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Immunophenotyping pancreatic cystic lesions for cancer risk stratification

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Ph.D. Thesis

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Declaration

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Abstract

Pancreatic cancer (PC) remains one of the most lethal malignancies worldwide, with a five-year survival rate of approximately 13%. This poor prognosis stems largely from the aggressive and asymptomatic nature of the disease which frequently results in late-stage clinical presentation. PC is generally poorly responsive to standard-of-care therapies, with patients achieving little to no improvements in overall survival, particularly due to the development of treatment resistance. One promising avenue for early detection and prevention of PC lies in the study of pancreatic cystic lesions (PCLs). PCLs are fluid-filled structures found within or on the surface of the pancreas. While many PCLs are benign, others such as intraductal papillary mucinous neoplasms (IPMNs) or mucinous cystic neoplasms (MCNs), possess the ability to undergo malignant transformation and can be regarded as precursor lesions of PC. The immune landscape of pre-malignant PCLs and its role in determining malignant potential remain poorly understood. Given that PC is typically considered an “immunologically cold” tumour, understanding the mechanisms involved in cystogenesis and tumourigenesis may be the key to identifying novel immune biomarkers within pre-malignant PCLs to further define existing stratification frameworks and provide high-risk patients with the appropriate surveillance and timely intervention. This investigative approach may also provide avenues of earlier PC diagnosis and intervention. Therefore, this study aims to 1) characterise the immune cell infiltrate and immunomodulatory secretome of risk-stratified PCL cyst fluid for improved cancer risk stratification, 2) investigate the influence of the soluble immunomodulatory secretome of risk-stratified pancreatic cyst fluid (PCF) on anti-tumour effector cell function and 3) elucidate key chemokine profiles in risk-stratified PCF to identify potential chemokine-based therapeutic targets that may enhance effector immune cell infiltration into the tumour microenvironment (TME).

This thesis aimed to characterise the local and systemic immune microenvironment of pre-malignant PCLs and to identify immune-related biomarkers and therapeutic targets associated with progression risk. Immunophenotyping was performed by flow cytometry on the immune cell infiltrate of PCF and patient-matched peripheral blood mononuclear cells (PBMCs). In parallel, the soluble immune microenvironment of PCLs was profiled using multiplex enzyme-linked immunosorbent assay (ELISA) platforms to quantify the expression of immunomodulatory mediators including cytokines, chemokines, growth factors, and adhesion molecules in risk-stratified PCF. Finally, co-culture assays and

lymphocyte killing assays were employed to assess the impact of PCF from benign pseudocysts, low-risk PCLs, and high-risk PCLs on effector immune cell phenotype and function.

Data from this study revealed that adaptive immune cell populations, including CD4⁺ T cells, CD8⁺ T cells, and natural killer (NK) cells, predominantly compose the immune cell infiltrate of pre-malignant PCF. However, these effector immune cells exhibited features of functional exhaustion, suggesting early immune dysfunction within the cyst microenvironment. Lymphocyte killing assays demonstrated that benign and low-risk PCF enhanced lymphocyte-mediated cytotoxicity against PC cells whereas high-risk PCF failed to do so, implying progressive immune silencing with disease progression. Furthermore, low-risk PCF was found to have an immune-active phenotype, characterised by abundant soluble cytokines, growth factors, and chemotactic factors, while high-risk PCLs exhibited diminished immune activity, consistent with the emergence of an immunosuppressive phenotype. Additionally, migration of T cells and NK cells was significantly increased towards PCF from patients with a low risk of PC development, a finding not observed with high-risk PCF or benign pseudocyst fluid. Collectively, these findings support a model in which immune-active low-risk lesions evolve into high-risk states over time, losing their immune-active phenotype and enabling malignant transformation.

In conclusion, findings from this thesis advance the current understanding of immune dysregulation in the pre-malignant PCL setting and highlights the potential of immune-based biomarkers for cancer risk stratification in patients with PCLs. By defining key immunological alterations associated with PCL progression, this study provides a rationale for early immunomodulatory intervention aimed at maintaining immune surveillance and preventing malignant progression.

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- **Establishment of a novel multi-omic biomarker panel in cyst fluid and blood for stratifying patient risk of pancreatic cancer.** Authors: Laura E. Kane, Gregory S. Mellotte, Rebecca Lyons, Eimear Mylod, Simone Marcone, Paul F. Ridgway, Finbar MacCarthy, Kevin C. Conlon, Joanne Lysaght, Barbara M. Ryan, Stephen G. Maher. *Cancer Research*, 2022.
- **Profiling the immune component of pancreatic cyst fluid to identify novel biomarkers of patient cancer risk.** Authors: Laura E. Kane, Gregory S. Mellotte, Rebecca Lyons, Eimear Mylod, Simone Marcone, Paul F. Ridgway, Kevin C. Conlon, Barbara M. Ryan, Joanne Lysaght, Stephen G. Maher. *Cancer Research*, 2022.

