Interactions between prostate cancer and monocytes in a 3D immunocompetent tumouroid model

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Declaration

I, Ebrahim Mohammed Abdal, certify that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Monocytes (U937) are innate immune cells having important roles in regulating progression in prostate cancer (PCA). Utilisation of three-dimensional (3D) culture models provides more realistic representations of tumour microenvironment (TME) compared to two-dimensional (2D) culture models. The aim of this study was to incorporate monocytes in a 3D tumouroid prostate cancer (PCA) model under different biophysical environments, to ultimately create a simple immunocompetent in vitro cancer model. The work built on the existing in-house model of a tumouroid, which is a 3D collagen construct containing cancer cells and compressed to physiological stiffness to mimic native tissues. Investigations focused on monocyte behaviour, cytokine secretion and effect on PCA spheroid growth.

U937 were added and their behaviour examined by microscopy over 7 days in three types of 3D constructs, under different biophysical conditions: acellular 3D collagen gels, either non-compressed hydrogels or compressed (RAFT method); and tumouroids, which consisted of PCA (LNCaP and PC3) cells within the compressed gel. Confocal microscopy determined fluorescently-tagged U937 remained viable and penetration depth was similar in all conditions, with that in non-compressed gels and immunocompetent tumouroids (i.e., gels already containing cancer cells) slightly higher (95µm and 114µm respectively, v. 61.6µm in compressed, NS). Interestingly, it appeared that monocyte numbers (i.e., fluorescence levels) were greater in non-compressed than compressed acellular gels, (day 7 85 vs. 56 AU, p<0.05).

Spheroid growth of LNCaP PCA cells over time (14 days), determined via fluorescent microscopy, increased more in immunocompetent tumouroids (4609µm²) than control tumouroids (4366µm²) (day 14, p<0.05). Further comparisons of experiments utilising PMA stimulated U937 monocytes (more differentiated) showed an increase in cancer spheroid diameter within tumouroids compared to un-stimulated monocytes (3114µm² v. 2568µm², day 10, p<0.05).

U937 cells cytokine production in gels was investigated via Luminex followed by ELISA. The Luminex analyses showed in compressed gels upregulated cytokines including: VEGF (20,015.3AU, vs. uncompressed 4,990AU, p<0.05); and to a lesser extent IL-10 (368 AU vs. uncompressed 343.3AU, p>0.05) and TNF-α (436 AU vs. uncompressed 386AU, p>0.05). Subsequent ELISA analyses showed that for LNCaP PCA tumouroids, VEGF and TNF-α levels were similar in simple and immunocompetent cultures (i.e., with added U937 monocytes), (Day 14: 0.52 and 0.23AU for simple; v. 0.52 and 0.21AU for immunocompetent respectively).

Hypoxia and cytokine expression in immunocompetent PCA tumouroids, constructed with two different cancer cell lines, was analysed. Under hypoxia, VEGF increased more in LNCaP than PC3 immunocompetent tumouroids (Day 14, 0.47 v. 0.25AU, p<0.05). Conversely, IL-10 peaked in PC3 than LNCaP immunocompetent tumouroids (Day 14, 0.26 v. 0.22 AU, p>0.05). TNF-α levels increased in LNCaP compared to PC3 immunocompetent tumouroids (Day 14, 0.30 v. 0.21AU, p>0.05) respectively. Additionally, in normoxia, PMA stimulated U937 cells in PCA (LNCaP) immunocompetent tumouroids compared to previous experiments on day 10 had elevated; VEGF (0.36 AU, p<0.05), IL-10 (0.24 AU, p<0.05) and TNF-α (0.28 AU, p<0.05). TNF-α secretion was higher than all previous experiments.

In conclusion, this is the first report of tumouroids successfully incorporating monocytes, a key immune cell in cancer progression. The work demonstrates monocytes promoting cancer growth and altered expression of key cytokines especially increase in protumourigenic VEGF. This is the basis for developing an immunocompetent tumouroid platform to eventually use as a drug testing platform. Furthermore, tumouroids have the real potential to reduce animal experimentation and provide insight into complex tumour interactions in a biomimetic environment.
Impact statement

Three-dimensional (3D) cancer constructs have allowed researchers to mimic to a high degree the tumour micro-environment (TME) in vivo. In addition, this technique has allowed research to be carried out reproducibly and in a non-technically demanding fashion which financially speaking means that it is cost-effective. Commercially speaking, in this study we demonstrate for the first time that PCA cells can be grown along with monocytes and constructed with the addition of different environmental exposures such as hypoxia, in a reproducible way. Hence, academic research can use this specific method of experimentation in studying cancer behaviour not only at a cellular level, with the ability of co-culturing cancer cells with a wide array of human cells be they immune or otherwise (enabling analysis of cell-cell interactions), but also using the same model and exposing it to different biophysical environments. This versatile model could be used for a variety of investigations, making the use of other potential models obsolete, resulting in effective cost management.

Scientifically speaking, this thesis investigates how the biophysical environment of a collagen based 3D model alters various parameters in the TME environment in vitro. The thesis reports for the first time how U937 monocytic cells increase in penetration in non-compressed versus compressed gels using a novel in-house method of measuring depth. Also this thesis demonstrates the first ever known use of PCA cells within the 3D model to analyse spheroid size and cytokinic release in the presence of monocytes, i.e., in immunocompetence. This work opens the door to further understanding of this disease which has not been extensively researched in complex in vitro models previously, and provides a starting ground for for the development of immunotherapies.
Medically speaking, PCA is a fatal disease that is considered as the second most frequent malignancy diagnosed in men [14]. In England the socio-economic costs for patients with PCA are considerable as they impact both their morbidity and financially cost healthcare institutions approximately £4699 at diagnosis.[1] It must be mentioned at this point that these cancer models could reduce morbidity of patients as well as both complications and cost by providing an in vitro route for therapeutic analysis and experimentation without harming patients. These models are amenable to being used as a considerable armament in the quest for anti-cancer drug production and optimisation as they give us considerable insight into the protumourigenic and antitumourigenic cytokinic release in PCA. This information could in turn be harnessed in cytotoxic or immunological drug development for cancer treatment. This is beneficial in the sense that it avoids patients being at risk of potentially harmful complications of these drugs due to their in vitro nature.

Thus, the immune system is a potential route in the therapeutic management of cancer that may harness the inherent ability that the body has in attacking tumours. Academically speaking, using immune cells in cancer research could also supplement our knowledge regarding the complex nature of the TME.
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Most importantly, I would like to dedicate this work to the benefit of human health in general and to all those who are afflicted with prostate cancer. I would also like to dedicate this work to my family, especially my father Mr Mohammed Abdal (may he rest in peace) who succumbed to cancer after a terrible battle. I also acknowledge my mother Sheikha Shahrazad Al-Sabah, who has supported me with her motherly care and affection throughout the toughest of moments. Also, I dedicate this work to my extremely patient wife Farah and my angel sons Mohammed and Fahad. They have all supported me in a multitude of ways to achieve my best in all situations.
Table of Contents

1 Chapter 1 ................................................................................................................................. 21

1.1 Overview of the prostate gland .......................................................................................... 22

1.2 PCA ................................................................................................................................................ 26

1.3 Risk factors for PCA .................................................................................................................... 28

1.4 Treatment options for PCA ........................................................................................................ 29

1.5 The immune system and its impact on PCA .............................................................................. 37

1.6 Monocytes and their role in cancer .......................................................................................... 40

1.7 Monocytes and PCA ..................................................................................................................... 46

1.8 The antitumourigenic effects of monocytes in PCA .............................................................. 47

1.8.1 Monocytes secrete cytokines which directly oppose PCA ................................................. 47

1.8.2 Monocytes secrete cytokines which cause other cells in turn to attack PCA .................... 48

1.8.3 Direct effect of monocytes on PCA cell death ........................................................................ 48

1.9 The protumourigenic effects of monocytes in PCA ............................................................... 50

1.9.1 Monocytes secrete cytokines that cause growth of PCA .................................................... 50

1.9.2 Monocytes inhibit cytotoxic cells and contribute to the growth of PCA .............................. 54

1.9.3 Monocytes contribute directly to the growth of PCA .......................................................... 54

1.10 Cytokines secreted by PCA ....................................................................................................... 56

1.11 In vitro models used for cancer research ................................................................................ 59

1.12 Applications of three-dimensional (3D) PCA (tumouroid) models ...................................... 62

1.12.1 3D PCA models investigating biology and progression of disease .................................. 62

1.12.2 3D PCA models to test therapeutic interventions .............................................................. 64

1.13 Monocytes in a three-dimensional (3D) model ....................................................................... 66

1.14 Monocytes in 3D collagen hydrogels ..................................................................................... 67
2.2.2 Luminex analysis of cytokines from supernatants of compressed vs. non-compressed U937 3D constructs ................................................................. 90
2.2.3 ELISA for VEGF .................................................................................. 93
2.2.4 ELISA for IL-10 .................................................................................. 94
2.2.5 ELISA for TNF-α ................................................................................ 95
2.2.6 Harvesting of supernatants of LNCaP CMs co-cultured with PMA-stimulated U937 cells ........ 96

2.3 Quantitative and semi-quantitative microscopy assays ........................................ 96
2.3.1 Thickness of compressed vs non-compressed 3D collagen constructs .................. 97
2.3.2 Depth of penetration of red cell tracked CM-Dil U937 in 3D cellular constructs (compressed vs. non-compressed) .......................................................... 98
2.3.3 Measurements of cancer spheroid size and invasion ........................................... 98
2.3.4 Measurements of fluorescence intensity ......................................................... 99

2.4 Methodological development for imaging distinct cell populations in 2D and 3D .......... 100
2.4.1 Imaging of LNCaP prostate cancer spheroids in 3D cancer masses and tumouroids, using Phalloidin and DAPI ................................................................. 100
2.4.2 Staining of LNCaP cancer cells with Quantum Dots ........................................ 101
2.4.3 Manufacture of gold nanoclusters conjugated to anti-CD68 antibody ...................... 103
2.4.4 Staining of 3D U937 constructs and complex tumouroids with anti-CD68 conjugated gold nanoclusters .................................................................................. 103
2.4.5 Staining of U937 cells and LNCaP cancer cells in 2D and 3D cultures, with anti-CD68-FITC... 105
2.4.6 Troubleshooting in the imaging of anti-CD68-FITC stained cultures ...................... 106
2.4.7 PMA treated U937 cells stained with anti-CD68-FITC and DAPI in 2D .................... 108
2.4.8 Staining used for imaging .............................................................................. 110

2.5 Statistical analysis ....................................................................................... 110

3 Chapter 3 ...................................................................................................... 111
3.1 Title: Comparison of U937 monocyte distribution and motility in non-compressed versus compressed 3D constructs ......................................................... 112
3.2 Introduction: .............................................................................................. 112
3.3 Methods: ........................................................................................................... 112

3.4 Results: ........................................................................................................... 112

3.4.1 CM-Dil tracked U937 cells began penetrating at 1 hour of incubation and increased in number at day 7 in compressed acellular collagen gels ........................................................................ 113

3.4.2 CM-Dil tracked U937 cells began penetrating at 1 hour of incubation and increased in number at day 7 in non-compressed acellular collagen gels ........................................................................ 114

3.4.3 Z-stack images of compressed vs. non-compressed acellular collagen gels incubated with CM-Dil U937 cells ........................................................................................................................................ 116

3.4.4 More CM-Dil tracked U937 cells penetrated at day 7 than at 1 hour in compressed gels, demonstrated by Z-stack imaging ........................................................................................................ 116

3.4.5 More CM-Dil tracked U937 cells penetrated at day 7 than at 1 hour in non-compressed gels compared to compressed gels demonstrated by Z-stack imaging ........................................................................ 117

3.4.6 Depth of penetration of CM-Dil U937 cells was higher in non-compressed than compressed acellular collagen gels ......................................................................................................................... 119

3.4.7 Fluorescence intensity of CM-Dil U937 cells was higher in non-compressed than compressed acellular collagen gels ........................................................................................................................................ 121

3.4.8 Cytokine analysis of compressed and non-compressed acellular collagen gels with U937 cells ........................................................................................................................................ 122

3.4.9 Increased secretion of protumourigenic cytokine VEGF in compressed acellular collagen gels incubated with U937 cells compared to non-compressed gels ........................................................................ 123

3.4.10 Increased secretion of antitumourigenic cytokine IL-10 in compressed U937 acellular collagen gels incubated with U937 cells compared to non-compressed gels ........................................................................ 125

3.4.11 Increased secretion of monocyte to macrophage differentiation indicator cytokine- TNF-α in compressed acellular collagen gels incubated with U937 cells compared to non-compressed gels .......... 126

4 Chapter 4 ............................................................................................................. 128

4.1 Title: Co-culture of U937 monocyte cells within LNCaP tumouroids results in increased depth of penetration of U937 cells and increased growth of LNCaP spheroids ....................................................... 129

4.2 Introduction: ...................................................................................................... 129

4.3 Methods: ......................................................................................................... 129
4.4 Results: ........................................................................................................130

4.4.1 CM-Dil U937 cells incorporated in CMFDA LNCaP tumouroids .................. 130

4.4.2 3D images of immunocompetent tumouroids containing CMFDA LNCaP cells and CM-Dil U937 cells ............................................................................................................. 132

4.4.3 U937 CM-Dil cells penetrated CMFDA LNCaP tumouroids, when LNCaP cancer cells were present as single cells or spheroids, days 1-10 ............................................................................................................. 134

4.4.4 U937 CM-Dil cells penetrated CMFDA LNCaP tumouroids with cancer spheroids, days 7-21 .......................................................................................................................... 135

4.4.5 LNCaP spheroid growth increased over time in simple tumouroids ............ 137

4.4.6 LNCaP spheroid growth increased with time in immunocompetent CMFDA-LNCaP tumouroids co-cultured with unstimulated CM-Dil U937 cells ............................................................................................................. 139

4.4.7 Cytokine expression in LNCaP simple tumouroids and LNCaP immunocompetent tumouroids co-cultured with U937 cells ............................................................................................................. 141

4.4.8 VEGF increased in both LNCaP only simple tumouroids and immunocompetent LNCaP tumouroids co-cultured with U937 cells ............................................................................................................. 141

4.4.9 IL-10 increased more in LNCaP immunocompetent tumouroids co-cultured with unstimulated U937 cells than in LNCaP only simple tumouroids .......................................................................................... 142

4.4.10 TNF-α increased in LNCaP only simple tumouroids than in LNCaP immunocompetent tumouroids co-cultured with U937 cells ............................................................................................................. 144

5 Chapter 5 ......................................................................................................... 147

5.1 Title: Immunocompetent prostate tumouroids in hypoxic and normoxic conditions ..148

5.2 Introduction ................................................................................................. 148

5.3 Methods ....................................................................................................... 148

5.4 Results ......................................................................................................... 149

5.4.1 Immunocompetent PCA tumouroids co-cultured with U937 cells formed spheroids in hypoxic and normoxic conditions but U937 cells appeared to decline in amount ................................................................. 149

5.4.2 LNCaP and PC3 spheroid growth in immunocompetent prostate tumouroids increased under hypoxic conditions compared to normoxic conditions ......................................................................... 154
5.4.3 Levels of cytokines secreted in immunocompetent LNCaP PCA tumouroids in hypoxia and normoxia conditions differed................................................................. 158

5.4.4 Secretion of protumourigenic VEGF cytokine was increased in hypoxia compared to normoxia in immunocompetent LNCaP tumouroids co-cultured with U937 cells ......................................................... 159

5.4.5 Secretion of antitumourigenic IL-10 cytokine was slightly elevated in immunocompetent LNCaP tumouroids co-cultured with U937 cells in normoxic compared to hypoxic conditions .......................... 160

5.4.6 Higher secretion of monocyte to macrophage differentiation indicator TNF-α cytokine in immunocompetent LNCaP tumouroids co-cultured with U937 cells in hypoxia than in normoxia at later stages of the experimental timeline................................................................. 162

5.4.7 Levels of cytokines secreted in immunocompetent PC3 PCA tumouroids in hypoxia and normoxia conditions differed ............................................................................................................... 164

5.4.8 Secretion of protumourigenic VEGF cytokine was increased in normoxia compared to hypoxia for immunocompetent PC3 tumouroids co-cultured with U937 cells at the later stages of the experimental timeline........................................................................................................... 164

5.4.9 Increased secretion of antitumourigenic cytokine IL-10 in hypoxia than in normoxia in PC3 tumouroids co-cultured with U937 cells ......................................................................................................... 165

5.4.10 Secretion of monocyte to macrophage differentiation indicator TNF-α is elevated at different stages in immunocompetent PC3 tumouroids under hypoxia and normoxia........................................ 167

5.4.11 Immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells (macrophages). ................................................................................................................................. 168

5.4.12 LNCaP spheroid growth increased with time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ......................................................................................................... 169

5.4.13 Cytokines secreted in immunocompetent LNCaP PCA tumouroids co-cultured with PMA-stimulated U937 cells (macrophages) ......................................................................................................... 171

5.4.14 VEGF secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ......................................................................................................... 171

5.4.15 IL-10 secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ......................................................................................................... 173

5.4.16 TNF-α secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ......................................................................................................... 174

6 Chapter 6 .................................................................................................................................................. 177
6.1 Discussion ........................................................................................................... 178

6.2 Effects of the physical state of collagen gels on the depth of penetration of U937 cells ......... 179

6.3 PCA spheroid formation and growth in a simple LNCaP tumouroid .................................. 183

6.4 The creation of an immunocompetent PCA (LNCaP) tumouroid model with the impregnation of either U937 cells or PMA-stimulated U937 cells (macrophages) has an effect on PCA spheroid growth .. 185

6.5 Incorporation of U937 cells and PMA-stimulated U937 cells in the spheroid structures in LNCaP immunocompetent tumouroids ............................................................................................. 187

6.6 PCA (LNCaP and PC3) spheroid growth is affected by hypoxia in the immunocompetent tumouroid model compared to normoxia ........................................................................................................ 189

6.7 Hypoxia leads to a reduction in U937 cell amount in immunocompetent PCA tumouroid models ........................................................................................................................................... 191

6.8 Cytokine Analysis ........................................................................................................ 191

6.8.1 Cytokine release by U937 alters in compressed (stiff) versus non-compressed (non-stiff) collagen gels ........................................................................................................................................... 191

6.8.2 VEGF secretion increased in LNCaP only simple tumouroids more than in immunocompetent tumouroids co-cultured with U937 cells ........................................................................................................ 193

6.8.3 IL-10 cytokine secretion increased more in LNCaP immunocompetent tumouroids co-cultured with U937 cells than in LNCaP only simple tumouroids ........................................................................ 194

6.8.4 TNF-α cytokine secretion increased in LNCaP only simple tumouroids more than in immunocompetent tumouroids co-cultured with U937 cells ................................................................................................. 195

6.8.5 VEGF increased in secretion in hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells ....... 196

6.8.6 IL-10 increased in secretion in normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells ........ 196

6.8.7 TNF-α increased in secretion in hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells ........ 197

6.8.8 VEGF increased in secretion in normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells ................. 198
6.8.9 IL-10 increased in secretion in hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells ........................... 200

6.8.10 TNF-α increased in secretion in normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells ............... 200

6.8.11 VEGF cytokine release increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ........................................................................................................................................ 202

6.8.12 IL-10 cytokine release is increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ........................................................................................................................................ 203

6.8.13 TNF-α cytokine release increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells ........................................................................................................................................ 205

6.8.14 PMA stimulation in macrophage differentiation ............................................................................... 206

Summary and conclusions ......................................................................................................................... 207

Future work ............................................................................................................................................... 209

Appendix .................................................................................................................................................. 211

References .............................................................................................................................................. 212
Table of Figures

Figure 1.1: The gross anatomy of the prostate. .......................................................................................................................... 24
Figure 1.2: Hormonal therapy .................................................................................................................................................... 32
Figure 1.3: Hormonal therapy using Androgen Deprivation Therapy ........................................................................................................ 33
Figure 1.4: Different types of immune cells involved in PCA. ............................................................................................................. 40
Figure 1.5: Subtypes of monocytes. .................................................................................................................................................... 41
Figure 1.6: The antitumourigenic effects of monocytes .................................................................................................................... 50
Figure 1.7: Diagrammatic difference between 2D and 3D static cell culture methods ................................................................. 62
Figure 1.8: Structure of a prostate cancer spheroid .......................................................................................................................... 65
Figure 1.9: Effects of collagen type 1 when monocytes are incubated in it ......................................................................................... 69
Figure 1.10: The multiple effects of hypoxia on cancer cells ............................................................................................................ 72
Figure 1.11: Effects of PMA stimulation on U937 monocytes. ................................................................................................................ 74
Figure 2.1: Light microscopy images of LNCaP cells and U937 cells ............................................................................................... 81
Figure 2.2: Diagram showing the construction of 3D in vitro cancer mass (CM). ............................................................................... 82
Figure 2.3: Diagram showing the construction of a complex tumoroid model ................................................................................... 83
Figure 2.4: Microscopic images of macrophage formation post 48 hr incubation of U937 cells with PMA. ........................................ 85
Figure 2.5: Application of CM-Dil (red cell tracker) to U937 cells ...................................................................................................... 86
Figure 2.6: CMFDA (green cell tracker) is added to LNCaP cells .......................................................................................................... 87
Figure 2.7: The hypoxic/normoxic experiment ................................................................................................................................ 89
Figure 2.8: Images of non-compressed (NC) and compressed (C) acellular collagen gels ........................................................................ 97
Figure 2.9: Process by which spheroids are identified and counted .................................................................................................. 99
Figure 2.10: LNCaP cells incubated with quantum dots .................................................................................................................... 102
Figure 2.11: Composite image of a complex tumouroid model ......................................................................................................... 105
Figure 2.12: 200,000 LNCaP cells in fluorodishes in 2D ..................................................................................................................... 109
Figure 2.13: 200,000 U937 cells in fluorodishes in 2D ....................................................................................................................... 109
Figure 3.1: Compressed acellular 3D gel with 25,000 CM-Dil U937 cells added ............................................................................. 114
Figure 3.2: Non-compressed acellular 3D gels with 25,000 CM-Dil U937 cells added ................................................................. 115
Figure 3.3: Z-stack of compressed acellular collagen gels, incubated with 25,000 CM-Dil U937 cells. ............................................ 117
Figure 3.4: Z-stack of non-compressed acellular collagen gels, incubated with 25,000 CM-Dil U937 cells. .............................. 118
Figure 3.5: Depth of penetration (μm) of 25,000 CM-Dil U937 cells added superficially to compressed and non-compressed acellular collagen gels ............................................................................................................. 120
Figure 3.6: Fluorescence intensity ....................................................................................................................................................... 121
Figure 3.7: VEGF production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells .................................................................................................................................................. 124
Figure 3.8: IL-10 production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells added. .............................................................................................................................................. 126
Figure 3.9: TNF-α production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells added. .............................................................................................................................................. 127
Figure 4.1: Images of simple tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 (red) cells at day 1 incubation. .............................................................................................................................................. 130
Figure 4.2: Images of simple tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 cells (red) at day 14 incubation. .............................................................................................................................................. 131
Figure 4.3: Images of tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 cells (red) at day 21 incubation. .............................................................................................................................................. 131
Figure 4.4: 3D image of day 1 tumouroids containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red) .............................................................................................................................................. 132
Figure 4.5: 3D image of day 14 tumouroids containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red). .............................................................................................................................................. 133
Figure 4.6: 3D image of day 21 tumouroid containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red). .............................................................................................................................................. 133
Figure 4.7: The depth of penetration measurements (µm). .............................................................................................................................................. 135
Figure 4.8: The depth of penetration measurements (µm). .............................................................................................................................................. 136
Figure 4.9: Image a) tumouroid containing 50,000 LNCaP cells stained with phalloidin (green) and DAPI (blue). .............................................................................................................................................. 138
Figure 4.10: Tumouroid with 50,000 LNCaP cells tracked with CMFDA (green) and 25,000 U937 cells tracked with CM-Dil (red) and DAPI (blue). .............................................................................................................................................. 140
Figure 4.11: VEGF secretion in LNCaP tumouroids containing 50,000 LNCaP cells and LNCaP immunocompetent tumouroids co-cultured with 25,000 U937 cells .............................................................................................................................................. 142
Figure 4.12: IL-10 secretion in LNCaP only simple tumouroids containing 50,000 LNCaP cells and LNCaP immunocompetent tumouroids co-cultured with 25,000 U937 cells. .............................................................................................................................................. 144
Figure 4.13: TNF-α secretion secretion in LNCaP only simple tumouroids containing 50,000 LNCaP cells and LNCaP immunocompetent tumouroids co-cultured with 25,000 U937 cells. .............................................................................................................................................. 146
Figure 5.1: Immunocompetent LNCaP tumouroids with 50,000 LNCaP prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions, day 1 .............................................................................................................................................. 150
Figure 5.2: Immunocompetent LNCaP tumouroids with 50,000 LNCaP prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions, day 14 .............................................................................................................................................. 151
Figure 5.3: Immunocompetent PC3 tumouroids with 50,000 PC3 prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions day 1 .............................................................................................................................................. 152
Figure 5.4: Immunocompetent PC3 tumouroids with 50,000 PC3 prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions, day 14........................................153

Figure 5.5: Cancer spheroid growth measured in μm² in immunocompetent LNCaP tumouroid samples co-cultured with U937 cells under hypoxic conditions. ................................................................. 155

Figure 5.6: Cancer spheroid growth measured in μm² in immunocompetent LNCaP tumouroids containing U937 cells under normoxic conditions. .............................................................................. 156

Figure 5.7: Cancer spheroid growth measured in μm² in PC3 immunocompetent tumouroids containing U937 cells under hypoxic conditions. .............................................................................. 157

Figure 5.8: Cancer spheroid growth measured in μm² in PC3 immunocompetent tumouroids containing U937 cells under normoxic conditions. .............................................................................. 158

Figure 5.9: VEGF secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added.............................................................. 160

Figure 5.10: IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added......................................................... 161

Figure 5.11: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added......................................................... 163

Figure 5.12: VEGF secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 PC3 cells and 25,000 U937 cells added ................................................................. 165

Figure 5.13: IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroid samples containing 50,000 PC3 cells and 25,000 U937 cells added ......................................................... 166

Figure 5.14: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroid samples containing 50,000 PC3 cells and 25,000 U937 cells added ......................................................... 168

Figure 5.15: Immunocompetent tumouroid with 50,000 LNCaP cells tracked with CM-Dil (red) and 25,000 PMA-stimulated U937 cells (macrophages)................................................................. 170

Figure 5.16: VEGF secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 PMA-stimulated U937 cells......................................................... 172

Figure 5.17: IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 PMA-stimulated U937 cells......................................................... 174

Figure 5.18: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 PMA-stimulated U937 cells......................................................... 176

Figure 6.1: Monocytes and macrophages infiltrate the spheroid structure through different chemokines. .............. 189
Table of Tables

Table 1: Characteristics of each of the anatomical human prostate zones .............................................................. 25
Table 2: Gleason scoring system .................................................................................................................................. 28
Table 3: Treatment options available for PCA ........................................................................................................... 36
Table 4: The subset of monocytes which have antitumourigenic functions ................................................................ 43
Table 5: The subset of monocytes which have protumourigenic functions ................................................................. 45
Table 6: Cytokines secreted by monocytes .................................................................................................................. 56
Table 7: Cytokines secreted by PCA .......................................................................................................................... 59
Table 8: Main differences between cellular models ....................................................................................................... 60
Table 9: Different types of cancers in which monocytes are included in 3D cell culture models and their respective research studies ................................................................................................................................. 67
Table 10: List of cytokines chosen for analysis with their classifications: protumourigenic, antitumourigenic and monocyte to macrophage differentiation indicator .......................................................................................... 92
Table 11: The specific cytokines chosen for ELISA analysis, and the subtypes of macrophages in the TME which were identified as the secreting cells ................................................................................................................................. 93
Table 12: Type of 3D culture used and cell types involved, as well as specific time-points from which supernatants were harvested for Luminex and/or ELISA analysis ......................................................................................................................... 93
Table 13: The difficulties encountered with anti-CD68 FITC staining and the troubleshooting methods I applied to try resolving them .................................................................................................................................................. 107
Table 14: Stains/agents used to delineate specific cell types within 2D and 3D cultures for imaging and analysis ....... 110
Table 15: All cytokines analysed and 3 cytokines were chosen for further ELISA analysis: VEGF, IL-10 and TNF-α. ................................................................................................................................................................. 123
Table 16: Spheroid size increased in all LNCaP tumouroids with or without U937 cells or PMA-stimulated U937 cells as time progressed. LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells had greater growth at day 10 compared with LNCaP immunocompetent tumouroids with U937 cells. ............................................................................. 187
**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ACM</td>
<td>Artificial Cancer Mass</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>CD68</td>
<td>Cluster of Differentiation 68</td>
</tr>
<tr>
<td>CMFDA</td>
<td>5-chloromethylfluorescein diacetate</td>
</tr>
<tr>
<td>CM-Dil</td>
<td>Carbocyanine membrane probe</td>
</tr>
<tr>
<td></td>
<td>Oxacarbocyanine</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
</tr>
<tr>
<td>EBRT</td>
<td>External Beam Radiotherapy</td>
</tr>
<tr>
<td>EMAP II</td>
<td>Endothelial Monocyte-Activating Polypeptide-II</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>EZH2</td>
<td>Enhancer of Zeste Homolog 2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>LAMPs</td>
<td>Lysosomal-Associated Membrane Proteins</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Lymph Node Carcinoma of the Prostate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>Milk fat globule-epidermal growth factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metallo-Proteinase</td>
</tr>
<tr>
<td>MSCs</td>
<td>Human bone marrow-derived mesenchymal stem cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor-kB</td>
</tr>
<tr>
<td>PCA</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-Myristate 13-Acetate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour Associated Antigens</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour Associated Macrophage</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc Finger E-Box Binding Homeobox 1</td>
</tr>
</tbody>
</table>
Chapter 1

Background and Introduction
1.1 Overview of the prostate gland

This thesis discusses the potential effect of the immune system on prostate cancer (PCA) on the cellular level. Specifically, the work employs a 3D in vitro model, a tumouroid, which incorporates both immune cells and cancer cells, to investigate the effects of the two cell populations on each other. This tumouroid would be one of the first immunocompetent in vitro models for prostate cancer.

However, to understand PCA, one should have a mental image of the anatomy of the prostate gland. The prostate is a gland which is exclusively found in males. It is referred to as ‘walnut-shaped’ and the gland envelopes the urethra as it exits the bladder. It is located in the pelvic cavity and specifically anterior to the rectum but posterior to the pubic symphysis. Anatomically speaking the prostate is made up of several surfaces. These include the following;

- base,
- apex,
- posterior,
- anterior and
- inferior-lateral surfaces.

The base of the prostate is attached to the bladder neck and the apex lies on the superior surface of the urogenital diaphragm and is in contact with the medial surface of the levator-ani muscles. The flat and triangular posterior surface lies on the anterior aspect of the rectum. The anterior surface of the prostate lies in close proximity to the prostatic urethra and is pierced by it. The infero-lateral surface of the prostate gland joins the anterior surface and rests on the levator ani fascia above the urogenital diaphragm [2].
In addition, the human prostate is composed of both glandular and stromal elements, which are tightly fused within a pseudo-capsule. The inner layer of the prostate capsule is composed of smooth muscle with the outer layer covering of collagen [3]. There are two anatomic defects in the prostatic capsule. These are located at the apex (anterior and anterolaterally) and at the site of entry of the ejaculatory ducts.

The arterial vascular supply of the prostate is delivered by the internal iliac arteries. The prostate receives its blood supply from the inferior vesical artery which is a branch of the internal iliac [4]. The inferior vesical artery passes obliquely downward, forward and medially along the anteroinferior surface of the bladder heading towards the prostate.

The venous drainage of the prostate occurs through many veins. These veins include; the prostatic venous plexus, the vesicular vein and the internal iliac vein [5]. The venous drainage of the prostate reaches its final point in the inferior vena cava. Also, PCA has been found to metastasise through the prostatic veins in what is known as Batson’s backward venous metastatic pathway [6].

The lymphatic drainage of the prostate starts at the level of prostatic acini forming larger lymphatic channels. These channels travel to the prostatic capsule level where they join to form what is known as the periprostatic plexus [7]. The lymphatic channels are then united and follow the drainage path of the vascular channels. Also, it is anatomiccally shown that the lymphatic drainage from the superolateral prostate flows into the internal iliac as well as the inferior vesical channels. Further, drainage posteriorly from presacral lymphatics and from the posterior surface of the prostate drain into the external iliac nodes [7]. It is well recognised that lymph nodes adjacent to the prostate tumour are the initial points of metastatic spread [8].

Moreover, the nerve supply of the prostate is an important factor to discuss when describing the anatomy of the gland. The prostate is supplied by the inferior hypogastric
plexus also known as pelvic plexus. This plexus contains sympathetic as well as parasympathetic fibres emanating from T11-L2 and S2-S4 segments respectively [9]. Furthermore, the prostate is divided anatomically into zones (Figure 1). These zones include the following:

1. Central zone
2. Transitional zone
3. Peripheral zone

![Figure 1.1: The gross anatomy of the prostate gland in the coronal plane. The enlarged image on the right shows the three zones of the prostate gland as they relate to the urethra (U) which include: Central (C), Transitional (T) and Peripheral zones (P). The central and peripheral zones are the origins of Benign Prostatic Hyperplasia (BPH) and Prostate Cancer (PCA) respectively. (Adapted from reference [10]).]

