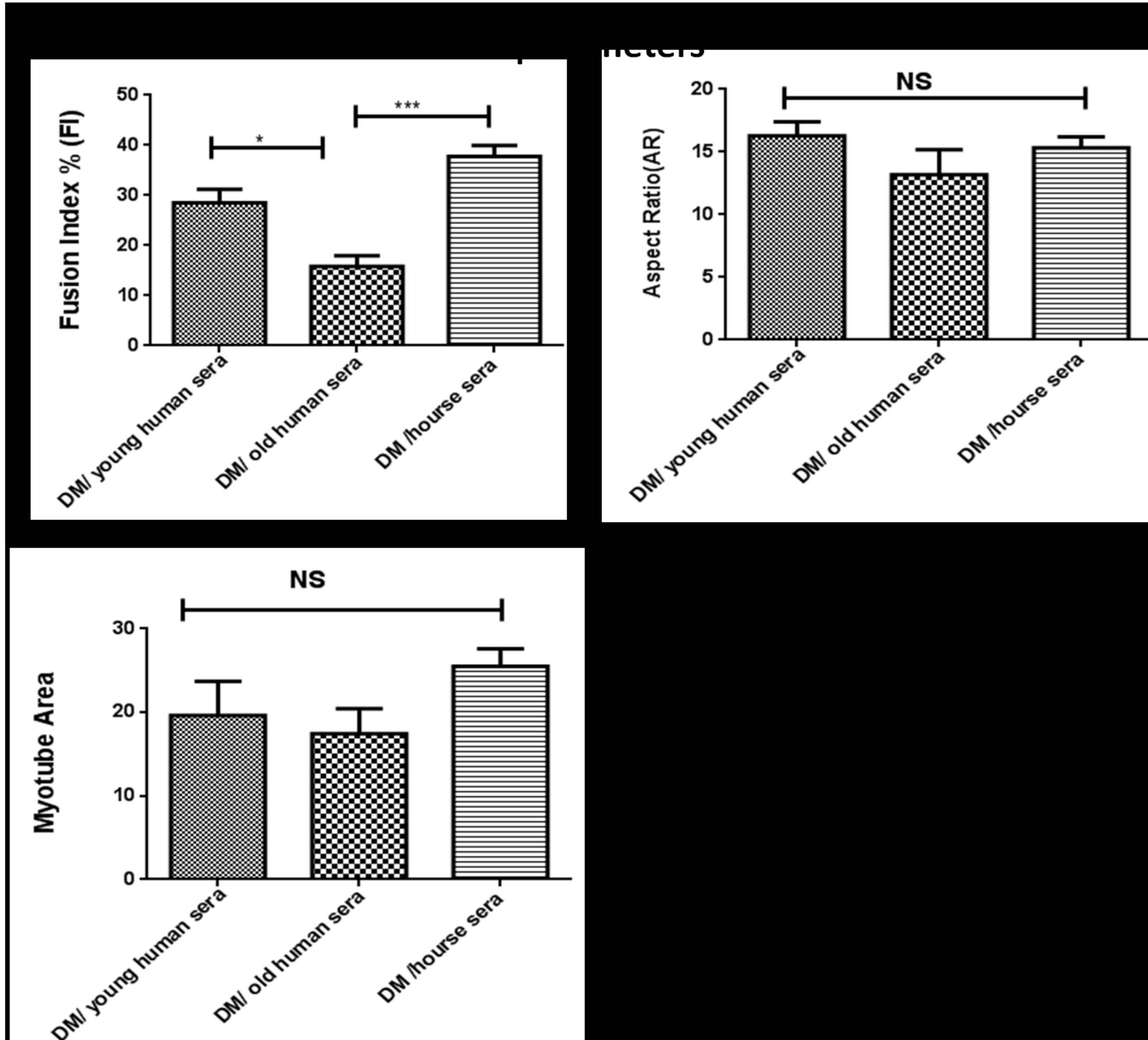


Figure 6.8: Immunofluorescence analysis of skeletal myosin heavy chain (MHC). A) C2C12 myoblasts treated with DM composed 2 %Young sera %(upper panel) or 2% old serum (middle panel) or 2% horse serum (lower panel). B) Differentiation parameters for myotubes development following 5 days of incubation with young, old and horse DM. C2C12 myoblasts were stained with the high-affinity MHC (marker of differentiation) -heavy-chain Alexa Fluor-488-MyHC (green) and DAPI (blue) nuclear counterstain. Images were taken from random fields by a Leica DMI6000B microscope at x10Mag. The data represents the means of ±SD of young men (n=7) and old men (n=7) in duplicates .

6.5 DISSCUSSION

6.5.1 MOPHOLOGICAL DIFFERENCES IN C2C12 MYOBLASTS TREAED WITH CM1 OF YOUNG AND OLD

The C2C12 cells treated with young CM1 were found to show markedly increased proliferation (as assessed by FLOW cytometry) whereas control treatments CM2-4, regardless of their formulation, displayed basal proliferation. Under these control treatments, C2C12 myoblasts maintained their ability to align, fuse and form elongated multi-nucleated myotubes, as occurs normally under low serum culture conditions (Blau et al., 1983) and was evident in myoblasts treated with old CM1 and all control conditions, regardless of the age of the donors.

In agreement with the above findings, anti-CD3- activated splenic T- cells significantly enhanced the proliferation of young satellite cells isolated from the gastrocnemius and plantaris compared to activated T lymphocytes from 32-mo-old rats (Dumke and Lees, 2011a). Similar to these findings, Mildner et al., (2013) demonstrated that the secretome from human mononuclear cells accelerated proliferation and wound healing of skin post injury (Mildner et al., 2013). Likewise, in another *in vivo* study conducted by Carlson & Faulkner (Carlson and Faulkner, 1989) old rat muscle transplanted to young rat exhibited a high potential for regenerative capacity, while young muscle transplanted into older rats did not regenerate due to inadequate proliferation signals. Consistently, the results of a study by Conboy et al. indicated that old progenitor satellite cells were activated upon exposure to the young secretome and in response to the secretome expressed high levels of Notch signalling proteins (Conboy et al., 2005)

This cross-talk between immune cells and muscle is biologically relevant, as cells *in vivo*are not stimulated with one cytokine in isolation, rather a cytokine cocktail instructs gene expression through the integration of multiple signalling pathways.

6.5.2 DIFFERENTIATION OF C2C12 FOLLOWING YOUNG CM1 WITHDRAWAL

Having observed increased proliferation rate in young CM1 treated myoblasts, it was investigated whether young CM1 treatment blocked differentiation. Muscle regeneration starts with activation of satellite cells which undergo several rounds of proliferation before they exit

the cell cycle to begin differentiation. Thus, differentiation is a necessary step in the regenerative process. Therefore, we sought to confirm that the Young CM1, which increased the rate of proliferation with minimal differentiation, did not terminally block differentiation in the myoblasts. Exchanging the Young CM1 for the usual differentiation media restored the ability of the C2C12 cells to differentiate, fuse and form myotubes.

6.5.3 MIGRATION STUDY

Migration is a critical step for successful muscle regeneration. Defective migration of transplanted myoblasts is one contributing factor that leads to ineffective therapy for muscular dystrophy (Price et al., 2007). In order to verify the effect of Young and Old CM1 on wound healing, C2C12 myoblasts were scratched and cultured 18 hours in Young, Old CM1 and control treatments.

Our data showed that Young CM1 induced an approximate 4-fold higher migration capacity of myoblasts compared to Old CM1 and control treatments (CM2 CM3 and CM4). These results were strongly supported by a study (Collins-Hooper et al., 2012),which suggested that satellite cells isolated from the extensor digitorum longus of young mice migrate significantly faster than old satellite cells. Moreover, Dumke and Lees (Dumke and Lees, 2011) reported that activated T-cells improved the regenerative capacity of satellite cells isolated from young rats by enhancing their migration capacity when compared to those isolated from aged animals. Taken together, these findings show intrinsic differences in key regulatory factors between young and old activated lymphocyte secretomes that regulate proliferative, migratory and ultimately the regenerative capacity of skeletal muscle satellite cells. Although we have not directly identified which factors might be principally involved in the effects shown here, the results from the previous chapter do provide some indications. We identified some potential candidates (cytokines and IGF-1) in the lymphocytes secretome. IGF-I is secreted by skeletal muscles and plays a key role in their growth, differentiation and repair. Previous reports showed that IGF-I is also secreted by activated T-lymphocytes (Leng et al., 2009; Merchav et al., 1988; Porras et al., 1998). Our results showed 30% higher IGF-I levels secreted by young activated lymphocytes compared to old when measured directly from the secretomes. However, the myoblasts were not exposed directly to these very high IGF-I concentrations, since our experimental approach used the secretomes to prepare the conditioned media, which reduced concentrations of IGF-I by around 20–fold. IGF -I is the only growth factor known to both

accelerate skeletal muscle proliferation (Machida and Booth, 2004) and differentiation (Musarò et al., 2001; Duan et al., 2010). It also limits fibrosis in injured muscle, potentiates the healing process by increasing the expression of myo-fibroblasts (Musaro et al., 2001; Werner and Grose, 2003) and prevents protein degradation (Karalaki et al., 2009). High IGF-I concentrations *in vitro* (50-200ng/ml) favour proliferation, whereas lower IGF-I concentrations (5-10ng/ml) promote differentiation (Sharon A. Coolican et al., 1997). Therefore, it is possible that the enhanced proliferation of myoblasts exposed to the young CM1 was in part accounted for by higher IGF-I concentrations (Foulstone et al., 2001).

The multiplex cytokine results presented in a previous chapter showed that IL-1β, TNF-α, IL-12, and IFN-γ were significantly higher in young activated lymphocytes compared to old, while IL-4, IL-5 and IL-8 were lower in young compared with old. Previous work demonstrated that TNF-α and IL-6 were decreased in old participants compared to young men following *in vitro* LPS stimulation (Bruunsgaard et al., 1999). TNF-α in combination with IL-6 is essential to maintain skeletal muscle strength and strongly associated with muscle repair after injury (Warren et al., 2002). TNF-α by activation of NFκ-β, a transcription factor in the cytosol, induces the expression of IL-6 leading to increased myoblasts proliferation and inhibits myogenic differentiation *in vitro* (Muñoz-Cánoves et al., 2013). TNF-α promotes myoblast proliferation directly and indirectly through IL-1β (Tidball and Villalta, 2010; Li, 2003) and endorses migration of mesoangioblasts, a subgroup of stem cells able to differentiate to skeletal muscle cells (Galvez et al., 2006). It is also essential for activation of P38 signalling in muscle regeneration (Chen et al., 2005). Recently, it was reported that pro-inflammatory cytokines (IL-6 and IL-1β) induce proliferation of C2C12 (Otis et al., 2014).

A study reported that INF-γ and IL-12 are potential candidates due to their anti-fibrotic effects (Mann et al., 2011). It is well documented that INF-γ regulates myogenesis and has potential mitogenic effects on C2C12 (Londhe and Davie, 2011; Villalta et al., 2011) as well as accelerating wound healing in skeletal muscle (De Rosa et al., 2004) and combating fibrosis (Cheng et al., 2008).Interestingly, this also supports our migration study findings, which demonstrated that the number of migrating cells to wound area was significantly higher in C2C12 treated with young secretome compared to old.

The results in previous chapter showed higher concentration of IL-4 in old secretome compared to young. It has been reported that IL-4 promotes myoblast fusion and increases myotubes size

(Horsley et al., 2003), which support the progression towards differentiation and myotube formation in old-CM1 treated myoblasts.

6.5.4 CULTURING CELLS IN DM SUPPLEMENTED WITH YOUNG OR OLD HUMAN SERUM

The main finding of this part of the study was that culturing C2C12 in DM containing young sera or old sera resulted in no difference in myotubes development. Generally, the three types of serum conditions induced strong differentiation, a robust MHC expression and a higher differentiation index including AR and MA%. Only FI% of nuclei inside myosin heavy chain myotubes was significantly lower in C2C12- treated old sera compared to young sera. These results are in agreement with a previous study, which demonstrated that, young and old serum equally sustain differentiation of human myoblasts extracted from muscle biopsy (George et al., 2010). However, our findings showing no age- related impairment in C2C12 myoblasts are in contrast with the results obtained from studies in mice (Carlson and Conboy, 2007; Brack et al., 2007) and humans (Carlson et al., 2009) where it was found that serum obtained from old participants inhibits the ability of satellite cells from young and old donors to express myogenic factors, Pax 7, MRF5, Myo D and desmin. The results of this experiment were not expected and consistent with our findings which showed that young secretome was a potent inducer for C2C12 proliferation compared to old secretome. However, it is possible that the small quantities of serum (2%) from young and old donors did not contain sufficient concentrations of effectors to impact on degree of differentiation. Also, it is important to note that the heat inactivation process could destroy heat-labile growth factors and cytokines eliminating any differences between young and old serum.

6.6 CONCLUSION

The conditioned media from old activated lymphocytes had no effect on proliferation and migration and instead C2C12 showed a high degree of differentiation, whereas conditioned media from young activated lymphocytes enhanced proliferation and migration of C2C12

myoblasts. Young secretome enhanced C2C12 proliferation and differentiation was restored successfully upon replacement of CM1 with DM. Both DM supplemented with young and old hi human serum was successfully able to induce differentiation and myotubes development of C2C12 that were comparable to the conventional differentiation media.

CHAPTER 7

7. C2C12: CELLULAR AND MOLECULAR ADAPTATION OF C2C12 CELLSTREATED WITH YOUNG AND OLD CONDITIONED MEDIA

7.1 INTRODUCTION

The findings from the study presented in the previous chapter demonstrated that young activated-lymphocyte secretomes stimulate proliferation and migration of C2C12 myoblasts, while old activated lymphocyte secretomes had no discernible effects on C2C12 proliferation and migration and thus behaved similar to the various control conditions (which included non-activated lymphocyte secretomes and denatured secretomes). These findings suggest that factors released from the activated lymphocytes of young adults can initiate signalling within C2C12 cells to up regulate proliferation and migration. . The results in chapter 5 showed that cytokines, IGF-1 and amphiregulin were highly expressed in the secretome of young activated lymphocytes but it is not clear which of these factors are directly involved in sustained proliferation and migration of young CM1- treated C2C12.

In this chapter the status of C2C12 anabolic signalling pathways were assessed after incubation in the presence of the young and old activated lymphocyte secretomes (CM1).

Since it is not yet known which signalling pathways within C2C12 cells might be affected by the lymphocyte secretome, several candidate molecules that regulate anabolic processes were assessed. This included the C2C12 cell mRNA expression of myogenic factors (MyoD and Myogenin) as well as the phosphorylated anabolic signalling proteins Akt/mTOR and Mapk/Erk. The protein kinase concentrations were assessed at several time points in preliminary experiments to identify an 'optimal' time-point in order to intervene by blocking selected signalling pathways. The Mapk inhibitor'UO126' was applied at different concentrations to block the Mapk/Erk pathway in C2C12 cells. The C2C12 IGF-IR receptor was also blocked and subsequent analyses of the cell cycle as well as Akt/mTOR levels were determined. Finally, in an effort to identify novel molecular regulators of the lymphocyte secretome-C2C12 interactions, a proteomics study was undertaken.

The blood samples obtained, for conditioned media preparations, to perform the experiments in this chapter were from young, n=15, male (aged 18-30 years) and old (n=15, male, aged 73-82 years) participants

7.2 AIMS AND OBJECTIVES

In these studies, the aim was to identify whether the C2C12 myogenic factors MRFs (Myogenin and MyoD) and phosphorylated proteins (Akt/mTOR and MAPK/Erk) were involved in the enhanced proliferation and migration of C2C12 cells after incubation with the young activated lymphocyte secretome. The objectives were: 1) to compare C2C12 expression of Myogenin and MyoD after incubation with young CM1 *vs* old CM1. 2) to investigate the phosphorylation status of Akt/mTOR and Mapk/Erk molecules, as potential regulators of the C2C12 proliferation and migration, after incubation with young CM1 *vs* old CM1. 3) to establish whether the enhanced proliferation of C2C12 cells observed after incubation with the young CM1 could be attenuated by blocking key anabolic signalling pathways in the C2C12 cells.

7.3 METHODS

7.3.1 CULTURE OF C2C12 MYOBLASTS AND TREATMENT WITH CM1-4

C2C12 myoblasts were maintained by growing cells in the usual growth media (GM) as described in 2.2.1.1 before culturing them with young or old CM1-4 as described in 2.4. CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media and CM4: no lymphocytes conditioned media. CMs-treated cells were incubated for 4 days in a humidified atmosphere using 5% CO_2 at 37°C.

7.3.2 RNA ISOLATION AND REAL-TIME PCR

RNA extraction and TaqMan ®PCR methods were performed as described in sections 2.8 and 2.9 respectively. The following predesigned primer sets were used in the TaqMan® detection method: GAPDH (mm99999915_m1), MyoD (mm00440387_m1) and Myogenin (mm00446194_m1). GAPDH was used as a reference gene for C2C12 myoblasts (Langen et al., 2004; Amabile et al., 2009) and performed in parallel with myogenic target genes. All reactions were performed in triplicate.

7.3.3 OPTIMAL TIME POINT TO ACTIVATE PHOSPHO-PROTEIN CASCADES

In order to determine the optimal time point for signalling proteins induction in response to CM1-2 and 4, C2C12 myoblasts were treated with Young CMs at 4 times points (5, 15, 25, and 60) min. Cell lysates were harvested for proteins estimation as described in section 2.10. The levels of Akt/mTOR /IRS-1 were determined using magnetic beads panel as described in 2.12.1. The results were analysed using a Luminex 200TM.

7.3.4DETERMINATION OF MYOBLAST LEVELS OF MAPK/ERK AND AKT/mTOR AFTER TREATMENT WITH YOUNG AND OLD CONDITIONED MEDIA

The measurement of MAPK/ERK and Akt/mTOR in the following experiments was conducted according to the optimal time point obtained. The MAPk inhibitor, UO126 was applied to Young CM1 at different concentrations (3, 5, 8 and 10μM). CM1-treated C2C12 cells (without UO126) were set as control conditions. For each experimental condition (3, 5, 8 and 10μM of UO126), the levels of pAkt and p-mTOR were determined following 25 min treatment using a magnetic beads panel and analysed by a Luminex 200TM plate reader as described in section

2.12.1. Phase contrast images of the C2C12 cultures were taken using a Leica DMI 6000B microscope after treatment with CM1+UO126 for 4 days at 37°C in a humidified atmosphere with 5% CO_2.

7.3.5 MULTIPLEX/IMMUNOASSAY PROTOCOL

The assays were conducted using the Luminex 200 system (Bio-Plex) running the xPONENT analytical software, the Milliplex MAP Kits of MAPK/ SPAK and Akt/mTOR signalling magnetic beads panel (EMD Millipore). The samples were tested as described in 2.12.1.

7.3.6 INVESTIGATING THE EFFECT OF IGF-1 ON C2C12 PROLIFERATION.

C2C12 treatment with IGF-1R inhibitor OSI-906 (Linsitinib)

C2C12 myoblasts were maintained by growing the cells in growth media (GM) as described in 2.2.1.1. CM1 was prepared as described in 2.4.2 and 2.4.3. In order to examine the role of IGF-1 on C2C12 proliferation, OSI-906 was reconstituted (as described in 2.2.1.4) and diluted with DMEM to final concentrations of 0.5µM and 0.8µM. Cell monolayers were washed with PBS and treated with the reconstituted inhibitor for 30 min in a humidified atmosphere of 5% CO_2 at 37^{0}C (Philippou et al., 2009). Following OSI-906 treatment, C2C12 cells were washed with PBS and CM1 or DM were added. C2C12 cells were incubated for 3 days in a humidified atmosphere of 5% CO2 at 37^{0}C. Phase contrast images were taken by a Leica microscope, cells were counted (as described in 2.3) and cell cycle analysis was performed (as described in 2.6.4). Protein estimation of cell lysate was performed prior to assessment of activated Akt and mTOR levels by multiplex immunoassay as described in 2.12.1.

7.4 STATISTICAL ANALYSIS

Statistical analysis of data was undertaken using GraphPad Prism. One-way ANOVA was used to compare the effects between all the experimental conditions followed by Benferroni post-test analysis. Two-way ANOVA was performed to investigate the effects of time points and the experimental conditions on phosphorylated protein levels, followed by Benferroni post-test analysis. Results were presented as mean ±SD. All values of $p< 0.05$ were considered statistically significant. The percentages of CV were determined for repeated independent experiments by dividing standard deviation by the mean for each group of treated cells .

7.5 RESULTS

7.5.1 GENE EXPRESSION ANALYSIS OF MYOD AND MYOGENIN FOR YOUNG AND OLD CM1-4- TREATED MYOBLASTS.

To investigate the effect of CMs on muscle specific regulatory factors (MyoD and Myogenin), RT-PCR was performed. The expression level of MyoD and Myogenin in myoblasts treated with (CM2-4) was not significantly different (Figure 7.1 and 7.2). The levels of MyoD and Myogenin in C2C12-treated with Young CM1 were significantly lower by over 5% compared to CM2-4). The expression level of MyoD and Myogenin in cells treated with old CM1 was significantly lower compared to CM4 –treated cells. Similarly, the gene expression of MyoD and Myogenin in C2C12 treated with Young CM1 was significantly lower compared to Old CM1- treated cells (0.46±0.12 and 0.34±0.05 *vs* 0.7±0.07 and 0.6±0.05, respectively $p<0.05$).

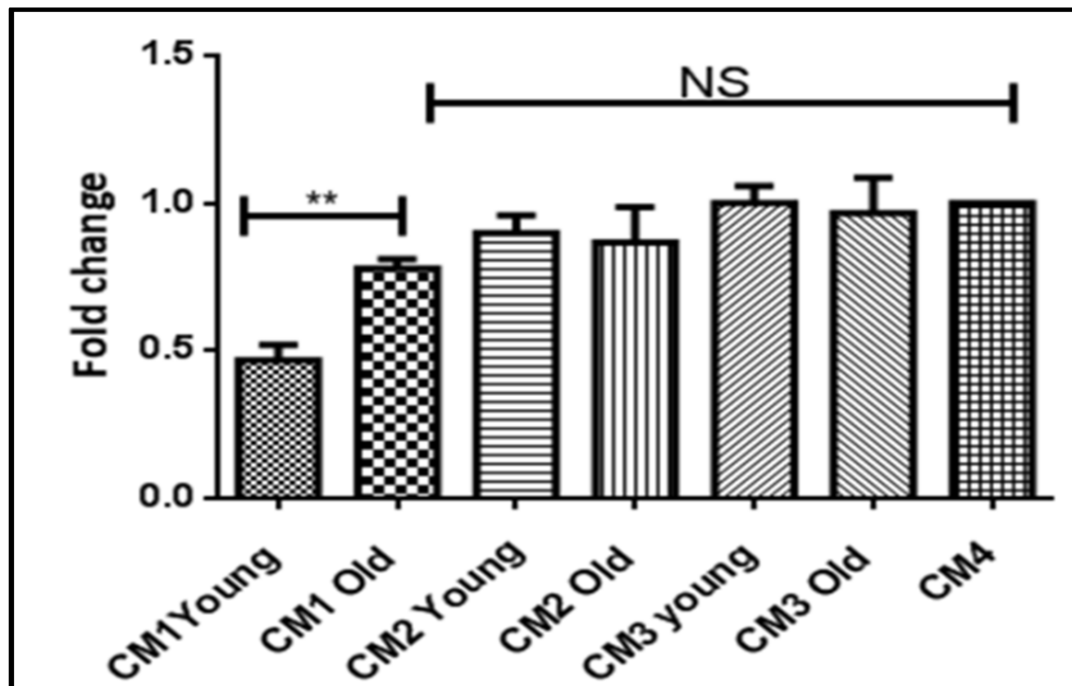


Figure 7.1: RT-PCR analysis of MyoD mRNA gene expression following 4 days of incubation with young and old CM1-4. C2C12 myoblasts were treated with CM1-4 for 96 hours before RNA collection. All values are represented as fold difference compared with the calibrator (CM4). None of the control conditions (CM2-4) were significantly different from CM4 ($p>0.05$). Expression of MyoD in Young CM1 treated myoblasts were significantly lower compared to Old CM1 treated myoblasts ($p<0.05$).Expression level of MyoD in Old CM1 treated cells were significantly lower compared to CM4 control condition. Data represent the means ±SD (n=5) in triplicates of CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media, CM4: no lymphocytes conditioned media. % CV: for CM1 Young; 27%, CM1 Old ;10%, CM2 Young; 10%, CM2 Old ; 21%, CM3 Young; 9% and CM3 Old;19% and CM4; 0%.