The zones vary in terms of their embryological origins, anatomical landmarks, histological characteristics and susceptibility to pathological conditions. Table 1, below, further delineates these differences. Prostate cancer, which will be discussed below, is a
pathological disease which has a predilection to the peripheral anatomical zone of the prostate.

<table>
<thead>
<tr>
<th>Embryonic Origin</th>
<th>Central Prostate Zone</th>
<th>Transitional Prostate Zone</th>
<th>Peripheral Prostate Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of normal prostate (%)</td>
<td>25</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>Origin of prostatic adenocarcinoma (%)</td>
<td>5</td>
<td>25</td>
<td>70</td>
</tr>
</tbody>
</table>

*Table 1: Characteristics of each of the anatomical human prostate zones and the predilection of each zone to prostate adenocarcinoma along with their embryonic origin and the volume that they occupy within the prostate gland. (Adapted from reference [1]).*

It is imperative at this point to mention the functions of the prostate. It produces prostatic fluid which consists of zinc, enzymes and citric acid. This fluid nourishes the spermatozoa (which are produced in testicles) and forms semen.

It is also necessary to mention that the prostate produces a vital protein known as prostate specific antigen (PSA). This is well established as a biomarker and has been widely used in the diagnosis and monitoring of prostate cancer [11].

Moreover, there are several diseases associated with the prostate, these include:

- **Prostatitis** describes a condition where the gland is inflamed or infected. The National Institutes of Health (NIH) has classified this condition into categories which include; Acute Bacterial Prostatitis (ABP), Chronic Bacterial Prostatitis (CBP), Chronic Prostatitis/chronic pelvic pain syndrome and Asymptomatic Inflammatory Prostatitis (AIP) [12]. The symptoms that patients present vary with each condition spanning from pain and fever to obstructive and irritative urinary symptoms.

- **Benign prostate hyperplasia, BPH**, which describes the non-malignant growth of prostatic tissue. The proliferation of these prostate cells leads to bladder outlet
obstruction and incomplete bladder emptying. The untreated disease, in the long term leads to the development of both chronic high pressure urine retention and long term bladder detrusor muscle changes such as reduced contractility and overactivity [13].

- PCA is described as a malignancy that develops symptoms at a late stage in the course of the disease, as there are no initial or early symptoms in the majority of cases [14].

1.2 PCA

PCA has been quoted to be the second most frequent malignancy diagnosed in men [15]. Further, this disease has been reported as being the fifth leading cause of death worldwide [16].

In the European Union, the predicted number of deaths from PCA in 2020 is 78,800 males. Whereas mortality in both the United Kingdom and Germany is approximately 11/100,000 men [17]. Moreover, research from the United States has provided data regarding the estimated deaths from prostate cancer and new cases of this disease. In 2020, the estimated approximate number of deaths and new cases were 33,330 and 191,930 respectively [18].

A diagnosis of PCA cannot be made without a proper medical history and clinical examination along with specific clinically based investigations. Patients may present with symptoms and signs some of which include; nocturia (night urination), dysuria (difficulty urinating), heamaturia (blood in urine), unplanned weight loss, bone pain, weak or interrupted urine flow, lymphoedeoma and renal insufficiency.

However, accurate diagnosis of PCA is in the main investigation based. Some of these investigations include the PSA (prostate specific antigen) levels in blood samples, with
the gold-standard being prostate gland biopsy. PCA3 levels in the urine have also been suggested to be correlated with positive biopsy samples [19].

Nevertheless, biopsy samples are important as histological examination is vital for confirmation of the diagnosis of PCA. In order to confirm the diagnosis one needs to establish the loss of the basal layer at a cellular level during histological viewing of samples [20]. The basal layer, i.e., the thin boundary of extracellular matrix proteins which is found immediately under the epithelial cells. The basal layer is made of collagen type 4 and Laminin and its breach signifies that the epithelial cells are invading outwards of their normal position. The Gleason histological score is an important scale used in assisting in this endeavour.

The Gleason grading system consists of numbers that correlate to the specific pattern of behaviour and morphology of cells in a sample of prostate biopsy. The grade that is given to the sample is dependent on a calculation which involves the addition of two numbers. The first and second numbers refer to the most prevalent pattern in the patient’s prostate biopsy (eg. 3) and the second most prevalent patterns (eg. 4) respectively, then these numbers are added to give a score of 7 which lands the patient in Gleason grade 2 category. The specific grades and their numbers are given below, in Table 2:

<table>
<thead>
<tr>
<th>Gleason Grade</th>
<th>Histological Description from prostate biopsies of PCA patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1 Gleason score ≤6</td>
<td>Discrete individual glands which are well-formed</td>
</tr>
<tr>
<td>Grade 2 Gleason score 3+4 = 7</td>
<td>Well-formed glands predominantly with lesser component of poorly fused/cribriform or formed glands</td>
</tr>
<tr>
<td>Grade 3 Gleason score 4 + 3 = 7</td>
<td>Poorly formed or cribriform or fused glands in the main with a lesser component of well-formed glands</td>
</tr>
<tr>
<td>Grade 4 Gleason score 4 + 4 = 8; 3 + 5 = 8; or 5 + 3 = 8</td>
<td>Refers to 3 patterns; i. poorly fused/cribriform/formed glands predominantly,</td>
</tr>
</tbody>
</table>
ii. Predominantly glands which are well-formed as well as a lesser component lacking glands, or

iii. Predominantly lacking glands with a lesser component of well-formed glands

| Grade 5 Gleason scores 9–10 | Lack of gland formation with the presence or absence of necrosis. May include poorly formed/fused/cribriform glands |

Table 2: Gleason scoring system which is used to stage prostate tumours based upon their microscopic appearances. Cancers with a higher Gleason score are more aggressive and have a worse prognosis. Pathological scores range from 2 to 10, with higher numbers indicating greater risks and higher mortality. (Adapted from reference [20]).

Furthermore, imaging is an important component in assisting the diagnosis of prostate cancer. One of those imaging techniques is transrectal ultrasound scanning (TRUS), which allows visualisation of the prostate in real-time. TRUS remains the standard of care as it permits suspicious areas in the prostate to be biopsied [22].

The other imaging technique imperative for prostate cancer diagnosis is magnetic resonance imaging (MRI). It has been described as the most superior of the imaging techniques as its main forte lies in its ability to image the entire prostate. This imaging modality has been described as having improved detection of PCA [23]. In addition, transformative research has been carried out by Moore et al., who have adopted MRI in surveillance of prostate cancer and in doing so have enabled the stratification of patients into low risk and high risk of PCA development. Hence, this leads to patients at low risk of developing prostate cancer avoiding radical treatment and its side effects [24].

1.3 Risk factors for PCA

There are several risk factors for PCA. Age is a factor which is associated with an increased risk of PCA, as approximately 6 in 10 cases of PCA are diagnosed at age of 65 or above [25].
In addition, ethnicity is an important risk factor and does have an impact on being afflicted with PCA. Although PCA occurs in men irrespective of race, African Americans are reported to have the highest risk of getting prostate cancer. Generally, it has been documented that people of African descent are at higher risk of developing PCA as opposed to those of non-African descent. Interestingly, the mortality rate in males of African descent has been reported as being twice that of Caucasian men [26].

Family history of PCA is also an important risk factor. Having a first-degree relative such as a son, brother or father with PCA imposes a three-fold increased risk of having this disease [25]. Although the familial link to PCA is well documented, there has been genetic research suggesting that PCA is weakly linked to BRCA1 but carriers with BRCA2 gene mutations puts patients not only at a high risk of developing PCA but also more aggressive PCA [27]. In addition, mutations in the HOXB13 gene have been found to be linked to hereditary PCA [28].

In terms of exogenous factors, both occupation and diet are risk factors for PCA, farmers who are exposed to pesticides have a two-fold risk of developing prostate tumours as opposed to those who are unexposed [29]. Regarding diet, an increased consumption of red meat and food rich in fat has been associated with an increased risk of developing PCA. Males who have adopted this particular diet as part of their lifestyle had a 12-fold increased risk of developing PCA [30].

1.4 Treatment options for PCA

Treatment for prostate cancer is manifold and depends on the stage and invasiveness of the disease. Surgery remains the gold-standard of management for prostate cancer, but treatment also encompasses; radiotherapy, High-Intensity Focused Ultrasound (HIFU), hormonal therapy, chemotherapy, oncolytic virotherapy and immunotherapy.

Surgery:
Radical prostatectomy in which the prostate gland is removed, is carried out when the cancer is localised in this gland. This is conducted using different techniques, i.e.,

- the **retropubic** (suprapubic) approach,
- the **laparoscopic radical prostatectomy** and
- the **robotic-assisted laparoscopic prostatectomy**.

Surgical intervention also involves pelvic lymph node dissection (PLND), and this intra-operative procedure removes lymph nodes for critical histopathological staging to determine prognosis. Notably, this has become the standard of care especially for patients at higher risk of lymph node disease [31].

It should be mentioned that there are complications associated with surgical intervention in PCA. These complications are divided as intra-operative, early and late complications and encompass: blood loss, rectal injuries, ureteral injuries, loss of sexual function, urinary incontinence and anterior urethral stricture (vesico-urethral anastomosis stricture) [32].

**Radiotherapy:**

There are two methods of radiotherapy used for PCA treatment: external also known as External Beam Radiotherapy (EBRT) and internal radiotherapy. EBRT has been shown to reduce the size of the prostatic tumour as well as preserving the urinary and sexual functions of the patient [33]. In addition, results from trials have shown that EBRT improves biochemical control of disease, this means that biochemical recurrence which is defined as a rise in PSA ≥0.2ng/ml is prevented, with increasing dose of radiation [34]. Internal radiotherapy also known as brachytherapy is where radiation is released within the prostate to treat the cancer. This can be in the form of seeds or capsules and can deliver higher doses of radiation than other radiotherapy modalities. Also, it can allow radiation to penetrate the whole prostate gland including the seminal vesicles [35].
Yet it must be known that radiation as a mode of treatment comes with its own set of side effects. These side effects of radiotherapy include; radiation proctitis, loss of hair in the pelvic region, skin irritation, lymphoedema, fatigue and erectile dysfunction [36].

**High Intensity Focal Ultrasound (HIFU):**

This method of PCA treatment uses high-frequency ultrasound energy to destroy cancer cells using heat. The malignant area is heated to a temperature of 65°C, resulting in coagulative necrosis. HIFU is recognised as being safer than surgical resection of the prostate as the rates of urinary incontinence and impotence are significantly lower [37].

**Hormonal therapy:**

PCA is a testosterone-dependent cancer, which means that testosterone aids in the growth of this cancer. Hormonal therapy is divided into two categories; prevention of testosterone production and blocking testosterone from reaching cancer cells.

The first category encompasses luteinizing hormone-releasing hormone (LHRH) agonists and gonadotropin-releasing hormone (GnRH) antagonists (*Figure 2*). LHRH agonists cause an increase of luteinizing hormone (LH) initially but continuous use causes suppression of LH and therefore testosterone [38]. Thus, having a negative impact on the growth of PCA. GnRH antagonists supress both GnRH and subsequently LH. Hence, testosterone secretion is blocked and the same aim of testosterone secretion inhibition is reached. These drugs have the added benefit over LHRH agonists in that they do not cause an initial LH surge [39].
Hormonal therapy using GnRH (gonadotropin release hormone) antagonists and continual use of LHRH (leutinising hormone releasing hormone) agonists are used to treat PCA. Both treatments suppress LH release and in turn suppress testosterone release. (Adapted from reference [40]).

The second category of hormonal therapy is obstructing testosterone reaching cancer cells. These drugs are known as androgen receptor blockers and androgen deprivation therapy (ADT). ADT causes a major drop in the level of androgens systematically as well as preventing activation of androgen receptors, making this treatment the mainstay for metastatic prostatic cancer treatment (Figure 1.3) [41].
Figure 1.3: **Hormonal therapy** using Androgen Deprivation Therapy (ADT) stops testosterone which is produced by the testes reaching the prostate gland by blocking the androgen receptors on the prostate gland.

**Chemotherapy:**

Chemotherapy employs anti-cancer (cytotoxic) drugs to destroy cancer cells and can be neoadjuvant and adjuvant. Neoadjuvant chemotherapy is offered to patients before surgery or radiotherapy to reduce micro-metastases, potentially shrink the primary tumour mass, and avoid treatment failure. Whereas adjuvant chemotherapy, where this treatment is given after radiation or surgery, is employed to eliminate micro-metastases. It has been shown in several studies that chemotherapy, in the form of docetaxel, in combination with; surgery, radiotherapy and androgen blockade may improve recurrence-free survival [42].
Although chemotherapy treatment has its benefits, it also has negative effects on patients. Side effects are numerous and include; hair loss (alopecia), headaches, facial flushing, cystitis (bladder inflammation), nausea, vomiting and diarrhoea.

**Oncolytic virotherapy:**

Oncolytic virotherapy refers to the treatment of tumours with replication-competent viruses that infect, replicate in and result in lysis of malignant tumour cells [43]. The other goal of this therapy is to minimise the harm to normal cells within patients afflicted with prostate cancer.

Interestingly, this therapeutic pathway harbours fewer side effects compared with other treatments for PCA. It has been reported that mild fever has been the most frequent minor side effect as well as low grade toxicity in some oncolytic viruses [44].

**Immunotherapy:**

Immunotherapy is a treatment modality used in patients with PCA which utilises a person’s own immune system to attack the tumour. Specifically, in PCA a host of immune cells play a major role in tumour killing and improved prognosis such as CD8+ T-cells and dendritic cells (DCs). The latter originate from monocytes.

Vaccine-based therapies as well as immune checkpoint inhibitors are ground-breaking treatments in immunotherapy. Sipuleucel-T, which is an example of vaccine centred therapy makes use of the patients Antigen Presenting cells (APCs), which are also of monocytic origin. When these cells are exposed to prostatic acid phosphatase (PAP) (this antigen is found in prostate cancer cells) induce a cytotoxic T-cell response which leads to tumour recognition and subsequent tumour extermination [45].

In addition, the immune check point inhibitors such as CTLA-4 inhibitors are important immunotherapy treatments. CTLA-4 is a protein bound to another protein called B7 found on T-cells which stops them from killing cancer cells. The employment of CTLA-4
inhibitors was found to cause a huge reduction in metastatic relapse after surgical resection of the tumour [46].
<table>
<thead>
<tr>
<th>Treatment Option for Prostate Cancer</th>
<th>Percentage Success Outcomes</th>
<th>Side Effects/ Complications</th>
<th>References</th>
</tr>
</thead>
</table>
| Surgery                            | 85% disease-specific survival rate at 10 years (organ confined disease)  
68% disease-specific survival rate at 10 years (focal capsular penetration)  
58% disease-specific survival rate at 10 years (established capsular penetration) | Urinary incontinence  
Erectile dysfunction  
Infertility  
Peripheral neurological injuries (obturator nerve injury) | [47, 48] |
| Radiotherapy                        | 92.3% disease-specific survival rate at 7 years (for localised disease)  
23.9% disease-survival rate at 7 years (for distant disease) | Urinary toxicities- Haematuria and increased obstructive and irritative symptoms including: increased frequency, urgency and nocturia.  
Bowel toxicities: diarrhoea, bleeding per rectum | [49, 50] |
| HIFU                               | 96.4% disease-specific survival rate at 7 years (for localised disease) | Incontinence  
Urinary retention  
Urethral stricture | [51] |
| Hormonal therapy                   | 89.1% disease-specific survival rate at 8 years (localised or locally advanced disease) | Development of coronary heart disease  
Development of Diabetes Mellitus  
Bone fractures | [52, 53] |
| Chemotherapy                        | Neoadjuvant chemotherapy- 80% disease-specific survival rate at 5 years  
Adjuvant chemotherapy- 72% disease- specific survival rate at 10 years | Gastrointestinal toxicity- nausea, vomiting, dysphagia and diarrhea  
Gonadal toxicity- damage to developing spermatocytes | [54, 55] |
| Oncolytic Virotherapy               | Intratumoural- >50% / >25% PSA reduction  
Intravenous- >25% PSA reduction | Flu-like symptoms,  
Mild liver inflammation,  
Disseminated intravascular coagulation (DIC)  
Thrombocytopenia | [56] |
| Immunotherapy                       | 30% reduction in PSA levels  
25.2% disease- specific survival rate at 2 years | Skin toxicity- maculopapular rash, pruritis, psoriatic rash, toxic necrolysis, | [57, 58] |

Table 3: Treatment options available for PCA with their percentage of success outcomes and their side effects and complications.
Despite the different modalities in treating prostate cancer, none come without the potential of devastating side effects for the patients. Thus, it is essential that platforms are fabricated to test treatment in order to minimise as much as possible the harm that may be inflicted on patients.

One technique by which treatments could be tested and therapeutic regimens modified with minimal harm to patients is by the use of 3D models. This is because they recapitulate to an extremely high degree the in vivo TME. 3D models are being used to test the efficiency of radiotherapy on LNCaP and PC3 spheroids [59]. Also, hormonal therapy is being used in the setting of hanging drop 3D models using the PCA LNCaP cell line in co-culture with fibroblasts [60]. In addition, chemotherapeutic agents such as docetaxel are being used in 3D LNCaP and DU145 to explore drug resistance [61, 62].

1.5 The immune system and its impact on PCA

The immune system is complex and has various cellular components (Figure 1.4). One key cell type which has a considerable influence on PCA is the monocyte. It has been reported that monocytes increase significantly in patients with PCA. In 2017, Hayashi et al. demonstrated that the serum monocyte fraction was elevated significantly (p<0.01) in men with positive prostate biopsy. They stratified patients into groups depending on their biopsy score as follows: patients with low Gleason score cancer and patients with high Gleason score cancer. They found that patients had varying serum monocyte fraction percentages and correlated them to their biopsy specimens as follows: 6.7% (low Gleason score PCA) and 7.6% (high Gleason score PCA) respectively. Hence, they concluded that the serum monocyte fraction is an essential factor that could predict a positive prostate cancer biopsy [63].

However, there are other immune cells that play different roles in PCA which are summarised briefly in this section.
The first cell type to be considered is the natural killer (NK) cell. NK cells are cytotoxic lymphocytes of the innate branch of the immune system. They reportedly play a role in antitumourigenic immunity in PCA. This has been confirmed in patients with PCA having a lower risk of progression with increased tumour-infiltration of NK cells. Pasero et al. found that patients with high NK cell infiltration in PCA tumours had a prolongation of time to castration resistance amounting to greater than 18 months [64, 65]. NK cells become cytotoxic on recognition of low or absent major histocompatibility complex (MHC) class I molecules. Once NK cells recognise this, they become activated, they start expressing death-inducing ligands including; FasL and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). They also release cytotoxic granules such as; granzyme and perforin leading to tumour cell death [66].

T-cells also play an important antitumourigenic role in PCA that should not be ignored. It has been reported that tumours with a high infiltration of these lymphocytes translated into higher survival rates vis-à-vis those tumours with reduced lymphocyte infiltration [67]. Yang et al., proved this to be the case as patients with PCA who had elevated levels of CD8+ T-cells infiltration in their prostates following radical prostatectomy had an improved 5-year overall survival of 98%. Whereas those patients with low CD8+ T cell density in their prostates had 5-year overall survival of 91% [68]. T-cells carry out their tumour cytotoxic effects through DCs also referred to as APCs. APCs present tumour antigens which are found in cellular debris to create what is known as tumour-specific CD4+ and CD8+ T-cells. It is however, the CD8+ T-cells which are considered tumour-fighting and help exterminate tumour cells through the production of; TRAILs, perforin and reactive oxygen species [69].

The other major cell type that have an antitumourigenic effect in PCA are the neutrophils. These cells are found in high densities not only within tumours but also in sites of
metastasis such as the lymph nodes. Further, tumour associated neutrophils (TANs) and especially those cells with N1 phenotype are cytotoxic to tumours. These N1 cells exert their tumour-exterminating effects through the activation of both innate and adaptive immune cells including; NK cells, T and B cells as well as DCs. Also, these cells have enhanced NADPH oxidase activity which in turn causes an increase in the production of ROS, thus leading to cytotoxicity to tumour cells [70]. However, this was not translated as would be expected into prolonged survival in PCA patients. A study which correlated the neutrophil level (in clinical terms using Neutrophil: Lymphocyte Ratio (NLR)) with patient survival was carried out by Lorente et al. and showed that patients with a higher NLR (NLR>3) had a lower overall survival compared to patients with a lower NLR (NLR<3) which amounted to 12.6 months compared to 15.9 months respectively [71].

Finally, Tumour Associated Macrophages (TAMS) which are derived from monocytes and are present in high numbers in the tumour micro-environment (TME), influence PCA tumours. These cells are classified into two distinct phenotypes either M1 TAMS or M2 TAMS.

The M1 TAMS are referred to as being antitumourigenic as they cause direct cytotoxicity to tumour cells as well as exerting antibody dependent cell-mediated cytotoxicity (ADCC). However, the M2 TAMS are tumour promoting as they lead to enhanced tumour angiogenesis, inhibition of T-cell antitumourigenic response as well as contributing to tumour metastases [72].

Yuri et al., have examined TAMS in prostate biopsies from patients with PCA post radical prostatectomy. These researchers found that increased TAMS in biopsy specimens was associated with a number of detrimental factors including: metastasis with a mean TAM number of 45.2 compared to no metastasis with a mean TAM number of 27.4, higher Gleason score ($\geq 7$) a mean TAM number of 38.4 compared to lower Gleason score (<7)
a mean TAM number of 24.4 and response to ADT was poor in patients with a mean TAM number of 37.1 compared to patients with a good response to ADT with a mean TAM number of 30.8 [73].

Figure 1.4: **Different types of immune cells** involved in PCA with their cell-specific clinical outcomes. Image a) Shows positive patient-based clinical outcomes related to Natural Killer (NK) cells and T-cells which translated into prolonged time to castration resistance and increased 5-year survival respectively. Image b) shows negative patient-based clinical outcomes related to monocytes, neutrophils and Tumour Associated Macrophages (TAMS) which were: increased Gleason score, reduced overall survival, increased metastases, increased Gleason score and poor ADT response respectively.

1.6 **Monocytes and their role in cancer**

Monocytes are cells which are part of the innate immune system that can differentiate into macrophages. They represent the third most abundant immune cell population in
human circulation and account for approximately 4-11% of leukocytes [74]. Also, monocytes constitute a major component of the stroma of tumours [75]. Monocytes are classified as; classical and non-classical (Figure 5). The ‘classical’ monocytes, in general, are observed to play a major role in promoting tumour growth and metastases. Whereas, the non-classical also referred to as ‘patrolling’ monocytes act against tumours and rapidly remove tumour material and may prevent the metastases of cancer cells [76].

![Figure 5: Subtypes of monocytes; classical and patrolling (non-classical) along with the proteins and chemokine (CCR2 and CX3CR1) receptors associated with them. (Adapted from reference [77]).](image)

However, monocyte behaviour and action within the cancer scenario is more complex than the original view points as described above. The current knowledge on monocyte action has emerged from studies, such as the one carried out by Chittezhath et al., where biopsies from patients with Renal Cell Carcinoma (RCC) showed that there was an elevation of classical (protumourigenic) monocytes with a corresponding elevation in VEGF secretion [78]. However, not all monocytes in cancers behave equally, as is shown by another study. Researchers found that breast cancer patients had a profound downregulation of VEGF
expression by their classical monocytes [79]. In addition, patients with melanoma were found to have a similar down regulation of VEGF expression by their classical monocytes as in breast cancer patients [80].

Moreover, to add to the complexity of the monocytic actions in cancer, researchers carried out experiments on gastric cancer tissue specimens and found that patients with elevated non-classical (classified as antitumourigenic) monocytes in the TME had worse stages of disease (stages II-IV) [81].

What is more, Sidibe et al., who carried out experiments on human colorectal cancer (HCT116) xenograft models also found that non-classical monocytes cause rapid cancer progression and promotes angiogenesis [82].

Studies have shown that both the classical and non-classical monocytes have anti-tumour functions in many cancers (Table 4). Further, it seems that the antitumourigenic activities of monocytes range from: tumour cytotoxicity, prevention of metastasis, recruitment of NK cells to inhibition of Tregs and engulfment of tumour material [83].
<table>
<thead>
<tr>
<th>Monocytic Subset</th>
<th>Antitumourigenic Function</th>
<th>Associated Cancer</th>
<th>Experimental model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>Cytotoxic to tumour cells</td>
<td>Ovarian cancer</td>
<td><em>in vitro</em> study using PBMCs and applied to ovarian cancer cells (OVCAR3) in 2D cell culture</td>
<td>[83, 84]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[85]</td>
</tr>
<tr>
<td>Non-Classical/patrolling</td>
<td>Cytotoxic to tumour cells</td>
<td>Ovarian cancer</td>
<td><em>in vitro</em> study using peripheral blood from epithelial Ovarian cancer patients and comparisons were made of cell surface subsets (CD14+ monocytes) and their cytokine production in normal donor peripheral blood and applied them to ovarian cancer cells (tumour cell line 2774)</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>Cancer cell death</td>
<td>Leukaemia</td>
<td><em>in vitro</em> study using peripheral blood from B-CLL (B cell Chronic Lymphocytic Leukaemia) patients and co-cultured with cancer cells from lung adenocarcinoma (A549), breast adenocarcinoma (SKBR3)</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td>NK cell recruitment</td>
<td>Lung cancer</td>
<td><em>in vitro</em> study using peripheral blood from patients with lung adenocarcinoma along with tumour samples and applying single-cell analysis of tumour and blood cells.</td>
<td>[88]</td>
</tr>
<tr>
<td></td>
<td>Inhibition of regulatory T-cells</td>
<td>Melanoma</td>
<td><em>in vitro</em> study using peripheral blood from patients with melanoma and correlating the results with the tissue samples.</td>
<td>[88]</td>
</tr>
</tbody>
</table>

*Table 4: The subset of monocytes which have antitumourigenic functions and examples of their action in different cancer types.*
What is more, these two subtypes of monocytes have been reported to have protumourigenic functions. These tumour promoting functions range from inhibiting the cytotoxic function of T cells, to angiogenesis and to formation of cancer promoting TAMs in malignant tumours (Table 5).
<table>
<thead>
<tr>
<th>Monocytic Subset</th>
<th>Protumourigenic Function</th>
<th>Associated Cancer</th>
<th>Experimental model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>Suppression of cytotoxic function of T cells</td>
<td>Hepatocellular carcinoma</td>
<td>Three murine HCC models were used as well as two patient cohorts analysed retrospectively and cellular analysis was carried out and correlation with tumour progression and growth. Murine animal models and human PBMCs from melanoma patients and cell analysis was carried out and correlation with tumour progression and growth.</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Differentiation into the pro-tumourigenic TAMS</td>
<td>Non-Small cell lung cancer</td>
<td>Murine animal models and <em>in vitro</em> culture models using transwell 2D models of human lung cancer cells (H69, H226 and H661) as well as hepatocellular carcinoma (Hep-G2) and <em>ex vivo</em> examination of human lung cancer specimens</td>
<td>[91]</td>
</tr>
<tr>
<td>Non-Classical/ Patrolling</td>
<td>Angiogenesis</td>
<td>Glioma</td>
<td>Murine animal models, human PBMCs, tissue specimens and cellular analysis was carried out and correlation with tumour progression and angiogenesis. Human xenograft model (mice) and human PBMCs and examination of pro-angiogenic cytokine (VEGF) secreted by monocytes</td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colorectal cancer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 5: The subset of monocytes which have protumourigenic functions and examples of their action in different cancer types.*
1.7 Monocytes and PCA

Monocytes are found in many cancers and thus it comes as no surprise that they are found in PCA and play a major role in this disease. They also comprise the majority of the infiltrating cells in the tumour. They are recruited and activated at sites within the tumour itself through signals expressed by chemokines and cytokines.

These immune cells are very important clinically as they provide an insight into prognosis of PCA. It has been found that the number of monocytes present within the prostate tumour can influence the prognosis of this particular cancer [94]. Wang et al. examined peripheral monocyte count in patients and found that patients who had a high monocyte count that they defined as $>0.425 \times 10^9\text{l}^{-1}$ faired worse than those with lower monocyte count defined as $<0.425 \times 10^9\text{l}^{-1}$. Specifically, patients with Gleason score $>7$ with higher monocyte count ($>0.425 \times 10^9\text{l}^{-1}$) had an overall survival of approximately 38% at 45 months. In comparison with patients in the same Gleason score category but with lower monocyte count ($<0.425 \times 10^9\text{l}^{-1}$), who had an overall survival of approximately 60% at 45 months [95].

Additionally, in the clinical setting, trans-rectal prostate biopsies are being used to detect monocytes in tumour tissue. This method has been used in correlation with the routine investigations of; PSA and Gleason stage scoring, to predict the metastatic potential of PCA [96]. Thus, highlighting the importance of monocytes in this disease.

On infiltrating the prostate tumour sites, the monocytes begin their journey in influencing the tumour behaviour. They can influence the progression of PCA by causing it to propagate and disseminate i.e., protumourigenic. However, these monocytes can also have a negative growth effect on the propagation of the tumour i.e., antitumourigenic.
1.8 The antitumourigenic effects of monocytes in PCA

1.8.1 Monocytes secrete cytokines which directly oppose PCA

Monocytes secrete several cytokines that directly oppose PCA. One such cytokine is IL-1β which has been found to be expressed by monocytes in PCA in increasing levels and it has anti-tumour properties in PCA. As demonstrated in a study carried out by Culig et al., who applied monocytes from PBMCs and cultured them in 2D with LNCaP cells [97]. They found that monocytes altered the morphology of LNCaP cells becoming more elongated and formed dendritic-like processes. They also found that in co-culture, monocytes reduced the proliferation of LNCaP androgen responsive PCA cells in a dose-dependent manner, such that 40% of the monocyte conditioned medium (MCM) reduced LNCaP proliferation by 50% [97]. Also, monocyte-derived IL-1β starkly reduced AR by 70% on co-culture with 40% of MCM and further reduced PSA levels by 80% in the LNCaP variant of PCA [97].

Another cytokine TNF-α is produced by monocyte-derived macrophages primarily in prostate tumours [98]. Although it may have a dual effect on PCA as it may be pro or antitumourigenic, I will focus in this section of the thesis on its antitumourigenic effect. It carries this out through increasing the levels of p53 which is a tumour suppressor protein [99].

Furthermore, CXCL-16 which is secreted by monocytes plays a dual role in tumour activity as it could be anti or protumourigenic. In this section, the antitumourigenic property of CXCL-16 will be considered. It is found that in some cases of PCA, including DU145 and PC3, this chemokine reduces metastases. Specifically, it has been found that CXCL-16 causes co-expression of CXCR6 and if the latter is found in concentrations of greater than 0.3ng/mL PCA cell migration is inhibited [100].
1.8.2 Monocytes secrete cytokines which cause other cells in turn to attack PCA

Moreover, IL-10 is a monocyte-secreted cytokine that can be considered as antitumourigenic when it comes to PCA. A study has reported that IL-10 causes a reduction of MMP-1 and MMP-2 which are known to be associated with a higher grade PCA [101]. Murine models have found that IL-10 can both increase the levels of CD8+ T-cells as well as stimulate the action of CD8+ T-cell antitumourigenic immunity which lead to tumour rejection [101]. Also, IL-10 has been reported to reduce angiogenesis and tumour growth by inhibiting the proangiogenic MMP-2 and reduces the progression of PCA [102].

What is more, the antitumourigenic effects of monocytes include the secretion of the cytokine IL-12. This cytokine exerts its antitumourigenic effect through a series of steps. It stimulates the cytotoxic effects and proliferation of NK and CD8+ T-cells as well as promoting the production of IFN-γ. It is this cytokine which then acts on tumour cells to induce anti-angiogenic effects, it also leads to enhanced antigen presentation of dendritic cells to cytotoxic T-cells [103].

In addition, CX3CL1 or what is also referred to as Fractalkine is a chemokine secreted by monocyte derived macrophages and has been found to reduce secretion of VEGF which leads to a reduction in tumour angiogenesis [104]. Its antitumourigenic effect is carried out through its recruitment of a variety of cells such as; NK cells, CD4+ and CD8+ T-cells into the tumour [104].

1.8.3 Direct effect of monocytes on PCA cell death

The antitumourigenic effects of monocytes have been documented. One particular study described that monocytes infused into tumours in mice developed into TAMs and were associated with reduced proliferation of the tumours [105]. Also, where PCA is
concerned, monocyte derived macrophages induced senescence as well as growth inhibition in LNCaP and PC3 cell lines [106].

Further, there has been research indicating that reactive oxygen species (ROS) released by monocytes can aid tumour extermination. PCA cells are described to be very sensitive to damage by high ROS leading to their apoptotic death (Figure 1.6). High levels of ROS are described as causing opening of the mitochondrial membrane which leads to mitochondrial membrane potential loss which is thought to lead to programmed cell death of PCA cells [107].

In addition, antitumourigenic effects of monocyte derived TAMs are heightened in conjunction with chemotherapy. Chemotherapy causes reprogramming of TAMs into M1-like and suppress tumour growth (Figure 1.6). Also, these monocyte-derived TAMs phagocytose dead cancer cells and in so-doing contribute to their antitumourigenic function [108].
1.9 The protumourigenic effects of monocytes in PCA

1.9.1 Monocytes secrete cytokines that cause growth of PCA

In advanced and metastatic PCA, monocytes secrete high levels of chitinase-3-like 1 (CHI3L1) which is a glycoprotein secreted specifically by innate immune cells. This glycoprotein has been linked to poor prognosis in PCA [109]. It has been found that CHI3L1 is responsible for increased PCA invasiveness through signalling between itself and another ligand, IL-13α2, expressed by PCA cells. [110].

Monocytes secrete IL-1β which is considered antitumourigenic but also protumourigenic and carries out this function through several pathways. Mainly, it acts in conjunction with NF-kB which leads to the activation of epithelium-specific ETS transcription factor-1
(ESE-1). This protein is known to have a strong connection with poor prognosis in patients with PCA [111].

However, the protumourigenic effects of IL-1β do not halt at this point. It has been shown that IL-1β induces the expression of endothelin-1 (ET-1) which has been shown to be linked to PCA progression [112].

In addition, it must be noted that IL-1β leads to the expression of an MMP in the LNCaP cell line of PCA. Matrisylin also referred to as MMP-7 expression has been found to be increased by IL-1β and this has been found to accelerate PCA progression [113]. IL-1β also contributes in a negative manner to the therapeutic management of PCA. This cytokine reduces AR expression in PCA leading to a reduction in efficacy of anti-androgen treatments [114].

What is more, monocytes secrete MMPs in PCA which collectively contribute to promoting the progression of this tumour. MMPs have been found to achieve this through their impact on several vital steps of tumour cell proliferation including; angiogenesis and epithelial to mesenchymal transition (EMT) [115].

MMPs have been associated with more advanced PCA with higher Gleason scoring. These MMPs include; MMP 2,3,7,8,9,10,12 and 13. However, according to the literature, MMP-1 is associated with a lower grade and a less invasive prostate tumour [115].

Further, monocytes secrete chemokine ligand 2 (CCL2) which is also known as monocyte chemoattractant protein (MCP-1). This, in turn, is responsible for the migration of monocytes into the prostate gland by the process of chemotaxis and leads to the progression of the cancer. It does so by promoting proliferation of PCA cells as well as inducing tumour angiogenesis [116].

The other monocytic-secreted protumourigenic cytokine to discuss here is CCL17 also referred to as Thymus and Activated regulated chemokine (TARC). This cytokine has
been found to increase the migration of PCA cells in the following cell lines; PC3, DU145 and LNCaP [117].

CCL18 also known as Pulmonary and activation-regulated chemokine (PARC) is also secreted by innate immune cells including monocytes. It exerts a protumourigenic effect on PCA, as researchers have noted that CCL18 promotes PCA cell growth and invasion as well as exerting a reduction in apoptotic rate in DU145 cells [118].

In addition, another protumourigenic cytokine is CCL22. It stimulates PCA progression through specific co-ordination with the CCL2-CCR2 axis. Further, this cytokine has been found to also be secreted by TAMs, namely M2 type macrophages and exert strong immunosuppression properties [117].

Moreover, the protumourigenic monocyte secreted chemokine CXCL1 (also known as KC) augments the growth of PCA via a fundamental process in tumour growth. This process is the epithelial-mesenchymal transition (EMT) which promotes the migration of PCA cells and leads to metastasis of this tumour [119].

In addition, CXCL-16 can be considered not only antitumourigenic (as mentioned above) but also protumourigenic and acts as a powerful monocyte derived chemokine which enhances the invasion and migration of cancer cells. It also leads to increased angiogenesis by up regulating the expression of MMPs. These MMPs, in the main, lead to secretion of pro-angiogenic factors (VEGF and TGF-β) and are considered important factors in cancer progression [100].

IL-1α is another cytokine secreted by monocytes and activated macrophages. This cytokine is considered to be protumourigenic and immunosuppressive. Also, this cytokine has been associated with a neuroendocrine phenotype of PCA and extremely poor prognosis of this cancer [120].
The other cytokine to discuss in this section of the thesis is IL-4. In PCA this cytokine is associated with a worse grade of disease. It carries this out through the activation of STAT6 which in turn leads to a marked increase in the colony-forming ability of PCA cells [121].

Further, IL-6 which is also monocyte-secreted, is linked to an aggressive PCA phenotype. Its danger is exemplified in its ability to direct cancer cells to bone. It is also observed to play a major role in converting PCA from hormone-dependent to Castrate Resistant Prostate Cancer (CRPC). In particular, it has been found to stimulate growth in the LNCaP lineage of PCA [122].

Moreover, IL-6 exerts its protumourigenic properties through up-regulating MMP-9 leading to increased metastatic potential of PCA as well as downregulating a tumour suppressor called Maspin. What is more, it is described as being a powerful inducer of EMT in LNCaP derived PCA, thus facilitating invasion and metastases [122].

Monocytes also secrete IL-7, which is considered to be a strong protumourigenic cytokine and its expression is markedly increased in PCA. It is reported to contribute greatly to the invasiveness of PCA by promoting EMT. This cytokine has also been found, when it comes to the PC3 lineage of PCA, to cause increased cellular invasion and metastasis to bone [123].

Another equally malicious protumourigenic monocyte derived cytokine is IL-8, which is not only associated with advanced stages and poor prognosis of PCA, but it is also detrimental to immunotherapeutic methods of PCA treatment [124].

It must be mentioned at this point that IL-10, which was previously discussed, not only has an antitumourigenic function but also is considered protumourigenic as well. IL-10, in its protumourigenic role, causes upregulation of PDL1 which through its interaction
with the immune cell inhibitory receptor PD1 acts to suppress antitumourigenic immunity [101].

Another monocyte derived cytokine that favours PCA growth is IL-23. This cytokine, has been found to cause PCA to become CRPC through making the prostate tumour insensitive to androgen deprivation therapy [125].

In addition, VEGF is another protumourigenic factor produced by monocytes and macrophages. This protein is known as ‘the major angiogenic marker’ [126]. It is responsible for several functions that aid in the proliferation and metastasis of PCA including; vascular permeation and endothelial cell proliferation.

It is imperative at this point to mention the monocyte-secreted cytokine TNF-α which plays a vital role in the growth of PCA. Researchers have shown that TNF-α leads to the induction of CCR7 (chemokine receptor type 7) expression and that the CCL21/CCR7 axis increases the metastatic potential of PCA cells. Hence, it is found that TNF-α induces PCA cell migration through the lymphatic system by its upregulation of CCR7 [127].

The above are tabulated in Table 6.

1.9.2 Monocytes inhibit cytotoxic cells and contribute to the growth of PCA

In addition to the secretion of cytokines, it has been shown that infiltrating monocytes into the prostate gland lead to creation of an immunosuppressive environment within the tumour. These monocytes have been found to be CD14+ and PD-L1+ and they inhibit the cytotoxic effect of infiltrating T- cells [128].

1.9.3 Monocytes contribute directly to the growth of PCA

Moreover, there has been data which suggested that monocytes are increased in number in patients with metastatic prostate cancer. Specifically results have indicated that there is a higher infiltration of CD68 positive monocytes in non-AR-driven CRPC [129].
Interestingly, infiltrating monocytes into the prostate gland also influence the pro-tumour activity of TAMs. It has been found that monocytes may be associated with enhanced extension and poorest prognosis of PCA as they could develop into protumourigenic M2 TAMs [96]. These M2 TAMS secrete VEGF which is associated with growth of prostate tumours.

<table>
<thead>
<tr>
<th>Monocyte secreted cytokine</th>
<th>Protumourigenic or Antitumourigenic or Monocyte to Macrophage differentiation indicator</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>MMP-1</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
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<td>MMP-9</td>
<td>Protumourigenic</td>
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<td>Protumourigenic</td>
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<td>Protumourigenic</td>
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<td>MMP-13</td>
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<td>[116]</td>
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<tr>
<td>VEGF</td>
<td>Protumourigenic</td>
<td>[127]</td>
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<td>IL-1β</td>
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<td>[98,112,113,114,115]</td>
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<td>TNF-α</td>
<td>Antitumourigenic and Protumourigenic and Monocyte to macrophage differentiation indicator</td>
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<td>CCL-17 (TARC)</td>
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<tr>
<td>CCL-18 (PARC)</td>
<td>Protumourigenic</td>
<td>[119]</td>
</tr>
</tbody>
</table>
Table 6: Cytokines secreted by monocytes with their classifications: protumourigenic, antitumourigenic and monocyte to macrophage differentiation indicator.

1.10 Cytokines secreted by PCA

The PCA mass produces an extensive list of cytokines and growth factors which drive a number of actions which are protumourigenic in the majority, while some appear to provide a degree of modulation of the disease such as the degree of aggressiveness of PCA (Table 7). A number of mechanisms have been described in the laboratory, while associations with disease state or severity has been reported in studies involving patients. VEGF is highly expressed and upregulated in PCA cells and is responsible for several devastating protumourigenic consequences. These include increased angiogenesis within the tumour and acceleration of cancer cell proliferation as well as bone destruction following PCA metastases [130]. Further, a study where levels of VEGF (measured as final immunoreactive score (IRS), where the percentage of positive tumour cells was combined with VEGF-A staining intensity) were correlated with patient biochemical
progession free survival showed that higher VEGF levels reduced patient survival. VEGF was measured and considered high when IRS score >5 and low when IRS score <5, the 8 year biochemical progression free survival was higher in patients with low VEGF IRS than with high VEGF IRS amounting to 68.6% and 67.8% respectively [131].

PCA cells also produce TNF-α. In the main, this factor is described as chronically produced but at low levels by cancer cells within the TME. However, the secretion of this factor by prostatic cancer cells has an impact on the prognosis of this disease, as shown by a study by Michalaki et al, who found that there was a statistically significant increase in TNF-α (6.3pg ml⁻¹) in patients with metastatic PCA compared with localised disease (3.9pg ml⁻¹) [132]. This finding confirmed that the metastatic potential of PCA is driven by TNF-α.

PCA cells also express a number of classical cytokines, such as IL-10. This cytokine as previously mentioned, has dual anti- and protumourigenic functions. Its cancer combative effects were observed in its ability to inhibit the function of MMP-1 and MMP-2 synthesis. These two metalloproteinases were correlated with poor PCA prognosis. Therefore, increased IL-10 is linked to reduced severity of PCA and improved prognosis [101]. On the other hand, the protumourigenic actions of IL-10 are also documented. As it inhibits the function of cytotoxic T-cells and it is thought to inhibit the immune anti-cancer response. Interestingly, high levels of IL-10 were detected in some cohorts of patients who had metastatic PCA, highly progressive disease and poor tumour morbidity [133].

IL-8 is also a protumourigenic cytokine expressed by prostate tumours. It prolongs tumour cell survival, causes chemoresistance, increases malignant cell proliferation and invasion. It has been found that IL-8 in the serum of patients with PCA is associated with extremely poor outcomes [134].
In addition, PCA cells produce three cytokines known as the proangiogenic cytokines. These are CXCL-1,2 and 3. They collectively contribute to the progression of PCA. It is thought that these cytokines maintain a pro-inflammatory environment within the prostate tumour and increase angiogenesis and dissemination of the cancer [135].

Also, the protumourigenic cytokine produced in large amounts by PCA cells is CXCL-16. It is found in patients who have a high-grade Gleason stage and is linked to very aggressive disease. It has been shown that CXCL-16 leads to an increase in PCA cell invasion and skeletal bone migration in three prostate cancer cell-lines which include; LNCaP, DU145 and PC3 [136].

PCA cells stimulate the secretion of RANKL which is also known as tumour necrosis factor-related activation-induced cytokine (TRANCE). This particular cytokine is responsible for accelerating migration of cancer cells and subsequent bone metastasis. Its danger is exemplified in its ability to protect cells from apoptosis and also has an influence in promoting epithelial-to-mesenchymal transition (EMT) which is a vital phenomenon underlying cancer metastasis [137].

What is more, the multi-functional peptide transforming growth factor beta (TGF-β) plays a major role in prostate cancer tumourigenesis. In PCA, this factor stimulates; angiogenesis, migration, invasion of malignant cells and maintains a reactive tumour micro-environment (TME) which is essential to the metastatic spread of prostate cancer. The maintenance of this reactive state of TME is carried out through TGF-β inhibiting T-cell induced cytotoxicity [138].

<table>
<thead>
<tr>
<th><strong>PCA secreted cytokine</strong></th>
<th><strong>Protumourigenic/ Antitumourigenic</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Protumourigenic</td>
<td>[131,132]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Protumourigenic</td>
<td>[133]</td>
</tr>
<tr>
<td>IL-10</td>
<td>Protumourigenic and Antitumourigenic</td>
<td>[102,134]</td>
</tr>
<tr>
<td>IL-8</td>
<td>Protumourigenic</td>
<td>[135]</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Classification</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>CXCL-1</td>
<td>Protumourigenic</td>
<td>[136]</td>
</tr>
<tr>
<td>CXCL-2</td>
<td>Protumourigenic</td>
<td>[136]</td>
</tr>
<tr>
<td>CXCL-3</td>
<td>Protumourigenic</td>
<td>[136]</td>
</tr>
<tr>
<td>CXCL-16</td>
<td>Protumourigenic</td>
<td>[137]</td>
</tr>
<tr>
<td>RANKL (TRANCE)</td>
<td>Protumourigenic</td>
<td>[138]</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Protumourigenic</td>
<td>[139]</td>
</tr>
</tbody>
</table>

Table 7: Cytokines secreted by PCA with their classifications: protumourigenic, antitumourigenic.

1.11 In vitro models used for cancer research

In the main there are two types of in vitro cancer cell culture methods; two-dimensional (2D) and three-dimensional (3D) (Table 8). The 2D method of culturing cells has been used since the early 20th Century [139]. Yet the limitations of this method are manifold including; inability to mimic the 3D in vivo organisation of tumours, cellular gene expression differences from that produced in in vivo tumours; and the inability to detect extracellular matrix (ECM) proteins with poor ECM-cell interactions.

<table>
<thead>
<tr>
<th>Cell Characteristics</th>
<th>2D cell culture</th>
<th>3D cell culture</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle stage</td>
<td>Most of the cells are in the same stage of the cell cycle</td>
<td>Spheroids mimic in vivo conditions and cells are; quiescent, hypoxic, proliferating and necrotic</td>
<td>[140, 141]</td>
</tr>
<tr>
<td>Cell junction</td>
<td>Cell junctions are not representative of real junctions and are less common</td>
<td>Cell junctions are highly frequent and allow cell to cell communication</td>
<td>[139]</td>
</tr>
<tr>
<td>Gene/protein expression</td>
<td>Gene and protein expression levels vary from in vivo</td>
<td>Cells express gene and proteins similar to in vivo tissues</td>
<td>[139]</td>
</tr>
<tr>
<td>Response to stimuli</td>
<td>Cells are unable to experience gravity and in turn unable to expand into the third dimension</td>
<td>Cells experience gravity and give a more accurate representation of cells in vivo There is an accurate representation to</td>
<td>[142, 143]</td>
</tr>
</tbody>
</table>
3D cell cultures are overtaking monolayer 2D cultures due to their ability to accurately mimic aspects of the complex in vitro tumour microenvironment and its 3D organisation. Generally speaking, 3D methods of cell culturing are divided under two headings; scaffold-based and non-scaffold based (Figure 1.7).

An example of a scaffold-based cell culture is hydrogels, where cells are seeded in a gel-based 3D structure such as collagen. The advantages of using hydrogels (e.g., collagen, alginate and laminin) are numerous such as: having the ability to grow different cell types, cells organising in a 3D manner spontaneously, and allowing for the visualisation of different zones, including necrotic [143]. Further, a strong advantage associated with hydrogels is the ability to create an ECM with components that are found in vivo. Gene expression and phenotype of the cells are very similar to those found in in vivo tumours [143].

Of particular interest for the current work, is the scaffold-based 3D model based on compressed collagen which has certain physicochemical properties. This type of matrix is the basis of the tumouroid model used throughout the thesis and is manufactured using a semi-automated method of compression (RAFT). The porous architecture with entangled and visible fibrils remained the same after compression [147]. A dense collagen structure obtained by fluid compression and mechanical loading leads to 97% of fluid being removed from the hydrogel [147]. This is reported to increase the mechanical

| Drug sensitivity | Cells have minor resistance to drug treatment, thus making drug treatment appear to be successful in a false manner | Cells have a higher resistance to drug treatment resembling the in vivo conditions and drugs are metabolised better | [144-146] |

Table 8: Main differences between cellular models in the two-dimensional (2D) and three-dimensional (3D) models.
integrity and strength of the hydrogel and is quoted as being comparable to tissue *in vivo*.

Also researchers have shown that there is high cellular viability for compressed collagen gels and minimal effect of fluid removal on cell survival [147].

Another example of a scaffold-based cell culture is what is known collectively as solid scaffolds and are either fibrous or porous and made from polymers. Fibrous scaffolds are usually produced by electro-spinning which produces a mesh of fibers by passing a polymer jet within an electric field [148]. This leads to cancer cells growing along the fibers and creating a 3D structure. To create a porous network a liquid such as a monomer is applied on a template which then solidifies [149]. This in turn contributes to the creation of a 3D environment where cells proliferate within these pores creating a more biomimetic model.

Non-scaffold methods for 3D cell culturing include the hanging drop where cells are placed in plates that are turned upside down. The cells in suspension form a hanging drop which is held by surface tension. Subsequently, spheroids are formed providing both a similar physicochemical environment and phenotypic properties as *in vivo* [150]. Magnetic levitation is another non-scaffold method which uses paramagnetic iron oxide nanoparticles to manipulate cells. These cells uptake the particles and a magnet is used to pull the labelled cells upwards leading to spheroid production. Such spheroids have been shown to express proteins such as N-cadherin that are produced *in vivo* thus providing a realistic reflection of the *in vivo* environment [151].

More advanced models incorporate fluid flow, usually in a non-scaffold system, where microfluidic flow occurs through channels which are connected to wells. These microchannels were originally produced by etching on the surface of materials such as; silicon or glass. Microfluidic systems allow for a number of cell types to be positioned in different channels/chambers to replicate the *in vivo* situation and fluid flow to follow a
specific order. This method tends to allow for cancer spheroid sizes to be homogenous and therefore is a good system to interrogate [152].

Figure 1.7: Diagrammatic difference between 2D and 3D static cell culture methods

1.12 Applications of three-dimensional (3D) PCA (tumouroid) models

1.12.1 3D PCA models investigating biology and progression of disease

Prostate tumours are one of many cancers in which researchers have utilised the 3D cell culture technique. Because of the morphology of cells within the 3D culture and due to a number of characteristics which mimic key aspects of cancer (Figure 1.8), various studies have focused on recapitulating and understanding the natural progression of the disease. For example, the use of PCA in 3D cultures allows cells to fully carry out tumourigenesis whereby they lose polarity and detach from the basement membrane forming a 3D structure [153]. This was illustrated in PCA (LNCaP) 3D cultures grown in a bilayer hydrogel platform where the PCA cells formed spheroids, therefore, providing an invaluable insight to the behaviour of native tumours in vivo [153].
Tumour invasion has been analysed using collagen-based hydrogel 3D models. A study using PCA cells has reported an increasing invasion of cancer cells into the surrounding ECM over time [154], thus, replicating the characteristic of invasion which is vital in tumour progression.

In addition, PCA 3D cultures replicate the epithelial to mesenchymal transition (EMT), a crucial change which is linked to disease aggressiveness, as well as the ECM remodelling that occurs in \textit{in vivo} conditions. In a Matrigel model, integrins, which are transmembrane receptors for ECM proteins such as: ITGB2, ITGB4 and ITGA10, were detected in PCA cells in 3D [155]. Other EMT markers found in PCA based 3D Matrigel models include; E-cadherin, connexin43 and laminin-5 [155]. Interestingly, determining the pattern of acinar invasion of PCA cells was highlighted using 3D models, where 1% agarose coated 24-well multi-well plates were used to form spheroids, through specific mesenchymal protein expression which encompass; Vimentin, Twist, and Slug [156].

Moreover, enzymes of the proteolytic nature are produced by PCA cells and have been studied in the context of 3D models. In a study of LNCaP PCA cells co-cultured with human osteoblast cells (hOB) in a 3D scaffold model which used a tissue engineered bone construct (TEB) and medical grade polycaprolactone-tricalciumphosphate (mPCL-TCP) scaffolds, it was observed that both MMP-2 and MMP-9 were secreted by these cancer cells [157]. It should be mentioned at this point that metalloproteinases are implicated in the proliferation as well as migration of cancer cells and degrade ECM components. Another important proteinase, namely MMP-1, or what is also known as metastasis-inducing metalloproteinase, was detected in 3D models of PCA cells. A study has reported that LNCaP cells in a 3D culture embedded in type 1 collagen hydrogels secrete MMP-1 and this leads to enhanced cell migration which mimics the \textit{in vivo} metastatic characteristic of PCA [158].
1.12.2 3D PCA models to test therapeutic interventions

Other studies have focused more on interventions and the resulting effects on the whole of the 3D structure. One of the strengths of using these 3D models in cancer research is their ability to accurately mimic the drug resistance which is characteristic of tumours. This characteristic is exemplified by these cultures modelling; hypoxia, low pH and low nutrient supply, which are all extremely vital factors in mimicking tumour drug resistance. Hypoxia is a factor which is replicated in 3D PCA models. In these models, cancer spheroids’ oxygen consumption varies from 2D models due to their larger surface area-to-volume ratio. In this sense it simulates the condition in vivo where only a small amount of tumour tissue is reached by blood vessels [158].

Further, an LNCaP based 3D model using Matrigel has provided researchers with information that can be utilised when exploring the delay in tumour death compared to monolayer cultures where the PCA cells were exposed to chemotherapeutic drugs such as mitoxantrone [156]. This is due to hypoxia inducing the release of vascular endothelial growth factor (VEGF) and hypoxia-inducible transcriptional mediator 1 (HIF-1). These factors in fact prolong spheroid survival and delay the onset of necrotic cellular death in the aggregate core [159] (Figure 1.8).
Figure 1.8: **Structure of a prostate cancer spheroid** in a 3D model which replicates the conditions of the in vivo prostate tumour cells. (Adapted from reference [155]).

A study demonstrated response to chemotherapy in 3D: LNCaP tumour cells cultured in hydrogels and treated with the chemotherapeutic agent Doxorubicin displayed an increase in expression of multidrug resistance proteins (MDR) [160]. This phenomenon accurately replicates what occurs *in vivo*. Generally, PCA cells in 3D culture models have been exposed to various chemotherapeutic agents, to study several spheroid parameters and monitor effectiveness of chemotherapy which can be translated to *in vivo* situations. These parameters include; growth by analysing the area of the spheroid, the spheroidal integrity by analysing the spheroid morphology and apoptosis through spheroid death [161].

Another mode of PCA treatment that has made use of 3D PCA models is radiotherapy. In one example, PCA spheroids were formed using the hanging drop technique (non-scaffold) and were subjected to various doses of radiation and using Surface Enhanced Raman Spectroscopy (SERS) the spheroid viability was analysed. The most effective
dose of radiation to kill PCA cells was determined by using fluorescent images of spheroids and analysing the disruption of morphology at different radiation doses as well. Also the researchers measured the doses of radiation against the pH of the spheroids and then concluded the optimum radiation dose (2x6 Gy) which lead to significant alkalosis and cell death [162].

The effects of hormonal drugs used for treatment of PCA were recently investigated in a 3D co-cultured model in hanging drop plates with PCA cells co-cultured with Cancer Associated Fibroblasts (CAF). The results showed that the anti-androgen resistance of spheroidic tumour cells diminished in co-culture samples compared to PCA samples with no co-culture [163].

1.13 Monocytes in a three-dimensional (3D) model

Monocytes comprise a vital component of the TME and have also been investigated using 3D model cell culture techniques (Table 9). One such example is the stirred 3D- 3 culture which uses alginate for cancer studies has also incorporated monocytes in their technique [164].

Furthermore, studies have employed 3D hydrogels using gelatine and hyaluronic acid derivatives to incorporate monocytes into their milieu. This method has shown that it is a suitable method for; cultivation, proliferation and encapsulation of the monocytes [165]. In addition, collagen has been used as a unique biomaterial for culturing monocytes. Researchers have used collagen hydrogels to incubate monocytes alongside cancer cells, by doing so they resemble the TME in a more realistic manner. They describe this method as being powerful as the monocytes in collagen hydrogels secrete cytokines leading to a protumourigenic environment [166].

What is more, 3D microfluidic cultures have been used to culture monocytes. The monocytic characteristics which were suspected to promote the metastatic cancer
progression investigated using this technique include; transmigration, differentiation of monocytes and intravascular migration [167].

Hanging drop 3D culture systems include monocytes in cancer research as well. In this method monocytes were used and differentiated into macrophages. Studies using this technique have found routes such as the Wnt pathway that promotes the creation of a pro-tumoural pathway in cancers (Table 9) [168].

<table>
<thead>
<tr>
<th>Type of Cancer that include monocytes in the study</th>
<th>3D culture type using monocytes and cancer cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian cancer</td>
<td>Hanging-drop spheroid model</td>
<td>[168]</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Gelatin Hydrogel</td>
<td>[169]</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Tumour-on-a-chip microfluidic model</td>
<td>[170]</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>Collagen Hydrogel</td>
<td>[171]</td>
</tr>
<tr>
<td>Hepatocellular cancer</td>
<td>3D microfluidic culture</td>
<td>[75]</td>
</tr>
</tbody>
</table>

Table 9: Different types of cancers in which monocytes are included in 3D cell culture models and their respective research studies.

1.14 Monocytes in 3D collagen hydrogels

Collagen has many unique effects on monocytes (Figure 1.9). It has been found that through integrins (specifically the αXβ2integrin), which are transmembrane proteins involved in cellular adhesion, collagen regulates the adhesion and diapedesis of monocytes. In essence, collagen has been found to increase the adhesion of monocytes [172].

What is more, the density of collagen has an effect on cytokine secretion by monocytes. Collagen with high fibril density caused an increase in the secretion of CCL2, also known as monocyte chemoattractant protein-1 (MCP-1) [173].
Moreover, other cytokines secreted by undifferentiated U937 cells such as the pro-inflammatory cytokines; IL-1β and TNF-α have also been detected when these specific types of monocytes are cultured in type 1 collagen [174].

It must be mentioned at this point that collagen has an influence on monocyte secretion of MMPs. A study showed that the MMPs gelatinase A and gelatinase B which correspond to MMP-2 and MMP-9 respectively were secreted by monocytes reaching a peak at day 3 of incubation [175].

In addition, collagen, specifically type 1, has been shown to differentiate monocytes to macrophages. An important study was carried out to prove this by measuring endocytosis of un-physiologic proteins [176]. Differentiation of monocytes to macrophages is exemplified by an increased ability of cells to endocytose. It was found that with increased incubation of undifferentiated monocytes in type 1 collagen there was increased endocytosis and hence macrophage differentiation.

Further, the mature macrophage marker CD71 has been found to be upregulated in monocytes incubated in collagen type 1 especially at 96hrs of incubation [176]. Whereas the so-called undifferentiated monocyte marker CD14 was found to be markedly reduced at 96hrs of collagen incubation. Thus, monocyte to macrophage differentiation is being influenced by collagen.

Interestingly, this differentiation can proceed further into favouring one type of macrophages over the other. This is due to collagen causing an increase in markers for the M1 (pro-inflammatory/ anti-tumour) phenotype including; IL-1, CD86 and IL-6. However, the levels of M2 (anti-inflammatory/ pro-tumour) phenotype did not seem to change in collagen as observed by their markers which encompass; CD206 and Arg1 [177].
Also, VEGF is found to be secreted by monocyte-derived-macrophages which are implanted in collagen scaffold models. Interestingly, these macrophages contribute to angiogenesis in this collagen environment in different ways. The M1 macrophages predominantly functioned to stimulate capillary sprouting. Whilst the M2 macrophages recruited pericytes and in so doing contribute to vascular stabilisation during angiogenesis [178].

It should be mentioned here that monocytes in 3D collagen environment undergo a process known as mechanosensing. Thus, these cells move through the collagen by integrins which bridge the cell membrane interacting with cytoskeletal proteins in the cell-matrix. This interaction with the actomysin cytoskeleton leads to cellular migration within the extracellular environment [179].

![Diagram of collagen type 1 effects](image)

**Figure 1.9**: Effects of collagen type 1 when monocytes are incubated in it. These influences include; monocyte to macrophage differentiation, MMP secretion, cytokine secretion and adhesion through integrins.

### 1.15 Studies incorporating monocytes and PCA in a 3D model

The co-culture of monocytes and PCA in a 3D model is an evolving field, with a modest number of studies. An interesting study described PCA spheroids harvested from 3D petri-dishes and transferred to agarose-coated dishes [180]. The spheroids had culture
media added to them which included U937 cells which were differentiated into macrophages. However, the researchers did not find any size changes in spheroids which were treated with this culture media as opposed to controls.

Microfluidic 3D models have been used also to co-culture monocytes and PCA cells. A study incorporating monocytes, after having them differentiated into macrophages, with prostate cancer cells was carried out by Kosoff et al. (2020). Researchers used a 3D microfluidic model as a platform to co-culture the afore mentioned cells. They were able to target the protumourigenic activity of these TAMs using Enhancer of Zeste Homolog 2 (EZH2) inhibitors. This led to an increase in cytotoxic T- cells infiltrating prostate tumour tissue, which the researchers analysed through migration studies supported by mRNA and protein expression analyses of the cell population, which may translate to a reduction in PCA tumour burden [181].

Another study used a multiple stacked microfluidic device in which co-culture of prostate cancer cells and monocytes resulted in tumour-cell-mediated differentiation of macrophages. Interestingly, the presence of two different PCA cells lines, LNCaP and C4-2, resulted in their associated macrophages secreting different cytokines [182].

1.16 Hypoxia and 3D models

Hypoxia has been used in determining the aggressiveness of cancers in several studies. One such study investigated hypoxia in a 3D model which utilised collagen in its production [183]. These researchers focused on breast cancer cell lines and discovered that hypoxia leads to the cancer cells displaying aggressive features. This included the overwhelming increase in cytokines indicative of bone metastatic potential including; RANK and JAG-1.

Moreover, it has been suggested that hypoxia in a 3D environment leads to spheroids mimicking, to a high degree of accuracy, the in vivo TME. This is exemplified in
spheroids expressing hypoxia inducible factors (HIF). HIF proteins cause an increase in VEGF-A, thus leading to angiogenesis and replicating tumours in vivo [184].

Also, another tumoroid model that has been used to study glioblastoma cells and hypoxia has been researched [185]. In this study, hypoxia was found to cause sprouting of blood vessels which is an event that happens in the tumour itself in the human body.

In addition, 3D models where Matrigel matrix has been used to culture breast cancer spheroids have been studied in the presence of hypoxia. In this study, hypoxic cells identified by a specific fluorescent stain, were shown to have a greater potential to metastasise when implanted in mice [186]. Thus, the implications of hypoxia in tumour cells in 3D models could be translated into the in vivo environment as well (Figure 1.10).

1.17 Hypoxia and PCA

PCA cells of the LNCaP variety, cultivated using the spheroid culture assay, have been used to analyse the effects of hypoxia on radiosensitivity. On treating these cancer spheroids with radiotherapy, proliferation and growth were reduced by only 39.2% [187]. The researchers attributed this observation to the hypoxic nature of the PCA spheroids which is correlated in vivo and translates into; poor prognosis, local recurrence of PCA as well as relapse after radiotherapy.

Spheroid models of PCA have also shown that hypoxia is an impediment to anti-androgen therapy such as bicalutamide [188]. Although initially, this method of treatment leads to cellular death, later on hypoxia overcomes this and leads to a restoration of vasculature, hypoxia resistant PCA cells grow and relapse occurs.

In addition, PCA spheroids were found to have accelerated EMT changes under hypoxic conditions. A study measured this EMT progression in these spheroids and proved, through the measurement of EMT markers which included; N-cadherin, fibronectin, vimentin and ZEB1, that hypoxia increased these markers greatly [189, 190].
The multiple effects of hypoxia are shown diagrammatically (Figure 1.10).

\[ \text{Figure 1.10: The multiple effects of hypoxia on cancer cells such as PCA. (Adapted from reference [190]).} \]

1.18 Hypoxia and monocytes

Hypoxia has been found to exert a negative influence on the proliferation of monocytes. A study was carried out whereby monocytes were exposed to both normoxic conditions and hypoxic conditions. It was observed that growth and proliferation of monocytes was dramatically reduced in hypoxic conditions as opposed to normoxic conditions [191]. Interestingly, in the same study, hypoxia caused an increase in a marker which is responsible for monocytic transmigration, namely CD49d.

Furthermore, hypoxic tumour cells have been shown to exert powerful chemotactic effects on monocytes by secretion of certain molecules. These chemoattractants which include; VEGF, CXCL-12, CCL5, CCL8 and CCL2 by hypoxic cancer cells attract monocytes into hypoxic regions within the tumour [192]. This, in turn, leads to monocytes
in the form of TAMs secreting further VEGF which leads to increased proliferation of the tumour.

1.19 PMA stimulation of U937 monocytes

It has been stated that PMA (Phorbol 12-Myristate 13-Aacetate), also known as a phorbol ester, has been widely used *in vitro* to differentiate U937 monocytes into macrophages (*Figure 1.11*) [193]. The mechanism by which PMA causes monocyte to macrophage differentiation occurs through the PKC pathway, this is a signalling pathway that leads to the phosphorylation of threonine and serine residues.

Specifically, in the immune system, PKC translocation to the nucleus in response to stimulation by PMA regulates the differentiation of monocytes to macrophages [194]. This signalling pathway causes not only the activation of PKC but also activates NF-kB. The role of NF-kB during maturation of monocytes to macrophages is unique, in that it is responsible for macrophages to secrete TNF-α [195].