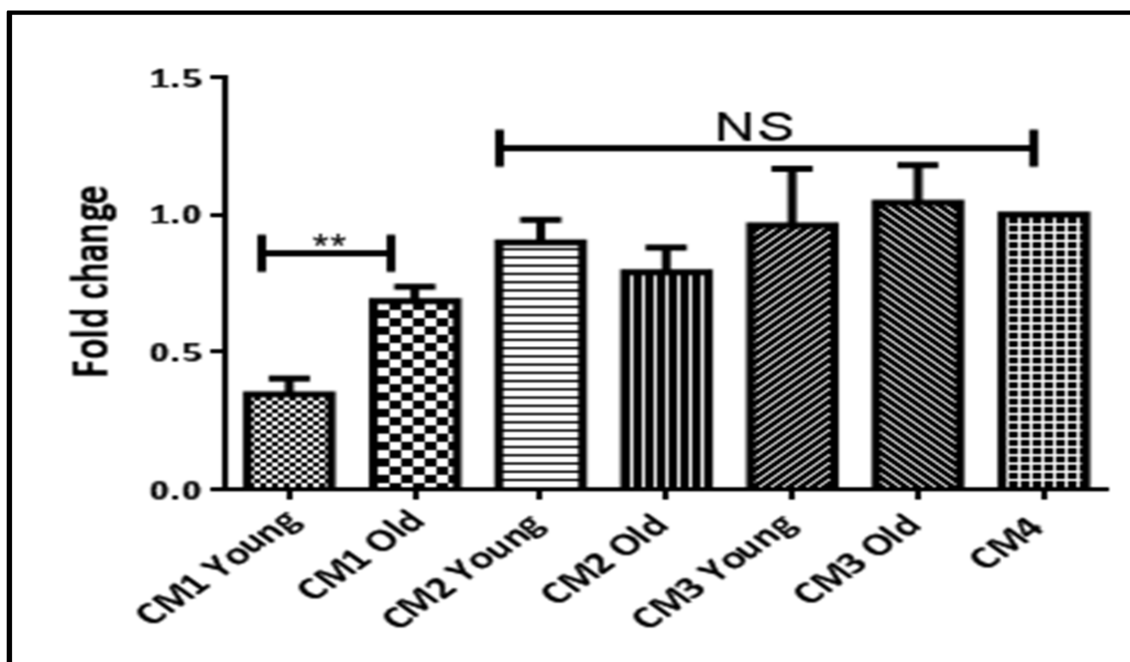


Figure 7.2: RT-PCR analysis of Myogenin mRNA gene expression following 4 days of incubation with young and old CM1-4. C2C12 myoblasts were treated with CM1-4 for 96 hours before RNA collection. All values are represented as fold difference compared with the calibrator

(CM4). None of the control conditions (CM2-4) were significantly different from CM4 ($p>0.05$). Expression of Myogenin in Young CM1 treated myoblasts were significantly lower compared to Old CM1 treated myoblasts ($p<0.05$).Expression level of Myogenin was in Old CM1 treated cells were significantly lower compared to CM4 control condition. Data represent the means ±SD (n=5) in triplicates of CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media and CM4: no lymphocytes conditioned media. % CV for CM1 Young; 10%, CM1 Old; 8%, CM2 Young; 8%, CM2 Old; 11%, CM3 Young; 22%, CM3 Old; 12% and CM4 0%.

7.5.2 OPTIMAL DURATION TIME TO ACTIVATE PHOSPHO-PROTEINS CASCADES

Having established that myogenic factors were downregulated in CM1-treated C2C12 cells, the level of phosphorylated proteins MAPK1/ERK1/2 and Akt/mTOR were investigated because they have a known role in C2C12 proliferation and diffrentiation. Initial experiements were undertaken to establish the optimal activation time ofpAktby comparing effects at 5, 15, 25 and 60min after treatment with Young CM1, CM2 and CM4. The results showed that the median of fluorescence intensity (MFI) of pAkt levels was significantly higher in myoblasts at 25min after first exposure to young CM1and CM2 compared to time points 5, 15 and 60min. The 25 min time point also had higherMFI of pAkt after treatment withCM4 compared to 5min. Data are shown in Figure 7.3.

The activation ofmTOR after exposure to CM2 and CM4 was significantly higher at 25min compared to 5 and 60 min. The mTOR levels in C2C12 cells after exposure to CM1 and CM2 were similar at 25 min compared with 15 and 60 min. Data are shown in Figure 7.4.

The MFI levels of phosphorylated IRS-1 were highest at 25min in CM1 and CM4-treated cells, while the 25 min and 60 min time points for CM2 were similar, and both were higher than time points 5 and 15 min. Data are shown in Figure 7.5.

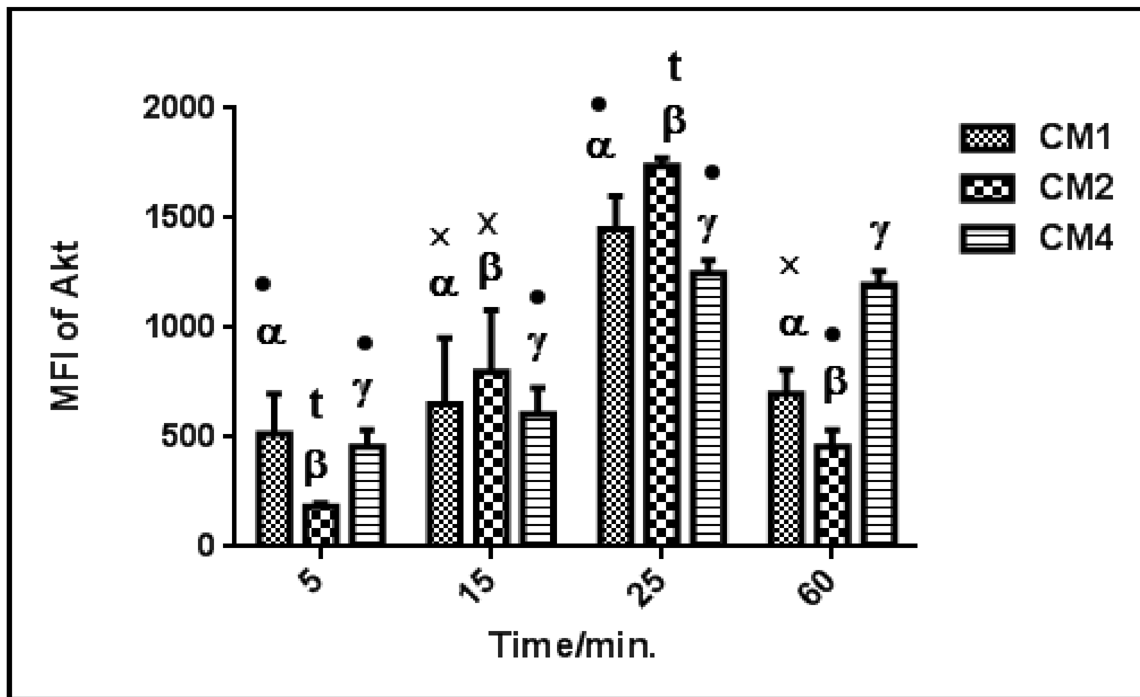


Figure 7.3: MFI of pAkt levels in myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25 and 60 min. MFI levels of pAkt in response to CMs treatment were highest after CM1 treatment at 25 min *vs* CM1 at 5 min ;($p<0.01$,represented as α•) and in CM1 at 15 and 60 min, ($p<0.05$, represented as αx).There was a significant increase in CM2 at 25min *vs* CM2 at 5 min; ($p<0.0001$, represented asβt)*vs* CM2 at 15($p<0.05$, represented as β x), *vs* CM2 at 60min($p<0.01$, represented as β•). The significant increase in CM4 at 25 min *vs* CM4 at 5min ($p<0.01$, is represented as y•). Data represent means ±SD (n=5) performed in duplicates.

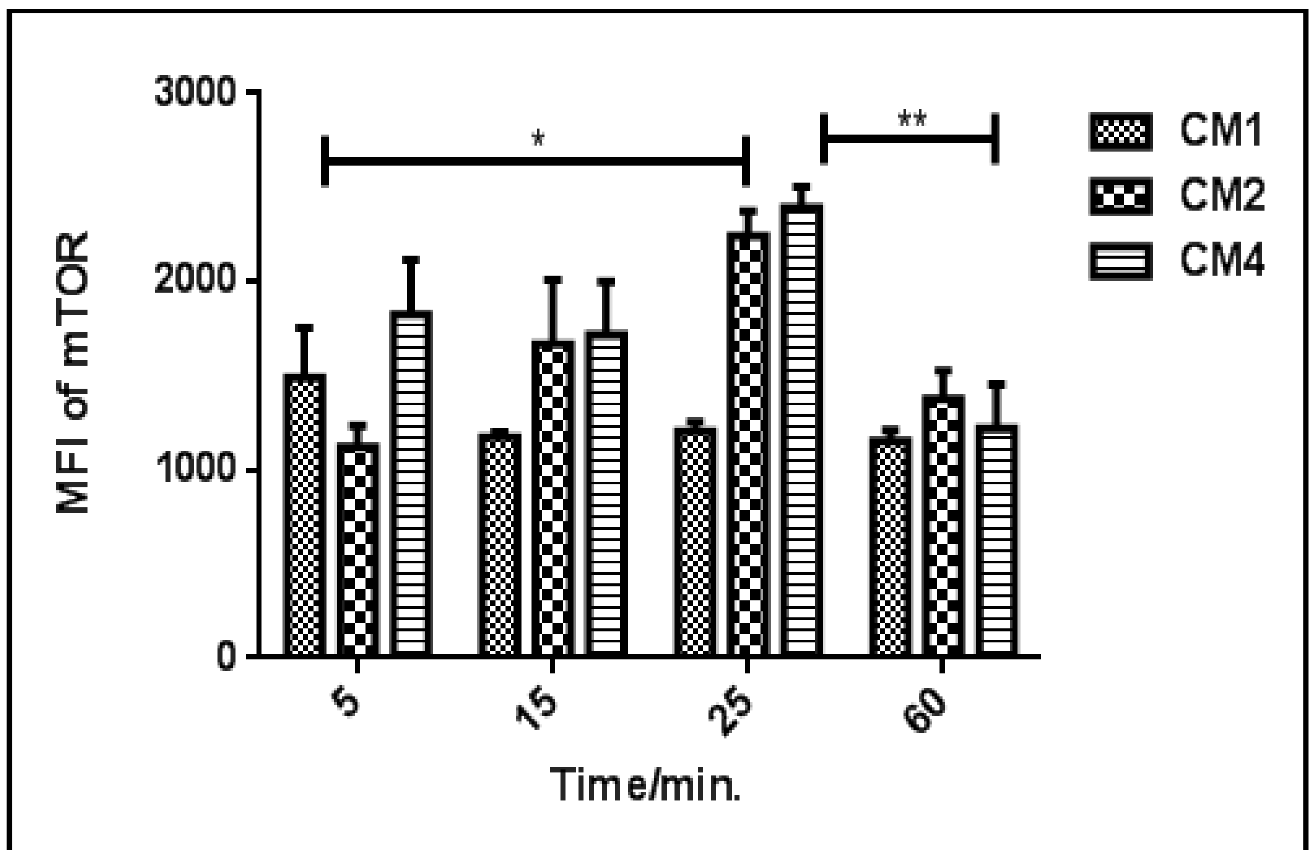


Figure 7.4: MFI of mTOR levels in myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25 and 60 min. MFI levels of mTOR in response to CMs treatment indicated a significant increase in CM2 at 25min *vs* CM1 at 5min ($p<0.05$). Similarly, CM4 had higher levels of mTOR at 25min *vs* 60min ($p<0.01$). No significant difference was observed in CM1 over different time points ($p>0.05$). Data represent means ±SD (n=5) in duplicates. CM1: Activated conditioned media, CM2: Non-activated conditioned media and CM4: no lymphocytes conditioned media

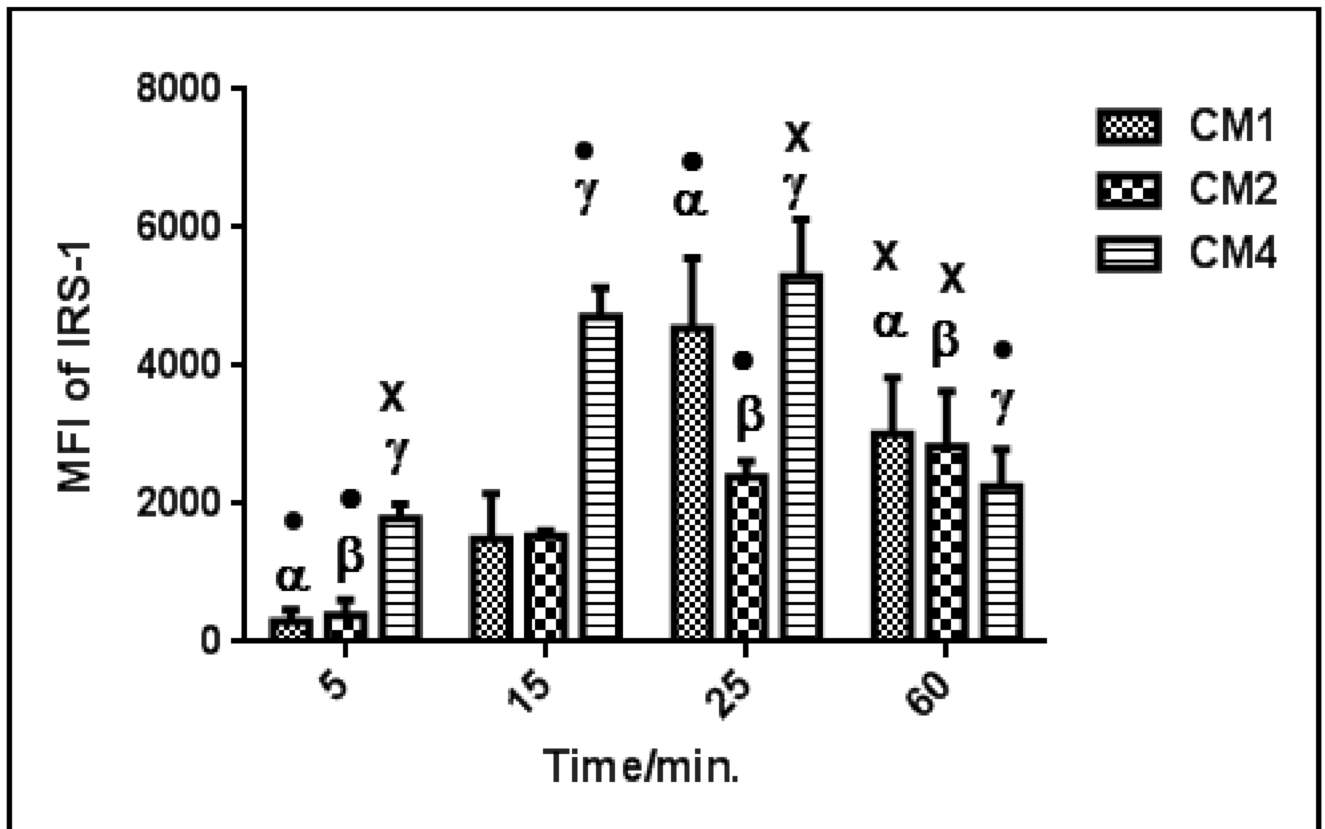


Figure 7.5: MFI of IRS-1 levels in myoblasts treated with CM1, CM2 and CM4 at 5, 15, 25 and 60 min. MFI levels of IRS in response to CM1, CM2 and CM3 treatment indicated a significant increase in CM1 at 25min *vs* 5 min ($p<0.01$, represented as α•). Also the level of IRS-1 in CM1was significantly higher at 60 min *vs* 5 min ($p<0.05$, represented as αx). A significant increase was observed in CM2 at 25min *vs* 5 min ($p<0.05$, represented as β•). Also, the IRS-1 level in CM2 at 60min was significantly higher *vs* 5 min ($p<0.05$, represented βx). The IRS-1 level was significantly higher in CM4 at 25 min *vs* 5 min ($p<0.001$, represented as yt). A significant difference was observed in CM4 at 15min compared to 60min ($p<0.01$, represented as y•). Significant difference in CM4 at 25 min *vs* 60 min was observed ($p<0.05$, represented as yx). Data represent means ±SD (n=5) in duplicates .CM1: Activated conditioned media, CM2: Non-activated conditioned media and CM4 : no lymphocytes conditioned media.

7.5.3 MEK1/ERK 1/2 SIGNALLING PATHWAYS ARE INDUCED IN RESPONSE TO YOUNG CM1BUT EFFECTS ARE ATTENUATED IN RESPONSE TO OLD CM1

Using the 25 min time point (identified above as the optimal time to measure signalling events), experiments were undertaken to examine the activation of MAPK/ERK1/2 and AKT/mTOR pathways after exposure to the various CMs from young and old.

The level of pMEK was significantly higher in Young CM1 compared to Old CM1(724.8±140 *vs* 528±80; $p<0.05$) and was higher in Young CM1 compared to Young CM2 and CM4 (724±140 *vs* 308±118; $p<0.001$ and 320±81, $p<0.005$, respectively). No significant difference was observed between theOld CM1, CM2, CM3 and CM4 (528±140 *vs* 311.8±98:$p>0.05$; 255±67:$p>0.05$ and 320±81:$p>0.05$, respectively, Figure 7.7).

Following the finding that 25min is the optimal timepoint to trigger the signalling pathway, the level of MAPK/ERK and AKT/mTOR were investigated. Our results showed that the level of pMEK was significantly higher in Young CM1 compared to Old CM1(724.8±140 *vs* 528±80; $p<0.05$). Also, the MFI of MEK indicated a significant increase in Young CM1 compared to CM2 and CM4 (724±140 *vs* 308±118; $p<0.001$ and 320±81, $p<0.005$ respectively). No significant difference was observed among Old CM1 and CM2, CM3 and CM4 (528±140 *vs* 311.8±98;$p>0.05$, 255±67; $p>0.05$ and 320±81; $p>0.05$ respectively)(Figure7.6).

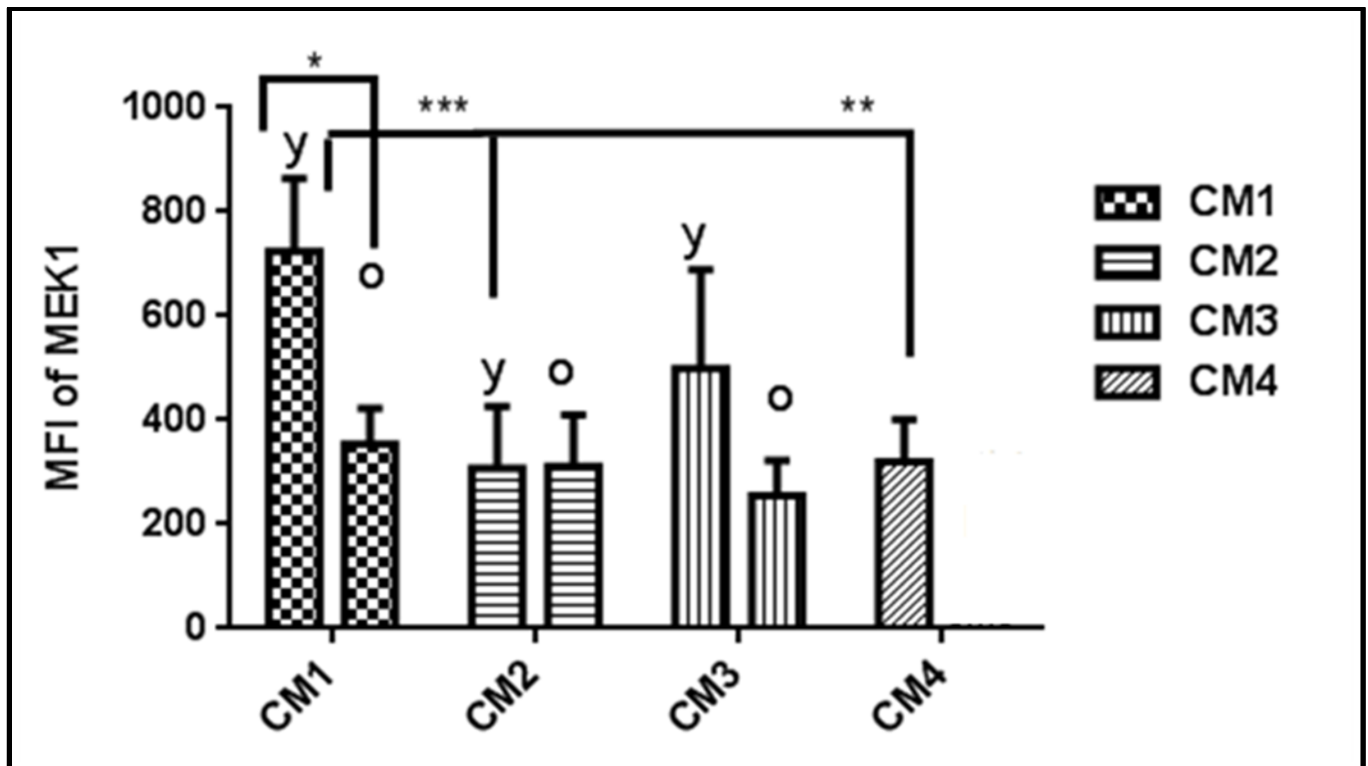


Figure 7.6: MFI for MEK1 in CM1 Young and Old treated C2C12. Levels of MEK1 were significantly higher in Young CM1 *vs* Old CM1, CM2 and CM4; $p<0.05$, 0.001 and 0.01 respectively. Data represent means ±SD (n=5) in triplicates: young and O: old.CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media and CM4: no lymphocytes conditioned media. %CV for CM1(y); 19%, (o) 19%), CM2(y); 38%, (o); 31%, CM3(y); 38%, (o); 26% and CM4; 25%.

The MFI of Erk1/2 was significantly higher in Young CM1 compared to Old CM1(3368±1105*vs*1141±668; $p<0.0001$) and compared to all other conditions. The Old CM1 was not significantly different compared to CM2-4 (1141±668 vs 1140±186, 933±510 and 914±98; $p>0.05$ respectively Figure 7.7).