In addition, PMA also leads to activation of the RhoA/ROCK signalling during the differentiation of monocytes to macrophages leading to changes in cellular morphology as well as increased cell adherence. Also, this pathway is responsible for the vital functions of macrophages which include; phagocytosis, motility, exocytosis, endocytosis and receptor surface expression [196].

It must be mentioned at this stage that PMA stimulation causes monocytes to differentiate into macrophages in the so called M0 stage. In order to obtain polarised macrophages either M1 or M2, there has to be further treatment with either IFN gamma and LPS or IL-4 and IL-3 respectively.[197]
Figure 1.11: **Effects of PMA stimulation on U937 monocytes and the immune pathways activated on their journey to differentiate into macrophages.**

**1.20 3D prostate tumouroids used in this thesis**

The 3D *in vitro* cancer model that is used in the work described here is the tumouroid. Generally, tumouroids are manufactured using collagen (rat tail collagen type 1) as the basic matrix protein. Collagen is mixed with cells of the desired cancer type and the resulting hydrogels are compressed using hydrophilic RAFT absorbers (LONZA 3D RAFT model) to remove excess fluid and produce simple tumouroids. If desired, simple tumouroids can be embedded in a further collagen compartment which is populated with connective tissue cells to mimic further aspects of the TME and again compressed. Tumouroids reach a reproducible stiffness of 5kPa which is of the order of solid cancers.
Furthermore, in-house work has demonstrated the presence of hypoxia in the middle of the central cancer mass (simple tumouroid) [198].

Our group has demonstrated the manufacture of tumouroids based on breast cancer, ovarian cancer and renal cancer, amongst others.

In some of the work carried out previously, tumouroids were used as drug testing platforms. For example, Stamati showed decrease of tumour masses and destruction of the stromal structures in renal cancer tumouroids, with the use of Pazopanib [198]. While Magdelin showed the anti-proliferative response of colorectal cancer tumouroids created with EGFR-expressing cells to Cetuximab [199].

Furthermore, as mentioned above collagen type 1 was used to create tumouroids, however the actual extracellular matrix could be composed of other constituents. For example, renal cancer tumouroids have an extracellular matrix (ECM) which in addition to collagen type 1, consists of collagen IV, laminin and fibronectin [198]. Interestingly, the addition of other specific ECM proteins may make a difference to the renal tumouroids, as shown in an example of the in-house work [197]. It was shown that only if laminin is added then any endothelial cells present can undergo cell-cell fusion to create a primitive vascular network.

Although tumouroids have been manufactured with cancer cells and a variety of other cell types, such as fibroblasts, endothelial cells and osteoblasts, to date none have incorporated immune cells.

The present work aims to incorporate U937 monocytes into a prostate cancer (PCA) tumouroid, towards a first version of an immunocompetent tumouroid. It is of course accepted that the inclusion of monocytes alone does not confer full immunocompetence, however these cells are key to both cancer progression and response to treatment, therefore they were chosen.
1.21 Cells chosen for this thesis

To create prostate tumouroids, two prostate cancer cell lines were chosen: LNCaP and PC3. LNCaP cell line is androgen receptor (AR) positive and has been widely used in research. The clinical importance of choosing this cell line lies in its AR positivity as it exemplifies the nature of PCA which relies on androgen signalling for tumourigenesis and anti-apoptosis [200]. Thus, in experimenting with this cell line we could have a better insight into the in vivo nature of PCA.

PC3 cells are also extensively used in PCA research. This cell line was used to compare the effects of immune cells on the PCA tumouroid. PC3 cells differ from LNCaP cells, as they are described as having a high metastatic potential whereas LNCaP tumours have a low metastatic potential [201]. It is also important that PC3 cells are AR negative unlike LNCaP cells [202].

The other cells used in the experiments were U937 cells. These cells are from a promonocytic cell line which resembles in its interaction those monocytes found in the human body. As monocytes form the bulwark of the immune response to PCA and influence tumour progression, angiogenesis and metastatic potential it seemed essential to utilise them.

1.22 Aims

The overarching aim of the thesis is to produce and characterise an immunocompetent 3D in vitro tumouroid model of prostate cancer. In the future, such a model can be used to further interrogate the interplay between immune cells and cancer, and for testing of immunotherapies.
The main hypothesis that this thesis revolves around is that the presence of monocytes has an impact on the tumour burden of PCA in a 3D immunocompetent tumouroid model setting.

The specific objectives are listed below, and results are presented in the relevant experimental chapters.

The first objective tackled was the depth of penetration / presence of monocytes in compressed (stiff) vs. non-compressed (non-stiff) collagen gels (Chapter 3) and whether it alters when collagen gels are populated with LNCaP cells in their single cell and spheroid forms respectively (Chapter 4). This required the development of a measurement/visualisation technique to track monocyte penetration within the gel (described in Chapter 2). Concurrently, cytokine/chemokines (classified as pro- and antitumourigenic) secreted by monocytes and differences between compressed and non-compressed collagen gels were investigated. Within this objective I also analysed whether cytokine secretion was influenced by the presence of LNCaP cells alone or in co-culture with U937 monocytes.

The second objective was to determine whether there was a change in growth of PCA spheroids when cultured alone or co-cultured with unstimulated monocytes or with PMA stimulated monocytes (macrophages). Cytokine expression was measured over the same timeline (Chapter 4).

The third objective was to observe the influence of hypoxia on 3D immunocompetent PCA tumouroids and whether this factor had an effect in terms of cytokine secretion, leaning towards a protumourigenic or antitumourigenic state (Chapter 5).
Finally, the fourth objective was to explore the effects that PMA stimulated U937 monocytes have on the PCA tumouroids. This was in order to see whether U937 macrophages generate a protumourigenic or antitumourigenic effect on PCA tumouroids, i.e., on growth and cytokine secretion, more so than unstimulated U937 cells (Chapter 5).
Chapter 2

Methods and Materials
2.1 Cell Culture

2.1.1 Cell lines and routine culture

LNCaP and PC3 prostate cancer cell lines were used as representative of prostate cell carcinoma. Respectively, they originated from a metastatic lymph nodes lesion of human PCA and are AR positive, and from bone metastases of grade IV PCA and are AR negative (Public Health England, England, UK). U937 cells were used to represent monocytes. U937 is a pro-monocytic human myeloid leukaemia cell line which differentiates and exhibits the characteristics of monocytes in culture (Public Health England, England, UK). All cell lines were cultured in Gibco RPMI 1640 Medium, fortified with 10% heat inactivated Fetal Bovine Serum (FBS), and 1% penicillin (5000 units/ml) and streptomycin (5000 μg/ml) solution (all from Gibco by Life Technologies, Thermo Fisher Scientific, UK).

Cells were routinely cultured either as 2D monolayers (Figure 2.1), or in 3D constructs (tumouroids) under aseptic conditions in a humidified atmosphere of 5% CO\textsubscript{2}/air, at 37°C. For 2D adherent cultures, LNCaP and PC3 cells were grown to approximately 85% confluence and then RPMI was removed, and cells washed with Phosphate Buffered Saline (PBS) (Gibco by Life Technologies). This was followed by addition of trypsin (Trypsin-EDTA solution 0.25% (Sigma-Aldrich, UK) for 5 minutes, which was then partly neutralised by adding RPMI containing serum (Gibco by Life Technologies). The supernatant containing cells was centrifuged (400g, 5 minutes) and the cell pellet was resuspended for propagation (1:10 split) or for setting up experiments. U937 cells are suspension cells and therefore were not trypsinised. To passage, cells were centrifuged (400g, 5 minutes) and resuspended in fresh RPMI. The U937 cells were also resuspended for propagation (1:10 split) or for setting up experiments.
Figure 2.1: Light microscopy images of a) LNCaP cells and b) U937 cells, in culture. Bright field images taken using EVOS microscope. Magnification bar= 1000μm.

2.1.2 Production of simple non-spheroid (U937) and spheroid (LNCaP) 3D constructs

The Raft 3D culture systems protocol (Lonza, Slough, UK) was followed, to manufacture the 3D in vitro compressed non-spheroid and spheroid cellular constructs. The basic formula and procedure for creating 3D constructs (and further on for complex 3D constructs, i.e., tumouroids) consisted of embedding cells in Type 1 collagen matrix and then subjecting them to plastic compression by using fluid absorbers. This resulted in 3D constructs with a reported thickness of c.200μm (collagen density, 9.6%) in 96 well plate wells [203].

The preparation of collagen hydrogels was carried out by creating a mixture containing Rat Tail Collagen Type I (1400μl, First Link UK Ltd. Custom Bio-Reagents, UK) and (170μl) 10x Medium Essential Medium (MEM) (used as colour/ pH indicator) (Gibco by Life Technologies), which was then neutralised using (99μl) of Neutralising solution (which consists of a mixture of 10M NaOH and HEPES buffer solution) (Gibco by Life Technologies).

Both LNCaP prostate cancer cells and U937 monocyte cells (71μl) were seeded separately into each collagen mix (total volume= 1740μl) at concentrations of 50,000 and 25,000 cells respectively. The final mixture of cells was plated in each well of a 96-well plate at
volumes of 240μl per well (TPP, Sigma Aldrich, UK). These constructs were then incubated at 37°C for 15 minutes to set, after which they were subjected to compression using absorbers (Lonza) at room temperature (RT) for 10 minutes (Figure 2.2). The absorbers were then removed and fresh media was added to each construct before they were finally placed in the incubator at 5% CO₂/air, 37°C. Media was changed daily and constructs were used as described further in this chapter. Cancer cells in 3D constructs were termed cancer masses (CMs) with cancer cells developing into spheroids over time, reflecting the nature of epithelial cancer, while monocyte cells did not, hence the spheroid/non-spheroid description respectively. For monocytes specifically, a number of uncompressed 3D constructs were also manufactured to provide controls (described further below).

Figure 2.2: **Diagram showing the construction of 3D in vitro cancer mass (CM).** The materials used to create a CM include: 10xMEM, collagen type 1 (of rat tail origin), neutralising solution (made from 10M of sodium hydroxide and HEPES buffer solution), along with the desired quantity of cells (in this example 50,000 LNCAP prostate cancer cells).

### 2.1.3 Prostate cancer complex tumouroid fabrication

This tumouroid model is a two-compartment 3D construct comprising of a central compressed cancer construct/ mass, ‘sandwiched’ within two layers of a collagen mix. The latter compartment, made in 24 well plates, surrounded the cancer mass which was
originally made in 96 well plates, and represents the cancer stroma. The stroma could be acellular or populated by non-cancer cells. The final 2-compartment construct was also compressed to result in a “stiff” tumouroid. The “stiffness” of tumouroids was measured at 5KPa [198].

CMs were manufactured in 96 well plates, according to the Lonza protocol as described above. The CMs were seeded with 50,000 LNCaP cells/construct. Their incorporation into the stromal compartment is described in 2.1.4 and 2.1.5 below and schematically represented in Figure 2.3.

**Figure 2.3:** Diagram showing the construction of a complex tumouroid model. The CM containing 50,000 LNCaP prostate cancer cells (made in 96 well plates, see Figure 13) is transferred into a larger compartment which mimics the cancer stroma (made in 24 well plates). In this example the stromal compartment contains 25,000 U937 monocyte cells. Subsequently, absorbers are added to the whole construct to form a complex tumouroid which is “stiff”.

### 2.1.4 Tumouroids with acellular stroma

In order to create the acellular stromal compartment, half of the total volume per well (650μl) of the stromal collagen mix (see 2.1.3) was inserted into each well of a 24 well plate (Corning) and allowed to set slightly in the incubator at 37°C for 2 minutes. The 3D cancer masses (see 2.1.3) were then removed from the 96 well plate and individually placed on the surface of the incompletely set stroma hydrogel in the 24 well plate, and
then topped-up with an additional 650μl of the stromal collagen mix (totalling 1300μl per well). This final complex construct was incubated at 37°C, 5% CO2/air for 15 minutes to set. Subsequently, the now well-set tumouroid hydrogels were compressed using absorbers (Lonza) for 10 minutes at RT. Upon removal of the absorbers, 1000μl of media (RPMI plus supplements) was added per well and plates were placed back in the incubator.

2.1.5 Tumouroids with cellular stroma

Tumouroids with cell populated stromal compartments were constructed using the same technique as described in 2.1.4 above. Here, 25,000 U937 cells were added to the stromal collagen mixture. The CMs, populated with 50,000 LNCaP cells, were set, compressed and transferred to wells of a 24 well plate containing slightly set 650μl of (cellular) stroma hydrogels containing U937 cells. The remaining cellular stroma 650μl, was added to each well, totalling 1300μl per well, and incubated at 37°C for 15 minutes. Subsequently, the now well-set tumouroid hydrogels were compressed with the use of absorbers for 10 minutes at RT. Upon removal of the absorbers, 1000μl of media (RPMI) was added per well and plates were returned to the incubator.

2.1.6 Use of PMA to differentiate U937 monocyte cells into macrophages

Phorbol 12-Myristate 13-Acetate (PMA) 100ng/ml (Fisher Scientific) was added to U937 cells in a T-75 flask (Corning) and incubated for 48 hours to induce differentiation, as quoted by previous researchers [204-206] (Figure 2.4). Subsequently, the U937 macrophages were scraped off the flask with specialised cell scrapers (Fisher Scientific), and the cells were counted and split (1:10 split) for either propagation or for experiments. To study penetration of macrophages into cancer in 3D, 25,000 of the now differentiated U937 cells were added to the surface of CMs containing 50,000 LNCaP cancer cells.
Microscopic images of; a) and b) macrophage formation post 48 hr incubation of U937 cells with PMA. The U937 macrophages are adherent to the flask, more rounded morphologically than monocytes, appear to have granular cytoplasm, and have a tendency for clumping. Magnification bar=200μm.

2.1.7 Use of red cell tracker CM-Dil in U937 cells

Red cell tracker CM-Dil (Molecular weight ~1,000 g/mol, Ex/Em 553/570nm; Thermo Fisher Scientific Inc., Waltham, MA, USA), 1mg powder, was mixed with 1000μl of anhydrous DMSO to make a stock solution of 1mg/ml. Working concentrations of 1μM (1μg/ml) and 2μM (2μg/ml) were produced using serial dilutions in Dulbecco’s PBS (DPBS, Sigma-Aldrich).

To determine optimal conditions, red cell tracker CM-Dil at 1μg/ml and 2μg/ml was applied to 200,000 U937 cells in fluorodishes with a central well (World Precision Instruments, USA). The samples were then fixed and imaged at 3 timepoints: days 1, 3 and 7, using the Olympus BX63 microscope (Olympus Corporation, Japan). The doses were equivalent in outcome and 1μg/ml was taken forward, for a much shorter time of incubation.

For further experiments, the cell-tracker, at 1μg/ml, and cells were incubated for 5 minutes at 37°C and for 15 minutes in the fridge immediately after. The cell suspension was centrifuged for 5 minutes at 400g, and the cellular pellet was washed with PBS twice and centrifuged. The final cell pellet with tracked U937 cells was resuspended in RPMI.
The simple process and the Ex/em spectra is shown in Figure 2.5. Cells were then used for co-cultures, either in tumouroids or added to CMs to determine penetration in 3D.

![Figure 2.5: Application of CM-Dil (red cell tracker) to U937 cells and spectrometry figure of the excitation/emission spectra (553/570nm).]

2.1.8 Use of cell trackers for LNCaP cells

Both red and green cell dyes were used to track LNCaP cancer cells, for different experiments.

2μg/ml of red cell tracker CM-Dil were added to LNCaP cells in T-75 flasks and placed in an incubator at 37°C for 5 minutes and then moved to the fridge for 15 minutes. Cells were disaggregated and used to create 3D CMs or tumouroids.

Green cell tracker CMFDA (Molecular weight 464.8 g/mol, Ex/Em 492/517nm) Thermo Fisher Scientific Inc., Waltham, MA, USA), 1mg powder, was mixed with (215μl) of anhydrous DMSO to make up a stock of 10 mM (~4.65 mg/ml). Then, a volume of 50μl of this stock was added to 20ml serum-free medium, and this was the 25μM working solution. This working solution was added 50-60% confluent LNCaP cells in a T-75 flask,
incubated for 45 minutes and subsequently removed (Figure 2.6). RPMI was added to the flask. Cells were trypsinised and used for creating 3D CMs or tumouroids.

![CMFDA (Green Cell Tracker)](image)

![T-75 flask with LNCaP cells](image)

![45 minutes incubation](image)

**Figure 2.6**: CMFDA (green cell tracker) is added to LNCaP cells and incubated for 45 minutes. The spectrometry figure shows the spectra (Ex/Em 492/517 nm) which should not interfere with CM-Dil (red cell tracker) used for U937 cells, to enable distinguishing both cell types.

For a specific set of experiments, CMs made with CMFDA tracked LNCaP cells and supernatants were collected at days 1,3,7,10,14 and stored at -20°C.

### 2.1.9 Use of cell trackers for each cell type in 3D co-cultures

3D CMs were constructed using 50,000 CMFDA-tagged LNCaP prostate cancer cells (see 2.1.2 and 2.1.8). 25,000 CM-Dil-U937 monocytes in 200µl were added on the surface of the CMs at days 1,3,7 and 10 post manufacture; and then at days 7,10,14 and 21 post manufacture. This ensured testing of CMs at different times of maturity, to capture both monodispered cancer cells and cancer spheroids. Subsequently, the CMs were incubated for 24 hours and fixed with Formalin (10%) (CellPath) solution. CMs were used for both imaging and collection of supernatants which were stored at -20°C. Controls were CMs without monocytes, at days 1,3,7, 10, 14 and 21.
2.1.10 Hypoxic reagent Image-iT™ Red in 3D PCA tumouroid models (LNCaP/PC3)

In order to analyse the level of hypoxia in prostate cancer spheroids, two experiments were conducted in parallel with the following time points days; 1, 3, 7, 10 and 14 in both normoxic and hypoxic conditions. The hypoxic reagent Image-iT™ Red (Thermofisher, UK) added to the live LNCaP/PC3 cells (50,000 cells/well) at the tumoroid construction phase as per the protocol at a dose of 5µM per tumouroid. The hypoxic reagent was added to the tumouroid samples in both experiments that included hypoxic and normoxic samples. In order to maintain the same conditions for media changing, a T-75 flask with RPMI was inserted into the hypoxia incubator with the cap slightly loosened. The media in the wells was changed using this. Cell culture under hypoxia was done in a humidified and temperature-controlled environment at 1% O₂ in a dedicated hypoxic incubator.

2.1.11 Addition of 25,000 Green Cell tracked U937 cells to Hypoxic 50,000 cell LNCaP and PC3 tumoroids

U937 cells were removed from T-75 flask and placed into a universal tube. This was then centrifuged (400g, 5 minutes) in order to attain a pellet. At this stage the media from the suspension was removed and the pellet was resuspended with green cell tracker CMFDA. The universal tube was then placed in the incubator for 45 minutes with cap slightly loose to enable gas exchange. Subsequently, the mixture was centrifuged (400g, 5 minutes) and the pellet was resuspended with RPMI as per protocol. This mixture was further centrifuged (400g, 5 minutes) to enable cell counting of the now CMFDA tracked U937 cells. In the hypoxic and normoxic conditions when samples containing 50,000 cell LNCaP/PC3 tumoroids reached the designated time point they were incubated with 25,000 green cell tracked CMFDA-U937 cells for 24 hours and then fixed and imaged.
using Olympus microscope. The supernatants were collected and stored at -20°C for subsequent ELISA analysis (see sections 2.2.3-2.2.5).

Figure 2.7: The hypoxic/normoxic experiment carried out with Hypoxic reagent Image-iT™ Red 50,000 LNCaP and PC3 cells and the green cell tracked (CMFDA) 25,000 U937 cells added superficially for 24hrs incubation throughout the time period; day 1, day 3, day 7, day 10 and day 14. The supernatants were collected per time point and analysed.

2.1.12 CM-Dil LNCaP CMs with PMA treated U937 cells stained with anti-CD68-FITC in 3D

50,000 LNCaP CMs were fabricated as previously mentioned (see section 2.1.2) and PMA stimulated U937 cells were added to them at days; 1, 3, 7 and 10. At each time-point the samples were fixed with formalin 10% 100µl/well, permeabilised with 0.3% Triton X and blocked with 1% BSA (PS) for 1 hour at RT and stained with anti-CD68-FITC (Thermo Fisher) 1:100µl (PS). The stained tumouroids were kept overnight in fridge at
4°C for 24 hours. Subsequently these samples were mounted on slides and DAPI (Vectashield, Vector Laboratories, USA) was added to samples. The samples were imaged with Olympus microscope.

2.2 Quantitative end point assays

2.2.1 Alamar blue viability assay

The Alamar Blue reagent (Invitrogen, Thermo Fisher Scientific) was used to determine cell viability in the 3D models. Alamar blue is a reagent that functions through its active component resazurin that changes colour from blue to pink when it is reduced by the metabolic activity of viable cells. Therefore, metabolic activity is taken as equivalent of growth.

The working solution was made by the addition of the dye to fresh culture media to 10% (v/v) dilution. The Alamar blue solution was added to each well, 100μl per well for 96 well plates and a volume of 300μl per well for 24 well plates. The plates were incubated for 4 hours at 37°C, 5% CO₂/air. At this point, volumes of 100μl and 300μl for 96 and 24 well plates respectively of the supernatants were transferred from each well into a corresponding well in a black-well plate (TPP, Sigma Aldrich). Fluorescence intensities were measured at the following wavelengths: Ex/Em 530nm/620nm, on a Fluoroskan ascent FL plate reader (Thermo Labsystems, UK).

2.2.2 Luminex analysis of cytokines from supernatants of compressed vs. non-compressed U937 3D constructs

Supernatants were collected from compressed and non-compressed 3D constructs from time points: 1hr, 3hrs, 6hrs, day 1, day 3 and day 7. Multiple samples for each time point, per each independent experiment, were pooled and sent to Labospace (Milan, Italy) for analysis of specific cytokines using Luminex plates (Biotechne, UK) (Table 10).
Cytokine and chemokine levels were assessed in human supernatant using two Human Discovery Assay (R&D System, Minneapolis, MN, USA) based on the multiplexability of the analytes.

The multiplex panel detect analytes via specifically mixed antibody-coated magnetic beads.

In brief, supernatant samples were diluted at 1:2 in Assay Buffer and incubated together with Microparticle Cocktail for 2 hours at RT, followed by washing the plates 3 times with Wash Buffer. Biotin-Antibody Cocktail was then added to each well, followed by incubation for 1 hour at room temperature, followed by washing as described previously. Finally, Streptavidin-PE was added to each well, followed by incubation for 30 minutes at room temperature. After washing 3 times with Wash Buffer, Sheath Fluid PLUS was added to each well and plates were read on the Luminex® FLEX MAP 3D platform and analyzed Bio Plex 6.2 Software (SN: MAGPX15334703, Version: 4.2). Median fluorescent intensity (MFI) data were obtained for further analysis. Standard curves were used to calculate concentrations of cytokines.

<table>
<thead>
<tr>
<th>Cytokine for Luminex analysis</th>
<th>Rationale for choosing cytokine: (Protumourigenic / Antitumourigenic / Monocyte to macrophage differentiation indicator)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Protumourigenic</td>
<td>[116]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Protumourigenic</td>
<td>[127]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Antitumourigenic and Protumourigenian</td>
<td>[98,112,113,114,115]</td>
</tr>
</tbody>
</table>
Table 10: List of cytokines chosen for analysis with their classifications: protumourigenic and antitumourigenic. TNF-α is a monocyte to macrophage differentiation indicator and is both protumourigenic and antitumourigenic.

The cytokines in the above table were selected from the literature and they were categorised into: protumourigenic, antitumourigenic and monocyte to macrophage differentiation indicator.

The Luminex data provided a baseline and identified a number of cytokines of interest, which were subsequently investigated using ELISAs. Three statistically significant cytokines secreted by monocytes were selected, each representing one of the three categories: VEGF to represent protumourigenic cytokine, IL-10 to represent antitumourigenic cytokine, and TNFα to represent the monocyte to macrophage differentiation indicator. This selection in turn reflected the cytokines secreted by the main subtypes of macrophages which are M1 and M2 in the TME (Table 11).
Macrophage subtype | Cytokine secreted for further ELISA analysis | References
--- | --- | ---
M1 | TNF-α | [207, 208]
M2 | VEGF, IL-10 | [209, 210]

*Table 11: The specific cytokines chosen for ELISA analysis, and the subtypes of macrophages in the TME which were identified as the secreting cells.*

ELISA investigations were undertaken in different types of 3D cultures, either with one cell type or with two cell types. For ease of reference, these are tabulated below and the investigations undertaken are also defined (*Table 12*). Supernatants were stored as aliquots to avoid freeze-thawing damage and allow for several investigations.

<table>
<thead>
<tr>
<th>3D culture type</th>
<th>Cell types</th>
<th>Time for supernatant harvest</th>
<th>Investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D monocyte constructs (compressed)</td>
<td>Unstimulated U937 monocytes</td>
<td>Hours; 1,3,6 Days; 1,3,7</td>
<td>Luminex</td>
</tr>
<tr>
<td>3D monocyte constructs (non-compressed)</td>
<td>Unstimulated U937 monocytes</td>
<td>Hours; 1,3,6 Days; 1,3,7</td>
<td>Luminex</td>
</tr>
<tr>
<td>CM</td>
<td>LNCaP cells only</td>
<td>Days; 1,3,7,10,14</td>
<td>ELISAs</td>
</tr>
<tr>
<td>Tumouroid</td>
<td>LNCaP cells only Unstimulated U937 monocytes</td>
<td>Days; 1,3,7,10,14</td>
<td>ELISAs</td>
</tr>
<tr>
<td>Tumouroid under hypoxia</td>
<td>LNCaP cells and unstimulated U937 monocytes PC3 cells and unstimulated U937 monocytes</td>
<td>Days; 1,3,7,10,14</td>
<td>ELISAs</td>
</tr>
<tr>
<td>Tumouroid</td>
<td>LNCaP and PMA-stimulated U937 monocytes (macrophages)</td>
<td>Days; 1,3,7,10,14</td>
<td>ELISAs</td>
</tr>
</tbody>
</table>

*Table 12: Type of 3D culture used and cell types involved, as well as specific time-points from which supernatants were harvested for Luminex and/or ELISA analysis.*

### 2.2.3 ELISA for VEGF

Reagents were prepared as per protocol in Human VEGF Quantikine™ ELISA kit (R and D systems, USA). Human VEGF standard was reconstituted with Calibrator Diluent.
RD5K and serial dilutions prepared in polypropylene tubes. The concentration of 1000pg/ml served as high standard. The calibrator diluent RD5K served as zero standard (0 pg/ml). 500,000µl of wash buffer was prepared and 400µl per well was used for washing, 3 times. To each well 50µl of Assay Diluent RD1W was added. Then, to each well 200µl of standard, sample or control was added. The detachable wells were covered with adhesive strip and incubated for 2 hours at RT. Following this, wells were aspirated and washed with 400µl of wash buffer per well, 3 times. The plate was inverted and blotted against clean paper towels after each wash.

Following this, 200µl of Human VEGF conjugate was added to each well and the plate was covered with a new adhesive strip and incubated for 2 hours at RT. Aspiration and washing were repeated as previously mentioned. Subsequently, 200µl of substrate solution (prepared from mixture of Colour reagent A and B) was added to each well. The plate was covered with aluminium foil to protect from light. The samples were incubated for 20 minutes, RT. Then 50µl of stop solution was added to each well. When the colour change appeared uniform, the plate was read on a microplate reader (Clariostar, Germany) set to 450 nm – 540nm. The ELISA readings were carried out using the Clariostar plate reader based on absorbance (Arbitrary Units) to establish expression of the cytokines.

It is important to mention that the ELISA readings, even though they were carried out extensively in the above conditions, did not come without limitations. It is difficult to determine whether the differences in measurements are due to phenotypic differences of cells or differences in cell numbers. This will be taken into account in further research.

2.2.4 ELISA for IL-10

Reagents were prepared as per protocol in Human IL-10 Quantikine™ ELISA kit (R and D systems, USA). Human IL-10 standard was reconstituted with distilled water. Serial
dilutions of Calibrator Diluent RD5C were prepared in polyprolene tubes. The concentration of 500pg/ml served as high standard. The calibrator diluent RD5C served as zero standard (0 pg/ml). 500,000µl of wash buffer was prepared and 400µl per well was used for washing, 3 times.

To each well 200µl of standard, sample or control was added. The detachable wells were covered with adhesive strip and incubated for 2 hours at RT. Following this, wells were aspirated and washed with 400µl of wash buffer per well, 3 times. The plate was inverted and blotted against clean paper towels after each wash.

Following this, 200µl of Human IL-10 conjugate was added to each well and the plate was covered with a new adhesive strip and incubated for 1 hour at RT. Aspiration and washing were repeated as previously mentioned. Substrate solution, 200µl (prepared from mixture of Colour reagent A and B) was added to each well.

The plate was covered with aluminium foil to protect from light. The samples were incubated for 20 minutes, RT. Then 50µl of stop solution was added to each well. When the colour change appeared uniform, the plate was read on a microplate reader (Clariostar, Germany) set to 450 nm – 540nm.

2.2.5 ELISA for TNF-α

Reagents were prepared as per protocol in Human TNF-α Quantikine™ ELISA kit (R and D systems, USA). Human TNF-α standard was reconstituted with distilled water. Serial dilutions of Calibrator Diluent RD6-12 were prepared in polypropylene tubes. The concentration of 1000pg/ml served as high standard. The calibrator diluent RD5C served as zero standard (0 pg/ml). 500,000µl of wash buffer was prepared and 400µl per well was used for washing, 3 times.
To each well 50µl of Assay Diluent RD1F was added. Then, to each well 50µl of standard, sample or control was added. The detachable wells were covered with adhesive strip and incubated for 2 hours at RT.

Following this, wells were aspirated and washed with 400µl of wash buffer per well, 3 times. The plate was inverted and blotted against clean paper towels after each wash. Following this, 200µl of Human TNF-α conjugate was added to each well and the plate was covered with a new adhesive strip and incubated for 2 hours at RT. Aspiration and washing were repeated as previously mentioned. Substrate solution, 200µl (prepared from mixture of Colour reagent A and B) was added to each well. The plate was covered with aluminium foil to protect from light. The samples were incubated for 30 minutes, RT. Then 50µl of stop solution was added to each well. When the colour change appeared uniform, the plate was read on a microplate reader (Clariostar, Germany) set to 450 nm – 540nm.

2.2.6 Harvesting of supernatants of LNCaP CMs co-cultured with PMA-stimulated U937 cells

The supernatants of the LNCaP CMs with co-cultured PMA stimulated U937 cells were harvested for subsequent cytokine analysis. The cytokine analysis was performed using the same protocols for; VEGF, IL-10 and TNF-α, as previously mentioned (sections 2.2.3-2.25).

2.3 Quantitative and semi-quantitative microscopy assays

This section of the methods chapter, outlines how microscopic imaging was used in order to gather quantitative/semi-quantitative readings. A variety of fluorescent molecules were used, as appropriate, and these are described in sections 2.1 and 2.4.
2.3.1 Thickness of compressed vs non-compressed 3D collagen constructs

Acellular collagen gels were prepared, either compressed or non-compressed. In order to measure the thickness of the samples, the compressed and non-compressed gels were placed on slides and a filter paper was cut and added to the top and bottom of the compressed and non-compressed gels (Figure 2.8). A fluorescent dye (Fluorescein) was incorporated into the filter paper and the fluorescence image of edge plane of the paper used as a guide to visualise the top of the gel and set the starting point. Using the Olympus BX63 microscope the visual field focus was adjusted downwards until the bottom of the gel was found and the difference measured. The thickness of the gel obtained for microscopic studies was approximately 200µm as established by previous studies obtained in this lab [202]. This was the cut-off measurement for both the compressed and non-compressed gels. Then casting of the collagen gels was obtained by using the wells of a tissue culture perkinlemer™ 96-well plate (dimension of each well is width 85.47mm, diameter 7.15mm, well depth 10.80mm). These approximate dimensions were assumed to be constant throughout the manufacture of the collagen gels.

Figure 2.8: Images of non-compressed (NC) and compressed (C) acellular collagen gels on slides. There was 240µl of collagen in the compressed gels and 120µl of collagen in non-compressed gels. The non-compressed (NC) and compressed (C) gels are on the left and right respectively in the slides in images a), b) and c). Filter paper pieces with fluorescent edges were placed on top and on the bottom of each gel in order to mark the top and bottom edges of the gels to serve as landmarks (boundary planes).
2.3.2 Depth of penetration of red cell tracked CM-Dil U937 in 3D cellular constructs (compressed vs. non-compressed)

96 well plates were used to place 6 acellular collagen constructs in each plate representing a specific time point. These constructs were arranged as 3 compressed and 3 non-compressed. The compressed samples had 240µl of collagen solution and non-compressed samples had 120µl. These samples had 25,000 cell-tracked with CM-Dil U937 cells added to them superficially and were designated time points; 1 hour, 3 hours, 6 hours, day 1, day 3 and day 7. These samples were subsequently fixed and mounted on slides with addition of DAPI after each time point for microscopic examination using the Olympus microscope.

In order to calculate the depth of penetration of U937 cells in 3D, Z-stack imaging was carried out from a fixed measurement at the top of the gel (18,932.7µm) and this was carried out deeper in the gel until we could see the best image of clear red cell tracked CM-Dil U937 cells. This was considered as the bottom of the gel, having moved within the dedicated 200µm of height that we have set as a standard for all gels. This was considered the variable value which changed according to the penetration distance of the CM-Dil U937 cells. Subsequently the difference between the variable value and the top of gel value was calculated and plotted for graphical analysis.