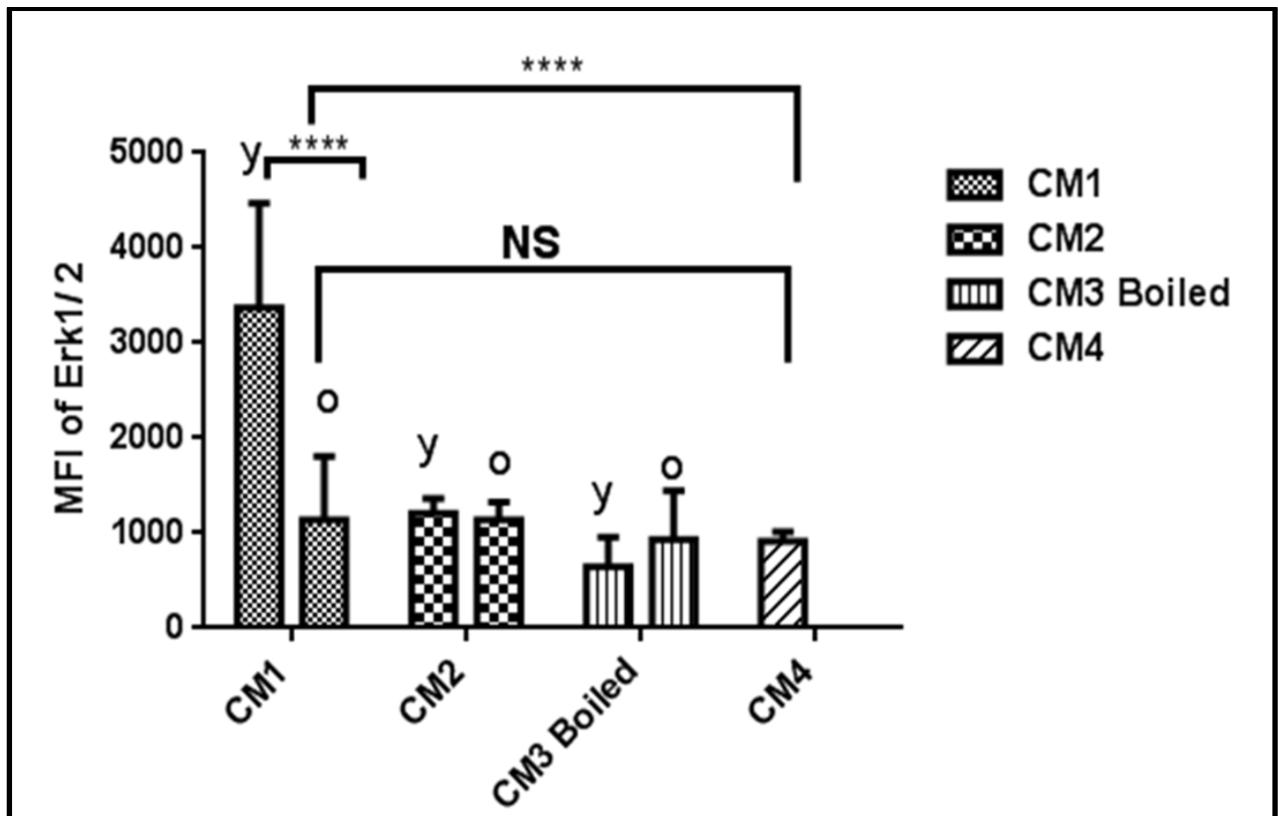


Figure 7.7: MFI levels for ERK1/2 in Young and OldCM1, CM2, CM3 and CM4- treated C2C12 myoblasts. A significant increase in ERK1/2 levels of Young CM1 *vs* Old CM1 and controls was found p<0.0001. No significant different in Old CM1 vs CM2-4; p<0.05 was found. Data represent means ±SD (n=5) in triplicates .Y: young and O: old.CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media, CM4: no lymphocytes conditioned media. %CV for CM1 (y); 33%,(o); 58%, CM2(y);13%, (o)16%, CM3 Boiled(y); 48%, (o); 55% and CM4 11%.

7.5.4. AKT/mTOR SIGNALLING PATHWAY IS LOWER IN RESPONSE TO YOUNG CM1 COMPARED WITH OLD CM1

pAkt was significantly lower in Young CM1-treated cells compared to Old CM1-treated cells.However, no significant differences existed between the Young or Old CM1 compared to their corresponding CM2-4s (Figure 7.8). Similarly, the phosphorylated mTOR levels were lowerin Young CM1 compared to Old CM1. The Phosphorylated mTOR did not differ across the Young CM1-4, while the Old CM1 was significantly higher than the Old CM2 and CM3 (Figure 7.9).

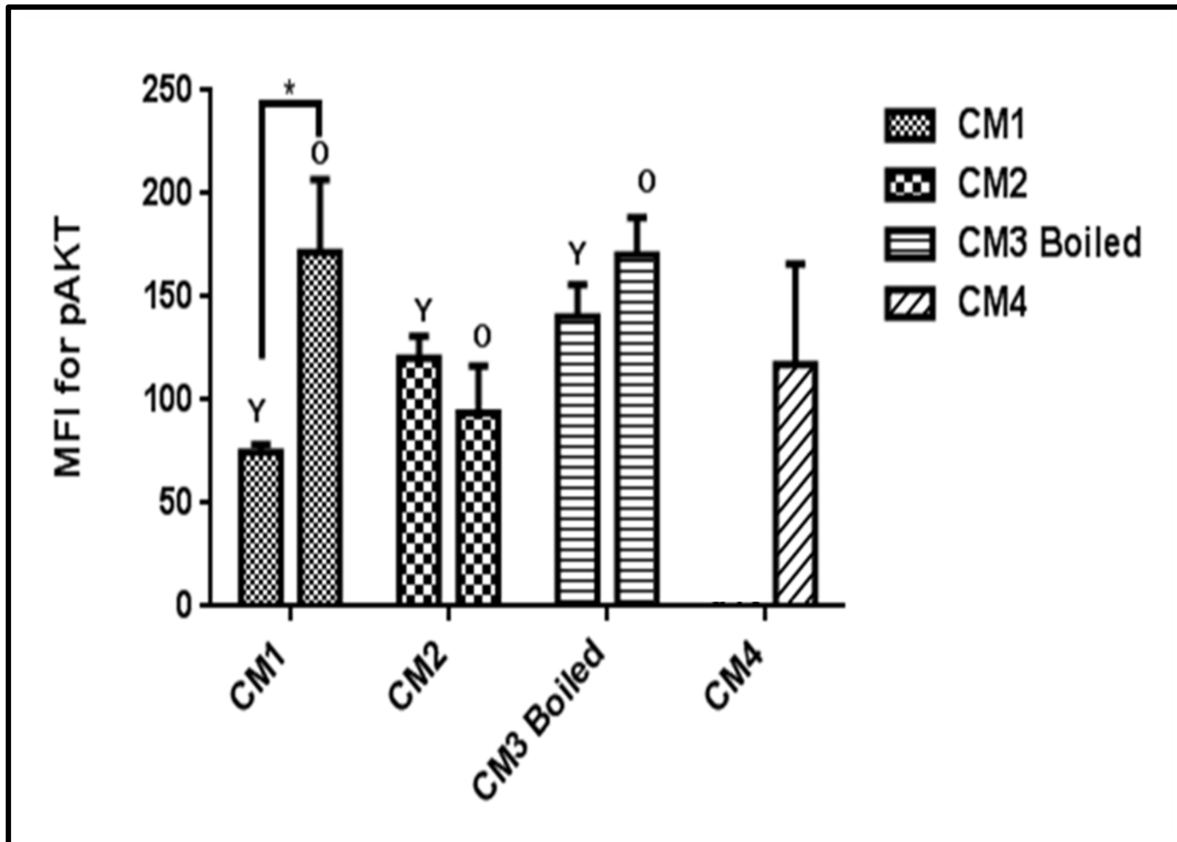


Figure 7.8: MFI of pAkt levels in Young and Old CM1, CM2, CM3 and CM4- treated C2C12 myoblasts. pAkt levels were significantly higher in Old CM1 compared to Young CM; p<0.05. Data represent means ±SD (n=4) in triplicates. : Young and O: old. CM1: Activated conditioned media, CM2: Non-activated conditioned media, CM3: Boiled activated conditioned media, CM4: no lymphocytes conditioned media. . % CV for CM1(y); 33%, (o); 58%, CM2(y); 13%, (o); 16%, CM3 Boiled (y); 48%, (o); 55% and CM4; 11%. .

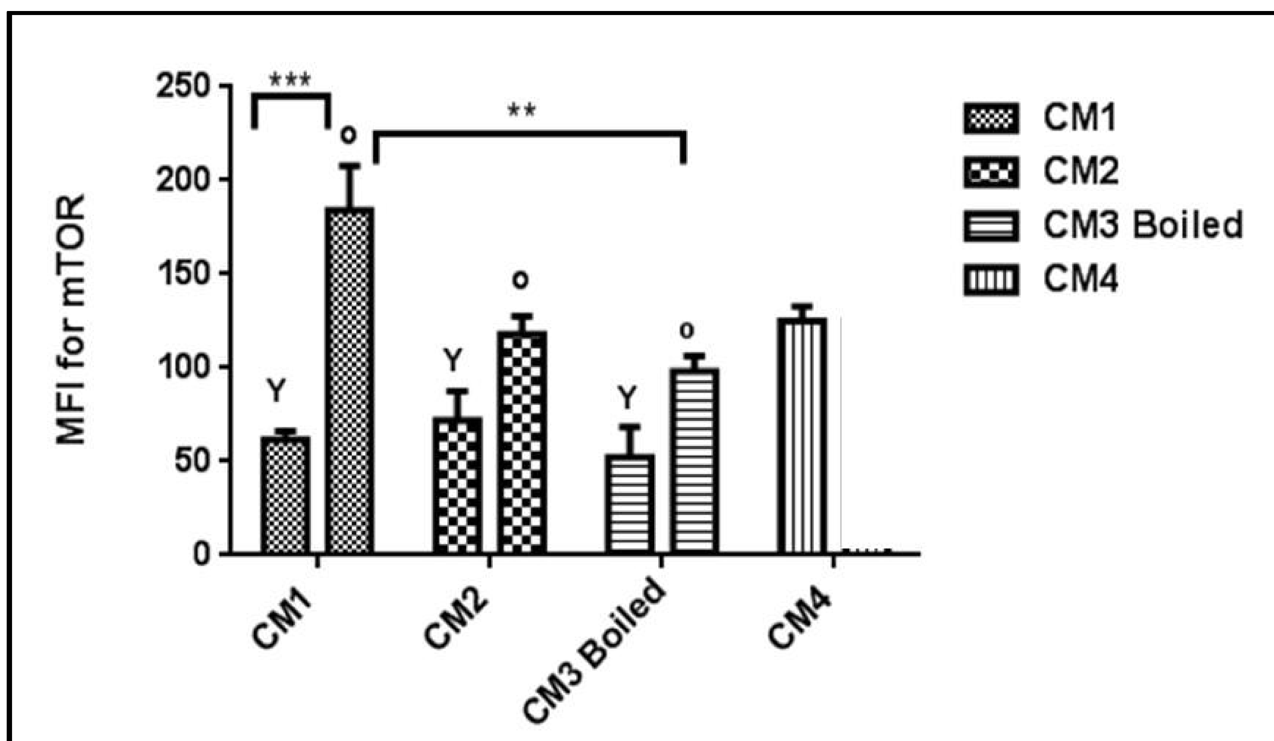


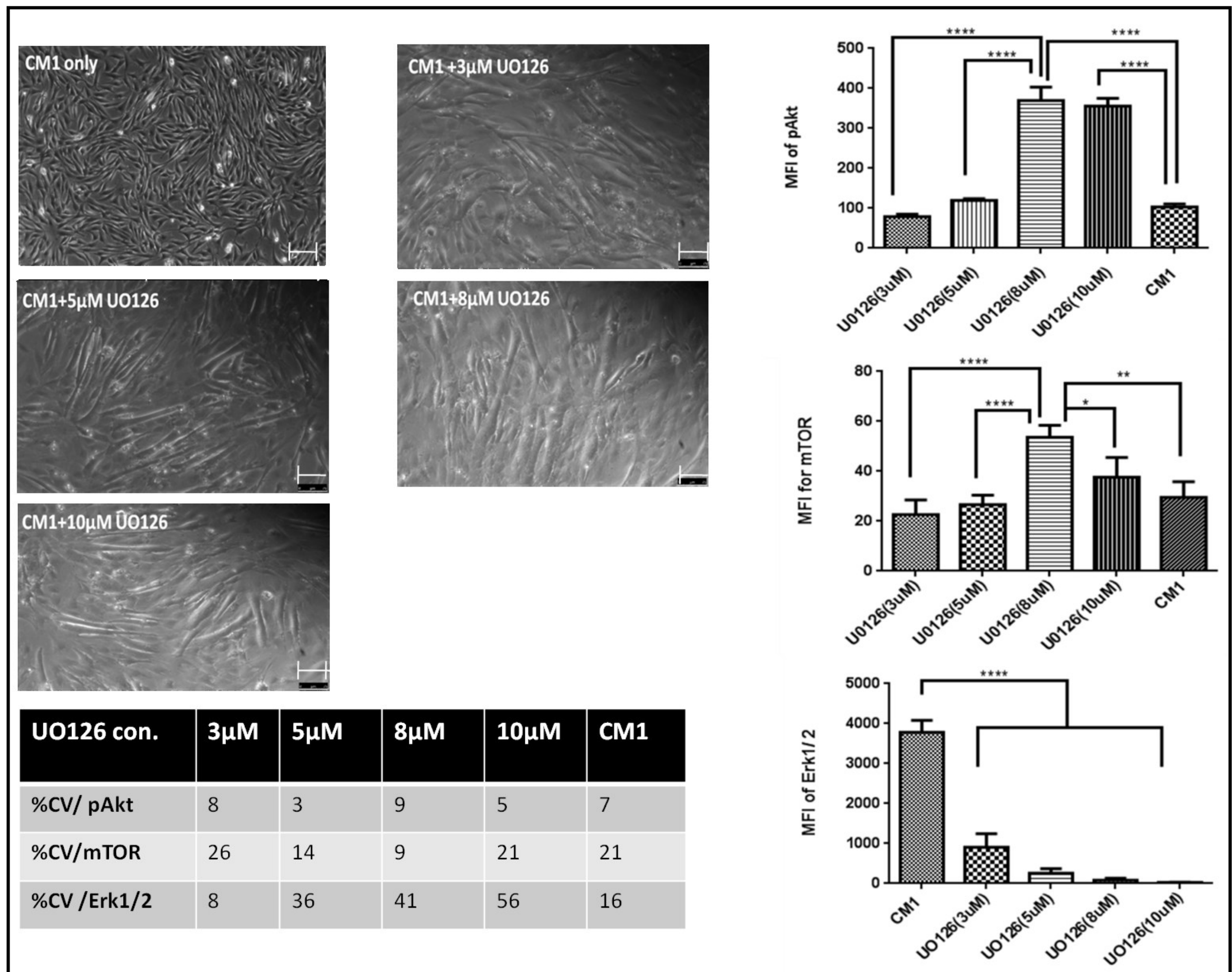
Figure 7.9: MFI of mTOR level in Young and Old CM1-4-treated C2C12 myoblasts. A significant increase in mTOR levels was found in Old CM1 *vs* Young CM; p<0.001 and compared to Old CM3;p<0.01. Data represent means ±SD (n=4) in triplicate: Y:young and O: old.CM1: Activated conditioned media, CM2: Non-activated conditioned media,CM3: Boiled activated conditioned media, CM4 : no lymphocytes conditioned media. %CV for CM1(y);12%, (o);22%, CM2(y)37%, (o); 14%, CM3 Boiled (y)52%, (o);14% and CM4;10%..

7.5.5THE MAPK INHIBITOR UO126 ATTENUATES THE YOUNG CM1-INDUCED MYOBLAST PROLIFERATION.

Initial trials to determine the optimum concentration of UO126 showed higher pAkt levels in C2C12cells treated with Young CM1+8μM UO126, compared with 3μM, 5μM and 10 μM UO126 (369.6±33.8 *vs* 78.9±6.11;p<0.001, 119.9±4.16; p<0.0001 and 103±7.6; p<0.0001). No significant difference was observed between treatments of CM1+8μM UO126 and CM1+10μM UO126 (561.4±110.5 *vs* 568±162; p>0.05; Figure 7.10). Similarly, phosphorylated mTOR was highest in C2C12 cells treated with CM1+8μM UO126 compared to other concentrations (Figure 7.10, right panels). All concentrations of UO126 (3,5,8 and 10μM) significantly decreased the level of Erk1/2 compared to myoblasts treated with CM1

alone (p<0.0001) in an apparent concentration-dependent manner, although no differences in ERK1/2 levels existed between the trials that included any concentration of UO126 (Figure 7.10, right panel,bottom).

Phase contrast imagesobtained following 4 days treatment with the CMs (Figure 7.10, left panels)showed greater myotube formation after incubation with Young CM1+UO126*vs* CM1 alone, with progressively larger myotubes developing with increasing UO126 concentrations.



UO126 con.	3μM	5μM	8μM	10μM	CM1
%CV/ pAkt	8	3	9	5	7
%CV/mTOR	26	14	9	21	21
%CV /Erk1/2	8	36	41	56	16

Figure 7.10: Levels of activated Akt/mTOR and ERK1/2 in C2C12 cells treated with CM1 and 3, 5, 8 and 10μM of UO126. pAkt levels in response to CM1+8μM of UO126 were significantly different compared to other concentrations applied ;p<0.0001. Similarly, mTOR levels in cells treated with CM1+8μM were significantly higher compared to CM1+3μM and CM1+5μM of UO126; p<0.0001 and higher than CM1+10μM and CM1 alone; p<0.05 and 0.001 respectively. Images indicated well-developed myotube formation in response to 8μM *vs* other concentrations and CM1. Data represent means ±SD (n=4) in triplicate. CV: Coefficient of variation. Scale bar=75μm

7.5.6 PROTEOMICS STUDY

In order to identify other novel candidate regulators of C2C12 myoblast proliferation or differentiations and the differences in protein concentrations in the secretomes of the CM1 between young and old, secretome samples were prepared for proteomics analyses. However, no results were obtained from these studies. The main reason was that the samples were dominated by a very small number of very highly abundant proteins, with serum albumin causing particular problems that prevented identification of any secreted, relatively low abundance proteins (see appendix 1).

7.5.7 THE PROLIFERATION OF YOUNG CM1-TREATED C2C1 CELLSWASPARTIALLY ATTENUATEDBY THE IGF-1R INHIBITOR OSI-906

The cell count of OSI-906 pre-treated cells cultured in CM1 was significantly lower compared to Young CM1-treated cells ($120 \pm 10x10^4$ *vs* $171 \pm 10x10^4$ cells/ml: $p<0.01$), but was higher than treatment with OSI-906+ DM alone or DM alone. The cell count of differentiating cells pre-treated with OSI-906+DM was significantly lower compared to CM1 treated cells (63 ± 15 x 10^4 *vs* 171 ± 10 x 10^4 cells/ml: $p<0.0001$ respectively). No significant difference in cell count existed between the control conditions of OSI-906 + DM and the DM-alone ($63.3 \pm 15x10^4$ *vs* $50 \pm 10x10^4$ cell/ml: $p>0.05$ respectively). Cell count data are shown in figure 7.11, bottom panel.

Flow cytometric cell cycle analysis was undertaken to assess the proliferation of cultured cells. Such analyses confirmed that %S phase of OSI-906 pre-treated cells cultured with Young CM1 was significantly lower compared to CM1 treated cells (36.6±2.8% *vs* 49.6±4.5% ; $p<0.01$ respectively). %S phase of OSI-906 pre-treated cells cultured with DM was significantly lower compared to CM1treated cells (24.6±1.5 *vs* 49.6±4.5; $p<0.0001$ respectively). A significant difference was observed between OSI-906 pre-treated cells cultured with CM1 and DM (36.6±2.8 %*vs* 24.6±1.5%; $p<0.01$ respectively). No significant difference was observed between OSI-906-treated and untreated cells cultured with DM (24.6±1.5%*vs* 18±2%; $p>005$). Cell cycle data are shown in Figure7.11, bottom panel.

Morphological assessment of OSI-906 + young CM1 treated cells showed that the usual increase of proliferation observed after Young CM1 treatment was attenuated after OSI-906 treatment. Nevertheless, the OSI-906 + young CM1 treated cells did not develop myotube

formation, as did occur in the control DM-alone conditions, indicating that blocking the IGF-1R was only partially effective at blunting the enhanced proliferation seen after treatment with Young CM1. Cells treated with OSI-906 alone did not grow well enough for any measurements to be taken.

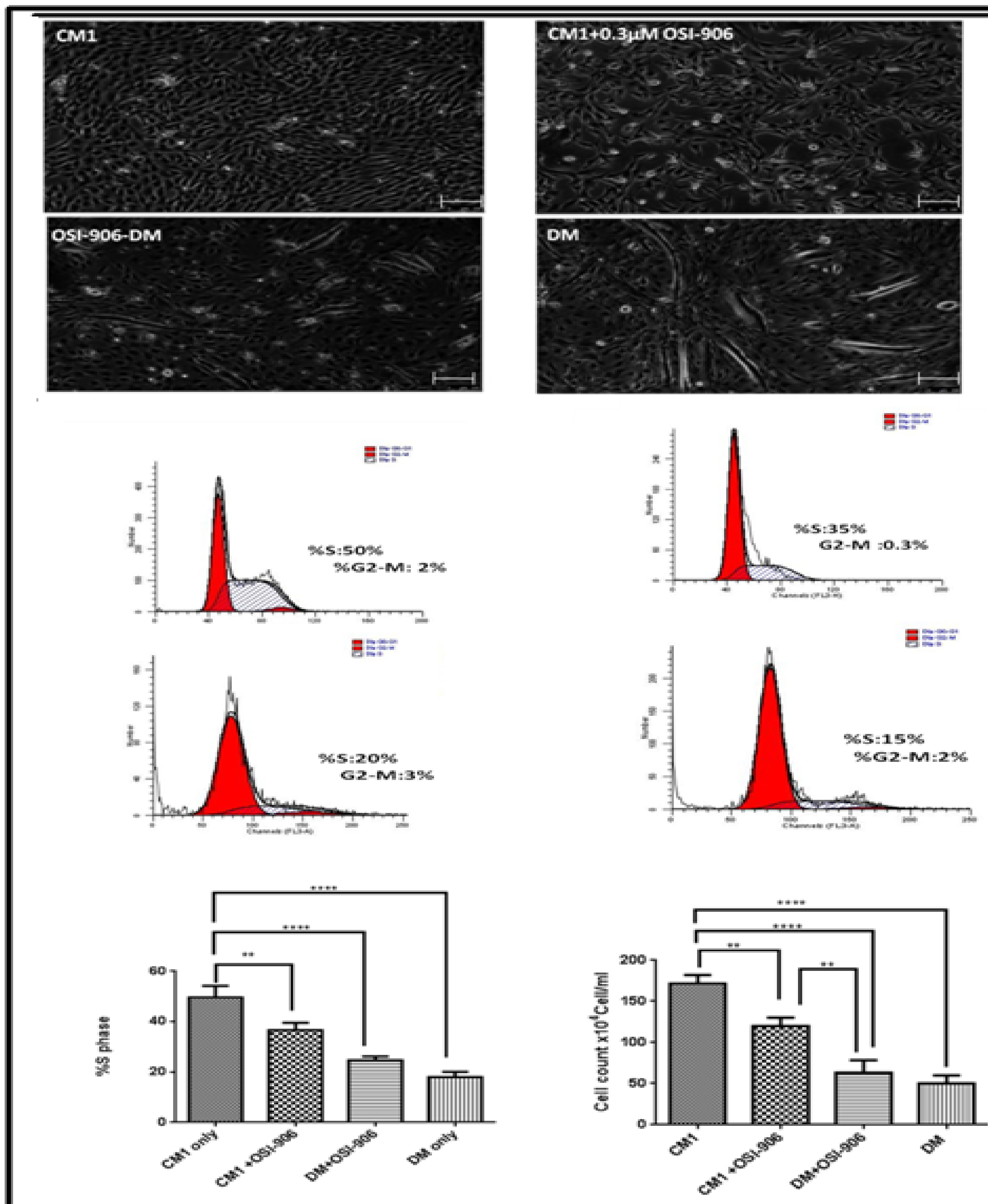


Figure 7.11: Morphological assessment, cell count and cell cycle analysis for OSI-906-pre-treated and un-treated cells cultured with CM1, DM and controls after 3 days. Scale bar=100µm. Data in graphs represent means ±SD (n=4) in triplicates.CM1:Activated conditioned media and DM: Differentiated media. % CV for cell cycle analysis in response to each treatment: % (6, 8, 5 and 9) respectively while %CV of the cell count data: % (6, 8, 12 and 5) respectively.