2.3.3 Measurements of cancer spheroid size and invasion

Cancer spheroids that were observed microscopically in 3D collagen constructs were counted in quadrant form as per the method adopted by previous researchers [211]. These quadrants divided the circular collagen construct into four distinct regions. These regions were allocated names; 3 o’clock, 6 o’clock, 9 o’clock and 12 o’clock. A total number of approximately 30 spheroids per construct were calculated from images captured by Olympus BX63 microscope. The size of each spheroid (area documented in µm²) was
calculated using open source Image J software (NIH, US), *Figure 2.9*. The same software was used to calculate the distance of invasion of spheroids into the stroma and recorded in µm.

*Figure 2.9: Process by which spheroids are identified and counted.* Schematic a) illustrates how a construct is divided in quadrant form in order to count the number of spheroids in each quadrant. The spheroid in the quadrant of interest is identified as per a) at the 3 o’clock region. Image b) is a spheroid in a day 14 CM of green cell tracked (CMFDA) LNCaP cells where red cell tracker (CM-Dil) U937 cells were added, DAPI was used as counterstain. Images were captured using Olympus BX63 microscope. Magnification bar=50µm.

### 2.3.4 Measurements of fluorescence intensity

Image J software was also used to analyse images of 2D samples and 3D constructs containing 50,000 LNCaP cells and 25,000 U937 cells. These samples were stained with anti-CD68-FITC, which should identify U937 cells, but not prostate cancer cells (please see section 2.4.8 for methodological development). Also, samples with CM-Dil U937 cells in compressed and non-compressed gels were analysed.

Fluorescence intensity was carried out measuring the mean fluorescence intensity of cells in 4 quadrants; 3 o’clock, 6 o’clock, 9 o’clock and 12 o’clock of each sample. The University of Chicago protocol was used to guide the accurate measurement of fluorescence, i.e., as pixel intensity. (University of Chicago. Basic Intensity...
2.4 Methodological development for imaging distinct cell populations in 2D and 3D

A number of staining agents and protocols were used to visualise cancer cells and monocytes/macrophages to determine biological behaviour in 2D and 3D. There were a number of challenges, especially when using two imaging agents/stains together to distinguish specific cell types in co-cultures. A number of agents/stains were either not taken up by the desired cell type, or they were taken up by both cell types. Methods and agents used for distinct experiments with specific outcomes are described below. A table with all staining agents used for the thesis (described in 2.1 and 2.4) and whether or not they were successfully used in the end is included at the end of the section for ease of reference.

2.4.1 Imaging of LNCaP prostate cancer spheroids in 3D cancer masses and tumouroids, using Phalloidin and DAPI

This protocol was used to visualise cancer cells and determine growth and/or depth of invasion into the stroma of cancer spheroids, in 3D monocultures. All chemicals used were purchased from Sigma-Aldrich, unless otherwise specified. LNCaP prostate cancer cells within either CMs or complex tumouroids developed into spheroids over time. Their morphology and spheroid formation were observed by fluorescent microscopy at different time points (days 1, 3, 7, 10 and 14) after the initial seeding, using Alexa Fluor 488 labelled Phalloidin (Molecular Probes, Life Technologies) and DAPI (Vectashield, Vector Laboratories, USA).
Phalloidin is used to visualise the filamentous actin in the cytoplasm of cells and 4’,6-diamidino-2-phenylindole DAPI which strongly binds to specific regions (Adenine-Thymine) in DNA allows the clear observation of cell nuclei.

The constructs (both 3D CMs and tumouroids) were fixed by the addition of Formalin (10%) for 30 minutes, followed by 3 PBS washes and permeabilised with a mixture of 1% BSA and 0.3% Triton X solution in PBS, followed by a further 3 PBS washes. After permeabilization, the constructs were stained with Phalloidin (1μl:200μl BSA/Triton-X solution for CMs, 1.5μl:300μl BSA /Triton-X solution for tumoroids) and left to incubate at RT for 90 minutes before washing with PBS, 3 times. At this stage the constructs were further stained with DAPI, 1.5μl/ml, for 5 minutes.

Imaging was carried out using the EVOS fluorescence inverted microscope (EVOS FL color, Life Technologies, Thermo Fisher Scientific) and Olympus BX63 microscope, with settings for Phalloidin (Ex/Em 495/518 nm) and DAPI (Ex/Em 340/488 nm). Analysis was carried out using ImageJ software, as appropriate.

2.4.2 Staining of LNCaP cancer cells with Quantum Dots

This protocol was used to visualise specifically LNCaP prostate cancer cells, by incubating them with fluorescent quantum dots (QDs). Ultimately, uptake was unsuccessful and the QDs were observed to be toxic to the LNCaP cells and this experimental avenue was not pursued further (Figure 2.10).

Fluorescent cadmium telluride (CdTe) QDs were kindly provided by Dr Bala Ramesh (UCL Division of Surgery and Interventional Science) and were synthesized by a one-pot procedure adopted in our laboratory. All the starting materials were obtained from commercial suppliers and were used without further purification. All reactions were carried out in a buffer solution composed of 15mM Na2B4O7 and 15mM citrate acid, pH adjusted to 7.0 with 1M HCL. The precursor material included solutions of CdCl2 (2mM),
Na₂TeO₃ (0.5mM) and MSA (4mM) in 50,000μl of the above buffer solution in a one-neck flask immersed in ice. The mixture was subjected to vigorous mixing using a magnetic stirrer for 5 minutes. At this time, solid NaBH₄ (15mg) was added rapidly and mixed for a further 5 minutes. Finally, the flask was connected to a condenser and refluxed with mixing at 100°C under aerobic conditions for 12 hours. Fluorescent CdTe/MSA QD solution was diluted with equal volume of cold 70% ethanol and centrifuged at 1500g for 20 minutes. The precipitated CdTe/MSA QDs were vacuum dried to obtain as a powder (approximately 1 mg) and re-suspended in 1ml phosphate buffer. This solution was then added to a T-75 flask with LNCaP cells. The flask was subsequently imaged post 48 hours with the EVOS microscope.

Figure 2.10: a i) T-75 flask of LNCaP cells incubated with 50μl of telluride quantum dots with calreticulin post 48 hours, a ii) FITC image, a iii) bright field image, b i) bright field image of flask, b ii) FITC image of another region of flask, b iii) bright field image. This technique for distinguishing LNCaP cells from U937 cells was not carried through as cells were not growing and the morphology of cells was altered, also there was a high amount of cellular debris. Imaging was carried out using EVOS microscope. Magnification scale= 200μm.
2.4.3 Manufacture of gold nanoclusters conjugated to anti-CD68 antibody

This series of experiments aimed to manufacture visualisable nanoclusters (NCs) which were targeted to U937 monocytes and test them in various cultures (see below). The target chosen was CD68, macrosialin, a glycoprotein expressed in lysosomes present inside monocytes.

Fluorescent gold (Au)/mercaptosuccinic acid (MSA) nanocluster (NC) solution (1000 µl) was kindly provided by Dr Bala Ramesh (UCL Division of Surgery and Interventional Science) [212]. This was diluted with equal volumes of cold ethanol and centrifuged at 4915g for 30 minutes.

The precipitated Au/MSA NCs were vacuum dried to obtain a powder. The dried NCs (approximately, 1mg) were re-suspended in 1000 µl PBS. The NC (1000 µl) solution was conjugated to anti-CD68 antibody (ABCAM, Cambridge, UK) using 1-Ethyl-3-(3-dimethylaminopropyl carbodiimide (EDC) as an acylating agent together with N-hydroxysuccinimide (NHS).

Briefly, 200 µl Au/MSA NC solution (1mg/ml) was mixed with 200 µl EDC (1 mg/ml) and 200 µl NHS (1mg/ml) in PBS for 30 minutes at RT. Then, 100 µl of Anti-CD68 antibody solution (5mg/ml) was added and mixed for 2 hours at RT. To separate the reagent and unconjugated Au/MSA NCs, membrane centrifugal columns (Centricon) were used with a cut off of 100 kDa with UV monitoring at 280 nm of the retained samples. The purified product, conjugated to anti-CD68 antibody and defined as Au/MSA/CD68 NCs, was collected and stored at 4 °C until further use.

2.4.4 Staining of 3D U937 constructs and complex tumouroids with anti-CD68 conjugated gold nanoclusters

Targeted NCs were applied in various cultures of monocytes and cancer cells. These did not yield positive outcomes and this avenue was not pursued further (Figure 2.11).
3D constructs containing 25,000 U937 monocyte cells were constructed as described above (section 2.1). The constructs were fixed and permeabilized as previously described (section 2.4.1) and washed 3 times with 100μl PBS. The constructs were incubated with anti-CD68 conjugated NCs for 1 hour at a dilution factor of 1μl in 1000μl BSA/triton X, washed with PBS, and imaged with both Olympus BX63 microscope and Leica confocal microscope (Leica Microsystems Gmbh, Germany).

Complex tumouroids comprising LNCaP prostate CMs embedded in U937 monocyte-populated stromal compartments, were manufactured as previously described (section 2.1.3). Constructs were fixed and permeabilised (section 2.4.1) at specific time-points; day 1, day 3 and day 7. These were stained with Phalloidin, anti-CD-68 conjugated gold NCs and DAPI. The protocol was as that described in 2.4.1, with a 90 minute incubation with Phalloidin, PBS washes, followed by incubation with anti-CD68 conjugated NCs at a dilution factor of 1μL:1000μL BSA /Triton-X solution, and then DAPI (see 2.4.1). Imaging was carried out with both Olympus BX63 microscope and Leica confocal microscope (Leica Microsystems Gmbh, Germany).
Figure 2.11: Composite image of a complex tumouroid model with 50,000 LNCaP cells in CM and 25,000 U937 cells with anti-CD68 conjugated gold nanoclusters in the extracellular matrix. Rows a), b) and c) represent day 1, 3 and 7 respectively. Columns; i, ii and iii illustrate 50,000 LNCaP cells stained with phalloidin (green), illustrates U937 cells with anti-CD68 conjugated gold nanoclusters (red) and merged images with DAPI (blue) respectively. Anti-CD68 conjugated gold nanoclusters stained non-specifically both cell lines and displayed high background fluorescence. Images were obtained using Leica confocal microscope (Leica Microsystems Gmbh, Germany). Magnification scale = 100μm.

2.4.5 Staining of U937 cells and LNCaP cancer cells in 2D and 3D cultures, with anti-CD68-FITC

As fluorescent gold nanoclusters conjugated to anti-CD68 did not yield desired results, a new approach was adopted. An anti-CD68 antibody conjugated to FITC (Fluorescein
Isothiocyanate) was purchased (Thermo Fisher) and optimised in different cultures of U937 monocytes and LNCaP prostate cancer cells.

(i) 2D cultures

LNCaP and U937 cells were plated in clear 96-well plates, at 50,000 cells per well for LNCaP and 25,000 cells U937 respectively, left to grow, and fixed at timepoints of day 1, 3 and 7. Cells were permealised with 100μl Permeabilising solution ((PS) 1% BSA and 0.3% Triton X solution) for 60 minutes RT and incubated with anti-CD68 antibody conjugated to FITC at 1μl of CD68FITC:100μl Permeabilising solution ((PS) 1% BSA and 0.3% Triton X solution) for 24 hours in fridge 4°C overnight. Imaging was then carried out with EVOS microscope.

(ii) 3D cultures

50,000 LNCaP and 25,000 U937 cells were put into wells in a 96 clear well plate after having split them and counted the exact number of cells respectively. The Lonza protocol, was referred to when 3D constructs were made. Fixation of wells was carried out per timepoints of days 1, 3 and 7. When each time point is reached, permeabilization of the sample occurs using the PS for 90 minutes RT. Subsequently, anti-CD68 FITC applied to both cell types respectively at (1μl of CD68FITC:100μl Permeabilising solution PS 1% BSA and 0.3% Triton X solution) for 24 hours in fridge 4°C overnight. Imaging was then carried out with EVOS microscope.

2.4.6 Troubleshooting in the imaging of anti-CD68-FITC stained cultures

The conditions in the above protocols (2.4.5) resulted in sub-optimal staining/imaging. Therefore a number of steps were taken to improve imaging (Table 13).

<table>
<thead>
<tr>
<th>Problems with 2D + 3D weak / no staining – possible defective steps</th>
<th>Proposed Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incorrect filter or light source</td>
<td>Made sure correct filter, x10 or x20 selected and matches objective lens.</td>
</tr>
<tr>
<td>Issue</td>
<td>Solution</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Light source (GFP) was chosen on EVOS microscope</td>
<td></td>
</tr>
<tr>
<td>Exposure/ Gain too low</td>
<td>Confirmed that exposure time was optimal in order to try to delineate cells and was same for all images (100ms)</td>
</tr>
<tr>
<td>Cells over fixed</td>
<td>Fixation was carried out using formalin 10% for 20 minutes for 2D and 30 minutes for 3D and no longer</td>
</tr>
<tr>
<td>Cells not permeabilised</td>
<td>All samples 2D and 3D were permeabilised with 1% BSA and 0.3% Triton-X</td>
</tr>
<tr>
<td>Not enough primary antibody</td>
<td>Used recommended anti-CD68-FITC concentration as per Thermofisher website</td>
</tr>
<tr>
<td>Cell samples dried out</td>
<td>All 2D and 3D samples were preserved with PBS in fridge either awaiting incubation or being incubated with antibody</td>
</tr>
<tr>
<td>Fluorescent antibody bleached and photo-deteriorated</td>
<td>I put the antibody on a slide and checked the fluorescence emission and it had not shown any photo-deterioration</td>
</tr>
<tr>
<td>Incubation for a short period</td>
<td>Samples were left in fridge 4°C overnight as per thermofisher website</td>
</tr>
<tr>
<td>Sample storage</td>
<td>All 2D and 3D samples were imaged and immediately stored at 4°C and in the dark</td>
</tr>
<tr>
<td>Insufficient washing</td>
<td>All samples were washed after application of antibody for 3x with PBS (2D- 5 minutes each time and 3D- 10 minutes each time)</td>
</tr>
<tr>
<td>Sample not blocked</td>
<td>All samples were blocked with 1%BSA</td>
</tr>
<tr>
<td>Over permeabilisation of sample which may be leading to destruction of cell membrane and affect CD68 receptors on cell membrane</td>
<td>Used BSA without Triton-X in order to preserve CD68 in cell membrane</td>
</tr>
<tr>
<td>Antibody not effective</td>
<td>Tried to internalise U937 cells with gold nanoparticles to overcome immunofluorescence issue with antibody</td>
</tr>
<tr>
<td>Cells not expressing enough of receptor (CD68)</td>
<td>Increase receptor expression (upregulation) by applying PMA</td>
</tr>
</tbody>
</table>

*Table 13: The difficulties encountered with anti-CD68 FITC staining and the troubleshooting methods I applied to try resolving them.*
2.4.7 PMA treated U937 cells stained with anti-CD68-FITC and DAPI in 2D

U937 cells were placed in the fluorodish (World Precision Instruments, USA) at a cell density of 200,000 cells. The cells were then treated with 25ng/ml of PMA (Fisher Scientific) for 24 hours [213]. The media was replaced with fresh RPMI and the cells were left to incubate for a further 24 hours. The cells were then fixed, permeabilised, washed and imaged using Olympus microscope. To prevent cell loss and due to the fact that U937 cells are suspension cells, we adapted a method of incubating cells in a universal tube. Cells were centrifuged (400g for 5 minutes) in order to get a pellet which was resuspended in 25ng/ml PMA and were left to incubate for 24hrs. Subsequently, the mixture of cells and PMA was centrifuged (400g for 5 minutes) and the supernatant was removed. The cells were suspended with fresh RPMI and left for an additional 24 hrs. The cells were designated 3 time points; days 1,3 and 7. Then the cells were subjected to centrifuging (400g for 5 minutes), washing, fixing, staining (with anti-CD68 FITC) and incubated for 24 hrs in fridge 4°C overnight at these specific time points. The stained cells, at each specific time point, were then removed and plated in a fluorodish plate and DAPI was added. The plated cells were imaged using Olympus microscope.

LNCaP cells in 2D were stained with anti-CD68-FITC as negative controls. LNCaP cells were plated at a density of 200,000 cells in fluorodishes. The cells were then fixed, permeabilised and stained with anti-CD68-FITC at specific time-points; day1,3,7. The plated cells were imaged under the Olympus microscope. The experimental outcomes demonstrated staining of U937 and LNCaP cells, which was not desired (Figures 2.12 and 2.13).
Figure 2.12: **200,000 LNCaP cells in fluorodishes in 2D**, at day 1, 3 and 7 which are represented in images i), ii) and iii) respectively. Images a) show LNCaP cells with anti-CD68-FITC (green), b) show LNCaP cells with anti-CD68-FITC and DAPI (blue) merged images. The images show that LNCaP cells have a positive signal when incubated with 1:100 anti-CD68-FITC using Olympus microscope. Magnification scale = 100μm.

Figure 2.13: **200,000 U937 cells in fluorodishes in 2D**, at day 1, 3 and 7 which are represented in images i), ii) and iii) respectively. Images a) show U937 cells cells with anti-CD68-FITC (green), b) show U937 cells with anti-CD68-FITC and DAPI (blue) merged images. The images show that U937 cells have a positive signal when incubated with 1:100 anti-CD68-FITC using Olympus microscope. Magnification scale = 100μm.
2.4.8 Staining used for imaging

In the course of this PhD multiple stains were used to aid in the optimisation of the imaging of cells in the tumouroid model. A summary of the stains used is in Table 14 below.

<table>
<thead>
<tr>
<th>Stain/agent used</th>
<th>Targeted cells for imaging</th>
<th>Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phalloidin</td>
<td>LNCaP and U937</td>
<td>Yes</td>
</tr>
<tr>
<td>DAPI</td>
<td>LNCaP and U937</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-CD68 FITC</td>
<td>U937</td>
<td>No (LNCaP stained also in co-culture)</td>
</tr>
<tr>
<td>Anti-CD68 conjugated gold nanoclusters</td>
<td>U937</td>
<td>No (LNCaP stained also in co-culture)</td>
</tr>
<tr>
<td>Cadmium telluride (CdTe) quantum dots (QDs)</td>
<td>LNCaP</td>
<td>No (Toxic to LNCaP cells and changes to morphology observed)</td>
</tr>
<tr>
<td>CM-Dil (red cell tracker)</td>
<td>LNCaP and U937</td>
<td>Yes</td>
</tr>
<tr>
<td>CMFDA (green cell tracker)</td>
<td>LNCaP and U937</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypoxic reagent Image-iT™ Red</td>
<td>LNCaP and PC3</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 14: Stains/agents used to delineate specific cell types within 2D and 3D cultures for imaging and analysis.

2.5 Statistical analysis

Cell culture experiments were performed routinely as three independent repeats, with duplicate or triplicate wells for each point. For cytokine analysis, supernatants were pooled from multiples of each experimental timepoint, for each of the independent experiments. The data were confirmed as parametric and statistical analysis was carried out using GraphPad Prism, with one-way ANOVA with Tukey’s posthoc analysis (cell behaviour experiments), and upaired T-Test (cytokine experiments). Significance was taken at p<0.05.
Chapter 3

Results
3.1 Title: Comparison of U937 monocyte distribution and motility in non-compressed versus compressed 3D constructs

3.2 Introduction:
This chapter describes the depth of penetration and cell load of CM-Dil tracked U937 cells in varying biophysical compositions of collagen gels, which include compressed (stiff) and non-compressed (non-stiff) gels. The biophysical structure of collagen constructs has been found to influence cellular motility (see Discussion). The hypothesis regarding cellular motility was that there will be an increase in number of cells and depth of penetration in non-compressed gels.

3.3 Methods:
In order to analyse depth of penetration, U937 monocyte cells were tagged with CM-Dil which is a red fluorescing cell tracker, a Chloromethylbenzamido derivative of a lipophilic carbocyanine (Dil). This marker was used in these and subsequent experiments, using the same technique as previously described (Methods chapter, section 2.1.7). It is noted by the manufacturer to have photostable fluorescence, minimal cytotoxicity and excellent cellular retention and is used in long-term labelling of cells.

CM-Dil tracked U937 monocyte cells were incubated with compressed and non-compressed acellular collagen gels at the following short-term and longer-term time points: 1, 3, and 6 hours, as well as 1, 3 and 7 days. Specifically, U937 cells were added on top of the gels and allowed to infiltrate freely. Analyses used were mainly by microscopy and detection of cytokines (Luminex and ELISAs).

3.4 Results:
It was observed that in non-compressed gels there was not only an increased depth of penetration of U937 cells with time but also an elevated number of U937 cells throughout the construct. The most upregulated cytokine linked to this was VEGF which was
recorded at higher levels in compressed and non-compressed gels compared to the other cytokines analysed namely: IL-10 and TNF-α (sections 3.4.9-3.4.11).

3.4.1 CM-Dil tracked U937 cells began penetrating at 1 hour of incubation and increased in number at day 7 in compressed acellular collagen gels

After having allowed the U937 cells to take up the CM-Dil red cell tracker (see Methods chapter, section 2.1.7), the cells (25,000) were applied to all the compressed acellular gels that had been already prepared. The gels, which now had the CM-Dil tracked U937, were left to incubate and microscopic examination took place at specific time points. From the initial time point of 1 hour there was penetration of the CM-Dil tracked U937 cells in the compressed gels. However, on microscopic imaging of all the gels for all the time points mentioned above, an obvious difference in cell number was seen in the day 7 samples which had a higher number of CM-Dil U937 cells versus the 1hour sample (Figure 3.1).
Figure 3.1: Compressed acellular 3D gel with 25,000 CM-Dil U937 cells added. Fluorescence images a) and c) show CM-Dil tracked (red) U937 cells at 1 hour and 7 days incubation respectively. Images b) and d) show merged images of CM-Dil tracked U937 cells at 1 hour incubation and 7 days incubation respectively, with DAPI (blue). The CM-Dil U937 cells started penetrating the compressed gels at 1 hour incubation. A higher number of CM-Dil U937 cells was observed to have penetrated to the bottom of the gels at day 7 compared to 1 hour incubation. Images were taken under the Olympus BX63 fluorescence microscope, at a set position at the bottom of the gel. Magnification bar=50μm.

3.4.2 CM-Dil tracked U937 cells began penetrating at 1 hour of incubation and increased in number at day 7 in non-compressed acellular collagen gels

The same protocol as in 3.1 was used, where the CM-Dil red cell tracked U937 cells (25,000) were incubated in non-compressed acellular gels that had been prepared at specific time points. From the initial time point of 1 hour there was penetration of the CM-Dil tracked U937 cells in the non-compressed gels. There was also, as in the compressed gels, more cells that penetrated in the day 7 samples than in the 1 hour
samples. Interestingly at both time points there were more cells that have penetrated in the non-compressed gels compared to the compressed gels (Figure 3.2).

![Image](image.png)

Figure 3.2: Non-compressed acellular 3D gels with 25,000 CM-Dil U937 cells added. Fluorescence images a) and c) show CM-Dil tracked (red) U937 cells at 1 hour and 7 days incubation respectively. Images b) and d) show merged images of CM-Dil tracked U937 cells at 1 hour incubation and 7 days incubation respectively with DAPI (blue). The CM-Dil U937 started penetrating the non-compressed gel at 1 hour incubation. A higher number of CM-Dil U937 cells was observed to have penetrated to the bottom of the gel at day 7 compared to 1 hour incubation. In both non-compressed samples at 1 hour and day 7 a higher number of CM-Dil U937 cells penetrated to the bottom of the gel compared to the compressed gels. Images were taken under the Olympus BX63 fluorescence microscope, at a set position at the bottom of the gel. Magnification bar=50μm.

Although images were taken at a number of other time points, presenting images from 1 hour and 7 days demonstrates the largest differences and were therefore chosen to include here.
3.4.3 Z-stack images of compressed vs. non-compressed acellular collagen gels incubated with CM-Dil U937 cells

3.4.4 More CM-Dil tracked U937 cells penetrated at day 7 than at 1 hour in compressed gels, demonstrated by Z-stack imaging

In Z-stacking, the slices of images from the 3D compressed gels which had been incubated with 25,000 CM-Dil tracked U937 cells were analysed microscopically from the top to the bottom of the gels, in order to analyse the depth of penetration from a visual perspective. In the compressed gels, more CM-Dil U937 cells were found at the bottom of the gel than at the top of the gel (Figure 3.3) at day 7 compared to post 1 hour incubation.
Figure 3.3: Z-stack of compressed acellular collagen gels, incubated with 25,000 CM-Dil U937 cells. Z-stack a) and b) represent 1 hour and day 7 incubation respectively. More CM-Dil U937 cells were found at the bottom of the day 7 gel than at the bottom of 1 hour gel. Images were taken on the Olympus BX63 fluorescence microscope.

3.4.5 More CM-Dil tracked U937 cells penetrated at day 7 than at 1 hour in non-compressed gels compared to compressed gels demonstrated by Z-stack imaging

In these Z-stack images, it was apparent by visual observation that more cells travelled to the bottom of the non-compressed gels at day 7 than to the bottom of the gels at 1 hour incubation. Also, in comparison to the same time-points in the compressed gels, there was
a higher number of U937 cells that travelled to the bottom of the gels at the same time points in the non-compressed gels. In other words, a higher number of CM-Dil U937 cells penetrated in non-compressed gels (Figure 3.4).

Figure 3.4: Z-stack of non-compressed acellular collagen gels, incubated with 25,000 CM-Dil U937 cells. Z-stack a) and b) represent 1 hour and day 7 incubation respectively. More CM-Dil U937 cells were found at the bottom of the day 7 gel than at the bottom of 1 hour gel. Images were taken on the Olympus BX63 fluorescence microscope.
3.4.6 Depth of penetration of CM-Dil U937 cells was higher in non-compressed than compressed acellular collagen gels

The depth of penetration of CM-Dil U937 cells necessitated quantification. This was carried out using the technique described in the methodology chapter (section 2.3.2). The same number of CM-Dil U937 cells (25,000) in all the gels was used for consistency. It was observed that overall, the CM-Dil tracked U937 cells travelled deeper in non-compressed gels than in compressed gels. The plotted data (Figure 3.5), show that CM-Dil tracked U937 cells travelled deeper in non-compressed gels specifically at 4 timepoints (1 hour, 6 hours, 24 hours and 72 hours) than the compressed gels. The pattern of the depth of penetration of the CM-Dil U937 cells was not always linear. However, throughout, the penetration measurements in non-compressed gels were always greater than compressed gels, for all time points apart from one where the numbers were similar. At 1 hour there was a greater penetration of U937 cells in non-compressed compared with the compressed gels, 78.4 µm and 65 µm respectively. At 3 hours, there was a very similar depth of penetration of CM-Dil U937 cells in non-compressed gels, 74 µm, compared to compressed gels, 74.2 µm. However, there was a marked increase at 6 hours, 24 hours and 72 hours in the depth of penetration of CM-Dil U937 cells in non-compressed gels compared to compressed gels amounting to: 95 µm, 66 µm and 88 µm compared to 86 µm, 61.6 µm and 81 µm respectively. The compressed gels had higher measurements of depths of penetration of CM-Dil U937 cells compared to non-compressed gels at 168 hours as well, which amounted to 88 µm vs. 65.4 µm respectively.
Figure 3.5: Depth of penetration (µm) of 25,000 CM-Dil U937 cells added superficially to compressed and non-compressed acellular collagen gels, at the following time-points: 1hr, 3hrs, 6hrs, 24hrs (day 1), 72hrs (day 3), 168hrs (day 7) incubation. The black columns represent compressed gels and grey columns represent non-compressed gels respectively. CM-Dil U937 cells had increased penetration in non-compressed gels at: 1, 6, 24 and 72 hours compared to compressed gels. The highest distance of depth of penetration was found in the non-compressed gels at 6 hours and amounted to a mean distance of 95µm. Statistical analysis was carried out using ANOVA one-way test with Tukey’s multiple comparisons analysis, no statistical significance found $p>0.05$ for each condition (mean±SD obtained from triplicates).

The observations above showed that U937 cells were able to travel the depth of any gel construct within an hour of incubation. Taking the shorter timeline of 1, 3, 6 hours, it appears that cells continue to accumulate at the bottom of each gel, although increases are not significantly different.

However, looking at the longer-term incubations, 1, 3, 7 days, the depth of penetration numbers are not different in any significant way from the short term incubations (and there is no significance in these sets either). This suggests that the cells easily move within the constructs. What is interesting however, is that all measurements for non-compressed
gels are always greater compared to compressed gels, suggesting greater ease of movement within the non-compressed gels.

3.4.7 Fluorescence intensity of CM-Dil U937 cells was higher in non-compressed than compressed acellular collagen gels

Fluorescence intensity (CM-Dil U937 cell fluorescence) analysis was carried out as described in the Methods section (section 2.3.4). The fluorescence intensity of the day 7 gels in non-compressed versus compressed gels was far higher than in the 1 hour gels [(56 and 85 AU) and (26 and 43 AU) respectively]. Furthermore, the non-compressed gels had greater fluorescence intensity than the compressed gels at 1 hour (43 vs. 26 AU) and day 7 (85 vs. 56 AU) respectively.

Figure 3.6: The effect of compression of collagen on CM-Dil U937 cells and their fluorescence intensity. Fluorescence intensity was measured (arbitrary units (AU), Y-axis) using Image J of microscopic images of compressed (black columns) and non-compressed (grey columns) gels incubated with 25,000 CM-Dil U937 cells at 1 hour and 168 hours (7 days). The fluorescence intensity was higher in the non-compressed gels with CM-Dil U937 cells at 1 hour and 7 days compared to the compressed gels at the
same time-points. Statistical analysis was carried out using ANOVA one-way test with Tukey’s multiple comparisons analysis. ** p<0.05, *** p<0.005, ****p<0.0001 for each condition (mean±SD obtained from triplicates).

3.4.8 Cytokine analysis of compressed and non-compressed acellular collagen gels with U937 cells

Supernatants from all 3D cultures, i.e., acellular gels which were incubated with U937 cells at different time points, were analysed for cytokine secretion, using a panel of pro and antitumourigenic molecules, by Luminex (see Methods, section 2.2.2). The list of cytokines is shown in Table 15 and the overall results are included in appendix (Figure A1). Of these panels, three molecules appeared to be of interest and were chosen for further analysis using ELISAs. The cytokines chosen were the protumourigenic VEGF, the antitumourigenic IL-10 and the monocyte to macrophage indicator TNF-α. The reason for selecting these specific cytokines was because some laboratories and scientific literatures have worked and discussed these cytokines specifically as they relate to U937 cells and PCA extensively (see Introduction). In addition, the unselected cytokines were in the main protumourigenic except for CXCL16 which is both protumourigenic and antitumourigenic. VEGF (also known as the major angiogenic marker), however, is widely accepted as the most powerful protumourigenic cytokine as well as being extensively studied and researched and thus is a vital cytokine to investigate and study (section 1.9.1). CXCL16 was not chosen as an antitumourigenic cytokine as it is not exclusively secreted by PCA cells but also by non-neoplastic or benign prostate cells (PrEC) and (RWPE-1) respectively [214]. IL-10 was chosen not only because it has been extensively researched in PCA but also because its potency comes from its influence on other cytokines and immune cells (see section 1.8.2). For each designated cytokine, supernatants were analysed from compressed gels and were compared with supernatants
from non-compressed gels for three time points: 1,3 and 7 days respectively following the relevant methodologies (Methods, section 2.2.2) and results for each are shown in the relevant sections below.

<table>
<thead>
<tr>
<th>Cytokines analysed</th>
<th>Cytokines of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-3</td>
<td>VEGF (chosen)</td>
</tr>
<tr>
<td>MMP-7</td>
<td>IL-10 (chosen)</td>
</tr>
<tr>
<td>MMP-8</td>
<td>TNF-α (chosen)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Chitinase 3 like 1 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>MMP-10</td>
<td>MMP-1 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>MMP-12</td>
<td>MMP-2 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>MMP-13</td>
<td>CCL-2 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CXCL16 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>CD-14 (protein)</td>
<td>IL-8 (upregulated) (not chosen)</td>
</tr>
<tr>
<td>CCL-17 (TARC)</td>
<td></td>
</tr>
<tr>
<td>CCL-18 (PARC)</td>
<td></td>
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<tr>
<td>CCL-22</td>
<td></td>
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<tr>
<td>CX3CL1 (Fractalkine)</td>
<td></td>
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<tr>
<td>CXCL1 (KC)</td>
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<tr>
<td>IL-1α</td>
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<td>IL-23</td>
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<td>TRANCE</td>
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</tr>
</tbody>
</table>

Table 15: All cytokines analysed and 3 cytokines were chosen for further ELISA analysis: VEGF, IL-10 and TNF-α.

3.4.9 Increased secretion of protumourigenic cytokine VEGF in compressed acellular collagen gels incubated with U937 cells compared to non-compressed gels

The supernatants from compressed and non-compressed gels which had 25,000 U937 cells added to them (at each specific time point for 24 hours) were isolated and processed as described previously (Methods, section 2.2.2, Luminex). At day 1, the non-compressed gels had a slightly higher secretion of VEGF compared to the compressed gels which was
recorded as 1112.3 vs. 1052 (arbitrary units [AU]) respectively. Interestingly, the VEGF cytokine was greatly elevated in compressed gels compared to non-compressed gels at days 3 and 7 with mean fluorescence intensity levels recorded at 2447 vs. 1284.3 and 20,015.3 vs. 4990 AU respectively (Figure 3.7). The differences in secretion amounted to 1163 AU at day 3, and 15,025.3 AU at day 7, between compressed and non-compressed gels, and both were statistically significant.

Figure 3.7: VEGF production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells added superficially for 24hrs at each time point. VEGF was measured as fluorescence intensity (arbitrary units [AU], Y-axis) using Luminex. At days 3 and 7, there was an increased secretion of VEGF in compressed gels compared to non-compressed gels. Statistical analysis using unpaired T-test was carried out and significant results are shown **p<0.05 for each condition (mean±SD obtained from triplicates).
3.4.10 Increased secretion of antitumourigenic cytokine IL-10 in compressed U937 acellular collagen gels incubated with U937 cells compared to non-compressed gels

The same supernatants generated from 3.4.9 were used to carry out investigations of IL-10, using Luminex. Briefly, compressed and non-compressed acellular collagen gels were incubated with U937 cells at different times points and supernatants were used for Luminex analysis. Unlike VEGF cytokine secretion, there was not a global increase of IL-10 in compressed versus non-compressed U937 incubated gels, at all time points throughout the experiment. There was a decrease in IL-10 secretion at day 1 in compressed compared to non-compressed gels with mean fluorescence intensities amounting to 454 vs 521.3 AU respectively (Figure 3.8). At days 3 and day 7 there was a slight but statistically insignificant rise in IL-10 secretion in compressed gels versus non-compressed gels, and the mean fluorescence intensities amounted to 447.2 vs. 416.3 AU at day 3 and 368 vs. 343.3 AU at day 7 respectively. These differences of 30.9 AU at day 3 and 24.7 AU at day 7 between compressed and non-compressed gels were not statistically significant.
Figure 3.8: **IL-10 production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells added** superficially for 24hrs at each time point. IL-10 was measured as fluorescence intensity (arbitrary units (AU), Y-axis) using. At days 3 and 7 there was a higher secretion of IL-10 in compressed gels compared to non-compressed gels. Statistical analysis using unpaired T-test was carried out but there was no statistical significance, \( p \geq 0.05 \) for each condition (mean±SD obtained from triplicates).

### 3.4.11 Increased secretion of monocyte to macrophage differentiation indicator cytokine- TNF-α in compressed acellular collagen gels incubated with U937 cells compared to non-compressed gels

The supernatants which were harvested from compressed and non-compressed gels incubated with U973 cells were also used to determine secretion of TNF-α (see sections 3.4.9 and 3.4.10). The pattern of TNF-α secretion by U937 cells in compressed gels
tended to be more similar to VEGF, rather than IL-10, with increases at days 3 and 7 of incubation. At day 1 the mean fluorescence intensity level in compressed gels amounted to 364.2 AU which was similar to that found in non-compressed gels at 388 AU. However, at days 3 and 7 the secretion of TNF-α in compressed vs. non-compressed gels was recorded at 388.3 vs. 356 and 436 vs. 386 AU respectively (Figure 3.9). These differences were of 32.3 AU at day 3 and 50 AU at day 7 between compressed and non-compressed gels, with only day 3 reaching a statistically significant difference.

Figure 3.9: TNF-α production in compressed vs. non-compressed acellular collagen gels with 25,000 CM-Dil U937 cells added superficially for 24hrs at each time point. TNF-α was measured as fluorescence intensity (arbitrary units (AU), Y-axis) using Luminex. At days 3 and 7 there was a higher secretion of TNF-α in compressed gels compared to non-compressed gels. Statistical analysis using unpaired T-test was carried out and statistical significance is shown, *p<0.05 (mean±SD obtained from triplicates).
Chapter 4

Results
4.1 Title: Co-culture of U937 monocyte cells within LNCaP tumouroids results in increased depth of penetration of U937 cells and increased growth of LNCaP spheroids

4.2 Introduction:
Following the observation that the depth of penetration of CM-Dil U937 cells may differ in varying biophysical states of collagen gels, compressed vs. non-compressed, experiments were subsequently carried out to examine whether the depth of penetration of U937 cells changed when co-cultured with PCA (LNCaP) cells in 3D. Furthermore, whether spheroid growth as well as cytokinic release would be altered in PCA tumouroids with co-cultured U937 cells was investigated. This was in an effort to determine that, as hypothesised, monocytes could have an antitumourigenic or protumourigenic influence.

4.3 Methods:
Firstly, extensive imaging was carried out of LNCaP simple tumouroids in co-culture with CM-Dil U937 cells. LNCaP cells were tracked using CMFDA (green cell tracker) to aid in microscopic visualisation using the techniques described in the methodology chapter. CMFDA, 5-chloromethylfluorescein diacetate, is a fluorescent dye used for monitoring cell movement or location. After loading into cells, the dye is well retained, allowing for multigenerational tracking of cellular movements. The dye is reported by the manufacturer to be stable, well retained in cells and nontoxic at working concentrations. Subsequently, microscopy was also used to analyse spheroid growth in the immunocompetent tumouroid to observe any changes in growth.

Cytokine analysis using ELISA was undertaken on supernatants from PCA tumouroids and immunocompetent PCA tumouroids for VEGF, IL-10 and TNF-α, the three cytokines of interest identified in Chapter 3.
4.4 Results:

4.4.1 CM-Dil U937 cells incorporated in CMFDA LNCaP tumouroids

On addition of CM-Dil U937 cells to LNCaP simple tumouroids, it was observed that the U937 cells continued to grow and increase in number throughout the specified time points of the experiments which included: 1, 3, 7, 10, 14 and 21 days. Furthermore, there was an incorporation of CM-Dil U937 monocytes into the spheroid structure at days 14 and 21 (Figures 4.2 and 4.3). In addition, CM-Dil U937 cells were seen at all time points, indicating that they continue to penetrate through the gel with progression of time.

Figure 4.1: Images of simple tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 (red) cells at day 1 incubation: i) U937 cells with CM-Dil, ii) LNCaP cells with CMFDA, iii) merged images with both cell types and DAPI (blue). Penetration of CM-Dil U937 through was observed at day 1 and CMFDA LNCaP cells were observed in their single cell state, as this was at day 1. Images were taken on the Olympus BX63 fluorescence microscope. Row a) magnification scale=100µm, row b) magnification scale=50µm.
Figure 4.2: Images of simple tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 cells (red) at day 14 incubation: i) U937 cells with CM-Dil, ii) LNCaP cells with CMFDA, seen as small and large spheroids iii) merged images with both cell types and DAPI (blue). The CM-Dil U937 cells incorporated within the spheroids and throughout the tumouroid structure. Images were taken on the Olympus BX63 fluorescence microscope. Row a) magnification scale=100µm, row b) magnification scale=50µm.

Figure 4.3: Images of tumouroid containing 50,000 CMFDA LNCaP cells (green) and 25,000 CM-Dil U937 cells (red) at day 21 incubation: i) U937 cells with CM-Dil, ii)
LNCaP cells with CMFDA, iii) merged images with both cell types and DAPI (blue). The CM-Dil U937 cells incorporated within the spheroids and throughout the tumouroid structure. Images were taken on the Olympus BX63 fluorescence microscope. Row a) magnification scale=100µm and row b) magnification scale=50µm.

4.4.2 3D images of immunocompetent tumouroids containing CMFDA LNCaP cells and CM-Dil U937 cells

3D images were taken from the top surface of the CMFDA LNCaP tumouroids co-cultured with CM-Dil U937 cells in order to visualise the gels from an additional perspective. Overall, CM-Dil U937 cells were seen at all time points of tumouroid growth (Figures 4.4 -4.6). These images also confirmed the observation stated in the previous section (section 4.4.1), that U937 cells were incorporated into cancer spheroids within the tumouroids as seen in days 14 and 21.

Figure 4.4: 3D image of day 1 tumouroid containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red), counterstained with DAPI (blue) taken from the top surface of the tumouroid. Image was taken on the Olympus BX63 fluorescence microscope. Magnification scale=100µm.
Figure 4.5: 3D image of day 14 tumouroid containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red), counterstained with DAPI (blue) taken from the top surface of the tumouroid. U937 cells are seen incorporated within the spheroid structure. Image was taken on the Olympus BX63 fluorescence microscope. Magnification scale=100µm.

Figure 4.6: 3D image of day 21 tumouroid containing 50,000 LNCaP CMFDA cells (green) and 25,000 U937 CM-Dil cells (red), counterstained with DAPI (blue) taken from the top surface of the tumouroid. U937 cells are seen incorporated within the spheroid structure. Image was taken on the Olympus BX63 fluorescence microscope. Magnification scale=100µm.
4.4.3 U937 CM-Dil cells penetrated CMFDA LNCaP tumouroids, when LNCaP cancer cells were present as single cells or spheroids, days 1-10

Simple tumouroids with 50,000 CMFDA LNCaP cells had 25,000 CM-Dil tracked U937 cells added superficially to them at designated time points, as described in the methodology chapter. The aim was to determine whether the depth of penetration of these cells alters in the presence of PCA cancer. Whether the LNCaP cells were in the single cell phase (i.e., days 1 and 3 of tumouroid manufacture) or spheroid phase (i.e., days 7 and 10 of tumouroid manufacture), the depth of penetration of U937 did not slow down but continued to rise as time increased (Figure 4.7). At day 1, 3 and 7 the U937 cells penetrated the tumouroids to a depth of 93.1µm, 97.6µm and 108µm respectively. This culminated at day 10 with a penetration depth of 112µm. Statistical significance was not present between the depths of penetration at the time points examined, which suggests that there is relatively free movement of monocytes within the 3D volume.
Figure 4.7: The depth of penetration measurements (µm) of 25,000 CM-Dil tracked U937 cells (added for 24 hours at each specific time point) embedded in 50,000 CMFDA tracked LNCaP CMs in the singular cell phase (day 1 and day 3 post tumouroid manufacture) and spheroid phase (day 7 and day 10 post tumouroid manufacture). The depth of penetration increased linearly as days of incubation increased. ANOVA one-way statistical analysis with Tukey’s multiple comparisons analysis was carried out and no statistical significance was found $p > 0.05$ for any condition (mean ± SD obtained from triplicates).

4.4.4 U937 CM-Dil cells penetrated CMFDA LNCaP tumouroids with cancer spheroids, days 7-21

As described in the methodology chapter, U937 CM-Dil cells were added to CMFDA LNCaP tumouroids exclusively at the spheroid formation phase which was determined to be day 7. The experiment was then carried out until day 21 (Figure 4.8). An increase in depth of penetration of U937 cells was noted as the time of incubation increased. There was a marked increase in depth of penetration of U937 cells from day 7 (84 µm) to day 10
(106μm) amounting to a rise of 22μm. However, there was very minor further increases at day 14 (113μm) and day 21 (114μm). Statistical significance was found in penetration depth between day 7 and all the time points in this experiment.

As a general comment, depth of penetration of CM-Dil tracked U937 cells continued to increase in LNCaP simple tumouroids to higher levels than the previous experiments with either non-compressed or compressed acellular gels which had been incubated with CM-Dil U937 cells.

Figure 4.8: The depth of penetration measurements (μm) of 25,000 U937 cells incorporated in 50,000 LNCaP tumouroids at times that cancer cells had formed spheroids in 3D (days 7-21). The depth of penetration of CM-Dil U937 cells continued to increase in the presence of LNCaP cells forming spheroids. The depth of penetration increased linearly as time of incubation increased. ANOVA one-way test with Tukey’s multiple comparisons analysis was carried out. Importantly, there is statistical significance between day 7 and day 10 in this experiment whereas, in the previous
experiment (Figure 40) there was no statistical significance between days 7 and 10, ***$p<0.05$, **** $p<0.0001$ for each condition (mean±SD obtained from triplicates)

4.4.5 LNCaP spheroid growth increased over time in simple tumouroids

LNCaP (50,000) spheroid growth was measured in tumouroids, using phalloidin staining and observed by microscopy as described in the methodology chapter. No U937 cells were added to these 3D cultures. Microscopic images were taken from the tumouroids at the initial stages where only single LNCaP cells were present and observation continued until well formed spheroids were seen at day 7 through to day 14 (Figure 4.9). At day 7, LNCaP spheroids grew to reach a size of 1271\(\mu\text{m}^2\). With increasing time of incubation the spheroid sizes increased linearly, reaching sizes of 3132\(\mu\text{m}^2\) and 4366\(\mu\text{m}^2\) at days 10 and 14 respectively. There was a greater increase in spheroid size between days 7 to 10 which amounted to 1861\(\mu\text{m}^2\) than between days 10 and 14 which amounted to 1234\(\mu\text{m}^2\). Statistical significance was present at different time points of the experiment.
Figure 4.9: Image of tumouroid containing 50,000 LNCaP cells stained with phalloidin (green) and DAPI (blue) a) at day 1 incubation, magnification scale=100µm. b) at day 14, magnification scale=100µm (images were taken on the Olympus BX63 fluorescence microscope) c) spheroid size measured in µm² increased with time, notably starting with the formation of LNCaP spheroids at day 7 and continuing up to day 14. Statistical analysis of graph using ANOVA one-way and Tukey analysis was carried out, statistical significance was found, *p<0.05, ***p<0.005, ****p<0.0001 for each condition (mean±SD obtained from triplicates).
4.4.6 LNCaP spheroid growth increased with time in immunocompetent CMFDA-LNCaP tumouroids co-cultured with unstimulated CM-Dil U937 cells

LNCaP tumouroids containing (50,000) CMFDA tracked LNCaP cells were co-cultured with (25,000) CM-Dil U937 cells over a time period of 21 days (Figure 4.10). There were marked increases in spheroid size measurements at days 7 and 10 which were recorded as 965µm² and 2568µm² respectively. This amounted to an increase in growth by 1603µm². Also, dramatic increases in spheroid sizes were found between days 10 and 14 at 2568µm² and 4609µm², respectively. This was equivalent to an increase in growth by 2041µm². There was another steep increase in spheroid growth between days 14 and 21 with sizes of 4609µm² and 9081µm², respectively. This was equivalent to an increase in growth by 4472µm². Statistical significance was also present between the time points. Generally, spheroid growth increased in the presence of CM-Dil U937 cells compared to control LNCaP tumouroids without U937 cells.
Figure 4.10: Tumouroid with 50,000 LNCaP cells tracked with CMFDA (green) and 25,000 U937 cells tracked with CM-Dil (red) and DAPI (blue). Images a) tumouroid at day 1 magnification scale=50µm, b) tumouroid at day 21 showing spheroid, magnification scale=50µm. Images were taken on the Olympus BX63 fluorescence microscope. Spheroid growth measured in µm² increased with time while in U937 co-culture, and this started at day 7 as there were no spheroids at day 1. Statistical analysis of graph using ANOVA one-way and Tukey analysis was carried out. Statistical significance was found, ***p<0.05, ****p<0.0001 for each condition (mean±SD obtained from triplicates).
4.4.7 Cytokine expression in LNCaP simple tumouroids and LNCaP immunocompetent tumouroids co-cultured with U937 cells

In this section of the chapter, experiments were carried out where supernatants from LNCaP simple tumouroids and LNCaP immunocompetent tumouroids with co-culture of U937 cells (for 24 hours at each time point) were harvested and analysed for the secretion of the specific cytokines which were previously chosen including: VEGF, IL-10 and TNF-α. The techniques used are described in the methods chapter (ELISAs, 2.2.3-2.2.5) and results are shown below.

4.4.8 VEGF increased in both LNCaP only simple tumouroids and immunocompetent LNCaP tumouroids co-cultured with U937 cells

VEGF secretion was seen to rise in both simple and immunocompetent tumouroids over the time points tested, days 1,3,7,10, 14. The most important observation was that day 1 VEGF secretion was much lower than that of days 3,7,10,14, for both simple and immunocompetent tumouroids. When comparing VEGF for the same time points across the two types of tumouroids, amounts were similar. LNCaP tumouroids secreted similar VEGF amounts to the tumouroids co-cultured with U937 cells, at specific time points, with slightly higher amounts at day 1 (0.22 vs.0.21 AU), day 10 (0.49 vs. 0.48 AU) and day 14 (0.52 vs. 0.51 AU) respectively (Figure 4.11). Whereas the LNCaP tumouroids co-cultured with U937 cells secreted slightly elevated levels of VEGF than LNCaP simple tumouroids at days 3 (0.46 vs. 0.44 AU) and day 7 (0.46 vs. 0.45 AU) respectively. There was no statistical significant differences between the cohorts throughout the experiment apart from day 1 versus the rest of the days, within each cohort.
4.4.9 IL-10 increased more in LNCaP immunocompetent tumouroids co-cultured with unstimulated U937 cells than in LNCaP only simple tumouroids

Overall, there were hardly any changes in IL-10 secretion from simple tumouroids, and only modest changes in immunocompetent tumouroids, over the time points tested, days 1, 3, 7, 10, 14. However, LNCaP immunocompetent tumouroids co-cultured with U937 cells secretion of IL-10 exceeded the secretion by LNCaP only simple tumouroids.
throughout the experiment (*Figure 4.12*). From the initial stages of the experiment, days 1 and 3, the LNCaP immunocompetent tumouroids had a higher secretion of IL-10 compared to the LNCaP only tumouroids, amounting to 0.24 vs. 0.23 AU and 0.25 vs. 0.22 AU respectively. The highest amount of IL-10 in the LNCaP immunocompetent tumouroids was observed at day 7 to be 0.26 AU compared to the LNCaP simple tumouroids which was 0.23 AU (the highest secretion on the simple tumouroids), a mere 0.03 AU rise which was not statistically significant. However, towards the end of the experiment there was a decline in IL-10 secretion in the LNCaP immunocompetent tumouroids at both day 10 (0.25 AU) and day 14 (0.24 AU) respectively. A reduction of IL-10 secretion by the LNCaP only tumouroids was seen after day 7, with 0.22 and 0.21 AU at days 10 and 14 respectively. Secretion of IL-10 was not statistically significant. Also, on statistical analysis within each group (i.e., LNCaP only tumouroids and LNCaP immunocompetent tumouroids) no statistical significance was recorded.
**Figure 4.12:** IL-10 secretion in LNCaP only simple tumouroids containing 50,000 LNCaP cells and LNCaP immunocompetent tumouroids co-cultured with 25,000 U937 cells added superficially for 24hrs at each time point. IL-10 was measured as absorbance units (AU, Y-axis) using ELISA. IL-10 secretion was found to be higher in LNCaP immunocompetent tumouroids co-cultured with U937 cells than in the LNCaP only simple tumouroids at specific time points. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried and no statistical significance was found, \( p > 0.05 \) for any condition (mean ± SD obtained from triplicates).

### 4.4.10 TNF-α increased in LNCaP only simple tumouroids than in LNCaP immunocompetent tumouroids co-cultured with U937 cells

The LNCaP only tumouroids secreted elevated TNF-α compared to the LNCaP immunocompetent tumouroids co-cultured with U937 cells, except for day 1. At this time point, the LNCaP immunocompetent tumouroids secreted more TNF-α at 0.26 AU, compared to LNCaP only tumouroids at 0.24 AU respectively. However, at day 3 the LNCaP only tumouroids took the lead in TNF-α secretion over the LNCaP...
immunocompetent tumouroids with levels recorded as 0.27 vs. 0.25 AU; and at day 7, 0.27 vs 0.22 AU, respectively. The maximum levels of TNF-α secretion in LNCaP only tumouroids and LNCaP immunocompetent tumouroids were 0.27 AU (at day 7) and 0.26 AU (at day 1) respectively. The reduction in TNF-α was continuous in LNCaP immunocompetent tumouroids co-cultured with U937 cells throughout the time period of the experiment, the TNF-α level started at 0.26 AU at day 1 and ended at day 14 with a level of 0.21 AU, a modest fall of 0.05 AU. The LNCaP only simple tumouroids also had a very slight decline in secretion from 0.24 at day 1 to 0.22AU at day 14 respectively. Interestingly, TNF-α levels were much higher in LNCaP only simple tumouroids than in LNCaP immunocompetent tumouroids at day 10 (0.24 vs. 0.21 AU) and 14 (0.22 vs 0.21 AU) respectively. Statistical analysis using ANOVA one way with Tukey’s multiple comparisons was carried out and results for all time points are shown below. In addition, when comparing the LNCaP only simple tumouroids alone, statistical significance was found at days 3 vs.14 and 7 vs.14 and on comparing the LNCaP immunocompetent tumouroids co-cultured with U937 alone, statistical significance was found at days 1 vs.10, 1 vs.14, 3 vs.7, 3 vs.10 and 3 vs.14.
Figure 4.13: TNF-α secretion in LNCaP only simple tumouroids containing 50,000 LNCaP cells and LNCaP immunocompetent tumouroids co-cultured with 25,000 U937 cells added superficially for 24hrs at each time point. TNF-α was measured as absorbance units (AU, Y-axis) using ELISA. TNF-α secretion was found to be higher in LNCaP only simple tumouroids than in the LNCaP immunocompetent tumouroids co-cultured with U937 cells at specific time points. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out and statistical significance found, *p<0.05, ***p<0.05 for specific conditions (mean±SD obtained from triplicates).
Chapter 5

Results
5.1 Title: Immunocompetent prostate tumouroids in hypoxic and normoxic conditions

5.2 Introduction

In this chapter, the effect of hypoxic and normoxic conditions on the immunocompetent PCA tumouroid model is presented. Specifically, the focus was on whether changes in oxygen levels promoted a pro or antitumourogenic environment within the PCA tumouroid.

The hypothesis was that hypoxia causes an increase in VEGF and this would in turn lead to an elevation in HIF, which would result in the creation of a protumourigenic environment within the tumouroid.

5.3 Methods

Image-iT Red Hypoxia reagent was used to stain the PCA cells from both cell lines (LNCaP and PC3). This reagent is unique in that it is not fluorescent when cells are at normal oxygen concentrations and becomes fluorescent when oxygen levels drop to less than 5%. The other advantage of using this stain is that it is formaldehyde fixable and this enabled fixing the stained samples and placing them on slides for imaging without losing the signal. Moreover, a vital advantage of using this Image-iT Red Hypoxia reagent was that its wavelength (Ex/Em 490/610nm) did not clash with the CMFDA wavelength (Ex/Em 492/517nm) which was used to stain the U937 cells. This meant that the differentiation on imaging between PCA cells and U937 cells was possible.

Furthermore, spheroid growth and cytokine secretion analyses were carried out in immunocompetent PCA tumouroids. In order to answer this, a step-wise approach was undertaken which included: microscopic images which were obtained from the LNCaP and PC3 tumouroids at all time points (days 1, 3, 7, 10 and 14), spheroid growth studies,
and cytokine expression analyses for VEGF, IL-10 and TNF-α levels in the supernatants of tumouroids.

As a final step, PMA stimulated U937 monocytes (i.e., differentiated) were used to construct immunocompetent tumouroids and spheroid growth and cytokine secretion were also determined, for comparison.

5.4 Results

5.4.1 Immunocompetent PCA tumouroids co-cultured with U937 cells formed spheroids in hypoxic and normoxic conditions but U937 cells appeared to decline in amount

LNCaP (50,000) and PC3 (50,000) tumouroids were created, with the PCA cells already stained with Hypoxia Image-iT reagent. These were co-cultured with CMFDA-tracked U937 (25,000) cells, which were added superficially to the tumouroids for 24 hours at each time point, as described in the methods chapter. Although both PCA cell lines continued to grow and formed spheroids over time (days 1-14), U937 cells appeared to decline in amount at day 14 in hypoxic samples only and not in normoxic samples and this was confirmed on visual microscopic examination (Figures 5.1, 5.2, 5.3, 5.4)
Figure 5.1: Immunocompetent LNCaP tumouroids with 50,000 LNCaP prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions. Tumouroids were stained with Hypoxia Image IT red stain at day 1 of incubation at the 6’oclock quadrant. Images a)c)e) and b)d)f) represent tumouroids under hypoxic conditions and normoxic conditions respectively. Images a) and b) show LNCaP cells stained with Hypoxia Image IT red stain in hypoxia and normoxia respectively. Images c) and d) show CMFDA tracked U937 cells in hypoxia and normoxia respectively. Images e) and f) show merged images with DAPI (blue). It was observed the LNCaP and U937 grew in co-culture and that there was no cell death at day 1 either in hypoxia or normoxia. For all images magnification scale=100µm. Images were taken on the Olympus BX63 fluorescence microscope.
Figure 5.2: Immunocompetent LNCaP tumouroids with 50,000 LNCaP prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions. Tumouroids were stained with Hypoxia Image IT red stain at day 14 of incubation at the 6’oclock quadrant. Images a)c)e) and b)d)f) represent tumouroids under hypoxic conditions and normoxic conditions respectively. Images a) and b) show LNCaP cells stained with Hypoxia Image IT red stain in hypoxia and normoxia respectively. Images c) and d) show CMFDA tracked U937 cells in hypoxia and normoxia respectively. Images e) and f) show merged images with DAPI (blue). It was observed there was a reduced amount of U937 cells in the hypoxic samples compared to the normoxic samples. LNCaP spheroids continued to grow in both hypoxic and normoxic
samples. For all images magnification scale=100µm. Images were taken on the Olympus BX63 fluorescence microscope.

Figure 5.3: Immunocompetent PC3 tumouroids with 50,000 PC3 prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions. Tumouroids were stained with Hypoxia Image IT red stain at day 1 of incubation at the 6’oclock quadrant. Images a)c)e) and b)d)f) represent tumouroids under hypoxic conditions and normoxic conditions respectively. Images a) and b) show PC3 cells stained with Hypoxia Image IT red stain in hypoxia and normoxia respectively. Images c) and d) show CMFDA tracked U937 cells in hypoxia and normoxia respectively. Images e) and f) show merged images with DAPI (blue). It was observed that PC3 and U937 grew in co-culture and that there was no cell death at day 1 either in
hypoxia or normoxia. For all images magnification scale=100µm. Images were taken on the Olympus BX63 fluorescence microscope.

Figure 5.4: Immunocompetent PC3 tumouroids with 50,000 PC3 prostate cancer cells (red) and 25,000 CMFDA (green) tracked U937 monocytes cells added, under hypoxic and normoxic conditions. Tumouroids were stained with Hypoxia Image IT red stain at day 14 of incubation at the 6’clock quadrant. Images a)c)e) and b)d)f) represent tumouroids under hypoxic conditions and normoxic conditions respectively. Images a) and b) show PC3 cells stained with Hypoxia Image IT red stain in hypoxia and normoxia respectively. Images c) and d) show CMFDA tracked U937 cells in hypoxia and normoxia respectively. Images e) and f) show merged images with DAPI (blue). It was observed there was a reduced amount of U937 cells in the hypoxic samples compared to the
normoxic samples. PC3 spheroids continued to grow in both hypoxic and normoxic samples. For all images magnification scale=100µm. Images were taken on the Olympus BX63 fluorescence microscope.

5.4.2 LNCaP and PC3 spheroid growth in immunocompetent prostate tumouroids increased under hypoxic conditions compared to normoxic conditions

Spheroid growth measurements were carried out on normoxic and hypoxic immunocompetent tumouroids as detailed in the Methods chapter (section 2.3.3). It was observed that in both LNCaP and PC3 immunocompetent tumouroids co-cultured with U937 cells, there was an acceleration of spheroid growth under hypoxic conditions compared to normoxic conditions. The LNCaP spheroids under hypoxia reached bigger spheroid sizes at days 7, 10 and 14 compared to PC3 spheroids: 1564μm² vs. 884.1μm², 2467.4μm² vs. 2360.2μm² and 4795.1μm² vs. 4260μm² respectively. These results correlated with studies which confirmed that in vivo hypoxia in PCA led to increased growth and angiogenesis which then led to progression of the tumour (see discussion).

In normoxia, however, this clear difference disappeared. LNCaP mean spheroid growth at day 7 exceeded PC3 mean spheroid growth 1107μm² compared to 922μm² respectively. At days 10 and 14, however, the mean spheroid growth in PC3 spheroids in normoxia was higher than mean LNCaP spheroid growth 2633μm² vs. 2203μm² and 3998μm² vs. 3812μm² respectively. Regardless, when statistical analysis was carried out there were no statistically significant differences between spheroid sizes LNCaP and PC3 immunocompetent tumouroids cells under hypoxic and normoxic conditions. All results are shown below (Figures 5.5, 5.6, 5.7, 5.8).
Figure 5.5: Cancer spheroid growth measured in µm² in immunocompetent LNCaP tumouroid samples co-cultured with U937 cells under hypoxic conditions. At day 7 spheroid formation occurs, the spheroids continued to grow as time of incubation progressed. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out and statistical significance was found, **p<0.05, ***p<0.05, ****p<0.0001 for each condition (mean±SD obtained from triplicates)
Figure 5.6: Cancer spheroid growth measured in µm² in immunocompetent LNCaP tumouroids containing U937 cells under normoxic conditions. At day 7 spheroid formation occurs, the spheroids continued to grow as time of incubation progressed. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found ***p<0.05, ****p<0.0001 for each condition (mean±SD obtained from triplicates).
Figure 5.7: Cancer spheroid growth measured in $\mu m^2$ in PC3 immunocompetent tumouroids containing U937 cells under hypoxic conditions. At day 7 spheroid formation occurs, the spheroids continued to grow as time of incubation progressed. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found, $**p<0.05$, $****p<0.0001$ for each condition (mean±SD obtained from triplicates).
Figure 5.8: Cancer spheroid growth measured in \( \mu m^2 \) in PC3 immunocompetent tumouroids containing U937 cells under normoxic conditions. At day 7 spheroid formation occurs, the spheroids continued to grow as time of incubation progressed. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found, ****\( p<0.0001 \) for each condition (mean±SD obtained from triplicates).

5.4.3 Levels of cytokines secreted in immunocompetent LNCaP PCA tumouroids in hypoxia and normoxia conditions differed

This section analyses the cytokines; VEGF, IL-10 and TNF-\( \alpha \) which represent protumourigenic, antitumourigenic and monocyte to macrophage differentiation indicator, respectively. These were secreted in supernatants harvested from immunocompetent LNCaP PCA tumoroids in the presence of hypoxia and normoxia. It was hypothesised that hypoxia leads to the creation of a protumourigenic environment
within the tumouroid model, as hypoxia is inherent within the TME *in vivo* and this leads to the progression of PCA.

5.4.4 Secretion of protumourigenic VEGF cytokine was increased in hypoxia compared to normoxia in immunocompetent LNCaP tumouroids co-cultured with U937 cells

VEGF levels markedly increased in hypoxia than in normoxia in immunocompetent LNCaP tumouroids co-cultured with U937 cells (*Figure 5.9*). Both cohorts in hypoxia and normoxia had elevations of VEGF which were linear as the time progressed. However, the hypoxic samples always secreted higher levels of VEGF compared to the normoxic samples at all time points: at day 1 0.17 vs. 0.16 AU, at day 3 0.22 vs. 0.19 AU, at day 7 0.27 vs. 0.20 AU and day 10 0.37 vs. 0.29 AU respectively. Also, a maximum VEGF level in hypoxia samples was recorded as 0.47 AU at day 14 and in normoxia samples the maximum secretion was 0.39 AU at day 14 respectively, a difference of 0.07 AU. Statistical significance was present at all time points from day 7 onwards. In addition to differences between the two conditions, the increase of VEGF secretion within tumouroids for each condition over time also showed significant changes. Notably, for hypoxia, starting from 0.16AU on day 1 to 0.46 AU on day 14, increases in VEGF amounts were significantly different throughout, apart from day 1 vs. day 3. Significance in normoxia was similarly pronounced. Starting from 0.16AU on day 1 to 0.39 AU on day 14, the only comparisons which were not significant were between day 1 and days 3 and 7 (*Figure 5.7*).
Figure 5.9: VEGF secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added superficially for 24hrs at each time point, over a time period of 14 days in both hypoxic (H) and normoxic conditions (N). VEGF was measured as absorbance units (AU, Y-axis) using ELISA. There was a profound increase in secretion of VEGF in hypoxic samples (H) as compared to the normoxic samples (N). Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found, **p<0.05, ***p<0.05 for each condition (mean±SD obtained from triplicates).

5.4.5 Secretion of antitumourigenic IL-10 cytokine was slightly elevated in immunocompetent LNCaP tumouroids co-cultured with U937 cells in normoxic compared to hypoxic conditions

There were hardly any differences in IL-10 secretions throughout these experiments, with only slight fluctuations. The secretion of IL-10 was slightly higher in normoxic immunocompetent LNCaP tumouroids than in the hypoxic samples at all time points except for day 3. At day 3 the IL-10 secretion in the normoxic samples slightly decreased
to 0.21 AU whereas the hypoxic samples were measured at 0.22 AU, respectively. The maximum IL-10 secretion in the normoxic tumouroids was recorded at day 1, at 0.23 AU. The maximum in hypoxic tumouroids was also found at day 1 and was recorded as 0.23 AU. Starting at day 7 and continuing to day 10 and day 14, there was a very slight lead in IL-10 secretion in the normoxic compared to the hypoxic tumouroids: 0.23 vs. 0.22 AU (for all three time points) with no statistical significance between the groups. In addition to differences between the two conditions, the IL-10 secretion within tumouroids for each condition over time showed no significant changes, apart from one comparison between day 1 and day 3 in normoxia (*Figure 5.10*).

*Figure 5.10: IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added superficially for 24hrs at each time point, over a time period of 14 days in both hypoxic (H) and normoxic conditions (N). IL-10 was measured as absorbance units (AU, Y-axis)
using ELISA. Overall, IL-10 increases more in normoxic (N) than in hypoxic (H) immunocompetent LNCaP tumouroids. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found *p<0.05 in normoxic cohort only (mean±SD obtained from triplicates).