7.5.8 DETERMININGTHE AKT/mTOR SIGNALLING PATHWAY WITHIN C2C12CELLS TREATED WITH THE IGF-1R INHIBITOR OSI-906

As shown above, pAkt and mTOR were significantly lower after treatment with Young CM1 compared with controls. Pre-treating the C2C12 cells with OSI-906 had no effect on either the phosphorylated Akt or mTOR levels after treatment with CM1, since both remained lower than control conditions. All data are shown in Figure 7.12.

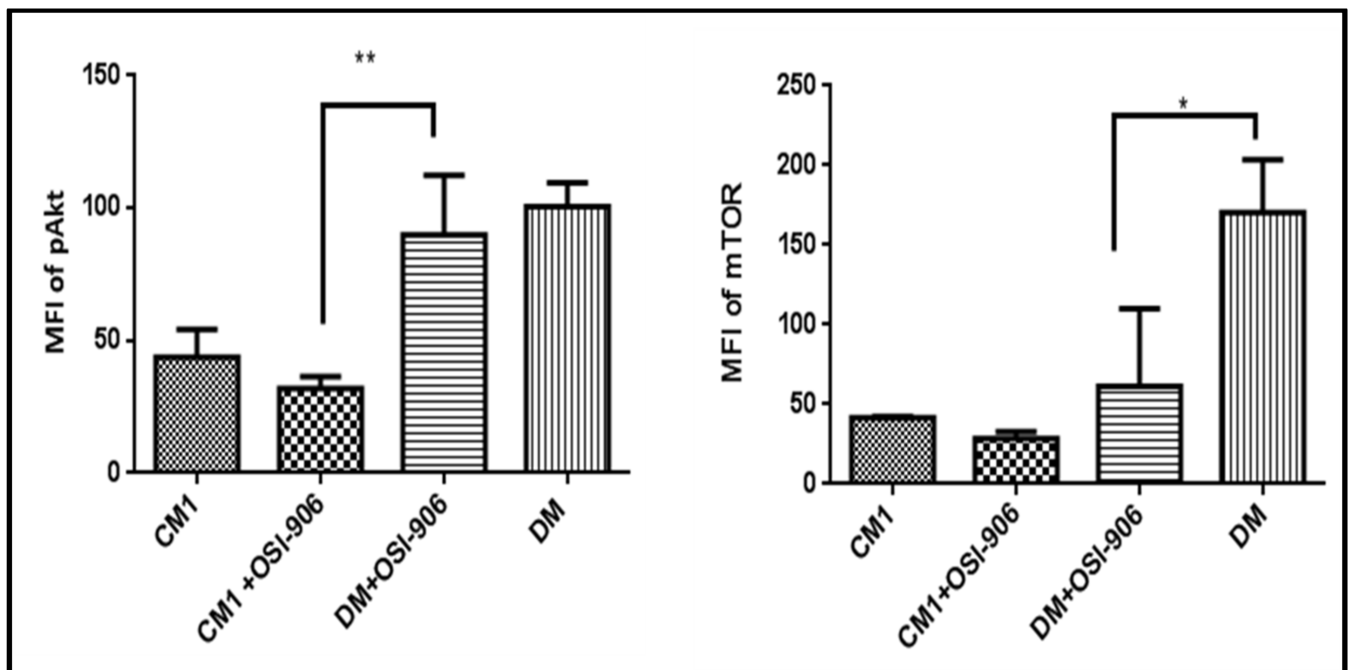


Figure 7.12: The level of phosphorylated Akt and mTOR for OSI-906- pre-treated and un-treated cells cultured with CM1, DM and controls. The level of Akt was significantly different between OSI-906 pre-treated and un-treated cells cultured with CM1 and DM ($p>0.01$). A significant difference was observed in mTOR level between OSI-9.6 pre-treated and un-treated cells cultured in DM ($p<0.05$). Data represent the means ± SD (n=4) in triplicates. %CV for MFI of pAkt: CM1; 20%, CM1+OSI-906 ; 14%, DM+OSI-906; 24% and DM ; 9%. CV for MFI of mTOR: CM12%, CM1+OSI-906; 16%, DM+OSI-906; 35% and DM; 20%.

7.6. DISCUSSION

It was shown in the previous chapter that C2C12 cells cultured with Young CM1 exhibited high levels of proliferation and lower differentiation. In contrast, C2C12 treated with Old CM1 exhibited fusion of myoblasts to form multinucleated myotubes, similar to those observed in control conditions. The work presented in this chapter reveals novel signalling pathways through which the Young CM1, but not the Old CM1 affects intracellular signalling of C2C12 cells, identifying possible mechanisms leading to enhanced C2C12 proliferation after treatment with Young CM1. These effects included MyoD and Myogenin being downregulated in C2C12 after exposure to Young CM1; phosphorylated MEK1 and ERK1/2 were upregulated, and phosphorylated Akt and mTOR were downregulated in Young CM1-treated C2C12 cells. Blocking MAPK eliminated the enhancement C2C12 proliferation after treatment with the Young CM1, and reversed the observations of cellular signalling by increasing pAkt and mTOR while decreasing ERK1/2. Blocking the IGF-1R partially attenuated the enhancement of C2C12 proliferation after treatment with the Young CM1, but had little effect on phosphorylated Akt or mTOR.

It is well established that MyoD is a marker of myogenic lineage, whereas Myogenin is a marker to drive activated myoblasts into the differentiation pathway (Knight and Kothary, 2011). The lower levels of MyoD and Myogenin in C2C12 cells treated with Young CM1 compared to Old CM1 was consistent with the morphological and cell cycle results, both of which showed supressed diffrentiation in Young CM1 compared to Old CM1-treated C2C12.

To determine what signalling cascades were activated or attenuated in Young and Old CM1 in association with the induced proliferation or differentiation, respectively, multiplex studies were performed following 25 min incubation with CMs. This time point was determined following initial studies which showed the maximal activation time of these phosphorylated proteins (Akt/mTOR and IRS-1) was 25 min, as opposed to slightly shorter or longer periods in Young CM1- treated cells and CMs controls. In the current study, it was found that pAkt and mTOR levels increased in Old CM1-4 treated cells; while MEK1and Erk1/2 decreased. Furthermore, our data showed Erk1/2 and MEK1 increased in young CM1; while a decrease in Erk1/2 and MEK1 in Young CM2-4 treated cells was accompained by increased in Akt/mTOR level. These findings are in line with previous studies that identified higher pAkt and mTOR levels in differentiating myoblasts, whereas MEK1 and Erk1/2were previously

shown to be higherin proliferating myoblasts (Creer et al., 2005; Al-Shanti and Stewart, 2012; Widegren et al., 1998; Al-Shanti et al., 2014; Jones et al., 2001).

In order to verify the role of the MEK1 /Erk1/2 signalling pathway for proliferation in CM1-treated myoblast, a selective ERK inhibitor, UO126 was used (Perkins et al., 2007; Winter et al., 2011). Skeletal muscle differentiation is marked by increased levels of Akt and mTOR and decreased in Erk1/2 (Al-Shanti et al., 2014), so the levels of these phosphorylated proteins were measured following incubation of CM1–treated myoblasts with different concentrations of UO126. The data showed that adding 8µMUO126 to the Young CM1 attenuated the enhanced proliferation that is normally observed in this condition, and instead more myotube formation was evidentwhen compared to C2C12 cultured with Young CM1 alone. The concentration of 10µM UO126 added to Young CM1also blunted proliferation and enhanced myotube formation, as previously described (Perkins et al., 2007). Both 8µM and 10µM UO126 significantly increased Akt levels compared to Young CM1 alone, while levels of mTOR were decreased most after adding 8µM UO126 to Young CM1 compared to other concentrations of UO126. These concentrations of UO126 all inhibited proliferation of myoblasts treated with Young CM1 via inhibition of Erk1/2 activation, inducingdifferentiation and myotube formation.

Our results in a previous chapter showed that the IGF -1 concentration in the young secretome was significantly higher compared to the old secretome. A previous study demonstrated that high IGF-I concentrations *in vitro* favoured proliferation, whereas lower IGF-I concentrations promoted differentiation (Sharon A Coolican et al., 1997). Therefore, it is possible that the observed enhanced proliferation in young CM1 is in part accounted for by increased IGF-1 concentrations (Foulstone et al., 2001). To investigate this, C2C12 myoblasts were treated with neutralising anti-IGF-1R antibodies. The outcomes showed that blocking IGF-1R resulted in decreased C2C12 cell counts and %S phase of C2C12 cultured with Young CM1 compared with cells treated with CM1 alone. The morphological results showed thatafter blocking the IGF-1R of C2C12 cells cultured with Young CM1, the cells did not proliferate as much as the usual Young CM1-treated cells, but the myotube formation was still attenuated (as normally occurs after treatment with Young CM1). These findings suggest that the effects of the Young CM1 (increasing the rates of proliferation of C2C12 cells) do not principally work through the IGF-1R, and instead implicate other, as yet unknown, cytokines secreted by activated lymphocytes in young secretomes. For example, we showed previously that IL-6 and TNF-α were more highly expressed by young lymphocytes compared to old. IGF-1 in combination

with TNF-α and IL-6 can achieve the maximal beneficial effect on skeletal myoblasts proliferation via increase ERK phosphorylation (Al-Shanti et al., 2008). Interestingly, this also supports our cell cycle findings and elevated level of activated Erk1/2. Furthermore, in a previous chapter we found elevated amphiregulin expression in the young secretome. Both of IGF-I and amphiregulin functioned synergistically to promote cell proliferation and migration through activation of the MAPK/ ERK pathway (Rundhaug and Fischer, 2010). Again, this supports our findings which demonstrated that CM1-treated cells showed higher MAPK and ERK1/2 phosphorylated proteins levels compared to cells treated with Old CM1. Also blocking IGF-1R did not have an effect on myotube formation in cells cultured with DM compared to normal cells which were grown in DM. Differentiation of myoblasts is strongly regulated by the Akt/mTOR signalling pathway. Our results showed that the level of activated Akt in C2C12 cells cultured with CM1 and DM was not influenced by the IGF-1R blocker. Thus our findingsconflict with a previous study which reported that differentiation in C2C12 is induced by endogenous IGF-1 (Yoshiko et al., 2002). However, the level of mTOR in C2C12 seems to be influenced by IGF-1R inhibitor.

Taken together, our data suggest that as yet unidentifiedcytokines and/or growth factors present in the young activated lymphocyte secretomescan induce a sustained proliferation via low expression of MyoD and myogenin and activation of MEK/ERK (Al-Shanti et al., 2014) signalling pathways, while proteins presentin the old secretomehave little effect, promoting the progression towards differentiaton via increased the levels of MyoD and Myogenin and activation of the Akt/mTOR signalling pathway (Al-Shanti et al., 2014).

7.8 CONCLUSION

In summary, 25min was the optimal time point to trigger Akt , mTOR and IRS-1 activation compared to 5, 15 and 60min in CM1, CM2 and CM4-treated cells. Old CM1-treated myoblasts expressed high levels of MyoD and Myogenin compared to Young CM1- treated cells. These were associated withhigh levels of Akt and mTOR in differentiating cells treated with Old CM1 and high levels of MEK1 and ERK1/2 in proliferating cells treated with Young CM1 . The induced diffrentiation of Young CM1 treated myoblasts following UO126 treatment was accompained by activation of Akt and mTOR. Activation of Akt/mTOR pathway was strongly achieved by 8μM and 10μM UO126 resulting in increased myotube formation and decreased

myoblast proliferation. Using OSI-906 blocker was associted with decreased proliferation with no myotube formation.

CHAPTER 8

8.1 GENERAL DISCUSSION

The aim of the work presented in this thesis was to investigate the differences between young and old immune cells (lymphocytes) for their impact) on skeletal muscle regeneration. To fulfil the aim, optimisation of lymphocyte culture conditions was undertaken. For this purpose young lymphocytes were isolated from fresh whole blood samples and activated using anti-CD3 for 2, 4 and 6 days. Our findings suggested that day 4 was the optimal culture time compared to days 2 and 6 with cells displaying higher proliferation, increased size of colony forming units and CD25 expression. Anti-CD28, a co- stimulatory molecule was added to the media to mimic *in vivo* stimulation. Anti-CD3/CD28 -activated T lymphocytes at day 4 released higher concentrations of cytokines and growth factors compared to day 2. The released growth factors and cytokines from T- lymphocytes are important in tissue repair and regeneration (Hawke, 2011), they contribute to the muscle cell response during proliferation and differentiation stages by affecting transcriptional factors (Tidball and Villalta, 2010).It is clearly important to optimise the volume of activated lymphocyte supernatant (secretome) to investigate the impact on muscle cell behaviour. To this wend it was shown that young conditioned media (i.e. the normal C2C12 cell media, enhanced with the lymphocyte secretome) was able to induce extended proliferation of C2C12 compared to non-stimulated lymphocyte secretomes and other control conditions (Al-Shanti et al., 2014b). These findings suggest that in our model, the young activated lymphocytes release key regulatory factors which are able to induce C2C12 proliferation compared to non-activated T- lymphocytes.

It has been well documented that immune cells undergo alterations in their intrinsic characteristics and marker expression during ageing. Previous studies showed that reduced thymic output in aged individuals negatively affects the function and phenotypes of T lymphocytes, including loss of T cell surface markers, TCR diversity and clonal expansion. A decline in the TCR diversity of T-cells was accompanied by oligoclonal expansion of naive T cells and memory cells. Consequently, all these changes contribute to alter the components of immune system declines in aged individuals (Goronzy and Weyand, 2005; Nikolich-Žugich, 2008).These age-related changes could affect cytokines production leading to alterations in the local microenvironment of satellite cells and hence changes in regeneration. Therefore attempts were made to investigate whether the key factors and CD marker expression of T lymphocytes which are involved in muscle modulation, are different in older lymphocytes compared with young. In the current study we determined CD25 and amphiregulin expression in young and old lymphocytes. These were selected, as FoxP3 Tregs and amphiregulin candidates have been

shown to contribute to muscle repair and regeneration (Burzyn et al., 2013). In addition, a recent study showed that muscular Treg FoxP3 cells potentially stimulate satellite cells proliferation, slow differentiation and enhance wound healing after CTX damage in mice (Castiglioni et al., 2015). The findings of this study are comparable to the present study results that show activated young T-lymphocytes secrete molecules (cytokines and growth factors) that enhance proliferation and inhibit differentiation (Al-Shanti et al., 2014). Therefore, further investigation of secreted Th1/Th2 cytokines from young and old lymphocytes was performed. Our findings strongly suggested that with ageing lymphocyte function and responses to challenge are altered. Our results show that old -activated lymphocytes express lower levels of CD25 marker and amphiregulin production and higher FoxP3 Tregs expression compared to young- activated lymphocytes. Also the present study demonstrates that old lymphocytes release lower concentration of cytokines including: TNF-α, INF-γ, IL-6, IL-1β, IL-12, IL-2 and IGF-I compared to young activated lymphocytes and that these may mediate skeletal muscle regeneration. These changes in lymphocytes with ageing had effects on C2C12 proliferation and migration. The present study shows remarkable proliferation and migration of Young CM1- treated C2C12 compared to cells treated with old CM1 which undergo differentiation, evident as elongated and multinucleated myotube formation that is comparable to the control conditions (CM2-CM4). The extended proliferation of Young CM1 treated cells was associated with a decrease in MyoD and Myogenin and increases in Mek1/Erk1/2 (Al-Shanti et al., 2014), while differentiation of myotubes treated with Old CM1 was associated with increased MyoD and myogenin expression and increased in Akt/ mTOR. A similar observation was recently made by Castiglioni and his colleagues (Castiglioni et al., 2015) who found that co-culture of activated T cells with murine satellite cells potentially enhanced skeletal muscle proliferation and wound healing compared to non-activated T-cells that did not show any myogenic effects on satellite cells. These findings strongly suggest that changes in lymphocyte behaviour and cytokine production with ageing affect skeletal muscle regeneration. It is possible that the competency of aged lymphocytes to respond to antigen challenge decreases and adversely affects downstream signalling pathways including tyrosin phosphorylation (Grossmann et al., 1995) and NFAT and NFҠB translocation (Haynes et al., 2004; Tamir et al., 2000). Consequently, these changes reduce the clonal expansion and cytokines production from naive T- cells (Salam et al., 2013; Haynes and Maue, 2009) as a compensatory response to thymus dwindling in aged individuals. It was found that naive CD4 T cells that have undergone homeostatic cell proliferation have lower diversity in TCR repertoire and produce less IL-2

compared to naive CD4 cells that have not undergone homeostatic replication (Swain et al., 2005). This was evident in our study where lower expression of the CD25 activation marker and IL-2 production were found which may contribute to the attenuated skeletal muscle cell proliferation and migration. It is possible that the decrease in cytokine and amphiregulin production from old T-helper cells results from an increased level of FoxP3 which was found to be associated with T-cell suppression (Fessler et al., 2013; Saito et al., 2010). Accordingly, the effect of FoxP3 Treg cells in promoting muscle repair and regeneration is excluded since its expression is inversely associated with amphiregulin production in young- activated lymphocytes. The results reported here are in contrast with a recent study that showed enhanced muscle repair and regeneration in young mice was generated from increased FoxP3 Treg which was accompanied by amphiregulin production, these differences warrant further investigation, however, they may be as a result of one study being performed *in vitro* and the other *in vivo*. It has been shown that a high concentration of IGF-I induces skeletal muscle proliferation (Coolican et al., 1997) and also accelerates differentiation of myoblasts, increasing the number of fused myonucleus in differentiated myofibres (Yin et al., 2013). Our results show that the proliferation of youngCM1-treated C2C12 cells was partially attenuated by the IGF-1R inhibitor OSI-906 and there was a delay in differentiation of myoblasts treated with OSI-906.The latter finding is comparable to the results of a previous study which showed that the functional inactivation of IGF-I R on C2C12 delayed the IGF-1 -induced differentiation (Cheng et al., 2000). In addition, the finding of extended proliferation of young- treated myoblasts suggests that IGF-I possibly in combination with high concentrations of TNF-α and IL-6 (Al-Shanti et al., 2008) and amphiregulin (Sharma et al., 2006), may contribute to sustained proliferation of C2C12. The mitogenic effect of the latter production is enhanced in the presence of IL-1 and TNF-α (Woodworth et al., 1995). It is not clear which of the cytokines (IGF-I and amphiregulin) are directly involved. But it is clear that the altered concentrations of these effectors in old secretomes impacted on the ability of the secretome to enhance C2C12 proliferation.

Future work should up- and down-regulate candidate proteins from the lymphocyte secretomes in order to identify those that are able to influence skeletal muscle cell signalling, particularly the Akt/mTOR, MyoD and Myogenin pathways. This would lay the foundation from which it should be possible to intervene by producing agents that can restore the capability of older lymphocytes to enhance skeletal muscle regeneration. Previous studies showed that increased level of p38 MAPK phosphorylation and reduced proliferation rate are associated with the

cellular senescence of T lymphocytes. These changes are actively regulated by the inhibitory signalling protein called programmed cell death protein (PD1) (Henson et al., 2012) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (Takahashi et al., 2000) which are both known directly to suppress the activity of T- cells. A recent study demonstrated that blocking these inhibitory molecules increased the functional activity of T cells of older humans (Henson et al., 2015).Thus these a aforementioned studies could open an avenue to restore the inhibitory effects of the aged immune system on skeletal muscle regeneration.It should be possible by reversing aged lymphocytes through interrupting the inhibitory signalling pathwaysthat may be able to enhance cytokines/growth factors–induced skeletal muscle regeneration.

8.2 LIMITATIONS

The results of the studies presented in this thesis may have been affected by a number of limitations which are described below.

- Using a rodent cell line such as C2C12 could be a limitation of the present study because C2C12 are immortalised cells and have been maintained under artificial culture conditions for many years, which can cause them to deviate from their original biological function. However, rodent cell lines are still commonly used to study the molecular mechanisms of muscle differentiation (Cui et al., 2009) and in drug discovery research (Sultan, Henkel et al. 2006, Cho, Kim et al. 2009). Nevertheless, another limitation is that the ageing of the mouse cell lines may not necessarily reflect the ageing of human cell lines, and therefore the work presented in this thesis should be repeated in human cell lines if the aim is to find relevance to human ageing.
- Another limitation could be that human cytokines were added to the murine skeletal muscle cells. This methodology was chosen due to the lack of availability of human immortalized muscle cells. Primary cell lines were unfeasible for the studies performed due to the need for muscle biopsies to be collected from human volunteers for each separate experiment. This model (human cytokines added to mouse cell lines) has been used by others

to investigate the inflammatory mediators secreted from A427 human lung cancer (Oraldi et al., 2013) serum creatine phosphokinase in choline-deficient humans in C2C12(Costa et al., 2004).Using human products with a murine cell line also helps to overcome some ethical and many technical handicaps on the *in vivo* study of human cells and tissues.

- A proteomics study was attempted to identify the potential candidates which induce proliferation and migration. However, the high abundant of albumin human serum in RPMI-1640 lymphocytes culture media was an obstacle to process our samples. Therefore, X-VIVO 15 serum free media was recommended to use for lymphocytes culture. This experiment was performed. Images were taken for lymphocytes after 4 days of stimulation (Figure8.1) and cells were counted (Figure8.2). Cell count results showed no significant difference between lymphocytes cultured in RPMI-1640 and X-VIVO 15. However, the time constrains and the budget limit prevented the performance of a proteomics study.

- The difficulty in recruiting healthy participants, particularly the elderly, for blood sample collection was another barrier that partially restricted the advancement of this work. This problem may be overcome in the future by establishing integrated clinical and academic teams with shared responsibilities for recruitment. It is also possible that the results presented in this thesis are not broadly relevant to all human ageing, since all of the blood donors were caucasian.

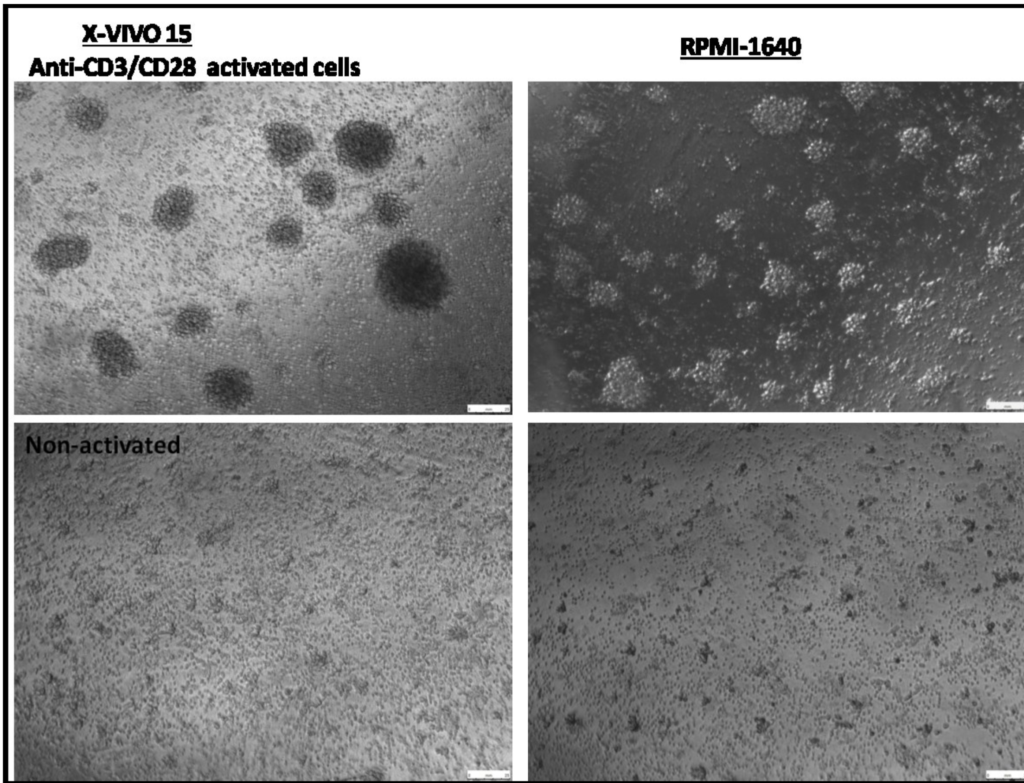


Figure 8.1: Young Lymphocytes growth in X-VIVO 15 (left panel) and RPMI-1640(left panel) at 10Mag.