5.4.6 Higher secretion of monocyte to macrophage differentiation indicator TNF-α cytokine in immunocompetent LNCaP tumouroids co-cultured with U937 cells in hypoxia than in normoxia at later stages of the experimental timeline

At the start of the experiment the TNF-α secretion was elevated in the normoxic samples more than in the hypoxic samples at days 1, 3 and 7 with levels recorded as 0.24 vs. 0.20 AU, 0.23 vs. 0.21 AU and 0.21 vs. 0.20 AU respectively. However, there was a surge in TNF-α secretion in both samples at day 10 but there was a higher surge in the hypoxic samples at day 10 and 14 than in the normoxic samples at the same time points which amounted to 0.25 vs. 0.23 AU and 0.29 vs. 0.24 AU respectively. Despite the trends, statistical analysis was carried out and no statistical significance was seen between the normoxic versus hypoxic samples, with–day 14 - the most obvious difference approaching, but not reaching, significance, at p=0.60.

However, comparisons between secretions of TNF-α for different days within the same condition, did show statistical significance between three hypoxia samples between days 1,3,7 and 14 but did not show any significance between normoxia samples (Figure 5.11).
Figure 5.11: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCaP cells and 25,000 unstimulated U937 cells added superficially for 24hrs at each time point, over a time period of 14 days in both hypoxic (H) and normoxic conditions (N). TNF-α was measured as absorbance units (AU, Y-axis) using ELISA. There was a higher secretion of TNF-α by hypoxic samples than normoxic samples at days 10 and 14 respectively, whereas at days 1,3 and 7 there was a higher secretion of TNF-α in the normoxic (N) samples than in the hypoxic (H) samples respectively. Statistical analysis using ANOVA one way with Tukey’s multiple comparisons was carried out, statistical significance was found*P<0.05 in hypoxic cohort only (mean±SD obtained from triplicates).
5.4.7 Levels of cytokines secreted in immunocompetent PC3 PCA tumouroids in hypoxia and normoxia conditions differed

5.4.8 Secretion of protumourigenic VEGF cytokine was increased in normoxia compared to hypoxia for immunocompetent PC3 tumouroids co-cultured with U937 cells at the later stages of the experimental timeline

At the initial stages of the experimental timeline, specifically at days 1 and 3, VEGF secretion was slightly higher in hypoxic compared to normoxic PC3 immunocompetent tumouroids and was recorded as 0.174 vs. 0.173 AU and 0.18 vs. 0.17 AU respectively. However, the normoxic immunocompetent PC3 tumouroids had an uptick of VEGF secretion which surpassed that of the hypoxic samples at day 7 and this was recorded as 0.28 AU compared to 0.23 AU respectively. The levels of VEGF secretion at day 10 and day 14 also showed that the normoxic tumouroids had higher secretion than hypoxic tumouroids at the same times and these were recorded as 0.28 vs. 0.24 AU and 0.28 vs. 0.25 AU respectively. The differences which were statistically significant were only for the normoxic vs hypoxic immunocompetent tumouroids for the later stages from days 7 to 14 (*Figure 5.12*).

Overall, VEGF continued to increase in PC3 immunocompetent tumouroids in hypoxia alone and normoxia alone throughout the time points of the experiment. PC3 immunocompetent tumouroids in hypoxia started at 0.17 AU at day 1 and ended at 0.25 AU at day 14. In normoxia PC3 immunocompetent tumouroids started at 0.17 AU at day 1 and ended at 0.28 AU at day 14. Statistical significance was found between the cohorts at specific time points and generally from day 7, as delineated below (*Figure 5.12*).
5.4.9 Increased secretion of antitumourigenic cytokine IL-10 in hypoxia than in normoxia in PC3 tumouroids co-cultured with U937 cells

IL-10 secretion was higher at day 1, 7, 10 and 14 in the PC3 immunocompetent tumouroids containing U937 cells under hypoxia conditions compared to normoxia conditions and the levels were as follows: 0.22 vs. 0.21 AU, 0.218 vs. 0.217 AU, 0.24 vs. 0.22 AU and 0.26 vs. 0.23 AU respectively. The exception was day 3 where the normoxic samples registered a higher IL-10 secretion level of 0.26 AU compared to hypoxic...
samples at 0.25 AU. Interestingly, the maximum difference between hypoxic and normoxic samples was found at day 14, recorded at 0.26 and 0.23 AU respectively. Statistical significance was found between the samples at day 14 (Figure 5.13).

Levels of IL-10 within the same condition, hypoxia or normoxia over time showed an interesting pattern. Overall there was an increase from day 1 to day 14, with 0.22 rising to 0.26 AU for hypoxic samples, and 0.21 rising to 0.23 AU with normoxic samples. However, for both conditions, there was a significant rise in IL-10 levels on day 3, compared to the values seen at the two timepoints either side, days 1 and 7.

Figure 5.13: *IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroid samples containing 50,000 PC3 cells and 25,000 U937 cells added superficially for 24hrs at each time point, over a time period of 14 days in both hypoxic (H) and normoxic conditions (N).* IL-10 was measured as absorbance units (AU, Y-axis)
using ELISA. IL-10 release in hypoxic (H) samples begin to increase more than in the normoxic (N) samples at days 7, 10 and 14. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found, ****p<0.0001 for each condition (mean±SD obtained from triplicates). Day 3 measurements (for both conditions) were significantly different from their equivalent day 1 and day 7 (p<0.05). This is not shown to avoid confusion in the graph.

5.4.10 Secretion of monocyte to macrophage differentiation indicator TNF-α is elevated at different stages in immunocompetent PC3 tumouroids under hypoxia and normoxia

TNF-α secretion at the initial stages of the experiment showed hypoxic immunocompetent PC3 tumouroids are slightly over normoxic samples at days 1 and 3, with levels amounting to 0.22 vs. 0.21 AU and 0.223 vs. 0.221 AU respectively. At days 7, 10 and 14, however, there was an increased secretion of TNF-α in the normoxic tumouroids compared to the hypoxic samples, at 0.22 vs. 0.21AU, 0.23 vs. 0.22 AU and 0.22 vs. 0.21 AU respectively. There was statistical significance only between day 14 hypoxic immunocompetent PC3 tumouroids and normoxic samples (Figure 5.14). However, looking at the TNF-α levels within the hypoxic and within the normoxic groups over the timeline of the experiment, day 1 to day 14, there was no statistically significant differences.

These findings, in other words, statistical significant difference between normoxia and hypoxia on day 14, albeit for a small difference in levels, and no real significance within the groups themselves, raises the question whether the numerical difference translates to biological significance. This will be discussed later on.
Figure 5.14: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroid samples containing 50,000 PC3 cells and 25,000 U937 cells added superficially for 24hrs at each time point, over a time period of 14 days in both hypoxic (H) and normoxic conditions (N). TNF-α was measured as absorbance units (AU, Y-axis) using ELISA. Overall, the maximum TNF-α cytokine release was found at day 14 in the normoxic (N) cohort compared to the hypoxic (H) cohort respectively. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found at between normoxia and hypoxia at day 14, *p<0.05 (mean±SD obtained from triplicates).

5.4.11 Immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells (macrophages)

In this section, the results of the final series of experiments are presented, where immunocompetent LNCaP tumouroids where created by co-culturing with PMA-stimulated U937 cells. This approach attempted to introduce a more biomimetic aspect to the 3D tumouroid. Subsequently, analyses were undertaken which focused on spheroid growth and also on cytokinic expression (VEGF, IL-10 and TNF-α) from the supernatants
harvested from the samples as described in the Methods chapter (sections 2.2.3-2.2.5). The results are shown below.

5.4.12 LNCaP spheroid growth increased with time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

Within immunocompetent tumouroids, LNCaP spheroids, when in co-culture with PMA-stimulated U937 cells, were found to form at day 7 and were recorded as having a size of 726µm² (Figure 5.15). The growth of the LNCaP spheroids continued to increase reaching a size of 3114µm² at day 10. The difference in size amounted to 2388µm². Statistical significance was found between day 7 and day 10.
Figure 5.15: Immunocompetent tumouroid with 50,000 LNCaP cells tracked with CM-Dil (red) and 25,000 PMA-stimulated U937 cells (macrophages), stained with anti-CD68-FITC (green) and DAPI (blue) taken at the 9 o’clock quadrant. Images a) tumouroid at day 1 magnification scale=50µm, b) tumouroid at day 10 magnification scale=50µm. Images were taken on the Olympus BX63 fluorescence microscope. Spheroids (red) were observed at later time points (b, day 10) and macrophages were seen in close proximity (green) to cancer cells/spheroids at both time points (a, day 1; b, day 10). There appeared to be more macrophages at day 10 (b), c) increase in spheroid growth measured in µm² as time increased, and this started at day 7 (beginning of spheroid formation). ANOVA one-way statistical test was carried out as well as multiple comparison Tukey, statistical significance was found, ****P<0.0001 (mean±SD obtained from triplicates).
5.4.13 Cytokines secreted in immunocompetent LNCaP PCA tumouroids co-cultured with PMA-stimulated U937 cells (macrophages)

5.4.14 VEGF secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

VEGF secretion was found to increase with progression of time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells. The VEGF secretion started at day 1 at a level of 0.19 AU and it continued to increase reaching a level of 0.36 AU at day 10, a total increase of 0.17 AU (Figure 5.16). The differences in VEGF secretion between day 1 (0.18 AU) and day 3 (0.23 AU) was 0.05 AU and between day 3 (0.23 AU) and day 7 (0.27 AU) was 0.04 AU. The greatest difference in secretion of VEGF, however, was found between day 7 (0.27 AU) and day 10 (0.36 AU), at 0.09 AU. Statistical significance was found between all time points (Figure 5.16). Of note, VEGF was the most upregulated cytokine compared to the other two cytokines analysed (IL-10 and TNF-α, described later).

A crucial observation that should be made here is a snapshot comparison between immunocompetent tumouroids created with PMA-stimulated versus unstimulated U937 cells.

Overall, VEGF secretion was higher in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells than the previous LNCaP immunocompetent PCA tumouroids which were co-cultured with unstimulated U937 samples at day 10 in normoxia only, and the PC3 immunocompetent PCA tumouroids which were co-cultured with unstimulated U937 samples at day 10 in both normoxia and hypoxia.
Figure 5.16: VEGF secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCAP cells and 25,000 PMA-stimulated U937 cells over a time period of 10 days. VEGF was measured as absorbance units (AU, Y-axis) using ELISA. There is an increased VEGF secretion as time progressed. Statistical analysis using ANOVA one-way with Tukey’s multiple comparisons was carried out, statistical significance was found,*p<0.05,**p<0.05,***p<0.05,****p<0.0001 (mean±SD obtained from triplicates).

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172
5.4.15 IL-10 secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

In immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated cells, IL-10 secretion was also found to increase with progression of time, similar to VEGF. Levels started at 0.22 AU at day 1 and ended at 0.24 AU at day 10 (*Figure 5.17*), a total increase of 0.02 AU. Throughout, the differences in IL-10 secretion between day 1 (0.22 AU), day 3 (0.23AU) and day 7 (0.23AU) were minimal, while day 10 amounts measured at 0.24 AU. These modest rises suggest that the production of IL-10 was reaching a plateau, unlike VEGF which continued to rise (*Figure 5.17*). Statistical significance was found at the majority of points throughout this experiment.

A crucial observation that should be made here is a snapshot comparison between immunocompetent tumouroids created with PMA-stimulated versus unstimulated U937 cells.

Overall, IL-10 secretion was higher in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells than the previous immunocompetent PCA tumouroids (LNCaP and PC3) which were co-cultured with unstimulated U937 samples at day 10, whether in normoxia or hypoxia conditions. Also IL-10 secretion in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells was higher than the initial experiment in LNCaP simple tumouroids at day 10.
Figure 5.17: IL-10 secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCAP cells and 25,000 PMA-stimulated U937 cells over a time period of 10 days. IL-10 was measured as absorbance units (AU, Y-axis) using ELISA. There is an increased IL-10 secretion as time progressed. Statistical analysis using ANOVA one-way with Tukey's multiple comparisons was carried out, statistical significance was found, \(*p<0.05, **p<0.05, ****p<0.0001\) (mean\(\pm\)SD obtained from triplicates).

5.4.16 TNF-\(\alpha\) secretion increased over time in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

In immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells, secretion of TNF-\(\alpha\) increased with progression of time. Levels at the start of the experiment were recorded at 0.21 AU at day 1 and ended at 0.28 AU at day 10, a total increase of 0.07 AU (Figure 5.18). The differences in TNF-\(\alpha\) secretion between day 1
(0.21 AU) and day 3 (0.22 AU) amounted to 0.01 AU, while that between day 3 (0.22 AU) and day 7 (0.24 AU) was larger at 0.02 AU. The largest difference in secretion of TNF-α was found between day 7 (0.24 AU) and day 10 (0.28 AU), at 0.04 AU. Statistical significance was found at different points throughout this experiment.

A crucial observation that should be made here is a comparison between immunocompetent tumouroids created with PMA-stimulated versus unstimulated U937 cells.

Overall, TNF-α secretion was higher in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells than all the previous immunocompetent PCA tumouroids (LNCaP and PC3) which were co-cultured with unstimulated U937 samples at day 10, whether in normoxia or hypoxia conditions.
Figure 5.18: TNF-α secretion (absorbance units, using ELISA) in immunocompetent tumouroids containing 50,000 LNCAP cells and 25,000 PMA-stimulated U937 cells over a time period of 10 days. TNF-α was measured as absorbance units (AU, Y-axis) using ELISA. There was an increased TNF-α secretion as time progressed. Statistical analysis using ANOVA one way with Tukey’s multiple comparisons was carried out, statistical significance was found, **p<0.05, ****p<0.0001 (mean±SD obtained from triplicates).
Chapter 6

Discussion
6.1 Discussion

This thesis revolved around the production of PCA immunocompetent tumouroids in an *in vitro* 3D platform, ensuring the survival and growth of both PCA cells and immune cells (U937 cells). The primary aim of the studies focused on how specific cells of the innate immune system, namely U937 cells, interacted with PCA spheroids. Experiments examined multiple factors within the PCA immunocompetent tumouroid platform. This was based on the literature indicating that monocytes exert antitumourigenic and protumourigenic influences on cancers within the TME.

A key factor studied was the biophysical aspect of the tumouroid model. This was explored and it was found that the depth of penetration of U937 cells added superficially and allowed to penetrate the tumouroid construct altered in compressed (stiff) compared to non-compressed (non-stiff) gels. To explore this, immunocompetent tumouroids manufactured with LNCaP prostate cancer cells were used. 

Spheroid growth was also an important aspect investigated in the course of this thesis. PCA spheroid growth was analysed in simple tumouroids and in immunocompetent tumouroids with co-culture of U937 cells (in hypoxia and normoxia) as well as PMA-stimulated U937 cells (macrophages). This was in an effort to see whether there was a protumourigenic or antitumourigenic influence of these immune cells on PCA spheroids. Moreover, cytokine secretion was analysed in compressed versus non-compressed gels impregnated with U937 cells. Cytokine analysis was also carried out in LNCaP immunocompetent tumouroids co-cultured with U937 cells to see whether there was a protumourigenic or antitumourigenic influence on cytokinic secretion. 

In addition, hypoxia was a factor focused on with respect to its implications on immunocompetent PCA tumouroids cytokinic secretion (pro/antitumourigenic). Both LNCaP (androgen receptor positive) and PC3 (androgen receptor negative) prostate...
cancer cell lines were used. Hypoxia in 3D cancer models has been shown to increase the proliferation and angiogenesis of cancer spheroids [215].

Finally, the influence that macrophages exert on the TME is profound and this was explored. The U937 cells were stimulated with PMA to convert them into macrophages. This was followed by PCA spheroid growth studies (as mentioned above) and cytokinic analysis to see whether there was a pro/antitumourigenic macrophagic influence on PCA cells.

6.2 Effects of the physical state of collagen gels on the depth of penetration of U937 cells

It was found that the non-compressed gels caused U937 monocytes to penetrate deeper than in compressed gels specifically at; 1 hour, 6 hours, 24 hours and 72 hours. On imaging, there were more CM-Dil U937 cells at the bottom of day 7 non-compressed gels than day 7 compressed gels, which also consolidated the finding that non-compressed gels favour cellular penetration. The highest distance of depth of penetration was found in the non-compressed gels at 6 hours and amounted to a mean of 95µm (section 3.4). This finding was attributed to the non-compressed gels being more viscous facilitating the depth of penetration of these cells as opposed to the compressed gels which were stiffer biophysically and perhaps more difficult for the U937 cells to negotiate.

This observation could be due to compressed gels being denser than their non-compressed counterparts. This is because compression causes the gels to lose water. What is more, there are mechanical factors in compressed collagen gels such as; intrahelical, interhelical and intermicrofibrillar covalent cross-links throughout the collagen gel that could impede the movement of cells [216].

Other researchers have also confirmed the finding of increased depth of penetration of cells in non-stiff collagen gels. They used a standard preparation of collagen (collagen
type 1), media (DMEM) and neutralised the acidic collagen with NaOH. The fabrication of collagen gels, in this PhD, also employed collagen type 1 and NaOH in neutralisation. The collagen gels were incubated and resulted in the formation of wedge-shaped gels which were then compressed vertically by a horizontal plate. This resulted in compressed collagen gels of approximately 0.1mm thickness [216]. The authors then allowed human dermal fibroblasts to infiltrate gels by adding a second layer of fibroblast-collagen solution to the first casting of acellular collagen, and reported that cells have increased locomotive abilities in non-stiff gels as opposed to stiff gels [217]. Cheema et al, have also seconded this observation that movement of cells is greater in non-stiff gels [218]. These researchers created compressed gels by a process known as ‘plastic compression’, where a forced flow of one direction from top to bottom compresses the gel leading to fluid leaving the surface of the gel by osmotic effects (this uses the same basic principle as RAFT constructs). The compressed gels recorded a stiffness of approximately 2,305kPa [217]. The researchers also described the movement of dermal fibroblast cells as ‘duro-taxis’ from a wedge area of non-compressed collagen to an area of compressed collagen. Hence, cells increase in mobility in non-compressed collagen gels. Depth of penetration of U937 cells alters in the presence of LNCaP cells in their single-cell form and in their spheroid form in PCA immunocompetent tumouroids. According to experiments carried out, it was found that U937 cells continued to penetrate in the LNCaP tumouroids as time increased over a 21 day period. The distance travelled by U937 cells reached a maximum of 114μm at day 21. However, on comparing the three experiments which analysed depth of penetration: U937 cells in compressed gels, U937 cells in LNCaP tumouroids (days 1-10) and exclusively spheroid phase (days 7-21), it was observed that there were differences in depth of
penetration. At days 1, 3 and 7 the depth of penetration of U937 cells only in compressed
gels was 61.6μm, 81μm and 88μm whereas in the co-culture within LNCaP tumouroids
it was 93.1μm, 97.6μm and 108μm respectively (sections 3.4.6 and 4.4.3). Also, this
increase in depth of penetration of U937 cells continued until the end of the experiment
at day 10 which culminated at 112μm. Thus, the experiment where U937 cells were added
to LNCaP tumouroids (1-10 days) showed higher levels of depth of penetration of U937
cells compared to the compressed constructs with U937 cells alone.

In addition, the experiments carried out with immunocompetent LNCaP tumouroids with
co-culture of U937 cells starting in the spheroid phase (day 7-21) (section 4.4.4), also
confirmed the finding of increasing depth of penetration as time of incubation progressed.
This showed that the depth of penetration continued to increase reaching a maximum of
114μm, even though initially, at day 7 and day 10 the depths of penetration were less in
the immunocompetent LNCaP tumouroids spheroid phase experiment (day 7-21) than the
days 1-10 experiment: 84μm vs. 108μm and 106μm vs. 112μm respectively (sections
4.4.3 and 4.4.4).

This difference in the depth of penetration between the immunocompetent tumouroid
experiments could be due to the fact confirmed in a study conducted by Sondag et al. who
showed that monocytes have a tendency to adhere together in collagen type 1 gels [219].
Additionally, the finding that immune cell movement in co-culture increased with cancer
cells has been corroborated in a study which used fluorescently coupled antibodies, in an
in vitro setting, and showed that T-cells increased in movement in human ovarian and
lung cancer tissue specimens [220].

Also, Grunewald et al. used T-cells in the setting of bioprinted 3D neuroblastoma models
and confirmed that depth of infiltration of T-cells increased in co-culture [221].
The correlation between increasing time of incubation leading to an increase in cellular depth of penetration has also been corroborated in another study, in which MSCs were seeded in PEG hydrogels which showed that with increasing time these cells increased their penetration depth. They recorded the depths of penetration at 200μm at 3 days and 500μm at 10 days of culture [222].

In the studies carried out in this PhD, the same method of plastic compression used by published literature carried out by Cheema et al., mentioned above, was used. This led to the creation of stiff or compressed gels. Also, as in this research paper, non-compressed hydrogels were created to analyse depth of penetration. However, the cellular constituents of the gels in the current studies differed as U937 cells were used as opposed to fibroblasts. Even though fibroblasts migrate through 3D models (in a amoeboidal, lobopodial or lamellipodial migration) as is the case with monocytes, the speed and extent to which these cells migrate comparatively is unknown [223, 224]. In that sense, and due to the fact that a different cellular type was used, the U937 depth of penetration may have differed. However, the concept that cellular locomotion increases in hydrogels or non-compressed gels has been found to remain true as has been shown in the results chapter of this thesis.

The other factor that should be mentioned, which could affect statistical significance is the different timelines used in the experiments I carried out compared to those experiments carried out by other researchers. For example, both Cheema et al. and Mudera et al, carried out experiments up to 6 days whereas I carried out experiments to 7 days and within my timeline I looked at hours as well (1,3,6 hours). Whereas, the studies quoted did not look at the hourly change in depth of penetration.

In terms of novelty of the research carried as part of this PhD, to date there has not been, to my knowledge, any experimental studies using microscopic analysis, with the aid of
fluorescent cell trackers to document to depth of penetration of specifically CM-Dil U937 cells in compressed and non-compressed gels formed following the RAFT Lonza technique.

Regarding the accuracy of the experiments that were carried out as part of this PhD in the domain of depth of penetration, although the depth of penetration of U937 cells increased in non-compressed vs. compressed gels, statistical significance was not found. Thus, in order to prove statistical significance and to better confirm this finding, a number of points should be built upon. Firstly, the technical limitation of our visual analysis was the equipment used and this could be improved if laser or even electron microscopy was used to further increase the precision of depth of measurement analyses. Also, the current analyses were based on fixed samples rather than real-time. Thus, it is hypothesised that real-time tracking in future of these samples could yield more statistical significant results. Secondly, the depth of penetration analyses were carried out on the basis of cellular movement within the gels whereas other studies such as that conducted by Mudera et al. (reference [216] above) showed statistical significance depended on manually counting cells in compressed and non-compressed gels and did not undertake the method in this thesis of microscopically manually moving down the gel. This technique was devised in-house (see methods chapter) and due to it being novel is still in the initial stages and may require in the future to be confirmed with manual cell counting or real-time cellular tracking to increase precision.

6.3  **PCA spheroid formation and growth in a simple LNCaP tumouroid**

In the experiments conducted, PCA (LNCaP) cells were successfully cultured in tumouroids and spheroids appeared well-formed at day 7 (see below). This is in line with a study that also analysed spheroids at this significant time point [225]. Both LNCaP and
C4-2B (cell line derived from LNCaP-derived C4-2 cells with epithelial-like morphology) cells were successfully cultured in a 3D microwell mesh platform using polydimethylsiloxane (PDMS) and spheroids appeared well-formed at day 7. LNCaP spheroid sizes were recorded up to 200μm in diameter at day 14.

Interestingly, from the experiments conducted in the course of this PhD, it was found that the mean size of LNCaP spheroids increased from the initial point of formation at day 7 and the sizes were as follows: 1,271μm² at day 7, 3,132μm² at day 10 and 4,366μm² at day 14 (Table 16). Many studies have aimed to measure the size of cancer spheroids in different culture types. LNCaP spheroids were harvested in Matrigel and their spheroid diameter was assessed after 7 days and displayed increasing diameters ranging from less than 80μm to greater than 160μm [226].

Another corroborating study, in which researchers adopted the 3D PDMS microwells, used the same cellular density that was used throughout our experiments, 50,000 LNCaP cell density and confirmed spheroid formation and recorded spheroid size formation at day 7 (as per our experiments) and that the size at this particular point was 120μm in diameter [227]. Compared to our experiments, spheroid sizes were measured in terms of their area (μm²), at day 7, they ranged in sizes from: 726-1271μm² (Table 16). In our hypoxia and normoxia experiments the LNCaP spheroids at day 7 were: 1564 and 1107.3μm² respectively and PC3 spheroids at day 7 were: 884.1 and 922μm² respectively (section 5.4.2).

Another study found that LNCaP PCA spheroids harvested from ultra-low attachment plates, which is a different technique to that which has been used in this research project, could grow to a size of approximately 400,000μm², a much larger size than what was recorded in this PhD [228]. Therefore, different culture platforms can have a great effect on tumour spheroid size, and ultra-low adhesion plates appear to provide the most
favourable conditions for growth, as demonstrated for other types of cancer spheroids, also harvested from ultra-low attachment plates, such as colorectal cancer spheroids could grow to an area corresponding to 600,000μm² [229].

However, until now to my knowledge PCA spheroid size analyses in compressed 3D collagen gels has not been undertaken.

6.4 The creation of an immunocompetent PCA (LNCaP) tumouroid model with the impregnation of either U937 cells or PMA-stimulated U937 cells (macrophages) has an effect on PCA spheroid growth

Based on the results of the experiments carried out during this PhD, there were differences in sizes of spheroids between PCA immunocompetent tumouroids co-cultured with U937 cells and LNCaP simple tumouroids. The differences were found at days: 7, 10 and 14. There was an increase in spheroid growth in the LNCaP simple tumouroids compared to those immunocompetent tumouroids with U937 cells at days 7 and 10. The difference at days 7 and 10 in spheroid size amounted to 306μm² and 564μm² respectively (Table 16).

However, at day 14 the growth of spheroids in the LNCaP immunocompetent tumouroids with U937 cells surpassed the growth in LNCaP simple tumouroids which amounted to an increase of 243μm². A study, which corroborates our finding of a greater spheroid size increase in cancer spheroids with monocyte co-culture consisted of artificially added monocytes into breast cancer spheroids which were formed in agarose coated wells and then implanted them in mice. The authors reported that the size of the co-cultured spheroids increased more than those spheroids lacking monocytes [230].

Moreover, when comparing to spheroid growth in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells at day 7, there was much reduced spheroid growth compared to the previously mentioned experiments (i.e., LNCaP simple tumouroids and
LNCaP immunocompetent tumouroids with U937 co-culture) with a size reduction amounting to $545\mu m^2$ and $239\mu m^2$ respectively (Table 16).

Nevertheless, at day 10, the spheroid growth in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells surpassed the size of immunocompetent tumouroids co-cultured with unstimulated U937 cells. This increase in size amounted to $546\mu m^2$. Owing to the massive increase seen on comparing the LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells with those immunocompetent tumouroids with unstimulated U937 cells it could be suggested that there was a protumourigenic effect exerted by the PMA-stimulated U937 cells on the LNCaP spheroids. This finding is corroborated by a study which showed that tumour growth is enhanced in prostate epithelial cells co-cultured with macrophages in a 3D Matrigel model [231].

However, if the LNCaP spheroid size in the immunocompetent tumouroids with PMA-stimulated U937 cells at day 10 is compared with the LNCaP simple tumouroids, it is noticed that the latter experiment had spheroid sizes of $3132\mu m^2$ vs. the former at $3114\mu m^2$. This variability in spheroid size is a known occurrence in in vitro 3D models and has been commented on in a study which used magnetic levitation and hanging drop techniques of cancer spheroid harvesting. The researchers cultured non-small cell lung cancer cells (A549) in both magnetic levitation and hanging drop cultures and found, through automated analyses using AnaSP program developed by them, that the spheroids varied in amount and diameter, where 25 spheroids recorded diameters of approximately $347\mu m$ vs 27 spheroids with diameters of approximately $359\mu m$, respectively. [232].

In addition, literature has stated that macrophages may increase prostate tumour growth and angiogenesis through the secretion of specific protumourigenic cytokines such as VEGF [233]. This corroborated the finding of increased spheroid size in the
immunocompetent tumouroids with PMA-stimulated U937 vis-à-vis the samples with unstimulated U937 cells at day 10.

Overall, from the current findings, it appears that any stimulation of cancer cell growth by monocytes is not evident early on in the experimental timeline, but takes place later on i.e., at 14 days. This is evident for U937 populated tumouroids versus simple tumouroids. Unfortunately, the measurements at this time point for the co-cultures with PMA stimulated U937 were not acquired. Further experiments with longer timelines, supported with cytokine expression levels, would be necessary to demonstrate whether this is true.

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>Mean spheroid size (µm²) in LNCaP simple tumouroids without U937 cells +/- Standard Deviation (SD)</th>
<th>Mean spheroid size (µm²) in LNCaP immunocompetent tumouroids co-cultured with U937 cells +/- Standard Deviation (SD)</th>
<th>Mean spheroid size (µm²) in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells +/- Standard Deviation (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1271 (+/-421)</td>
<td>965 (+/- 435.4)</td>
<td>726 (+/-379.3)</td>
</tr>
<tr>
<td>10</td>
<td>3132 (+/- 1681)</td>
<td>2568 (+/- 1109.1)</td>
<td>3114 (+/-1745)</td>
</tr>
<tr>
<td>14</td>
<td>4366 (+/- 2612)</td>
<td>4609 (+/- 1602.1)</td>
<td>N/A</td>
</tr>
<tr>
<td>21</td>
<td>N/A</td>
<td>9081(+/- 4823)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 16: Spheroid size increased in all LNCaP tumouroids with or without U937 cells or PMA-stimulated U937 cells as time progressed.

6.5 Incorporation of U937 cells and PMA-stimulated U937 cells in the spheroid structures in LNCaP immunocompetent tumouroids

In the experiments in which LNCaP cells formed spheroids in immunocompetent tumouroids with U937 cells and PMA-stimulated U937 cells, there was an incorporation of these cells within the spheroid structure. This finding has been corroborated in a study, which used ultra-low attachment plates for spheroid formation, in which monocyte infiltration was shown to infiltrate spheroids of various cancer cell-lines including:
PANC-1 as well as MIA PaCa-2 (pancreatic cancers), MCF-7 (breast cancer) and HT-29 (colon cancer) [166].

A study has also reported that monocytes are recruited into tumours by chemotaxis. This is caused by cancer cell driven chemokine secretion which include: VEGF, CXCL-12, CCL5, CCL8 and CCL2 (Figure 6.1) [234].

In addition, PMA-stimulated U937 cells (macrophages) may have a distinct process in which they infiltrate the cancer spheroids. Murdoch et al. explained its occurrence being due to the hypoxic tumour cells producing chemoattractants that specifically lead macrophages into the hypoxic core within the cancer, these include: Endothelin, EMAP II and VEGF (Figure 6.1) [192]. The same study explained that these macrophages now referred to as TAMs remain in the hypoxic area due to the absence of chemotactic signals and due to the inhibition of migration by Macrophage migration Inhibitory Factor (MIF).
Figure 6.1: Monocytes and macrophages infiltrate the spheroid structure through different chemokines. Monocytes infiltrate spheroids through specific cytokines secreted by tumour cells including: VEGF, CXCL-12, CCL5, CCL8 and CCL2. Macrophages infiltrate into the hypoxic core within the cancer through the actions of the following chemoattractants including: Endothelin, EMAP II and VEGF. (Adapted from reference [235]).

6.6 PCA (LNCaP and PC3) spheroid growth is affected by hypoxia in the immunocompetent tumouroid model compared to normoxia

In experiments where PCA immunocompetent tumouroids were subjected to hypoxia, there was an increase in spheroid growth in both LNCaP and PC3 cell lines. LNCaP spheroids under hypoxic conditions exceeded the size of LNCaP spheroids under normoxic conditions at day 7 (1564μm² vs. 1107.3μm²), day 10 (2467.4μm² vs. 2203.1μm²) and day 14 (4795.1μm² vs. 3812μm²) respectively (section 5.4.2). On the other hand, PC3 spheroid sizes also increased more in hypoxia at day 14 than in normoxia,
4260µm² vs. 3998µm² respectively. However, on analysis of the day 7 and day 10 spheroid sizes, there was an increased growth in the normoxic PC3 samples compared to the hypoxic samples: 922µm² vs. 884.1µm² and 2633 vs. 2360.2µm² respectively. The elevation in PC3 spheroid growth in days 7 and 10 in normoxic samples compared to the hypoxic samples was found to be non-conformant with the LNCaP samples and current literature which solidified the fact that hypoxia forms a driving force for tumour progression and spheroid growth. However, researchers have found that PC3 cells actually produce less HIF-1α in certain hypoxic conditions which they referred to as ‘intermittent hypoxia’ [236]. Moreover, the finding that hypoxia leads to an increase in PCA growth has been extensively studied. PCA has been quoted to rank high among the malignancies in which hypoxia plays a major role in increasing its tumour evolution, growth and metastases [236]. Further support for the findings that were observed in the experiments that were carried out comes from a study which reported that hypoxia influenced cancer spheroids, specifically with the aid of monocytes through VEGF upregulation which led to an increased angiogenesis along with neovascularisation and ultimately contributed to tumour progression [184]. The other factor that should be considered here is the production of HIF which also contributes to cell growth and could explain the increase in PCA spheroid growth in hypoxia. This is because HIF maintains the survival and growth of PCA cells as well as preventing cellular apoptosis [237].
6.7 Hypoxia leads to a reduction in U937 cell amount in immunocompetent PCA tumouroid models

The LNCaP and PC3 immunocompetent tumouroid models were created as per the exact method outlined in the methodology chapter (sections 2.1.10 and 2.1.11). On imaging the models, it was apparent that there was a reduction in U937 cell amounts, i.e., flurorescence in the hypoxic compared to the normoxic samples at day 14. Similarly, the proliferation of U937 cells in a 2D setting was found to be suppressed in the presence of hypoxia [191].