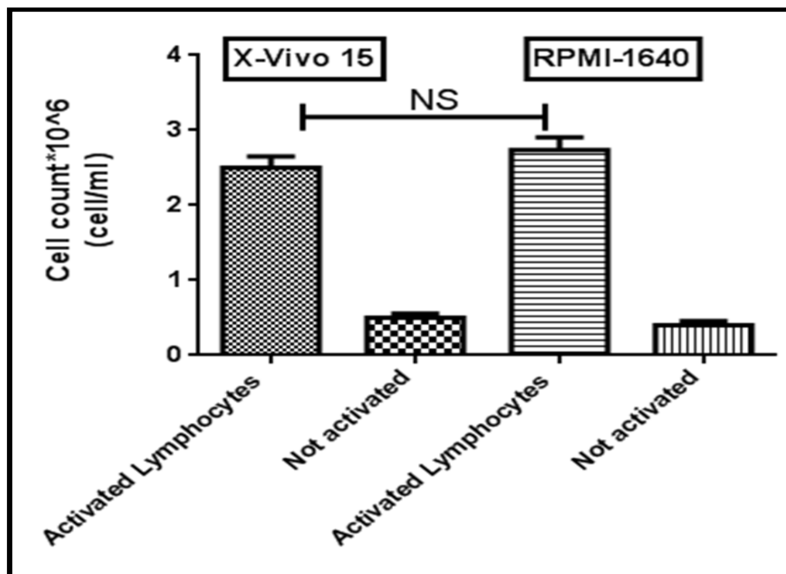


Figure 8.2: cell count of lymphocytes cultured with X-VIVO 15 and RPMI-1640.

8.3 FUTURE WORK

From the results obtained in this current study, the following future studies are suggested:

- To set up larger scale studies to investigate the effect of immune system secretions (growth factors and cytokines) on old and young human skeletal muscle cells *in vitro.*
- To reduce the methodological problems associated with identifying one or more candidate molecules from the hundreds secreted by lymphocytes to induce muscle proliferation and migration, more elaborate studies are needed. For example, we should carry out the Cignal Pathway reporter array which allows for the comprehensive analysis of the 45 most common transcriptional factors signalling pathways that are perturbed by specific genes. This will enable us to identify the potential proteins secreted by activated young lymphocytes, which induce proliferation, migration of C2C12 myoblasts and influence on signalling pathways and the expression of myogenic factors expression. This would allow the possibility to intervene by producing agents that can reverse aged T- cells function to enhance skeletal muscle regeneration.
- Based on the results of the previous aim, targeted-discovery mass spectrometry proteomics can be applied to identify the effector proteins that are secreted by young lymphocytes which induce proliferation and migration.

8.4 CONCLUSION

In summary, the findings presented in this thesis uncovered the novel regulatory role of proteins secreted from activated young T lymphocytes on C2C12 proliferation and migration. These observations provide insights into the interaction between T cells and skeletal muscle cells which may ultimately help us to provide therapeutic strategies to enhance wound healing and promote proliferation of skeletal muscle cells. Our findings also revealed that ageing is associated with changes in intrinsic characteristics and cytokine production of T cells, which in turn attenuated skeletal muscle regeneration.

Our results extended the data obtained from several studies using parabiosis-models. However, our model is more able to examine lymphocyte-specific mediators as we are not dealing with the thousands of substances which exist in the blood and which need to be examined to identify the candidate(s) which may induce such effect. Therefore, our model could be used as fundamental research tool which may provide the essential knowledge needed to develop novel therapeutic strategies to tackle underlying mechanisms of muscle wasting.

REFERENCES

- Al-Shanti, N. and Stewart, C. E. (2009) 'Ca2+/calmodulin‐dependent transcriptional pathways: potential mediators of skeletal muscle growth and development.' *Biological Reviews*, 84(4) pp. 637-652.

- Al-Shanti, N. and Stewart, C. E. (2012) 'Inhibitory effects of IL-6 on IGF-1 activity in skeletal myoblasts could be mediated by the activation of SOCS-3.' *Journal of cellular biochemistry*, 113(3), pp. 923-933.

- Al-Shanti, N., Durcan, P., Al-Dabbagh, S., Dimchev, G. A. and Stewart, C. E. (2014) 'Activated Lymphocytes Secretome Inhibits Differentiation and Induces Proliferation of C2C12 Myoblasts.' *Cellular Physiology and Biochemistry*, 33(1) pp. 117-128.

- Al-Shanti, N., Faulkner, S. H., Saini, A., Loram, I. and Stewart, C. E. (2011) 'A semi-automated programme for tracking myoblast migration following mechanical damage: manipulation by chemical inhibitors.' *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 27(6) pp. 625-636.

- Al-Shanti, N., Saini, A., Faulkner, S. H. and Stewart, C. E. (2008) 'Beneficial synergistic interactions of TNF-α and IL-6 in C2 skeletal myoblasts—potential cross-talk with IGF system.' *Growth Factors*, 26(2) pp. 61-73.

- Amabile, G., D'Alise, A., Iovino, M., Jones, P., Santaguida, S., Musacchio, A., Taylor, S. and Cortese, R. (2009) 'The Aurora B kinase activity is required for the maintenance of the differentiated state of murine myoblasts.' *Cell Death & Differentiation*, 16(2) pp. 321-330.

- Andres, V. and Walsh, K. (1996) 'Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis.' *The Journal of cell biology*, 132(4), Feb, pp. 657-666.

- Arnalich, F., Hernanz, A., Vázquez, J. and Amores, A. (1994) 'Cell-mediated immune response and cytokine production in idiopathic senile anorexia.' *Mechanisms of ageing and development*, 77(1) pp. 67-74.

- Arnold, C. R., Wolf, J., Brunner, S., Herndler-Brandstetter, D. and Grubeck-Loebenstein, B. (2011) 'Gain and loss of T cell subsets in old age—age-related reshaping of the T cell repertoire.' *Journal of clinical immunology*, 31(2) pp. 137-146.

- Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Van Rooijen, N., Plonquet, A., Gherardi, R. K. and Chazaud, B. (2007) 'Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis.' *The Journal of experimental medicine*, 204(5) pp. 1057-1069.

- Aurora, A. B. and Olson, E. N. (2014) 'Immune modulation of stem cells and regeneration.' *Cell stem cell*, 15(1) pp. 14-25.

- Barberi, L., Scicchitano, B. M., De Rossi, M., Bigot, A., Duguez, S., Wielgosik, A., Stewart, C., McPhee, J., Conte, M. and Narici, M. (2013) 'Age-dependent alteration in

muscle regeneration: the critical role of tissue niche.' *Biogerontology*, 14(3) pp. 273-292.

- Bendich, A. (1993) 'Physiological role of antioxidants in the immune system.' *Journal of Dairy Science*, 76(9) pp. 2789-2794.

- Bentzinger, C. F. and Rudnicki, M. A. (2014) 'Rejuvenating aged muscle stem cells.' *Nature medicine*, 20(3) pp. 234-235.

- Bernet, J. D., Doles, J. D., Hall, J. K., Tanaka, K. K., Carter, T. A. and Olwin, B. B. (2014) 'p38 MAPK signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice.' *Nature medicine*, 20(3) pp. 265-271.

- Biressi, S., Molinaro, M. and Cossu, G. (2007) 'Cellular heterogeneity during vertebrate skeletal muscle development.' *Dev Biol*, 308(2), Aug 15, pp. 281-293.

- Blau, H. M., Webster, C., Chiu, C.-P., Guttman, S. and Chandler, F. (1983) 'Differentiation properties of pure populations of human dystrophic muscle cells.' *Experimental cell research*, 144(2) pp. 495-503.

- Blotnick, S., Peoples, G. E., Freeman, M. R., Eberlein, T. J. and Klagsbrun, M. (1994) 'T lymphocytes synthesize and export heparin-binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts: differential production and release by CD4+ and CD8+ T cells.' *Proceedings of the National Academy of Sciences*, 91(8) pp. 2890-2894.

- Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C. and Glass, D. J. (2001) 'Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo.' *Nature cell biology*, 3(11) pp. 1014-1019.

- Bondesen, B. A., Mills, S. T. and Pavlath, G. K. (2006) 'The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms.' *American Journal of Physiology-Cell Physiology*, 290(6) pp. C1651-C1659.

- Borg, T. K. and Caulfield, J. B. (1980) 'Morphology of connective tissue in skeletal muscle.' *Tissue and Cell*, 12(1) pp. 197-207.

- Born, J., Uthgenannt, D., Dodt, C., Nünninghoff, D., Ringvolt, E., Wagner, T. and Fehm, H.-L. (1995) 'Cytokine production and lymphocyte subpopulations in aged humans. An assessment during nocturnal sleep.' *Mechanisms of ageing and development*, 84(2) pp. 113-126.

- Bosurgi, L., Corna, G., Vezzoli, M., Touvier, T., Cossu, G., Manfredi, A. A., Brunelli, S. and Rovere-Querini, P. (2012) 'Transplanted mesoangioblasts require macrophage IL-10 for survival in a mouse model of muscle injury.' *The Journal of Immunology*, 188(12) pp. 6267-6277.

- Brack, A. S., Bildsoe, H. and Hughes, S. M. (2005) 'Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy.' *J Cell Sci*, 118(Pt 20), Oct 15, pp. 4813-4821.

- Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C. and Rando, T. A. (2007) 'Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis.' *Science*, 317(5839) pp. 807-810.

- Brocardo, M. G., Schillaci, R., Galeano, A., Radrizzani, M., White, V., Guerrico, A. G., Santa-Coloma, T. A. and Roldan, A. (2001) 'Early effects of insulin-like growth factor-1 in activated human T lymphocytes.' *Journal of leukocyte biology*, 70(2), Aug, pp. 297-305.

- Bruunsgaard, H., Pedersen, A. N., Schroll, M., Skinhoj, P. and Pedersen, B. (1999) 'Impaired production of proinflammatory cytokines in response to lipopolysaccharide (LPS) stimulation in elderly humans.' *Clinical and experimental immunology*, 118(2) pp. 235-241.

- Buckingham, M. (2006) 'Myogenic progenitor cells and skeletal myogenesis in vertebrates.' *Current opinion in genetics & development*, 16(5) pp. 525-532.

- Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, D. and Relaix, F. (2003) 'The formation of skeletal muscle: from somite to limb.' *Journal of anatomy*, 202(1) pp. 59-68.

- Burzyn, D., Benoist, C. and Mathis, D. (2013) 'Regulatory T cells in nonlymphoid tissues.' *Nature immunology*, 14(10) pp. 1007-1013.

- Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J. L., Cerletti, M., Jang, Y., Sefik, E., Tan, T. G., Wagers, A. J. and Benoist, C. (2013) 'A special population of regulatory T cells potentiates muscle repair.' *Cell*, 155(6) pp. 1282-1295.

- Cannon, J. G. and St Pierre, B. A. (1998) 'Cytokines in exertion-induced skeletal muscle injury.' *Molecular and cellular biochemistry*, 179(1-2), Feb, pp. 159-167.

- Carlson, B. and Faulkner, J. (1989) 'Muscle transplantation between young and old rats: age of host determines recovery.' *American Journal of Physiology-Cell Physiology*, 256(6) pp. C1262-C1266.

- Carlson, B. M. and Faulkner, J. A. (1996) 'The Regeneration of Noninnervated Musele Grafts and Marcaine-Treated Muscles in Young and Old Rats.' *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 51(1) pp. B43-B49.

- Carlson, M. E. and Conboy, I. M. (2007) 'Loss of stem cell regenerative capacity within aged niches.' *Aging cell*, 6(3) pp. 371-382.

- Carlson, M. E., Suetta, C., Conboy, M. J., Aagaard, P., Mackey, A., Kjaer, M. and Conboy, I. (2009) 'Molecular aging and rejuvenation of human muscle stem cells.' *EMBO molecular medicine*, 1(8-9) pp. 381-391.

- Carolan, L. A., Butler, J., Rockman, S., Guarnaccia, T., Hurt, A. C., Reading, P., Kelso, A., Barr, I. and Laurie, K. L. (2014) 'TaqMan real time RT-PCR assays for detecting ferret innate and adaptive immune responses.' *Journal of virological methods*, 205 pp. 38-52.

- Carosio, S., Berardinelli, M. G., Aucello, M. and Musaro, A. (2011) 'Impact of ageing on muscle cell regeneration.' *Ageing Res Rev*, 10(1), pp. 35-42.

- Castiglioni, A., Corna, G., Rigamonti, E., Basso, V., Vezzoli, M., Monno, A., Almada, A. E., Mondino, A., Wagers, A. J. and Manfredi, A. A. (2015) 'FOXP3+ T Cells Recruited to Sites of Sterile Skeletal Muscle Injury Regulate the Fate of Satellite Cells and Guide Effective Tissue Regeneration.' *PloS one*, 10(6) p. e0128094.

- Chambard, J.-C., Lefloch, R., Pouysségur, J. and Lenormand, P. (2007) 'ERK implication in cell cycle regulation.' *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1773(8) pp. 1299-1310.

- Chao, K.-H., Wu, M.-Y., Yang, J.-H., Chen, S.-U., Yang, Y.-S. and Ho, H.-N. (2002) 'Expression of the interleukin-2 receptor α (CD25) is selectively decreased on decidual CD4+ and CD8+ T lymphocytes in normal pregnancies.' *Molecular human reproduction*, 8(7) pp. 667-673.

- Charge, S. B. and Rudnicki, M. A. (2004) 'Cellular and molecular regulation of muscle regeneration.' *Physiological reviews*, 84(1) pp. 209-238.

- Chen, J., Mo, R., Lescure, P. A., Misek, D. E., Hanash, S., Rochford, R., Hobbs, M. and Yung, R. L. (2003) 'Aging is associated with increased T-cell chemokine expression in C57BL/6 mice.' *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 58(11) pp. B975-B983.

- Chen, S.-E., Gerken, E., Zhang, Y., Zhan, M., Mohan, R. K., Li, A. S., Reid, M. B. and Li, Y.-P. (2005) 'Role of TNF-α signaling in regeneration of cardiotoxin-injured muscle.' *American Journal of Physiology-Cell Physiology*, 289(5) pp. C1179-C1187.

- Cheng, M., Nguyen, M. H., Fantuzzi, G. and Koh, T. J. (2008) Endogenous interferon-gamma is required for efficient skeletal muscle regeneration. (May) *American journal of physiology. Cell physiology*. 2008/03/21 ed., Vol. 294, pp. C1183-1191.

- Cheng, Z., Adi, S., Wu, N., Hsiao, D., Woo, E., Filvaroff, E., Gustafson, T. and Rosenthal, S. (2000) 'Functional inactivation of the IGF-I receptor delays differentiation of skeletal muscle cells.' *Journal of endocrinology*, 167(1) pp. 175-182.

- Chiu, B. C., Shang, X., Frait, K. A., Hu, J. S., Komuniecki, E., Miller, R. A. and Chensue, S. W. (2002) 'Differential effects of ageing on cytokine and chemokine responses during type-1 (mycobacterial) and type-2 (schistosomal) pulmonary granulomatous inflammation in mice.' *Mechanisms of ageing and development*, 123(4) pp. 313-326.

- Chiu, B.-C., Stolberg, V. R., Zhang, H. and Chensue, S. W. (2007) 'Increased Foxp3+ Treg cell activity reduces dendritic cell co-stimulatory molecule expression in aged mice.' *Mechanisms of ageing and development*, 128(11) pp. 618-627.

- Collins-Hooper, H., Woolley, T. E., Dyson, L., Patel, A., Potter, P., Baker, R. E., Gaffney, E. A., Maini, P. K., Dash, P. R. and Patel, K. (2012) 'Age-Related Changes in Speed and Mechanism of Adult Skeletal Muscle Stem Cell Migration.' *Stem Cells*, 30(6) pp. 1182-1195.

- Conboy, I. M. and Rando, T. A. (2002) 'The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis.' *Developmental cell*, 3(3) pp. 397-409.

- Conboy, I. M., Conboy, M. J., Smythe, G. M. and Rando, T. A. (2003) 'Notch-mediated restoration of regenerative potential to aged muscle.' *Science*, 302(5650) pp. 1575-1577.

- Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L. and Rando, T. A. (2005) 'Rejuvenation of aged progenitor cells by exposure to a young systemic environment.' *Nature*, 433(7027) pp. 760-764.

- Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J. and Florini, J. R. (1997) 'The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways.' *Journal of Biological Chemistry*, 272(10) pp. 6653-6662.

- Cooper, L. J., Topp, M. S., Pinzon, C., Plavec, I., Jensen, M. C., Riddell, S. R. and Greenberg, P. D. (2004) 'Enhanced transgene expression in quiescent and activated human CD8+ T cells.' *Human gene therapy*, 15(7) pp. 648-658.

- Corsetti, G., Pasini, E., D'Antona, G., Nisoli, E., Flati, V., Assanelli, D., Dioguardi, F. S. and Bianchi, R. (2008) 'Morphometric changes induced by amino acid supplementation in skeletal and cardiac muscles of old mice.' *The American journal of cardiology*, 101(11) pp. S26-S34.

- Creer, A., Gallagher, P., Slivka, D., Jemiolo, B., Fink, W. and Trappe, S. (2005) 'Influence of muscle glycogen availability on ERK1/2 and Akt signaling after resistance exercise in human skeletal muscle.' *Journal of applied physiology*, 99(3), Sep, pp. 950-956.

- Cui, Z., Chen, X., Lu, B., Park, S. K., Xu, T., Xie, Z., Xue, P., Hou, J., Hang, H. and Yates, J. R. (2009) 'Preliminary quantitative profile of differential protein expression between rat L6 myoblasts and myotubes by stable isotope labeling with amino acids in cell culture.' *Proteomics*, 9(5) pp. 1274-1292.

- Curti, B. D., Ochoa, A. C., Urba, W. J., Alvord, W. G., Kopp, W. C., Powers, G., Hawk, C., Creekmore, S. P., Gause, B. L. and Janik, J. E. (1996) 'Influence of interleukin-2 regimens on circulating populations of lymphocytes after adoptive transfer of anti-CD3-stimulated T cells: results from a phase I trial in cancer patients.' *Journal of Immunotherapy*, 19(4) pp. 296-308.

- da Costa, K.-A., Badea, M., Fischer, L. M. and Zeisel, S. H. (2004) 'Elevated serum creatine phosphokinase in choline-deficient humans: mechanistic studies in C2C12 mouse myoblasts.' *The American journal of clinical nutrition*, 80(1) pp. 163-170.

- Day, K., Shefer, G., Shearer, A. and Yablonka-Reuveni, Z. (2010) 'The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny.' *Dev Biol*, 340(2) pp. 330-343.

- de la Serna, I. L., Roy, K., Carlson, K. A. and Imbalzano, A. N. (2001) 'MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes.' *The Journal of biological chemistry*, 276(44), Nov 2, pp. 41486-41491.

- De Rosa, S. C., Lu, F. X., Yu, J., Perfetto, S. P., Falloon, J., Moser, S., Evans, T. G., Koup, R., Miller, C. J. and Roederer, M. (2004) 'Vaccination in humans generates broad T cell cytokine responses.' *J Immunol*, 173(9), Nov 1, pp. 5372-5380.

- Dejaco, C., Duftner, C. and Schirmer, M. (2006) 'Are regulatory T-cells linked with aging?' *Experimental gerontology*, 41(4) pp. 339-345.

- Delmas, C., Manenti, S., Boudjelal, A., Peyssonnaux, C., Eychène, A. and Darbon, J.-M. (2001) 'The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells.' *Journal of Biological Chemistry*, 276(37) pp. 34958-34965.

- Deng, B., Wehling-Henricks, M., Villalta, S. A., Wang, Y. and Tidball, J. G. (2012) 'IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration.' *The Journal of Immunology*, 189(7) pp. 3669-3680.

- Dhawan, J. and Rando, T. A. (2005) 'Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment.' *Trends in cell biology*, 15(12) pp. 666-673.

- Dinarello, C. A. (2006) 'Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process.' *The American journal of clinical nutrition*, 83(2) pp. 447S-455S.

- Dreyer, H. C., Blanco, C. E., Sattler, F. R., Schroeder, E. T. and Wiswell, R. A. (2006) 'Satellite cell numbers in young and older men 24 hours after eccentric exercise.' *Muscle & nerve*, 33(2) pp. 242-253.

- Duan, C., Ren, H. and Gao, S. (2010) 'Insulin-like growth factors (IGFs), IGF receptors, and IGF-binding proteins: roles in skeletal muscle growth and differentiation.' *General and comparative endocrinology*, 167(3) pp. 344-351.

- Dumke, B. R. and Lees, S. J. (2011) 'Age-related impairment of T cell-induced skeletal muscle precursor cell function.' *American journal of physiology. Cell physiology*, 300(6), pp. C1226-1233.

- Duran, E. M., Shapshak, P., Worley, J., Minagar, A., Ziegler, F., Haliko, S., Moleon-Borodowsky, I. and Haslett, P. (2005) 'Presenilin-1 detection in brain neurons and FOXP3 in peripheral blood mononuclear cells: normalizer gene selection for real time reverse transcriptase pcr using the deltadeltaCt method.' *Front Biosci*, 10(9) pp. 2955-2965.

- Duran, E.M., Shapshak, P., Worley, J., Minagar, A., Ziegler, F., Haliko, S., Moleon-Borodowsky, I. and Haslett, P.A., 2004. Presenilin-1 detection in brain neurons and FOXP3 in peripheral blood mononuclear cells: normalizer gene selection for real time reverse transcriptase pcr using the deltadeltaCt method. *Frontiers in bioscience: a journal and virtual library*, *10*, pp.2955-2965.
- Egerman, M.A., Cadena, S.M., Gilbert, J.A., Meyer, A., Nelson, H.N., Swalley, S.E., Mallozzi, C., Jacobi, C., Jennings, L.L., Clay, I. and Laurent, G., 2015. GDF11 increases with age and inhibits skeletal muscle regeneration. *Cell metabolism*, *22*(1), pp.164-174.

- Fagiolo, U., Cossarizza, A., Scala, E., Fanales-Belasio, E., Ortolani, C., Cozzi, E., Monti, D., Franceschi, C. and Paganelli, R. (1993) 'Increased cytokine production in

mononuclear cells of healthy elderly people.' *European journal of immunology*, 23(9) pp. 2375-2378.

- Farini, A., Meregalli, M., Belicchi, M., Battistelli, M., Parolini, D., D'Antona, G., Gavina, M., Ottoboni, L., Constantin, G. and Bottinelli, R. (2007) 'T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse.' *The Journal of pathology*, 213(2) pp. 229-238.
- Fessler, J., Ficjan, A., Duftner, C. and Dejaco, C. (2013) 'The impact of aging on regulatory T-cells.' *Frontiers in immunology*, 4
- Foster, W., Li, Y., Usas, A., Somogyi, G. and Huard, J. (2003) 'Gamma interferon as an antifibrosis agent in skeletal muscle.' *Journal of orthopaedic research*, 21(5) pp. 798-804.
- Foulstone, E. J., Meadows, K. A., Holly, J. M. and Stewart, C. E. (2001) 'Insulin-like growth factors (IGF-I and IGF-II) inhibit C2 skeletal myoblast differentiation and enhance TNFα-induced apoptosis.' *Journal of cellular physiology*, 189(2) pp. 207-215.
- Fritsch, R. D., Shen, X., Sims, G. P., Hathcock, K. S., Hodes, R. J. and Lipsky, P. E. (2005) 'Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27.' *The Journal of Immunology*, 175(10) pp. 6489-6497.
- Frommer, S. A. (2008) *Investigating the use of multipotent adult progenitor cells for treatment of Duchenne muscular dystrophy: A translational approach.* ProQuest.
- Fu, X., Han, B., Cai, S., Lei, Y., Sun, T. and Sheng, Z. (2009) 'Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-α and its possible role in wound healing.' *Wound repair and regeneration*, 17(2) pp. 185-191.
- Fuentes, E. N., Björnsson, B. T., Valdés, J. A., Einarsdottir, I. E., Lorca, B., Alvarez, M. and Molina, A. (2011) 'IGF-I/PI3K/Akt and IGF-I/MAPK/ERK pathways in vivo in skeletal muscle are regulated by nutrition and contribute to somatic growth in the fine flounder.' *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 300(6) pp. R1532-R1542.
- Galvez, B. G., Sampaolesi, M., Brunelli, S., Covarello, D., Gavina, M., Rossi, B., Constantin, G., Torrente, Y. and Cossu, G. (2006) 'Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability.' *The Journal of cell biology*, 174(2) pp. 231-243.
- Gamell, C., Osses, N., Bartrons, R., Rückle, T., Camps, M., Rosa, J. L. and Ventura, F. (2008) 'BMP2 induction of actin cytoskeleton reorganization and cell migration requires PI3-kinase and Cdc42 activity.' *Journal of cell science*, 121(23) pp. 3960-3970.
- Garland, R. J., El-Shanti, N., West, S. E., Hancock, J. P., Goulden, N. J., Steward, C. G. and Rowbottom, A. W. (2002) 'Human CD8+ CTL recognition and in vitro lysis of herpes simplex virus-infected cells by a non-MHC restricted mechanism.' *Scandinavian journal of immunology*, 55(1), Jan, pp. 61-69.

- Gayraud-Morel, B., Chrétien, F., Flamant, P., Gomès, D., Zammit, P. S. and Tajbakhsh, S. (2007) 'A role for the myogenic determination gene Myf5 in adult regenerative myogenesis.' *Dev Biol*, 312(1) pp. 13-28.

- Geissmann, F., Jung, S. and Littman, D. R. (2003) 'Blood monocytes consist of two principal subsets with distinct migratory properties.' *Immunity*, 19(1) pp. 71-82.

- George, T., Velloso, C. P., Alsharidah, M., Lazarus, N. R. and Harridge, S. D. (2010) 'Sera from young and older humans equally sustain proliferation and differentiation of human myoblasts.' *Experimental gerontology*, 45(11) pp. 875-881.

- Glass, D. J. (2003) 'Molecular mechanisms modulating muscle mass.' *Trends Mol Med*, 9(8), Aug, pp. 344-350.

- Glass, D. J. (2010) 'Signaling pathways perturbing muscle mass.' *Current Opinion in Clinical Nutrition & Metabolic Care*, 13(3) pp. 225-229.

- Goldspink, G., Fernandes, K., Williams, P. E. and Wells, D. J. (1994) 'Age-related changes in collagen gene expression in the muscles of mdx dystrophic and normal mice.' *Neuromuscular Disorders*, 4(3) pp. 183-191.

- Goronzy, J. J. and Weyand, C. M. (2005) 'T cell development and receptor diversity during aging.' *Current opinion in immunology*, 17(5) pp. 468-475.

- Goronzy, J. J., Lee, W.-W. and Weyand, C. M. (2007) 'Aging and T-cell diversity.' *Experimental gerontology*, 42(5) pp. 400-406.

- Gottenberg, J.-E., Lavie, F., Abbed, K., Gasnault, J., Le Nevot, E., Delfraissy, J.-F., Taoufik, Y. and Mariette, X. (2005) 'CD4 CD25 high regulatory T cells are not impaired in patients with primary Sjögren's syndrome.' *Journal of autoimmunity*, 24(3) pp. 235-242.

- Gregg, R., Smith, C., Clark, F., Dunnion, D., Khan, N., Chakraverty, R., Nayak, L. and Moss, P. (2005) 'The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age.' *Clinical & Experimental Immunology*, 140(3) pp. 540-546.

- Gros, J., Manceau, M., Thomé, V. and Marcelle, C. (2005) 'A common somitic origin for embryonic muscle progenitors and satellite cells.' *Nature*, 435(7044) pp. 954-958.

- Grossmann, A., Maggio-Price, L., Jinneman, J. C. and Rabinovitch, P. S. (1991) 'Influence of aging on intracellular free calcium and proliferation of mouse T-cell subsets from various lymphoid organs.' *Cellular immunology*, 135(1) pp. 118-131.

- Grossmann, A., Rabinovitch, P. S., Kavanagh, T. J., Jinneman, J. C., Gilliland, L. K., Ledbetter, J. A. and Kanner, S. B. (1995) 'Activation of Murine T-cells via Phospholipase-Cγl-Associated Protein Tyrosine Phosphorylation Is Reduced With Aging.' *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 50(4) pp. B205-B212.

- Grounds, M. D. (1998) 'Age-associated Changes in the Response of Skeletal Muscle Cells to Exercise and Regenerationa.' *Annals of the New York Academy of Sciences*, 854(1) pp. 78-91.

- Grounds, M. D. (2002) 'Reasons for the degeneration of ageing skeletal muscle: a central role for IGF-1 signalling.' *Biogerontology*, 3(1-2) pp. 19-24.
- Grubišić, V., Gottipati, M. K., Stout Jr, R. F., Grammer, J. R. and Parpura, V. (2014) 'Heterogeneity of myotubes generated by the MyoD and E12 basic helix-loop-helix transcription factors in otherwise non-differentiation growth conditions.' *Biomaterials*, 35(7) pp. 2188-2198.
- Gutmann, E. and Carlson, B. M. (1976) 'Regeneration and transplantation of muscles in old rats and between young and old rats.' *Life sciences*, 18(1) pp. 109-114.
- Haddad, F., Zaldivar, F., Cooper, D. M. and Adams, G. R. (2005) 'IL-6-induced skeletal muscle atrophy.' *Journal of Applied Physiology*, 98(3) pp. 911-917.
- Halevy, O., Piestun, Y., Allouh, M. Z., Rosser, B. W., Rinkevich, Y., Reshef, R., Rozenboim, I., Wleklinski-Lee, M. and Yablonka-Reuveni, Z. (2004) 'Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal.' *Developmental Dynamics*, 231(3) pp. 489-502.
- Hara, M., Yuasa, S., Shimoji, K., Onizuka, T., Hayashiji, N., Ohno, Y., Arai, T., Hattori, F., Kaneda, R. and Kimura, K. (2011) 'G-CSF influences mouse skeletal muscle development and regeneration by stimulating myoblast proliferation.' *The Journal of experimental medicine*, 208(4) pp. 715-727.
- Hawke, T. J. (2011) 'T-cells and muscle just don't talk like they used to: focus on "Age-related impairment of T cell-induced skeletal muscle precursor cell function".' *American journal of physiology. Cell physiology*, 300(6), Jun, pp. C1223-1225.
- Hawke, T. J. and Garry, D. J. (2001) 'Myogenic satellite cells: physiology to molecular biology.' *Journal of applied physiology*, 91(2) pp. 534-551.
- Hawke, T. J., Meeson, A. P., Jiang, N., Graham, S., Hutcheson, K., DiMaio, J. and Garry, D. J. (2003) 'p21 is essential for normal myogenic progenitor cell function in regenerating skeletal muscle.' *American Journal of Physiology-Cell Physiology*, 285(5) pp. C1019-C1027.
- Haynes, L. and Maue, A. C. (2009) 'Effects of aging on T cell function.' *Current opinion in immunology*, 21(4) pp. 414-417.
- Haynes, L., Eaton, S. M., Burns, E. M., Rincon, M. and Swain, S. L. (2004) 'Inflammatory cytokines overcome age-related defects in CD4 T cell responses in vivo.' *The Journal of Immunology*, 172(9) pp. 5194-5199.
- Haynes, L., Linton, P.-J., Eaton, S. M., Tonkonogy, S. L. and Swain, S. L. (1999) 'Interleukin 2, but not other common γ chain–binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice.' *The Journal of experimental medicine*, 190(7) pp. 1013-1024.
- Haynes, L., Linton, P.J., Eaton, S.M., Tonkonogy, S.L. and Swain, S.L., 1999. Interleukin 2, but not other common γ chain–binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. *The Journal of experimental medicine*, *190*(7), pp.1013-1024.

- Henson, S. M., Macaulay, R., Franzese, O. and Akbar, A. N. (2012) 'Reversal of functional defects in highly differentiated young and old CD8 T cells by PDL blockade.' *Immunology*, 135(4) pp. 355-363.

- Henson, S. M., Macaulay, R., Riddell, N. E., Nunn, C. J. and Akbar, A. N. (2015) 'Blockade of PD-1 or p38 MAP kinase signaling enhances senescent human CD8+ T-cell proliferation by distinct pathways.' *European journal of immunology*, 45(5) pp. 1441-1451.

- Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., Rando, T. A. and Chawla, A. (2013) 'Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration.' *Cell*, 153(2) pp. 376-388.

- Hoetzenecker, K., Zimmermann, M., Hoetzenecker, W., Schweiger, T., Kollmann, D., Mildner, M., Hegedus, B., Mitterbauer, A., Hacker, S., Birner, P., Gabriel, C., Gyongyosi, M., Blyszczuk, P., Eriksson, U. and Ankersmit, H. J. (2013) 'Mononuclear cell secretome protects from experimental autoimmune myocarditis.' *European heart journal*, Jan 14,

- Holmes, D. A. (2008) *Modulation of HIV-1 Replication and T Cell Activation by FoxP3.* ProQuest.

- Horsley, V., Jansen, K. M., Mills, S. T. and Pavlath, G. K. (2003) 'IL-4 acts as a myoblast recruitment factor during mammalian muscle growth.' *Cell*, 113(4) pp. 483-494.

- Jerome, K. R. (2008) 'Viral modulation of T-cell receptor signaling.' *Journal of virology*, 82(9) pp. 4194-4204.

- Jersmann, H. P. (2005) 'Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells.' *Immunology and cell biology*, 83(5) pp. 462-467.

- Jetten, N., Verbruggen, S., Gijbels, M. J., Post, M. J., De Winther, M. P. and Donners, M. M. (2014) 'Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo.' *Angiogenesis*, 17(1) pp. 109-118.

- Johnson, E., Jones, L. and Kozak, R. (1992) 'Expression and function of insulin-like growth factor receptors on anti-CD3-activated human T lymphocytes.' *The Journal of Immunology*, 148(1) pp. 63-71.

- Jones, N. C., Fedorov, Y. V., Rosenthal, R. S. and Olwin, B. B. (2001) 'ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion.' *Journal of cellular physiology*, 186(1) pp. 104-115.

- Josefowicz, S. Z., Lu, L. F. and Rudensky, A. Y. (2012) 'Regulatory T cells: mechanisms of differentiation and function.' *Annu Rev Immunol*, 30 pp. 531-564.

- Junnila, R. K., List, E. O., Berryman, D. E., Murrey, J. W. and Kopchick, J. J. (2013) 'The GH/IGF-1 axis in ageing and longevity.' *Nature Reviews Endocrinology*, 9(6) pp. 366-376.

- Karalaki, M., Fili, S., Philippou, A. and Koutsilieris, M. (2009) 'Muscle regeneration: cellular and molecular events.' *In vivo*, 23(5) pp. 779-796.

- Karanfilov, C. I., Liu, B., Fox, C. C., Lakshmanan, R. R. and Whisler, R. L. (1999) 'Age-related defects in Th1 and Th2 cytokine production by human T cells can be dissociated from altered frequencies of CD45RA+ and CD45RO+ T cell subsets.' *Mech Ageing Dev*, 109(2) pp. 97-112.

- Kasprzak, A. and Adamek, A. (2012) 'The insulin-like growth factor (IGF) signaling axis and hepatitis C virus-associated carcinogenesis (Review).' *International journal of oncology*, 41(6) pp. 1919-1931.

- Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V. and Tajbakhsh, S. (2004) 'Mrf4 determines skeletal muscle identity in Myf5: Myod double-mutant mice.' *Nature*, 431(7007) pp. 466-471.

- Katsimpardi, L., Litterman, N. K., Schein, P. A., Miller, C. M., Loffredo, F. S., Wojtkiewicz, G. R., Chen, J. W., Lee, R. T., Wagers, A. J. and Rubin, L. L. (2014) 'Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors.' *Science*, 344(6184) pp. 630-634.

- Katz, S. C., Ryan, K., Ahmed, N., Plitas, G., Chaudhry, U. I., Kingham, T. P., Naheed, S., Nguyen, C., Somasundar, P. and Espat, N. J. (2011) 'Obstructive jaundice expands intrahepatic regulatory T cells, which impair liver T lymphocyte function but modulate liver cholestasis and fibrosis.' *The Journal of Immunology*, 187(3) pp. 1150-1156.

- Kim, H. O., Kim, H.-S., Youn, J.-C., Shin, E.-C. and Park, S. (2011) 'Serum cytokine profiles in healthy young and elderly population assessed using multiplexed bead-based immunoassays.' *J Transl Med*, 9(113) pp. 1-7.

- Kitzmann, M. and Fernandez, A. (2001) 'Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts.' *Cellular and Molecular Life Sciences CMLS*, 58(4) pp. 571-579.

- Kleinschnitz, C., Kraft, P., Dreykluft, A., Hagedorn, I., Göbel, K., Schuhmann, M. K., Langhauser, F., Helluy, X., Schwarz, T. and Bittner, S. (2013) 'Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature.' *Blood*, 121(4) pp. 679-691.

- Knapp, J. R., Davie, J. K., Myer, A., Meadows, E., Olson, E. N. and Klein, W. H. (2006) 'Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size.' *Development*, 133(4) pp. 601-610.

- Knight, J. D. and Kothary, R. (2011) 'The myogenic kinome: protein kinases critical to mammalian skeletal myogenesis.' *Skeletal muscle*, 1 p. 29.

- Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Okamoto, I., Usui, M., Ikeda, M. and Kurimoto, M. (1997) 'IFN-gamma-inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12.' *The Journal of Immunology*, 158(4) pp. 1541-1550.

- Krug, N., Madden, J., Redington, A. E., Lackie, P., Djukanovic, R., Schauer, U., Holgate, S. T., Frew, A. J. and Howarth, P. H. (1996) 'T-cell cytokine profile evaluated

at the single cell level in BAL and blood in allergic asthma.' *American journal of respiratory cell and molecular biology*, 14(4) pp. 319-326.

- Kuswanto, W., Burzyn, D., Panduro, M., Wang, K.K., Jang, Y.C., Wagers, A.J., Benoist, C. and Mathis, D., 2016. Poor Repair of Skeletal Muscle in Aging Mice Reflects a Defect in Local, Interleukin-33-Dependent Accumulation of Regulatory T Cells. *Immunity*, *44*(2), pp.355-367.
- Lages, C. S., Suffia, I., Velilla, P. A., Huang, B., Warshaw, G., Hildeman, D. A., Belkaid, Y. and Chougnet, C. (2008) 'Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation.' *The Journal of Immunology*, 181(3) pp. 1835-1848.
- Lai, J.-P., Yang, J.-H., Douglas, S. D., Wang, X., Riedel, E. and Ho, W.-Z. (2003) 'Quantification of CCR5 mRNA in human lymphocytes and macrophages by real-time reverse transcriptase PCR assay.' *Clinical and diagnostic laboratory immunology*, 10(6) pp. 1123-1128.
- Langen, R. C., Van der VELDEN, J. L., Schols, A. M., Kelders, M. C., Wouters, E. F. and Janssen-Heininger, Y. M. (2004) 'Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization.' The FASEB Journal, 18(2) pp. 227-237.
- Le Grand, F., Jones, A. E., Seale, V., Scimè, A. and Rudnicki, M. A. (2009) 'Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells.' *Cell stem cell*, 4(6) pp. 535-547.
- Leng, S. X., Hung, W., Cappola, A. R., Yu, Q., Xue, Q. L. and Fried, L. P. (2009) 'White blood cell counts, insulin-like growth factor-1 levels, and frailty in community-dwelling older women.' *The journals of gerontology. Series A, Biological sciences and medical sciences*, 64(4), Apr, pp. 499-502.
- Leong, J. W., Chase, J. M., Romee, R., Schneider, S. E., Sullivan, R. P., Cooper, M. A. and Fehniger, T. A. (2014) 'Preactivation with IL-12, IL-15, and IL-18 induces CD25 and a functional high-affinity IL-2 receptor on human cytokine-induced memory-like natural killer cells.' *Biology of Blood and Marrow Transplantation*, 20(4) pp. 463-473.
- Levings, M. K., Bacchetta, R., Schulz, U. and Roncarolo, M. G. (2002) 'The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells.' *International archives of allergy and immunology*, 129(4) pp. 263-276.
- Li, J. and Johnson, S. E. (2006) 'ERK2 is required for efficient terminal differentiation of skeletal myoblasts.' *Biochemical and biophysical research communications*, 345(4) pp. 1425-1433.
- Li, Y. and Kurlander, R. J. (2010) 'Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation.' *J Transl Med*, 8 p. 104.
- Li, Y.-P. (2003) 'TNF-α is a mitogen in skeletal muscle.' *American Journal of Physiology-Cell Physiology*, 285(2) pp. C370-C376.

- Licona-Limón, P., Kim, L. K., Palm, N. W. and Flavell, R. A. (2013) 'TH2, allergy and group 2 innate lymphoid cells.' *Nature immunology*, 14(6) pp. 536-542.

- Linton, P. J. and Dorshkind, K. (2004) 'Age-related changes in lymphocyte development and function.' *Nature immunology*, 5(2) pp. 133-139.

- Loffredo, F. S., Steinhauser, M. L., Jay, S. M., Gannon, J., Pancoast, J. R., Yalamanchi, P., Sinha, M., Dall'Osso, C., Khong, D. and Shadrach, J. L. (2013) 'Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy.' *Cell*, 153(4) pp. 828-839.

- Londhe, P. and Davie, J. K. (2011) 'Gamma interferon modulates myogenesis through the major histocompatibility complex class II transactivator, CIITA.' *Molecular and cellular biology*, 31(14) pp. 2854-2866.

- Lu, H., Huang, D., Saederup, N., Charo, I. F., Ransohoff, R. M. and Zhou, L. (2011) 'Macrophages recruited via CCR2 produce insulin-like growth factor-1 to repair acute skeletal muscle injury.' *The FASEB Journal*, 25(1) pp. 358-369.

- Lutz, C. T. and Quinn, L. S. (2012) 'Sarcopenia, obesity, and natural killer cell immune senescence in aging: altered cytokine levels as a common mechanism.' *Aging (Albany NY)*, 4(8) p. 535.

- Machida, S. and Booth, F. W. (2004) 'Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation.' *Proceedings of the Nutrition Society*, 63(02) pp. 337-340.

- Madaro, L. and Bouché, M. (2014) 'From Innate to Adaptive Immune Response in Muscular Dystrophies and Skeletal Muscle Regeneration: The Role of Lymphocytes.' *BioMed research international*, 2014

- Malm, C., Nyberg, P., Engström, M., Sjödin, B., Lenkei, R., Ekblom, B. and Lundberg, I. (2000) 'Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies.' *The Journal of physiology*, 529(1) pp. 243-262.

- Mann, C. J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A. L. and Muñoz-Cánoves, P. (2011) 'Aberrant repair and fibrosis development in skeletal muscle.' *Skelet Muscle*, 1(1) pp. 21-21.

- Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004) 'The chemokine system in diverse forms of macrophage activation and polarization.' *Trends in immunology*, 25(12) pp. 677-686.

- Marquez-Curtis, L. A. and Janowska-Wieczorek, A. (2013) 'Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis.' *BioMed research international*, 2013

- Martinez, C. O., McHale, M. J., Wells, J. T., Ochoa, O., Michalek, J. E., McManus, L. M. and Shireman, P. K. (2010) 'Regulation of skeletal muscle regeneration by CCR2-activating chemokines is directly related to macrophage recruitment.' *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 299(3) pp. R832-R842.

- Matsui, T., Nagoshi, T. and Rosenzweig, A. (2003) 'Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival.' *Cell Cycle*, 2(3) pp. 219-222.

- McPhee, J. S., Hogrel, J. Y., Maier, A. B., Seppet, E., Seynnes, O. R., Sipila, S., Bottinelli, R., Barnouin, Y., Bijlsma, A. Y., Gapeyeva, H., Maden-Wilkinson, T. M., Meskers, C. G., Paasuke, M., Sillanpaa, E., Stenroth, L., Butler-Browne, G., Narici, M. V. and Jones, D. A. (2013) 'Physiological and functional evaluation of healthy young and older men and women: design of the European MyoAge study.' *Biogerontology*, 14(3), Jun, pp. 325-337.

- Merchav, S., Tatarsky, I. and Hochberg, Z. (1988) 'Enhancement of human granulopoiesis in vitro by biosynthetic insulin-like growth factor I/somatomedin C and human growth hormone.' *The Journal of clinical investigation*, 81(3), Mar, pp. 791-797.

- Messina, G. and Cossu, G. (2009) 'The origin of embryonic and fetal myoblasts: a role of Pax3 and Pax7.' *Genes & development*, 23(8) pp. 902-905.

- Mildner, M., Hacker, S., Haider, T., Gschwandtner, M., Werba, G., Barresi, C., Zimmermann, M., Golabi, B., Tschachler, E. and Ankersmit, H. J. (2013) 'Secretome of peripheral blood mononuclear cells enhances wound healing.' *PloS one*, 8(3) p. e60103.

- Minet-Quinard, R., Farges, M. C., Thivat, E., Deleine, C., Mayot, G., Brtko, J., Ribalta, J., Winklhofer-Roob, B., Rock, E. and Vasson, M. P. (2010) 'Neutrophils are immune cells preferentially targeted by retinoic acid in elderly subjects.' *Immun Ageing*, 7(10) p. 10.1186.

- Moelling, K., Schad, K., Bosse, M., Zimmermann, S. and Schweneker, M. (2002) 'Regulation of Raf-Akt cross-talk.' *Journal of Biological Chemistry*, 277(34) pp. 31099-31106.

- Morrison, Partridge, T. and Bou-Gharios, G. (2005) 'Nude mutation influences limb skeletal muscle development.' *Matrix Biol*, 23(8), Jan, pp. 535-542.

- Mosmann, T. R. and Sad, S. (1996) 'The expanding universe of T-cell subsets: Th1, Th2 and more.' *Immunology today*, 17(3) pp. 138-146.

- Muñoz-Cánoves, P., Scheele, C., Pedersen, B. K. and Serrano, A. L. (2013) 'Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?' *FEBS Journal*, 280(17) pp. 4131-4148.

- Münz, C., Steinman, R. M. and Fujii, S.-i. (2005) 'Dendritic cell maturation by innate lymphocytes coordinated stimulation of innate and adaptive immunity.' *The Journal of experimental medicine*, 202(2) pp. 203-207.

- Murray, J. S. (1998) 'How the MHC selects Th1/Th2 immunity.' *Immunology today*, 19(4) pp. 157-162.

- Musarò, A., McCullagh, K. J., Naya, F. J., Olson, E. N. and Rosenthal, N. (1999) 'IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1.' *Nature*, 400(6744) pp. 581-585.

- Musarò, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E. R., Sweeney, H. L. and Rosenthal, N. (2001) 'Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle.' *Nature genetics*, 27(2) pp. 195-200.

- Nagelkerken, L., Hertogh-Huijbregts, A., Dobber, R. and Dräger, A. (1991) 'Age-related changes in lymphokine production related to a decreased number of CD45RBhi CD4+ T cells.' *European journal of immunology*, 21(2) pp. 273-281.