This finding was also reported by Strese et al, who experimented in an *in vitro* 2D setting, with U937 cells exposed to hypoxia and they concluded that these cells had a profound reduction in proliferation when exposed to hypoxia compared to normoxia [238]. The effects of hypoxia specifically on proliferation of monocytes in a 3D PCA immunocompetent tumouroid model are reported here for the first time.

6.8 Cytokine Analysis

6.8.1 Cytokine release by U937 alters in compressed (stiff) versus non-compressed (non-stiff) collagen gels

On cytokine measurement of U937 impregnated collagen gels, it was found that there were three cytokines which had statistically significant increases in compressed gels containing U937 cells. These cytokines were; VEGF (protumourigenic), IL-10 (antitumourigenic) and TNF-α (monocyte to macrophage indicator).

The mean fluorescence intensity of VEGF levels was higher in compressed gels than in non-compressed gels at days 3 and 7 (2447 vs. 1284.3 AU) and (20015.3 vs. 4990 AU) respectively (section 3.4.9). The finding that VEGF is secreted by U937 cells is well established in several studies [239, 240].
IL-10 mean fluorescence intensity levels increased in compressed U937 gels specifically at days 3 (447.2 AU) and 7 (368 AU) vis-à-vis non-compressed gels at the same time points (416.3 AU) and (343.3 AU) respectively (section 3.4.10). This finding was similar to a paper which applied compression using a ceramic hip ball and multi-axial load bioreactor to a 3D agarose gel construct containing THP monocytes and found that not only IL-10 levels but also TNF-α levels were significantly raised [241].

TNF-α levels also increased in compressed U937 gels more than non-compressed gels. At days 3 and 7 the mean fluorescence intensity in compressed gels was 388.3 and 436 AU respectively (section 3.4.11). Whereas in non-compressed gels at days 3 and 7 the mean fluorescence intensity was 356 and 386 AU respectively. Monocytes have been reported to secrete elevated TNF-α levels on compression. Researchers have conducted experiments on monocytes (THP1-Blue), which they harvested on agarose gels. They used a custom built multi-axial bioreactor with a 32mm ceramic hip ball to apply compression to the gels. The researchers then proceeded to quantify TNF-α levels using ELISA assays following multiaxial loading for 3 days and found that there was an increase in TNF-α compared to those gels without compression (controls).[241].

It is imperative to mention at this point that external forces influence cytokine release by U937 cells. Literature has mentioned that on application of mechanical strain on these cells cultured on collagen type 1 membranes, an increase in cytokine secretion such as IL-6 was recorded. This supports what was found on applying compressive pressure to the collagen gels in the current experiments which led to an increase in cytokinic release [242].

Interestingly, other researchers have conducted studies on the effects of compression of 3D collagen scaffolds on cellular cytokine mRNA expression. It was found that with
increasing compression of collagen hydrogels embedded with fibroblasts there was an increase in cytokine expression such as; RANK-L and IL-6 [243]. Shimomura et al. also analysed the effect of compression on cellular impregnated collagen based hydrogels and its effect on cytokine release [244]. They found that the levels of IL-6 and IL-8 secreted by fibroblasts were significantly higher in the compressed samples.

6.8.2 VEGF secretion slightly increased in LNCaP only simple tumouroids compared to immunocompetent tumouroids co-cultured with U937 cells

Analysis of VEGF cytokine expression was carried out on supernatants of both LNCaP simple tumouroids and LNCaP immunocompetent tumouroids co-cultured with U937, following the technique mentioned in the methods chapter (section 2.2.3). The levels of VEGF were elevated in both groups at different time points but culminated with an elevation in LNCaP simple tumouroids at days 10 and 14 (0.49 and 0.52 AU), slightly higher than the VEGF secretion in LNCaP immunocompetent tumouroids co-cultured with U937 cells at those time points (0.48 and 0.51 AU) respectively (section 4.4.9).

It is well known that LNCaP cells produce VEGF [245, 246]. It is also well established, that VEGF is the most secreted cytokine by PCA cells and studies have reported that VEGF expression in PCA cells is found in approximately all PCA tissue specimens on immuno-histochemistry (IHC) analysis [247]. Researchers have shown that this holds true in a 3D model where LNCaP cells were cultivated in a hyaluronic acid based hydrogel and secreted VEGF [155], supporting my findings.

Also, from the current results, VEGF secretion was reduced when LNCaP cells were co-cultured with U937 cells relative to LNCaP only simple tumouroids, specifically towards the tail-end of the experiment (see above). This result goes against what was found in compressed constructs of U937 alone where there was a high level of secretion of VEGF.
One would expect there to be a synergistic or additive effect of U937 cells on VEGF secretion leading to higher levels of VEGF in co-culture.

VEGF secreted by U937 has been well established by researchers in the cardiovascular realm of medicine [248]. Hajighasemi et al., seconded this by proving that U937 secrete VEGF on their own without stimulation with LPS [249]. However, does the co-culture of LNCaP with U937 cells in immunocompetent tumouroids diminish their secretion of VEGF compared to LNCaP only simple tumouroids? This was apparent from the current results.

In addition, there was a xenograft study where PCA cells and U937 cells were inoculated in mice and this was found to cause an increase in angiogenesis of the tumour accompanied with an increase in VEGF [250]. Unfortunately, there are no publications to date, to my knowledge, that study the cytokinic profile of this cellular co-culture in a 3D simple or immunocompetent stiff 3D in vitro model. These findings merit further investigation.

6.8.3 IL-10 cytokine secretion increased more in LNCaP immunocompetent tumouroids co-cultured with U937 cells than in LNCaP only simple tumouroids

On analysis of IL-10 secretion, the level was higher in LNCaP immunocompetent tumouroids co-cultured with U937 cells and exceeded the levels in LNCaP only simple tumouroids throughout the whole time period of the experiment: day 1 (0.23 vs. 0.22 AU), day 3 (0.24 vs. 0.21 AU), day 7 (0.26 vs. 0.22 AU), day 10 (0.25 vs. 0.21 AU) and day 14 (0.24 vs. 0.21 AU) respectively (section 4.4.10).

A study has confirmed that high levels of IL-10 is strongly associated with prostate cancer recurrence and progression [251]. Interestingly, Yu et al., showed in their study using a
2D transwell model that IL-10 reduced the proliferation of LNCaP cells [252]. This could explain why the levels of IL-10 were not elevated in the LNCaP simple tumouroids due to the negative cellular growth impact of this cytokine.

6.8.4 TNF-α cytokine secretion slightly increased in LNCaP only simple tumouroids more than in immunocompetent tumouroids co-cultured with U937 cells

TNF-α was also analysed and at day 1 only there was a rise in its level in the co-culture sample surpassing that found in the LNCaP simple tumouroids (0.26 vs. 0.24 AU) respectively. However, following day 1 and throughout the whole time period of the experiment the levels in the LN CaP simple tumouroids had slightly higher TNF-α expression than in co-culture samples: day 3 (0.26 vs. 0.25 AU), day 7 (0.26 vs. 0.22 AU), day 10 (0.2486 vs. 0.21 AU) and day 14 (0.22 vs. 0.21 AU) respectively (section 4.4.11). Essentially, LNCaP is known to produce low levels of TNF-α in culture medium [253]. Also, it was reported that TNF-α exerted an anti-proliferative effect on LNCaP cells [253]. In effect this means that TNF-α secretion leads to a reduction in number of LNCaP cells and in turn further decreases the cellular cytokinic secretion. Yet, this was not the case in the LNCaP simple tumouroids cohort presented here.

Moreover, U937 cells were found to secrete TNF-α without the addition of stimulation in the form of LPS [254]. This may explain why the TNF-α levels in co-culture samples, except for day 1, were lower than the LNCaP only samples owing to the fact TNF-α is secreted by two cell types and thus more TNF-α causes death of LNCaP cells and in turn leads to an overall reduction of cytokinic secretion in the co-culture samples. The fact that TNF-α causes LNCaP death was also corroborated by Ricote et al, using a 2D model of culturing LNCaP cells to 60% confluence and added to these cells a TNF-α stock solution (50mg/ml) in distilled water. The researchers confirmed the apoptosis of LNCaP
cells following the addition of TNF-α solution by fluorescence and flow cytometry, they considered cells as apoptotic as those with fragmented chromatin (30% of apoptotic LNCaP cells recorded with addition of 100ng/ml of TNF-α solution) [255].

6.8.5 VEGF increased in secretion in hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells

There was a profound increase in VEGF secretion in hypoxic LNCaP immunocompetent tumouroid samples compared to the normoxic samples reaching the maximum level at day 14 of 0.46 vs. 0.39 AU respectively (section 5.4.4). This observation confirmed what has been reported in several studies, that VEGF increases in hypoxia. One such study that Raina et al. carried out, showed HIF-1-α expression produced in hypoxia increased VEGF levels in TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) models [256]. Another study that corroborated the current findings, used LNCaP spheroids, produced in ultra-low adherent wells, which were placed in hypoxic versus normoxic conditions. This resulted in a stark increase in VEGF in hypoxic but not normoxic samples [257]. The researchers attributed this difference to the influence associated with high levels of HIF-1α which is known to promote angiogenesis in PCA and worsen the prognosis.

6.8.6 IL-10 slightly increased in secretion in normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells

From the experimental results it was observed that IL-10 secretion was slightly higher in normoxic samples in all time points: day 1 (0.23 vs. 0.22 AU), day 7 (0.23 vs. 0.22 AU), day 10 (0.23 vs.0.22 AU) and day 14 (0.22 vs. 0.21 AU) except for day 3 where hypoxic samples had more IL-10 expression than normoxic samples (0.22 vs. 0.21 AU) respectively (section 5.4.5).
IL-10 secretion was shown to correlate with the aggressiveness of PCA and hypoxia caused HIF-1α to stimulate and enhance transcription of IL-10 [258]. Another study also stated that IL-10 was upregulated by prostate cancer cells in hypoxia [259]. Thus, it would be hypothesised that IL-10 secretion would be increased in the hypoxic environment but this was not seen in the LNCaP immunocompetent tumouroids.

The other issue to consider here is whether hypoxia affected U937 cells in a way that led to reduction of their IL-10 secretion. A study showed that on removing myeloid HIF-1α, which is an important factor produced in hypoxia, there was a stark elevation of IL-10 secretion by monocyte derived macrophages. This may indicate that HIF-1α reduces IL-10 secretion by monocytes (or their macrophage derivatives due to collagen differentiation within the tumouroid model) which corroborates the findings in our experiments where IL-10 was elevated in the normoxic samples at nearly all the time-points except for day 3 [260].

Another study, which supports our finding that IL-10 levels were reduced in hypoxia relative to normoxia samples used PBMCs that were cultured in hypoxia [261]. However, to this date and according to my knowledge no specific study has analysed the effect that hypoxia has on U937 expression of IL-10 within a compressed PCA tumouroid model.

6.8.7 TNF-α increased in secretion in hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells

With regards to TNF-α, it was observed that at the initial stages of the experiment (day 1-7) there was a higher secretion in normoxic compared to hypoxic samples: day 1 (0.23 vs. 0.20 AU), day 3 (0.22 vs. 0.20 AU) and day 7 (0.21 vs. 0.20 AU) respectively (section 5.4.3). However, at days 10 and 14, the mean absorbance levels were elevated in hypoxia.
It has been reported in the literature that under hypoxic conditions through HIF-1α stimulated TNF-α secretion in prostate tumours there is increased invasiveness of PCA through stabilising the transcriptional repressor of E-cadherin expression (Snail). This, in turn, led to an up-regulation of genes such as; vimentin and MMP-9 which are responsible for the invasive potential of PCA [262]. This is in line with what was found with the elevation of TNF-α in hypoxic samples surpassing the normoxic samples at later stages of this experiment.

Additionally, another study has reported that TNF-α caused monocytes to differentiate into macrophages [263]. This differentiation into macrophages in specifically hypoxic conditions may explain why there was a gross increase in TNF-α secretion at day 10 and 14. It is important to note that macrophages have been quoted to be a major source of TNF-α secretion [264]. It has been established in literature that TNF-α is increased by monocyte-derived macrophages in hypoxia [265]. This may explain the reason behind TNF-a secretion, as per the current results, increasing in hypoxic conditions.

**6.8.8 VEGF increased in secretion in normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells**

Overall, the maximum mean absorbance level of VEGF secretion in hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells was less than that secreted by hypoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells (0.25 compared with 0.46 AU) respectively. Further, normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells had lower maximum levels of VEGF secretion
compared to normoxic LNCaP immunocompetent tumouroids co-cultured with U937 cells (0.27 compared to 0.39 AU) respectively.

Researchers have noted that PC3 cells secreted higher VEGF than LNCaP [266]. However, their research utilised a 2D model rather than a 3D model and they did not use collagen type 1 as a scaffold. Additionally, these cells were not co-cultured with U937. Thus, it would be inferred that there might be an influence of the U937 cells as well as collagen on the secretion of VEGF by PC3 cells.

In addition, the VEGF levels were observed in PC3 co-cultured samples to be very similar in hypoxic than normoxic samples at the early stages: day 1 (0.174 vs. 0.173 AU) and day 3 (0.18 vs. 0.17 AU) respectively, but the normoxic samples had more VEGF expression at the later stages of the experiment: day 7 (0.27 vs. 0.22 AU), day 10 (0.27 vs. 0.24 AU) and day 14 (0.27 vs. 0.25 AU) respectively (section 5.4.8).

However, on analysing relevant literature, it was found that hypoxia exerted an additive effect on the secretion of VEGF in PC3 cells. This is probably via TGF-β1 stimulation leading to an increased VEGF expression in PC3 cells [267]. This would explain the initial surge of VEGF in hypoxic samples, however, this was not sustained throughout the PC3 hypoxic samples over time.

Furthermore, researchers have shown that monocytes were induced to secrete VEGF in hypoxia through HIF-1-α [268]. It was shown in the initial experiments that U937 cells do secrete VEGF. Yet the effect of hypoxia on these cells may lead to a reduction of their VEGF secretion. This was confirmed on observing the microscopic images of the hypoxic samples where there was a reduction in the number of U937 cells which may explain the lower VEGF levels in hypoxic PC3 immunocompetent tumouroids (section 5.4.1).
6.8.9 IL-10 increased in secretion in hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells

Overall, the IL-10 levels increased in all time points in hypoxic samples more than normoxic samples: day 1 (0.22 vs. 0.21 AU), day 7 (0.22 vs. 0.21 AU), day 10 (0.24 vs. 0.22 AU) and day 14 (0.26 vs. 0.23 AU) except for day 3 where normoxic samples had higher secretion than hypoxic samples (0.25 vs. 0.24 AU) respectively (section 5.4.9). This was unlike the LNCaP co-cultured samples where the IL-10 levels were higher in normoxic samples than in hypoxic samples also except for day 3.

Moreover, the PC3 cell line of PCA has been quoted to increase secretion of IL-10 when cultured alone, but in co-culture with PBMCs the secretion of IL-10 was augmented [269]. In addition, the same researchers did not find that LNCaP cells had a significant cytokine secretion when cultured alone or in co-culture with PBMCs. This observation correlates with the results of my experiments where the maximum mean absorbance of IL-10 was higher in PC3 (0.26 AU) than in LNCaP (0.23 AU) respectively.

The increased IL-10 secretion in hypoxia could be due to monocytes adding to the high IL-10 levels by secreting this cytokine themselves. It has been suggested that in hypoxic conditions monocytes develop a more cytotoxic phenotype and impede cancer progression through IL-10 secretion [270].

6.8.10 TNF-α increased in secretion in normoxic PC3 immunocompetent tumouroids co-cultured with U937 cells vis-à-vis hypoxic PC3 immunocompetent tumouroids co-cultured with U937 cells

Secretion of TNF-α was higher in hypoxic PC3 samples than in normoxic PC3 samples at days 1 (0.22 vs. 0.21 AU) and 3 (0.23 vs. 0.22 AU) respectively. However, at a later stage of the experiment, starting from day 7 until day 14, the TNF-α levels were slightly
higher in the normoxic than in the hypoxic samples: day 7 (0.22 vs. 0.21 AU), day 10 (0.22 vs. 0.21 AU) and day 14 (0.22 vs. 0.21 AU) respectively (section 5.4.10). This finding is the opposite of what was found in the LNCaP samples where TNF-α levels were higher in hypoxia at a later stage of the experiment. Interestingly, statistical significance was only found between the hypoxic and normoxic samples at day 14 and within the normoxic samples between day 1 and 14 only but not within the hypoxic samples at any given time point. The biological significance of this difference will be discussed below.

Biologically speaking, the TNF-α levels, only at the initial stages of the experiment, correlate with what has been reported that hypoxia increases the levels of TNF-α by PC3 cells, through the up-regulation of HIF-1α [271]. However, this did not translate into statistical significance.

In addition, the statistical significance within normoxic samples between day 1 and 14 and its increased TNF-α secretion vis-à-vis hypoxic samples (at day 14) goes against the literature which states that hypoxia is the main driver for TNF-α release by PC3 cells. The other cellular component to consider here is the U937 cells and whether hypoxia reduces their TNF-α secretion potential. A study by Sampson et al., showed that hypoxia did not induce TNF-α secretion in unstimulated U937 cells [272].

This corroborates what was found in these sets of experiments where TNF-α levels were not increased in hypoxic samples throughout the entirety of the experiment. Thus, it is inferred that any added amount of TNF-α was not secreted by the U937 cells in hypoxia which may have contributed to the low TNF-α levels in the hypoxic samples at the later experimental stages.
6.8.11 VEGF cytokine release increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

The VEGF secretion in the experiment in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells in normoxia showed a linear increase in VEGF secretion as follows: day 1 (0.18 AU), day 3 (0.23 AU) and day 7 (0.27 AU) with the maximum VEGF secretion attained on day 10, at 0.36 AU (section 5.4.14). Compared to that, when LNCaP immunocompetent tumouroids with unstimulated U937 cells were exposed to hypoxia and normoxia, the levels of VEGF at day 10 were 0.37 and 0.29 AU respectively.

Thus, it was evident from my results that when PMA-stimulated U937 cells were added to the LNCaP tumouroids, the VEGF level surged to a level higher than in LNCaP immunocompetent tumouroids with U937 cells in normoxic (0.36 vs 0.29 AU) but not hypoxic conditions. This is supported by a study where PMA-stimulated U937 cells were compared with U937 cells in a 2D model, with the results showing a much higher VEGF secretion in the PMA-stimulated U937 cells than in U937 cells [273].

Moreover, comparing the maximum mean absorbance level of VEGF attained in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells with the experiment where PC3 immunocompetent tumouroids were co-cultured with unstimulated U937 cells, it was found that VEGF levels at day 10 reached a maximum level of 0.24 and 0.27 AU in hypoxia and normoxia respectively. Hence, the levels of VEGF secretion in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells exceeded VEGF levels in PC3 immunocompetent tumouroids with co-culture of unstimulated U937 cells at day 10 in both hypoxic and normoxic samples.

However, when LNCaP simple tumouroids were compared to LNCaP immunocompetent tumouroids co-cultured with U937 cells, at day 10 the mean absorbance levels were 0.489
and 0.488 AU respectively. Thus, there was an increase in VEGF beyond the level found when LNCaP immunocompetent tumouroids were co-cultured with PMA-stimulated U937 cells.

The reason why the VEGF secretion, in LNCaP immunocompetent tumouroids co-cultured with PMA stimulated U937 cells, did not increase to levels higher than all the previous experiments, may be due to several factors, one of these factors could be the reduced number of M2 macrophages. This particular phenotype of macrophages is known to secrete high amounts of VEGF [274]. In addition, the polarisation into M2 phenotype is dependent on secretion of MFG-E8 this is known to be produced by PCA cells in vivo [275]. However, whether LNCaP cells within the 3D immunocompetent tumouroid model produce a large amount of this factor to cause an elevated number of M2 macrophages and subsequently a major surge in VEGF was difficult to decipher from the current experiments.

It is well known, as previously mentioned, that hypoxic environments lead to a surge in VEGF as is the case in the TME in vivo. As this condition was not present in this experiment, this may form another reason as to why a profound surge in VEGF was not evident. Macrophages, specifically, have been quoted to respond to hypoxic environments by inducing a heightened secretion of VEGF through upregulation of HIF-1 and HIF-2 [276].

**6.8.12 IL-10 cytokine release slightly increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells**

Levels of IL-10 in LNCaP immunocompetent tumouroids with co-culture of PMA-stimulated U937 cells increased linearly throughout the time period of the experiment: day 1 (0.22 AU), day 3 (0.23 AU) and day 7 (0.24 AU) respectively, reaching a plateau at day 10 with a mean absorbance level of 0.24 AU (section 5.4.15). In comparison, at
day 10 LNCaP immunocompetent tumouroids co-cultured with unstimulated U937 in hypoxia and normoxia the maximum secretion of IL-10 was 0.22 and 0.23 AU respectively. Whereas, at day 10 of PC3 immunocompetent tumouroids with unstimulated U937 in hypoxia and normoxia, IL-10 secretion reached mean absorbance levels of 0.24 and 0.22 AU respectively. Thus, these results showed that co-culture of PMA-stimulated U937 cells in LNCaP immunocompetent tumouroids led to slightly higher amounts of IL-10 than the levels recorded in immunocompetent tumouroids with both PCA cell lines and U937 in both hypoxic and normoxic conditions.

In addition, on comparing the results of the LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells with the initial experiment of LNCaP simple tumouroids and LNCaP immunocompetent tumouroids with co-culture of unstimulated U937 cells, the mean absorbance levels at day 10 were 0.21 and 0.25 AU respectively. Even though the IL-10 levels in the experiments with PMA-stimulated U937 cells yielded higher quantities than the LNCaP simple tumouroids, the opposite was true when the IL-10 levels were analysed in the LNCaP immunocompetent tumouroids were co-cultured with U937 cells.

Macrophages are known to be profound producers of IL-10 [277]. This may explain why overall, except for the initial co-cultured PCA tumouroid experiment, that the levels in the PCA tumouroid experiments with PMA-stimulated U937 were higher than those recorded in the previous experiments. In addition, it has been documented that macrophages of the M2 phenotype release elevated levels of IL-10 in PCA [278]. This could imply that M2 macrophages were present in the cellular milieu of the PCA tumouroid model.
6.8.13 TNF-α cytokine release increased in immunocompetent LNCaP tumouroids co-cultured with PMA-stimulated U937 cells

The levels of TNF-α secretion in immunocompetent LNCaP tumouroids with co-culture of PMA-stimulated U937 cells at day 10 was greatly elevated compared to all the previous experiments with U937 co-culture. Here, TNF-α secretion was observed to increase linearly with advancement of time: day 1 (0.21 AU), day 3 (0.22 AU) and day 7 (0.24 AU) reaching a maximum mean absorbance of 0.28 AU on day 10 (section 5.4.16).

The levels secreted by LNCaP immunocompetent tumouroids co-cultured with PMA-stimulated U937 cells was higher than the TNF-α secretion in both hypoxic and normoxic LNCaP immunocompetent tumouroids co-cultured with unstimulated U937 cells where the mean absorbance levels at day 10 were 0.25 and 0.22 AU respectively.

Also, the levels of TNF-α at day 10 in LNCaP immunocompetent tumouroids with PMA-stimulated U937 co-culture were much higher than PC3 immunocompetent tumouroids with unstimulated U937 cells. In the latter, TNF-α secretion at day 10 amounted to 0.21 AU in hypoxia and 0.22 AU in normoxia respectively.

Further, if the TNF-α secretion is compared with LNCaP simple tumouroids in the initial experiments, the mean absorbance level at day 10 in these experiments was 0.24 AU. Whereas, in the initial LNCaP immunocompetent tumouroid co-cultured with U937 cells at day 10 the mean absorbance level attained was 0.21 AU. In comparing these two experiments, the LNCaP immunocompetent tumouroids co-cultured with PMA-stimulated U937 showed a greater level of TNF-α secretion.

Overall, TNF-α secretion was augmented on addition of PMA-stimulated U937 cells to LNCaP tumouroids to a level higher than all the other experiments where U937 cells were added. These results are in line with literature which indicates that macrophages produce high levels of TNF-α [264]. Interestingly, within the TME, M1 macrophages are noted to
be the main secretors of TNF-$\alpha$ [279]. Thus, according to our results it seems that the macrophagic milieu in these immunocompetent tumouroids may consist of a higher number of M1 phenotype macrophages.

This surge in TNF-$\alpha$ by macrophages in the PCA setting is corroborated in a study where this cytokine secretion correlated with a higher Gleason score of PCA in human prostate biopsy specimens [280]. Thus, the 3D immunocompetent tumouroid setting could determine the severity of PCA as is the case in vivo. However, to date there has not been, to my knowledge, any studies that measure TNF-$\alpha$ secretion in PCA 3D compressed collagen models with U937 derived macrophages.

6.8.14 PMA stimulation in macrophage differentiation

An important consideration is that there has been evidence in literature stating that monocytes could polarise into macrophages depending on the collagen environment that they are placed within (see section 1.14). However, the research that reported this used PBMCs and not U937 cells as is the case in this PhD. The mixture of cells (PBMCs) may have provided the environment which enabled the monocyte to macrophage differentiation [176]. The researchers also used a different time-line to that used in the course of my research, which was 120hrs (5 days) as opposed to 14 days.

In this PhD, after experiments using (unstimulated) U937 cells yielded generally modest effects, the decision was taken to use PMA to differentiate U937 cells to include in tumouroids.

On doing so, it was observed from the cytokinic analyses of the PCA tumouroids with the PMA-stimulated monocytes that TNF-$\alpha$ secretion was elevated to a higher degree than the levels recorded when unstimulated monocytes were added into the tumouroids. On this basis it was considered that the PMA stimulation of the monocytes favoured one subset of macrophages over the other, i.e., the M1 macrophage, as it is the predominant
secretor of TNF-α. Whilst the VEGF and IL-10 did not exceed in their levels the previous experiments with the unstimulated monocytes. This led to the conclusion that the M2 phenotype of macrophages might be less in amount and therefore exceeding a much less influence on secretion of VEGF and IL-10 than the M2 phenotypyope on TNF-α. However, other researchers (section 1.14) used M1 and M2 specific markers to demonstrate that collagen polarizes monocytes into M1 and M2 macrophages, this analysis was not performed in this PhD - a limitation that will be addressed in future work.

**Summary and Conclusion**

Producing an immunocompetent model using the 3D Raft model for studying cancer at a cellular level is an important tool for cancer research. Not only does it offer a replicable environment *in vitro* but it also allows for a deeper technical knowledge of the model itself and cellular interactions within it.

In addition, the biophysical nature of the collagen model and its effect on cellular motility was explored. In non-compressed gels U937 cells travelled more than in compressed gels. When the U937 cells were co-cultured with LNCaP cells it was observed that the depth of penetration of U937 cells increased with time.

When U937 cells were co-cultured with LNCaP cells in 3D immunocompetent tumouroid models, it was observed that there were differences in spheroid growth compared to LNCaP simple tumouroids. Interestingly, PCA spheroidal growth continued until day 21. On implanting PMA-stimulated U937 cells (macrophages) in the PCA tumouroid model, differences were also found in spheroid growth vis-à-vis co-culture with U937 cells and again spheroids continued to grow.

It was also found that cytokinetic secretion by U937 increases on compression of collagen gels to statistically significant levels, in particular: VEGF, IL-10 and TNF-α. Also,
monocytes in the form of U937 cells and PMA-stimulated U937, as per our findings, have shown that if cultured in 3D models influence the cytokinic profile of these tumouroids. In the initial experiments, VEGF was elevated more in LNCaP simple tumouroids than in co-culture tumouroids. IL-10 expression, on the other hand, showed an elevation in co-culture tumouroids for the whole period of incubation compared to LNCaP simple tumouroids. However, TNF-α levels were reduced in co-cultured tumouroids compared to LNCaP simple tumouroids.

Furthermore, on subjecting the LNCaP immunocompetent tumouroids co-cultured with U937 cells to hypoxic environments, there were changes in cytokinic secretion vis-à-vis normoxic conditions. VEGF, the protumourigenic cytokine, was elevated in hypoxic conditions compared to normoxic conditions. The finding that VEGF increases in hypoxia mimics what was found in the TME in vivo [281]. Thus, it could be concluded that the 3D collagen models do, to a high degree, mimic in vivo conditions. IL-10, on the other hand, showed there was a higher secretion at day 14 in normoxic than hypoxic samples respectively. TNF-α levels were elevated markedly at days 10 and 14 in hypoxic compared to normoxic LNCaP immunocompetent tumoroids.

The PC3 immunocompetent tumouroids were also subjected to hypoxic and normoxic conditions and their cytokine secretion was analysed. In the normoxic immunocompetent tumouroids there was an increase in VEGF levels more than in the hypoxic immunocompetent tumouroids at days 7, 10 and 14.

IL-10 cytokine release in PC3 immunocompetent hypoxic tumouroids was increased more than in the normoxic tumouroids at days; 1, 7, 10 and 14. TNF-α levels were higher in normoxic samples than in hypoxic samples at days; 7, 10 and 14.

Moreover, the final set of experiments where cytokine analysis occurred on subjecting LNCaP immunocompetent tumouroids co-cultured with PMA-stimulated U937 cells also...
provided interesting results. With regards to VEGF, the levels attained with PMA-stimulated U937 cells were higher than in normoxia in LNCaP and PC3 immunocompetent tumouroids at day 10. Also, the LNCaP immunocompetent tumouroids co-cultured with PMA-stimulated U937 samples secreted VEGF to levels that exceeded those levels secreted in hypoxic, day 10, PC3 but not LNCaP immunocompetent tumouroids co-cultured with U937 cells.

IL-10, however, in the LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells far exceeded all the previous experiments except the initial experiment with LNCaP immunocompetent tumouroids co-cultured with U937 cells. In addition, the TNF-α levels secreted in LNCaP immunocompetent tumouroids with PMA-stimulated U937 cells were higher than all the readings analysed in all the previous experiments.

Overall, the above findings are complex and warrant further investigation. It is important that the thesis demonstrated viable co-cultures of monocytes/macrophages and prostate cancer cells in a 3D compressed collagen (tumouroid) model over 2-3 weeks. What appear the most optimal conditions is the use of PMA-stimulated U937 cells in tumouroids, the condition of hypoxia and an awareness that cultures of immunocompetent tumouroids are quite “active” around day 7 and beyond.

**Future work**

Future directions in the field of immunocompetent tumouroids, should include both technical advances and cellular complexity.

The utilisation of confocal microscopy should be extensively adopted, to enhance the image quality and enable a better visualisation of cellular morphology as well as spheroid growth analysis in the 3D model, supported by robust semi-quantitation. Live imaging, early on and at later points would be ideal. Electron microscopy could be used to add more detailed imaging to analyse the interaction between cancer and immune cells.
Due to the complex phenotypic divisions of immune cells such as monocytes and macrophages in the TME, it would be useful to examine whether the phenotypes of immune cells in the 3D tumouroid model correspond to those found *in vivo*. FACS could be used to examine the levels of these subsets of cells.

Although it has been reported that pro-monocytic U937 cells are very similar in their behaviour to PBMCs, it would also be interesting to co-culture the latter cells with PCA cells and see whether the results in the realms of depth of penetration within the 3D model, spheroid growth and cytokinic milieu are comparable to the *in vivo* conditions. In addition, the use of fibrillin and laminin are key components of the extracellular matrix in the TME *in vivo* and could be incorporated within the tumouroid model.

What is more, I propose that further cell types are added within the 3D tumouroid model. Although the monocyte is the initiator of the immune response in the field of carcinogenesis, it would be more comparable to the TME if more cells were added into the cellular milieu within the 3D model. In addition to stromal cells, such as fibroblasts and endothelial cells, of interest would be to incorporate T-cells, as these cells through the recognition of Tumour Associated Antigens (TAAs) could be directed to eliminate cancer cells through cytotoxicity. This would enable cellular crosstalk to mimic the *in vivo* scenario and would also harness more cell types involved in immunotherapies.

Hence, correlation of the results attained from 3D models with *in vivo* tumour models would enable us to gain further insight into the degree that *in vitro* models compare to *in vivo* models. As all elements of tumouroids are easily reproducible, such as concentrations of matrix proteins, cell numbers added and stiffness, in addition to using the RAFT system which is semi-automated, therefore it is not difficult to scale up to create a tumouroid platform. Thus, truly immunocompetent tumouroids would be novel tools for the testing of immunotherapies.
Figure A1: Heatmap of all cytokines measured using Luminex. All measurements are normalized against internal standard curves. The numbers in the heatmaps are in pg/ml. Results are shown for uncompressed and compressed collagen gels over 6 time-points: 1hour, 3hours, 6 hours, day 1, day 3 and day 7. On the y-axis the molecules are grouped as follows: regulatory ILs (IL-23 and IL-7), protumourigenic ILs (IL-4, IL-10 and IL-12) and suppressive ILs (IL-1α, IL-1β, IL-6 and IL-8). MMPs and miscellaneous cytokines that are known to be secreted by PCA are also shown. All experiments were done in 3 independent repeats.
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