- Nijhuis, E., Remarque, E., Hinloopen, B., POUW-KRAAN, T., LIER, R., Ligthart, G. and Nagelkerken, L. (1994) 'Age-related increase in the fraction of CD27− CD4+ T cells and IL-4 production as a feature of CD4+ T cell differentiation in vivo.' *Clinical & Experimental Immunology*, 96(3) pp. 528-534.

- Nikolich-Žugich, J. (2008) 'Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections.' *Nature Reviews Immunology*, 8(7) pp. 512-522.

- Nishioka, T., Shimizu, J., Iida, R., Yamazaki, S. and Sakaguchi, S. (2006) 'CD4+ CD25+ Foxp3+ T cells and CD4+ CD25− Foxp3+ T cells in aged mice.' *The Journal of Immunology*, 176(11) pp. 6586-6593.

- Nolan, Y., Maher, F. O., Martin, D. S., Clarke, R. M., Brady, M. T., Bolton, A. E., Mills, K. H. and Lynch, M. A. (2005) 'Role of interleukin-4 in regulation of age-related inflammatory changes in the hippocampus.' *Journal of Biological Chemistry*, 280(10) pp. 9354-9362.

- Olguin, H. C., Yang, Z., Tapscott, S. J. and Olwin, B. B. (2007) 'Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination.' *The Journal of cell biology*, 177(5) pp. 769-779.

- Oraldi, M., Maggiora, M., Paiuzzi, E., Canuto, R. A. and Muzio, G. (2013) 'CLA reduces inflammatory mediators from A427 human lung cancer cells and A427 conditioned medium promotes differentiation of C2C12 murine muscle cells.' *Lipids*, 48(1) pp. 29-38.

- Otis, J. S., Niccoli, S., Hawdon, N., Sarvas, J. L., Frye, M. A., Chicco, A. J. and Lees, S. J. (2014) 'Pro-inflammatory mediation of myoblast proliferation.' *PloS one*, 9(3) p. e92363.

- Pallafacchina, G., Calabria, E., Serrano, A. L., Kalhovde, J. M. and Schiaffino, S. (2002) 'A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification.' *Proceedings of the National Academy of Sciences*, 99(14) pp. 9213-9218.

- Parker, M. H., Seale, P. and Rudnicki, M. A. (2003) 'Looking back to the embryo: defining transcriptional networks in adult myogenesis.' *Nature Reviews Genetics*, 4(7) pp. 497-507.

- Paul, W. E. and Seder, R. A. (1994) 'Lymphocyte responses and cytokines.' *Cell*, 76(2) pp. 241-251.

- Paul, W. E. and Zhu, J. (2010) 'How are TH2-type immune responses initiated and amplified?' *Nature Reviews Immunology*, 10(4) pp. 225-235.

- Pedersen, B. K. and Toft, A. D. (2000) 'Effects of exercise on lymphocytes and cytokines.' *British journal of sports medicine*, 34(4), Aug, pp. 246-251.

- Perkins, K. J., Basu, U., Budak, M. T., Ketterer, C., Baby, S. M., Lozynska, O., Lunde, J. A., Jasmin, B. J., Rubinstein, N. A. and Khurana, T. S. (2007) 'Ets-2 Repressor Factor Silences Extrasynaptic Utrophin by N-Box–mediated Repression in Skeletal Muscle.' *Molecular biology of the cell*, 18(8) pp. 2864-2872.

- Philippou, A., Papageorgiou, E., Bogdanis, G., Halapas, A., Sourla, A., Maridaki, M., Pissimissis, N. and Koutsilieris, M. (2009) 'Expression of IGF-1 isoforms after exercise-induced muscle damage in humans: characterization of the MGF E peptide actions in vitro.' *In vivo*, 23(4) pp. 567-575.

- Piccirillo, C. A. and Shevach, E. M. (2004) *Naturally-occurring CD4+ CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance.* Vol. 16: Elsevier.

- Podkowka, R., Wiśniewska, J., Korybalska, K., Wieczorowska-Tobis, K. and Knapowski, J. (2001) '[Plasma level of interleukin-6 and interleukin-8 in the elderly].' *Przeglad lekarski*, 59(4-5) pp. 230-233.

- Porras, A., Alvarez, A. M., Valladares, A. and Benito, M. (1998) 'p42/p44 mitogen-activated protein kinases activation is required for the insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation, in rat fetal brown adipocytes.' *Molecular endocrinology*, 12(6), Jun, pp. 825-834.

- Price, F., Kuroda, K. and Rudnicki, M. (2007) 'Stem cell based therapies to treat muscular dystrophy.' *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1772(2) pp. 272-283.

- Qi, Q., Zhang, D. W., Weyand, C. M. and Goronzy, J. J. (2014) 'Mechanisms shaping the naïve T cell repertoire in the elderly—Thymic involution or peripheral homeostatic proliferation?' *Experimental gerontology*, 54 pp. 71-74.

- Qi, Y., Operario, D. J., Georas, S. N. and Mosmann, T. R. (2012) 'The acute environment, rather than T cell subset pre-commitment, regulates expression of the human T cell cytokine amphiregulin.' *PloS one*, 7(6)

- Qi, Y., Operario, D. J., Oberholzer, C. M., Kobie, J. J. and Mosmann, T. R. (2009) 'The expression and regulation of amphiregulin on human basophils and CD4 T cells.' *The Journal of Immunology*, 182(Meeting Abstracts 1) p. 36.16.

- Rao, P. K., Kumar, R. M., Farkhondeh, M., Baskerville, S. and Lodish, H. F. (2006) 'Myogenic factors that regulate expression of muscle-specific microRNAs.' *Proceedings of the National Academy of Sciences*, 103(23) pp. 8721-8726.

- Rea, I., McNerlan, S. and Alexander, H. (1999) 'CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-α, IFN-γ, and sIL-2R levels in aging.' *Experimental gerontology*, 34(1) pp. 79-93.

- Relaix, F. and Zammit, P. S. (2012) 'Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage.' *Development*, 139(16), Aug, pp. 2845-2856.

- Ren, K., Crouzier, T., Roy, C. and Picart, C. (2008) 'Polyelectrolyte multilayer films of controlled stiffness modulate myoblast cell differentiation.' *Advanced functional materials*, 18(9) pp. 1378-1389.

- Renshaw, M., Rockwell, J., Engleman, C., Gewirtz, A., Katz, J. and Sambhara, S. (2002) 'Cutting edge: impaired Toll-like receptor expression and function in aging.' *The Journal of Immunology*, 169(9) pp. 4697-4701.

- Ricotti, L., Taccola, S., Bernardeschi, I., Pensabene, V., Dario, P. and Menciassi, A. (2011) 'Quantification of growth and differentiation of C2C12 skeletal muscle cells on PSS–PAH-based polyelectrolyte layer-by-layer nanofilms.' *Biomedical Materials*, 6(3) p. 031001.

- Rink, L., Cakman, I. and Kirchner, H. (1998) 'Altered cytokine production in the elderly.' *Mechanisms of ageing and development*, 102(2) pp. 199-209

- Ríos, R., Carneiro, I., Arce, V.M. and Devesa, J., 2002. Myostatin is an inhibitor of myogenic differentiation. *American Journal of Physiology-Cell Physiology*, *282*(5), pp.C993-C999.
- Rogel, M. R., Soni, P. N., Troken, J. R., Sitikov, A., Trejo, H. E. and Ridge, K. M. (2011) 'Vimentin is sufficient and required for wound repair and remodeling in alveolar epithelial cells.' *The FASEB Journal*, 25(11) pp. 3873-3883.

- Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., Yancopoulos, G. D. and Glass, D. J. (2001) 'Mediation of IGF-1-induced skeletal myotube hypertrophy by PI (3) K/Akt/mTOR and PI (3) K/Akt/GSK3 pathways.' *Nature cell biology*, 3(11) pp. 1009-1013.

- Ruby, C. E. and Weinberg, A. D. (2009) 'OX40-enhanced tumor rejection and effector T cell differentiation decreases with age.' *The Journal of Immunology*, 182(3) pp. 1481-1489.

- Ruckh, J. M., Zhao, J.-W., Shadrach, J. L., van Wijngaarden, P., Rao, T. N., Wagers, A. J. and Franklin, R. J. (2012) 'Rejuvenation of regeneration in the aging central nervous system.' *Cell stem cell*, 10(1) pp. 96-103.

- Rundhaug, J. E. and Fischer, S. M. (2010) 'Molecular mechanisms of mouse skin tumor promotion.' *Cancers*, 2(2) pp. 436-482.

- Saclier, M., Cuvellier, S., Magnan, M., Mounier, R. and Chazaud, B. (2013) 'Monocyte/macrophage interactions with myogenic precursor cells during skeletal muscle regeneration.' *FEBS Journal*, 280(17) pp. 4118-4130.

- Saini, A., Al-Shanti, N., Faulkner, S. H. and Stewart, C. E. (2008) 'Pro-and anti-apoptotic roles for IGF-I in TNF-α-induced apoptosis: a MAP kinase mediated mechanism.' *Growth Factors*, 26(5) pp. 239-253.

- Saito, S., Nakashima, A., Shima, T. and Ito, M. (2010) 'REVIEW ARTICLE: Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy.' *American journal of reproductive immunology*, 63(6) pp. 601-610.
- Sajko, Š., Kubínová, L., Cvetko, E., Kreft, M., Wernig, A. and Eržen, I. (2004) 'Frequency of M-cadherin-stained satellite cells declines in human muscles during aging.' *Journal of Histochemistry & Cytochemistry*, 52(2) pp. 179-185.
- Salam, N., Rane, S., Das, R., Faulkner, M., Gund, R., Kandpal, U., Lewis, V., Mattoo, H., Prabhu, S. and Ranganathan, V. (2013) 'T cell ageing: Effects of age on development, survival & function.' *The Indian journal of medical research*, 138(5) p. 595.
- Sartori, R., Milan, G., Patron, M., Mammucari, C., Blaauw, B., Abraham, R. and Sandri, M., 2009. Smad2 and 3 transcription factors control muscle mass in adulthood. *American Journal of Physiology-Cell Physiology*, *296*(6), pp.C1248-C1257.
- Schaerli, P., Britschgi, M., Keller, M., Steiner, U. C., Steinmann, L. S., Moser, B. and Pichler, W. J. (2004) 'Characterization of human T cells that regulate neutrophilic skin inflammation.' *The Journal of Immunology*, 173(3) pp. 2151-2158.
- Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis.' *Nature methods*, 9(7) pp. 671-675.
- Schwartz, R. H. (1990) 'A cell culture model for T lymphocyte clonal anergy.' *Science*, 248(4961) pp. 1349-1356.
- Seres, I., Csongor, J., Mohácsi, A., Leövey, A. and Fülöp, T. (1993) 'Age-dependent alterations of human recombinant GM-CSF effects on human granulocytes.' *Mechanisms of ageing and development*, 71(1) pp. 143-154.
- Sharma, S., Dominguez, A. L. and Lustgarten, J. (2006) 'High accumulation of T regulatory cells prevents the activation of immune responses in aged animals.' *The Journal of Immunology*, 177(12) pp. 8348-8355.
- Shavlakadze, T., McGeachie, J. and Grounds, M. D. (2010) 'Delayed but excellent myogenic stem cell response of regenerating geriatric skeletal muscles in mice.' *Biogerontology*, 11(3) pp. 363-376.
- Sheard, P., Duxson, M. and Harris, A. (1991) 'Neuromuscular transmission to identified primary and secondary myotubes: a reevaluation of polyneuronal innervation patterns in rat embryos.' *Dev Biol*, 148(2) pp. 459-472.
- Shefer, G., Van de Mark, D. P., Richardson, J. B. and Yablonka-Reuveni, Z. (2006) 'Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle.' *Dev Biol*, 294(1), Jun 1, pp. 50-66.
- Shen, Q., Zhong, W., Jan, Y. N. and Temple, S. (2002) 'Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts.' *Development*, 129(20) pp. 4843-4853.

- Shephard, R. J. (1998) 'Immune changes induced by exercise in an adverse environment.' *Canadian journal of physiology and pharmacology*, 76(5), May, pp. 539-546.

- Sindermann, J., Kruse, A., Frercks, H.-J., Schütz, R. M. and Kirchner, H. (1993) 'Investigations of the lymphokine system in elderly individuals.' *Mechanisms of ageing and development*, 70(1) pp. 149-159.

- Singh, K. and Dilworth, F. J. (2013) 'Differential modulation of cell cycle progression distinguishes members of the myogenic regulatory factor family of transcription factors.' *FEBS Journal*, 280(17) pp. 3991-4003.

- Sinha, M., Jang, Y. C., Oh, J., Khong, D., Wu, E. Y., Manohar, R., Miller, C., Regalado, S. G., Loffredo, F. S. and Pancoast, J. R. (2014) 'Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle.' *Science*, 344(6184) pp. 649-652.

- Sjögren, K., Liu, J.-L., Blad, K., Skrtic, S., Vidal, O., Wallenius, V., LeRoith, D., Törnell, J., Isaksson, O. G. and Jansson, J.-O. (1999) 'Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice.' *Proceedings of the National Academy of Sciences*, 96(12) pp. 7088-7092.

- Smith, K. A. and Cantrell, D. A. (1985) 'Interleukin 2 regulates its own receptors.' *Proceedings of the National Academy of Sciences*, 82(3) pp. 864-868.

- Sonntag, W. E., Lynch, C., Thornton, P., Khan, A., Bennett, S. and Ingram, R. (2000) 'The effects of growth hormone and IGF-1 deficiency on cerebrovascular and brain ageing.' *Journal of anatomy*, 197(04) pp. 575-585.

- Sprent, J. and Surh, C. D. (2011) 'Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells.' *Nature immunology*, 12(6) pp. 478-484.

- Streicher, K. L., Willmarth, N. E., Garcia, J., Boerner, J. L., Dewey, T. G. and Ethier, S. P. (2007) 'Activation of a nuclear factor κB/interleukin-1 positive feedback loop by amphiregulin in human breast cancer cells.' *Molecular cancer research*, 5(8) pp. 847-861.

- Sukho, L. (2003) 'Insulin-like growth factor-1 induces skeletal muscle hypertrophy.' *Journal of Exercise Science and Fitness*, 1(1) pp. 47-53.

- Swain, S., Clise-Dwyer, K. and Haynes, L. (2005) *Homeostasis and the age-associated defect of CD4 T cells.* Vol. 17: Elsevier.

- Tajbakhsh, S. (2013) 'Losing stem cells in the aged skeletal muscle niche.' *Cell Res*, 23(4), 04//print, pp. 455-457.

- Takahashi, A., Kureishi, Y., Yang, J., Luo, Z., Guo, K., Mukhopadhyay, D., Ivashchenko, Y., Branellec, D. and Walsh, K. (2002) 'Myogenic Akt signaling regulates blood vessel recruitment during myofiber growth.' *Molecular and cellular biology*, 22(13) pp. 4803-4814.

- Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. and Sakaguchi, S. (2000) 'Immunologic self-tolerance maintained by CD25+ CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte–associated antigen 4.' *The Journal of experimental medicine*, 192(2) pp. 303-310.

- Tamir, A., Eisenbraun, M. D., Garcia, G. G. and Miller, R. A. (2000) 'Age-dependent alterations in the assembly of signal transduction complexes at the site of T cell/APC interaction.' *The Journal of Immunology*, 165(3) pp. 1243-1251.

- Te Velde, A., Huijbens, R., Heije, K., De Vries, J. and Figdor, C. (1990) 'Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis.' *Blood*, 76(7) pp. 1392-1397.

- Tedesco, F. S., Dellavalle, A., Diaz-Manera, J., Messina, G. and Cossu, G. (2010) 'Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells.' *The Journal of clinical investigation*, 120(1) p. 11.

- Tidball, J. G. (1995) 'Inflammatory cell response to acute muscle injury.' *Medicine and science in sports and exercise*, 27(7), Jul, pp. 1022-1032.

- Tidball, J. G. (2005) 'Inflammatory processes in muscle injury and repair.' *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 288(2) pp. R345-R353.

- Tidball, J. G. and Villalta, S. A. (2010) 'Regulatory interactions between muscle and the immune system during muscle regeneration.' *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 298(5) pp. R1173-R1187.

- Tsoukas, C. D., Landgraf, B., Bentin, J., Valentine, M., Lotz, M., Vaughan, J. H. and Carson, D. A. (1985) 'Activation of resting T lymphocytes by anti-CD3 (T3) antibodies in the absence of monocytes.' *The Journal of immunology*, 135(3) pp. 1719-1723.

- Turner, N. J. and Badylak, S. F. (2012) 'Regeneration of skeletal muscle.' *Cell Tissue Res*, 347(3) pp. 759-774.

- Tzachanis, D. and Boussiotis, V. A. (2009) 'Tob, a member of the APRO family, regulates immunological quiescence and tumor suppression.' *Cell Cycle*, 8(7) pp. 1019-1025.

- Valenzuela, J., Schmidt, C. and Mescher, M. (2002) 'The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells.' *The Journal of Immunology*, 169(12) pp. 6842-6849.

- Van der Pouw Kraan, T., Boeije, L., de Groot, E. R., Stapel, S. O., Snijders, A., Kapsenberg, M. L., Van Der Zee, J. and Aarden, L. A. (1997) 'Reduced production of IL-12 and IL-12-dependent IFN-gamma release in patients with allergic asthma.' *The journal of immunology*, 158(11) pp. 5560-5565.

- Vasto, S., Carruba, G., Lio, D., Colonna-Romano, G., Di Bona, D., Candore, G. and Caruso, C., 2009. Inflammation, ageing and cancer. Mechanisms of ageing and development, 130(1), pp.40-45.
- Venuti, J. M., Morris, J. H., Vivian, J. L., Olson, E. N. and Klein, W. H. (1995) 'Myogenin is required for late but not early aspects of myogenesis during mouse development.' *The Journal of cell biology*, 128(4) pp. 563-576.

- Verdijk, L. B., Snijders, T., Drost, M., Delhaas, T., Kadi, F. and van Loon, L. J. (2014) 'Satellite cells in human skeletal muscle; from birth to old age.' *Age*, 36(2) pp. 545-557.

- Villalta, S. A., Deng, B., Rinaldi, C., Wehling-Henricks, M. and Tidball, J. G. (2011) 'IFN-γ promotes muscle damage in the mdx mouse model of Duchenne muscular dystrophy by suppressing M2 macrophage activation and inhibiting muscle cell proliferation.' *The Journal of Immunology*, 187(10) pp. 5419-5428.

- Villalta, S. A., Nguyen, H. X., Deng, B., Gotoh, T. and Tidball, J. G. (2009) 'Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy.' *Human molecular genetics*, 18(3) pp. 482-496.

- Villarino, A. V., Tato, C. M., Stumhofer, J. S., Yao, Z., Cui, Y. K., Hennighausen, L., O'Shea, J. J. and Hunter, C. A. (2007) 'Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals.' *The Journal of experimental medicine*, 204(1) pp. 65-71.

- Villeda, S. A., Luo, J., Mosher, K. I., Zou, B., Britschgi, M., Bieri, G., Stan, T. M., Fainberg, N., Ding, Z. and Eggel, A. (2011) 'The ageing systemic milieu negatively regulates neurogenesis and cognitive function.' *Nature*, 477(7362) pp. 90-94.

- Vivanco, I. and Sawyers, C. L. (2002) 'The phosphatidylinositol 3-kinase–AKT pathway in human cancer.' *Nature Reviews Cancer*, 2(7) pp. 489-501.

- Warren, G. L., Hulderman, T., Jensen, N., McKinstry, M., Mishra, M., Luster, M. I. and Simeonova, P. P. (2002) 'Physiological role of tumor necrosis factor α in traumatic muscle injury.' *The FASEB Journal*, 16(12) pp. 1630-1632.

- Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R. and Hollenberg, S. (1991) 'The myoD gene family: nodal point during specification of the muscle cell lineage.' *Science*, 251(4995) pp. 761-766.

- Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. and Miller, A. D. (1989) 'Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD.' *Proceedings of the National Academy of Sciences*, 86(14) pp. 5434-5438.

- Weng, N.-p. (2006) 'Aging of the immune system: how much can the adaptive immune system adapt?' *Immunity*, 24(5) pp. 495-499.

- Werner, S. and Grose, R. (2003) 'Regulation of wound healing by growth factors and cytokines.' *Physiological reviews*, 83(3) pp. 835-870.

- White, J. D., Scaffidi, A., Davies, M., McGeachie, J., Rudnicki, M. A. and Grounds, M. D. (2000) 'Myotube formation is delayed but not prevented in MyoD-deficient skeletal muscle: studies in regenerating whole muscle grafts of adult mice.' *Journal of Histochemistry & Cytochemistry*, 48(11) pp. 1531-1543.

- Widegren, U., Jiang, X. J., Krook, A., Chibalin, A. V., Bjornholm, M., Tally, M., Roth, R. A., Henriksson, J., Wallberg-henriksson, H. and Zierath, J. R. (1998) 'Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal

muscle.' *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 12(13), Oct, pp. 1379-1389.

- Winter, J. N., Jefferson, L. S. and Kimball, S. R. (2011) 'ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling.' *American Journal of Physiology-Cell Physiology*, 300(5) pp. C1172-C1180.

- Woodworth, C. D., McMullin, E., Iglesias, M. and Plowman, G. D. (1995) 'Interleukin 1 alpha and tumor necrosis factor alpha stimulate autocrine amphiregulin expression and proliferation of human papillomavirus-immortalized and carcinoma-derived cervical epithelial cells.' *Proceedings of the National Academy of Sciences*, 92(7) pp. 2840-2844.

- Wynn, T. A., Chawla, A. and Pollard, J. W. (2013) 'Macrophage biology in development, homeostasis and disease.' *Nature*, 496(7446) pp. 445-455.

- Yablonka-Reuveni, Z. (2011) 'The Skeletal Muscle Satellite Cell Still Young and Fascinating at 50.' *Journal of Histochemistry & Cytochemistry*, 59(12) pp. 1041-1059.

- Yaffe, D. and Saxel, O. (1977) 'Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle.' *Nature*, (270) pp. 725-727.

- Yamamoto, H., Sohmiya, M., Oka, N. and Kato, Y. (1991) 'Effects of aging and sex on plasma insulin-like growth factor I (IGF-I) levels in normal adults.' *Acta endocrinologica*, 124(5) pp. 497-500.

- Yeh, S.-S. and Schuster, M. W. (1999) 'Geriatric cachexia: the role of cytokines.' *The American journal of clinical nutrition*, 70(2) pp. 183-197.

- Yin, H., Price, F. and Rudnicki, M. A. (2013) 'Satellite cells and the muscle stem cell niche.' *Physiological reviews*, 93(1) pp. 23-67.

- Yoshiko, Y., Hirao, K. and Maeda, N. (2002) 'Differentiation in C2C12 myoblasts depends on the expression of endogenous IGFs and not serum depletion.' *American Journal of Physiology-Cell Physiology*, 283(4) pp. C1278-C1286.

- Zaiss, D. M., Yang, L., Shah, P. R., Kobie, J. J., Urban, J. F. and Mosmann, T. R. (2006) 'Amphiregulin, a TH2 cytokine enhancing resistance to nematodes.' *Science*, 314(5806) pp. 1746-1746.

- Zhang, C., Li, Y., Wu, Y., Wang, L., Wang, X. and Du, J. (2013) 'Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration.' *Journal of Biological Chemistry*, 288(3) pp. 1489-1499.

- Zheng, S. G., Wang, J. and Horwitz, D. A. (2008) 'Cutting edge: Foxp3+ CD4+ CD25+ regulatory T cells induced by IL-2 and TGF-β are resistant to Th17 conversion by IL-6.' *The Journal of Immunology*, 180(11) pp. 7112-7116.

- Zhou, X., Tang, J., Cao, H., Fan, H. and Li, B. (2015) 'Tissue resident regulatory T cells: novel therapeutic targets for human disease.' *Cellular & molecular immunology*,

- Zhou, Y., Lin, G., Baarsch, M. J., Scamurra, R. W. and Murtaugh, M. P. (1994) 'Interleukin-4 suppresses inflammatory cytokine gene transcription in porcine macrophages.' *Journal of leukocyte biology*, 56(4) pp. 507-513.

Appendix 1

	A	B	C	D	E	F	G
1	prot_hit_num	prot_acc	prot_desc	prot_score	prot_mass	prot_matche	prot_cover
2	**1**	**ALBU_HUMAN**	**Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2**	**12568**	**71317**	**865**	**76.5**
3	1	FETA_HORSE	Alpha-fetoprotein OS=Equus caballus GN=AFP PE=2 SV=1	41	70130	34	1.1
4	**2**	**ALBU_FELCA**	**Serum albumin OS=Felis catus GN=ALB PE=1 SV=1**	**2077**	**70611**	**212**	**22.9**
5	3	IGHG_RABIT	Ig gamma chain C region OS=Oryctolagus cuniculus PE=1 SV=1	43	35952	1	2.2
6	3	IGHG1_HUMAN	Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1	1703	36596	119	52.4
7	4	TRFE_HUMAN	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	1587	79294	119	50.4
8	4	TRFE_MOUSE	Serotransferrin OS=Mus musculus GN=Tf PE=1 SV=1	114	78841	4	2.2
9	4	TRFE_RAT	Serotransferrin OS=Rattus norvegicus GN=Tf PE=1 SV=3	114	78512	4	2.1
10	**5**	**ALBU_BOVIN**	**Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4**	**1405**	**71244**	**132**	**23.7**
11	**5**	**ALBU_CAPHI**	**Serum albumin (Fragments) OS=Capra hircus GN=ALB PE=1 SV=2**	**0**	**10048**	**1**	**12.2**
12	**5**	**ALBU_SHEEP**	**Serum albumin OS=Ovis aries GN=ALB PE=2 SV=1**	**42**	**71139**	**9**	**13**
13	6	CO3_BOVIN	Complement C3 OS=Bos taurus GN=C3 PE=1 SV=2	58	188675	11	2.8
14	6	CO3_HUMAN	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	959	188569	74	29.2
15	6	CO3_MOUSE	Complement C3 OS=Mus musculus GN=C3 PE=1 SV=2	75	187904	13	4
16	7	IGKC_HUMAN	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	928	11773	68	80.2
17	8	APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	910	30759	34	38.6
18	9	HPT_BOVIN	Haptoglobin OS=Bos taurus GN=HP PE=2 SV=1	505	45629	23	7.2
19	9	HPT_CAPIB	Haptoglobin OS=Capra ibex GN=HP PE=2 SV=1	503	45411	19	5.2
20	9	HPT_HUMAN	Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	901	45861	57	34
21	9	HPT_PONAB	Haptoglobin OS=Pongo abelii GN=HP PE=2 SV=1	700	38985	46	32
22	9	HPTR_HUMAN	Haptoglobin-related protein OS=Homo sapiens GN=HPR PE=2 SV=2	613	39518	32	23.9
23	9	HPTR_PANTR	Haptoglobin-related protein (Fragment) OS=Pan troglodytes GN=HPR PE=3 SV=1	774	39242	43	26.3
24	10	IGHG3_HUMAN	Ig gamma-3 chain C region OS=Homo sapiens GN=IGHG3 PE=1 SV=2	824	42287	62	29.4
25	11	IGHG2_HUMAN	Ig gamma-2 chain C region OS=Homo sapiens GN=IGHG2 PE=1 SV=2	772	36505	63	47.2
26	**12**	**ALBU_EQUAS**	**Serum albumin OS=Equus asinus GN=ALB PE=1 SV=1**	**727**	**70490**	**89**	**11.2**
27	**12**	**ALBU_HORSE**	**Serum albumin OS=Equus caballus GN=ALB PE=1 SV=1**	**727**	**70550**	**90**	**13.8**
28	13	LAC2_HUMAN	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	707	11458	35	74.5
29	13	LAC6_HUMAN	Ig lambda-6 chain C region OS=Homo sapiens GN=IGLC6 PE=4 SV=1	627	11441	32	46.2
30	13	LAC7_HUMAN	Ig lambda-7 chain C region OS=Homo sapiens GN=IGLC7 PE=1 SV=2	602	11467	29	32.1

An example of results generated from proteomics analysis of young secreatome. The sample is predominated with albumin human serum**(bold)**

Appendix 2

Application Number…………….. **(facultycoding)**

(Sep 2007)

Date………………………………..

MANCHESTERMETROPOLITANUNIVERSITY

FACULTY OF SCIENCE AND ENGINEERING

APPLICATION FOR ETHICAL APPROVAL

Introduction

All university activity must be reviewed for ethical approval. In particular, all undergraduate, postgraduate and staff research work, projects and taught programmes must obtain approval from their Faculty Academic Ethics committee (or delegated Departmental Ethics Committee).

APPLICATION PROCEDURE

The form should be completed legibly (preferably typed) and, so far as possible, in a way which would enable a layperson to understand the aims and methods of the research. Every relevant section should be completed. Applicants should also include a copy of any proposed advert, information sheet, consent form and, if relevant, any questionnaire being used. The Principal Investigator should sign the application form. Supporting documents, together with one copy of the full protocol should be sent to the Administrator of the appropriate Faculty Academic Ethics Committee.

Your application will require external ethical approval by an NHS Research Ethics

Committee if your research involves staff, patients or premises of the NHS (see guidance notes)

Work with children and vulnerable adults

You will be required to have a Criminal Disclosure, if your work involves children or vulnerable adults.

The Faculty Academic Ethics Committee meets every (insert period) and will respond as soon as possible, and where appropriate, will operate a process of expedited review. Applications that require approval by an NHS Research Ethics Committee or a Criminal Disclosure will take longer - perhaps 3 months.

1. DETAILS OF APPLICANT (S)

1.1 Principal Investigator: (Member of staff or student responsible for work)

1) Dr Nasser Al-SHANTI (DoS), IRM, School of Healthcare Science, Manchester Metropolitan University, E226, 01612475712, n.al-shanti@mmu.ac.uk

2) Sarah Al-dabbagh , Bsc. MSc, PhD Postgraduate research student, e-mail: sarah.aldabbagh@mmu.ac.uk

1.2 Co-Workersand their role in the project: (e.g. students, external collaborators, etc)

Supervisors and internal collaborators:

Professor Claire Stewart (C.E.Stewart@ljmu.ac.uk)

Dr. Chris Murgatroyd - 0161 2471212 (c.murgatroyd@mmu.ac.uk)

Dr Jamie S. McPhee – 0161 247 5675 (j.s.mcphee@mmu.ac.uk)

1.3 University Department/Research Institute/Other Unit:

School of Healthcare Science, Manchester Metropolitan University

School of Sport and Exercise Sciences, Liverpool John Moores University

2. DETAILS OF THE PROJECT

2.1 Title: Investigating the effect of immune cells on skeletal muscle cells C2C12

2.2 Description of Project: (please **outline** the background and the purpose of the research project, 250 words max.)

Title: Investigating the effect of immune environment on skeletal muscle stem cells proliferation and differentiation

Background

Skeletal muscle cell has a unique ability for maintenance and repair following major damage or muscle disease. The regenerative ability of skeletal muscle cells are highly dependent the presence of high population of mononucleotide and myogenic cells called satellite cells that maintain their ability to proliferate and differentiate to new fibers. (James *et al*., 2010). The process of differentiation involves three major stages which are proliferative , fusion and transition and terminal differentiation stages) ,all of which are regulated in part by the activities of a family of muscle-specific transcription factors in the basic helix-loophelix family (bHLH).(James*et al* ., 2010). However, the regenerative ability of skeletal muscle cells are significantly reduced in elderly people, due to reduced systemic environment (Conboy et al.,2005), satellite cells numbers and hormonal imbalance but also the immune system efficiency decreased (Rafi et al., 2003). Myeloid cells play crucial role in muscle cells repair and maintenance through release of cytokines and exogenous growth factors. The most common cytokines produced during inflammatory response are Interleukin -1 (IL-1), (IL-6) and tumour necrosis factor –α (TNF-α). Recent published study has shown that during inflammatory response, Monocytes /Macrophages shift their phenotype to participate in muscle regeneration. Strong evidence also shows the role of cytokines particularly IL-6 in recruitment of myeloid cells and satellite cells to the site of skeletal muscle injury. (Pedersen *et al*., 2000).In light of such evidence, biological interactions between immune system and skeletal muscle cells can be proposed which may induce a detrimental effect in maintaining an adequate muscle mass and function in elderly people. To date, most researchers have focused on investigating the impact of these cytokines alone on skeletal muscle proliferation and differentiation. **We hypothesised** that combined effects of these cytokines which are present in the systemic environment may have detrimental effect on skeletal muscle regeneration. Therefore, **our aim** is to explore how immune cells cross-talk with skeletal muscle cells with **objectives** 1) to examine the effects of the supernatant of the culture immune cells on skeletal muscle cells proliferation, fusion and differentiation. Therefore, better understanding of such interactions could not only be used to study the skeletal muscle wasting in elderly people but also could lead research into new directions and highlight possible therapeutic strategies for both prevention and treatment of muscle wasting conditions with age and with disease

Describe what type of study this is (e.g. qualitative or quantitative; also indicate how the data will be collected and analysed).

Both quantitative and qualitative data will be collected in the purposed study . 60 participants will be recruited to take part in this study .Contribution of participants involves only blood drawn which consumes mostly no more than 5 minutes .During recruitment , potential participants will be provided with details of this study. Information of this study will be provided in verbal and written formats in order for participants to make an informed decision on whether to participate . Participants will be encouraged to understand the information provided and make a decision away from the researchers .A copy of the signed consent form will be provided to the participants for their information . Participants have the right to withdraw from the experiment at any time . Participants will also complete a medical questionnaire in which all records details are completely anonymised (free from any potential identifier) . This will be achieved by allocating each participant with non –identifiable Participant Number (PN) . The participants will be asked to give blood by phlebotomist . The needle will be inserted in to antecubital vein to obtain 10 ml baseline blood sample .After obtaining blood , the needle will be removed and guaze applied at the site of venepuncture . The method of blood sampling is routinely used in clinical and research setting . The participants then will be free to leave the laboratory .

Blood samples will be stored at 4 C $^{\circ}$ until analysis in strict accordance with the Human Tissue Act (2004) and university regulations and procedures .poly morphonuclear cells (PMNCs) will be separated by density gradient centrifugation using conical tubes prior to washing with 10 ml of Phosphate Buffer Saline . PMNCs will be cultured for 4 days in the presence of IL-2 .Following culture , the supernatant will be harvested and cultured with young C2C12 (p5) and artificially aged C2C12 p55 . Gene expression of myogenic regulatory factors of C2C12 p5 & p55 will be measured . Wound healing and migration assays for of C2C12 will be carried out . The level of protein expression will be determined using western blot. Flow cytometry will be used to examine the proliferating and cell cycle of C2C12 cells . creatinine kinase (CK) and protein concentration will be assessed.

As mentioned previously, all raw data will be stored anonymously under a participant number (PN) rather than name .Data storage will comply with the Data Protection Act (1998) . The data will be analysed to study the effect of immune cells on young and aged skeletal muscle cells during proliferation and differentiation stages using *******.If a participant decided to withdraw from the study at any time after the experimental procedure commences , all samples and data will be destroyed including, paper and electronic format .

2.3 Are you going to use a questionnaire? YES

2.4 Start Date / Duration of project:

Start date 10/10/2012
Duration (3 years)

2.5 Location of where the project and data collection will take place:

School of Healthcare Science laboratories, first floor labs in John Dalton Building, Manchester Metropolitan University

2.6 Nature/Source of funding

None

2.7 Are there any regulatory requirements? NO

If yes, please give details, e.g., from relevant professional bodies

3. DETAILS OF PARTICIPANTS

3.1How many?

60

3.2Age: 20-75

3.3Sex: Both sexes

3.4 How will they be recruited?

Participants will be approached verbally, via email

3.5 Status of participants: (e.g. students, public, colleagues, children, hospital patients**,** prisoners, including young offenders, participants with mental illness or learning difficulties.) ?

The majority of participants will be healthy staff and students at Manchester Metropolitan University. Some healthy participants may be recruited from the general public.

3.6 Inclusion and exclusion from the project: (indicate the criteria to be applied).

Participants will be healthy individuals who are not taking medications, particularly anti-inflammatory drugs

3.7 Payment to volunteers: (indicate any sums to be paid to volunteers).
No payment

3.8 Study information:
Have you provided a study information sheet for the participants?

Please attach a copy of the information sheet, where appropriate

YES

3.9 Consent:
(A written consent form for the study participants MUST be provided in all cases, unless the research is a questionnaire.)

Have you produced a written consent form for the participants to sign for your records? YES

4. RISKS AND HAZARDS

Please respond to the following questions if applicable

4.1 Are there any risks to the researcher and/or participants?

(Give details of the procedures and processes to be undertaken, e.g., if the researcher is a lone-worker.)

Potential risks to participants:

The risks involved in this study are minimal. Some minor discomfort may arise in the participant from collection of the blood samples. Any adverse effects will be treated by first aid and/or medical intervention as necessary. The method to be used is common practice in clinical settings, and in the literature, and members of staff supervising this project have previously used this method of blood collection. No major adverse effects have been recorded with this procedure provided sterile materials are utilised for the blood collection. Commercially-available, quality-assured, sterile materials will be utilised throughout this study.

Potential risks to researchers:

As this investigation would involve collection and handling of biological samples, there is some degree of risk to researchers which can be minimized with good laboratory practice and adherence to health and safety procedures. Researchers will utilise standard laboratory personal protective equipment (gloves, laboratory coat and safety glasses) whilst in the laboratory. Researchers will not be left alone with participants at any time and academic staff will supervise the participant while he/she is in the laboratory (with the exception of when a urine sample is provided due to need for privacy). The participant will not be observed during this procedure but will go to the lavatory, collect the urine sample and return to the laboratory before handing the sample to the researcher.

4.2 State precautions to minimise the risks and possible adverse events:

Participants will be seated while the blood sample is taken in order to minimize any risk that may occur due to the participant suddenly feeling unwell. A small plaster will be provided to cover the puncture site at the end of the experimental trial once the cannula has been removed.

Student researchers will be taught how to handle equipment safely.

Researchers will follow good laboratory practice and adhere to laboratory health and safety procedures. This will include wearing gloves and safety glasses whenever handling biological samples and wearing a white laboratory coat at all times.

4.3 What discomfort (physical or psychological) danger or interference with normal activities might be suffered by the researcher and/or participant(s)? State precautions which will be taken to minimise them:

As this investigation would involve collection of biological samples, there is a small risk of infection to both participant and researcher. This risk will be minimized by following standard aseptic techniques such as using an unused sterile needle for each participant and the wearing of appropriate safety apparel

5. PLEASE DESCRIBE ANY ETHICAL ISSUES RAISED AND HOW YOU INTEND TO ADDRESS THESE:

Ethical issues

- The study involves collecting sensitive data i.e. blood samples from number of participants .Therefore to protect the participant's personal data .the study will be in agreement with the Data Protection Act 1998 . The blood samples collected during this study will be kept confidential and will be identified using serial number coding so that the participants will remain anonymous , no hospital records will be accessed for this study and after analysis the sample will be disposed of.

- Blood sample will be obtained from healthy young and aged people, both sexes .

- As safety precaution, the researcher must wear an appropriate laboratory coat, disposal latex gloves and safety spectacles to reduce the risk of contamination when handling blood samples. Also, the participants are required to fill out medical screening

questionnaire to ensure they are free from infections and it is safe to take a blood sample.

- The blood sample will be in accordance with the Human Tissue Act 2004. Each participants is required to formally give their consent before taking blood sample .Also the samples will be identified using serial number coding in order for the participants to remain anonymous and after analysis the blood samples will then be disposed.

6. SAFEGUARDS /PROCEDURAL COMPLIANCE

6.1Confidentiality:

(a) Indicate what steps will be taken to safeguard the confidentiality of participant records. If the data is to be computerised, it will be necessary to ensure compliance with the requirements of the Data Protection Act.

All data will be kept electronically and will be stored anonymously under participant number, rather than participant name. Any identifiable details will not be recorded. Raw data sheets and records will be stored in a locked cupboard or drawer and access will be restricted to the involved investigators only.

(b) If you are intending to make any kind of audio or visual recordings of the participants, please answer the following questions:

a. How long will the recordings be retained and how will they be stored? N/A

b. How will they be destroyed at the end of the project? N/A

c. What further use, if any, do you intend to make of the recordings? N/A

6.2 Human Tissue Act:

The Human Tissue Act came into force in November 2004, and requires appropriate consent for, and regulates the removal, storage and use of all human tissue.

a. Does your project involve taking tissue samples, e.g., blood, urine, hair, etc., from human subjects? YES

b. Will this be discarded when the project is terminated? YES

If NO – Explain how the samples will be placed into a tissue bank under the Human Tissue Act regulations:

6.3 Insurance:

The University holds insurance policies that will cover claims for negligence arising from the conduct of the University's normal business, which includes research carried out by staff and by undergraduate and postgraduate students as part of their courses. This does **not** extend to clinical negligence. There are no arrangements to provide indemnity and/or compensation in the event of claims for non-negligent harm.

Will the proposed project result in you undertaking any activity that would not be considered as normal University business? If so, please detail below:

No

6.4 Notification of Adverse Events (e.g., negative reaction, counsellor, etc):

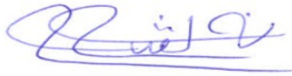
(Indicate precautions taken to avoid adverse reactions.)

Please state the processes/procedures in place to respond to possible adverse reactions.

In the case of clinical research, you will need to abide by specific guidance. This may include notification to GP and ethics committee. **Please seek guidance for up to date advice**, e.g., see the NRES website at http://www.nres.npsa.nhs.uk/

Although adverse reactions are highly unlikely, any incidents will be reported to the laboratory supervisor and necessary medical intervention will be provided immediately.

SIGNATURE OF PRINCIPAL INVESTIGATOR DATE:



....

SIGNATURE OF FACULTY ACADEMIC ETHICS   DATE:

COMMITTEE CHAIRPERSON:

....

School of Healthcare Science

Informed Consent

Project title:Investigating the effect of immune environment on skeletal muscle cells proliferation and differentiation

Investigators:
Sarah Al-Dabbagh

To be completed by participant:

The investigators have provided me with an information sheet on the above investigation which I have read and have had the opportunity to ask questions regarding the purpose and protocol of the investigation. The investigators have outlined any potential risks or discomforts associated with the experimental protocol.

The investigators have informed me of my right to withdraw from this investigation at any time without giving any reason for doing so.

I fully and freely give my consent to participate in this investigation as it has been described to me and understand that participation in this investigation may not benefit me in any way.

I understand that the investigators may publish the findings gathered from this investigation in abstracts, journals, conferences or other media formats. However, I understand that all my personal information will remain confidential so that nobody can identify me from such publications.

Signature: **Date:**

To be completed by researcher:

I have explained the experimental protocol to the participant and have informed the participant of any risks or discomforts associated with the protocol. I have informed the participant of the purpose of the experiment and of their right to withdraw from the investigation without giving any reason for doing so.

Signature: **Date:**

Title: Investigating the effect of Immune Environment on skeletal muscle cells proliferation and differentiation

Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This is to ensure that the study protocol will not exacerbate any existing conditions of the participant. Please answer the following questions as accurately as possible.

Age:

Sex: male female

Are you currently taking any prescribed medication ie. Corticosteroids?
YES/NO

Are you currently attending your GP?
YES/NO

Have you ever suffered from a cardiovascular problem?

i.e. high blood pressure, anaemia, heart attack etc
YES/NO

Have you ever suffered from a neurological disorder?

i.e. epilepsy, convulsions etc

YES/NO

Have you ever suffered from an endocrine disorder?

i.e. diabetesetc.

YES/NO

Have you ever suffered from a chronic gastrointestinal disorder?

i.e. Crohn's disease, irritable bowel syndrome etc YES/NO

Have you ever suffered from a skin disorder?

i.e. eczema etc YES/NO

Do you suffer from any allergies?

i.e. any medications, foods etc YES/NO

Are you aware of any other medical condition that may prevent you from participating in this investigation? i.e. immunological disorders, numbness in extremities, asthma etc YES/NO

Are you currently taking any supplement? i.e. creatine
YES/NO

If you have answered "yes" to any of these questions, please provide details below:

Participant's details

Code	Age	Height	Weight	BMI

RM-CWI	26	1.81	75	22.89307
JC_ CWI	31	1.73	94	31.40766
JG_CW1	26	1.75	81	26.44898
TW_CWI	30	1.73	75	25.05931
AB_CWI	26	1.835	74	21.97655
OB_CWI	23	1.835	67.7	20.10558
KS_CWI	32	1.795	87.6	27.18787
JO.C_CWI	30	1.835	90	26.72824
MR-253	32	1.8	65.4	20.18519
SR-256	27	1.76	89.3	28.82877
FD-562	28	1.785	91.6	28.74875
DU-596	20	1.705	61	20.98365
MUH-214	20	1.74	72.9	24.07848
FRS-824	29	1.815	69.6	21.12788
JAM-596	23	1.8	68	20.9
LAL-458	26	1.5	55	24.12
SAM-895	25	1.6	85	34
CLI-256	24	1.62	50	22.05
MAH-895	24	1.62	50	19.05197
HAK-568	28	1.74	75	24.7721
ALS-595	32	1.59	75	29.66655
MAT-591	29	1.55	52	21.64412
STV-879	23	1.94	80	21.25624
JAZ-353	24	1.67	64.8	23.23497
SAR-25	26	167	70	25.09
KM-26	31	179	70	21.84
MAR-56	22	180	85	26.23
SJK-85	30	169	75	26.25
KW/CIOO	30	1.78	81	25.56495
A5632	24	1.66	73	26.49151
A4577	28	1.79	84	26.21641
M5896	25	1.82	86	25.96305
V4556	31	1.68	66	23.38435
Hl1589	28	1.75	73	23.83673
F8965	23	1.81	85	25.94548
Q5896	22	1.62	58	22.10029
R8981	33	1.8	74	22.83951
ZASIA	26	1.81	75	22.89307
RIA-85	31	1.73	94	31.40766
GB22	26	1.75	81	26.44898
SAR	30	1.73	75	25.05931
BAR	26	1.835	74	21.97655
CL-25	23	1.835	67.7	20.10558

Ras3a	32	1.795	87.6	27.18787
BA4a	30	1.835	90	26.72824
MAQ-1	32	1.8	65.4	20.18519
ABEE-6	27	1.76	89.3	28.82877
ANTO-8	20	1.8	78.5	24.2284
UND-3	28	1.785	91.6	28.74875
Yong22	20	1.705	61	20.98365
POR-2C	20	1.74	72.9	24.07848
B89536	29	1.815	69.6	21.12788
CWR-53	71	1.735	80	26.57609
NKL-5	76	1.7	85.1	29.44637
JWN-52	77	1.79	66.6	20.78587
JW77	77	1.695	77.3	26.90544
DJ-84	82	1.75	82.3	26.87347
VPU-48	73	1.735	89	29.5659
LNR-89	68	1.84	63.6	18.78544
A5623	78	1.66	71	25.76571
YS-76	76	1.695	80	27.84522
Bo3-72	72	1.84	67.4	19.90784
ROS-75	74	1.745	83.05	27.274
AKM-88	75	1.8	85.6	26.41975
FLO-89	75	1.605	59.4	23.05878
X71-81	81	1.61	74.5	28.74118
H1-80	82	1.75	82.3	26.87347
GB0082A	75	1.9	120	25.95
GB0092A	81	1.79	77	24
GB0072A	72	1.76	106	34.3042
MOR-58	88	1.6	86	26.56