Manuscripts in preparation

- **Chemotactic effects of risk-stratified pre-malignant pancreatic cyst fluid on effector immune cells and its role in anti-tumour immunity.** Authors: Rebecca Lyons, Gregory Mellotte, Brendan Moran, Aoife Kilgallon, Laura E. Kane, Barbara M. Ryan, Stephen G. Maher, Joanne Lysaght. **Under preparation for submission.*
- **The impact of risk-stratified pre-malignant pancreatic cyst fluid on T cell function and phenotype: Implications for anti-tumour immunity.** Authors: Rebecca Lyons, Gregory Mellotte, Brendan Moran, Aoife Kilgallon, Laura E. Kane, Barbara M. Ryan, Stephen G. Maher, Joanne Lysaght. **Under preparation for submission.*
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Table of contents

Declaration	ii
Abstract.....	iii
Acknowledgements.....	v
PhD outputs	viii
Table of contents.....	xii
List of figures	xix
List of tables.....	xxiii
Abbreviations.....	xxv
 Chapter 1: General Introduction	 1
1.1 Pancreatic cancer.....	2
1.1.1 Overview	2
1.1.2 Clinical presentation.....	2
1.1.3 Tumour staging.....	3
1.1.4 Current treatments	4
1.1.4.1 Surgery	4
1.1.4.2 Radiation therapy	5
1.1.4.3 Chemotherapy	5
1.1.4.4 Immunotherapy	6
1.1.5 Desmoplastic microenvironment of PC	7
1.1.6 Risk factors of PC	10
1.2 PCLs as potential avenues for enhanced cancer risk stratification and earlier PC diagnosis.....	10
1.2.1 Pancreatic cystic lesions.....	10
1.2.1.1 Non-neoplastic lesions	10
1.2.1.1.1 Pseudocysts	10
1.2.1.2 Neoplastic lesions.....	11
1.2.1.2.1 Pancreatic intraepithelial neoplasms	13
1.2.1.2.2 Intraductal papillary mucinous neoplasms	14
1.2.1.2.3 Mucinous cystic neoplasms.....	15
1.3 Immunology of PCLs	15
1.4 Co-morbidities associated with increased incidence of PCLs	17

1.4.1 Inflammation-driven co-morbidities.....	17
1.4.1.1 Pancreatitis.....	17
1.4.1.2 Diabetes mellitus.....	18
1.4.2 Genetically-driven co-morbidities	19
1.4.2.1 Von Hippel-Lindau disease	19
1.5 Biological framework for cancer risk stratification and management of pre-malignant PCLs.....	19
1.5.1 Clinical evaluation of PCLs.....	19
1.5.2 Diagnostic guidelines.....	20
1.5.3 Lack of consensus in PCL management and stratification guidelines.....	22
1.6 Potential immunobiological biomarkers for cancer risk stratification of PCLs	24
1.7 Summary.....	29
1.8 Hypothesis and aims	29
1.8.1 Overall hypothesis	29
1.8.2 Overall aim	29
1.8.3 Specific aims.....	29
Chapter 2: Materials and methods.....	30
2.1 Ethical approval	31
2.2 Patient sample acquisition	31
2.3 Patient PCF collection and storage	31
2.4 Patient blood collection and storage	32
2.5 Patient demographics	32
2.6 Materials	36
2.6.1 Preparation of buffers and media	36
2.7 Methods	36
2.7.1 PBMC isolation by density gradient centrifugation	36
2.7.2 Cell counting.....	37
2.7.3 Anti-CD3 and anti-CD28 antibody T cell activation	38
2.7.4 Co-culture of PBMCs with risk-stratified PCF.....	38
2.7.5 Flow cytometric staining	38
2.7.5.1 Extracellular flow cytometric staining.....	38
2.7.5.2 Intracellular flow cytometric staining.....	39
2.7.5.3 Flow cytometric acquisition and analysis	41

2.7.5 Boyden chamber chemotaxis assay	41
2.7.7 Quantification of soluble immune factors by flow cytometry-based AssayGenie multiplex ELISA	42
2.7.8 Quantification of soluble immune factors by MSD multiplex ELISA.....	43
2.7.9 Quantification of soluble immune factors by sandwich ELISA	44
2.7.10 Lymphocyte-mediated cytotoxicity assay	44
2.7.11 Cell culture	45
2.7.11.1 Pancreatic cell line maintenance	45
2.7.11.2 Cell line sub-culture	46
2.7.11.3 Cryopreservation and reconstitution of frozen cell stocks	47
2.7.11.4 Mycoplasma testing.....	47
2.7.12 Preparation of whole cell lysates from pancreatic cell lines	48
2.7.13 Cytoplasmic and nuclear extraction from pancreatic cell lines.....	49
2.7.14 Total protein quantification by BCA protein assay	50
2.7.15 SDS-PAGE	50
2.7.16 Western blot analysis to determine fractionation efficiency	51
2.7.17 Statistical analysis	53

Chapter 3: Immunophenotyping the immune cell infiltrate of pancreatic cystic lesions and the associated role these cellular immune profiles play in malignant progression

3.1 Introduction	55
3.2 Hypothesis	57
3.3 Specific aims	57
3.4 Results	58
3.4.1 PCF has a high proportion of CD4 ⁺ T cells and CD8 ⁺ T cells.	58
3.4.2 PCF has a significantly lower proportion of adaptive immune cells compared to matched PCL-associated PBMCs.	60
3.4.3 PCL-associated PBMC samples have a significantly higher proportion of CD4 ⁺ T helper cells compared to PBMCs from healthy, non-PCL donors.	62
3.4.4 PCF has a significantly lower proportion of innate immune cell populations compared to matched PCL-associated PBMC samples.	64
3.4.5 PCL-associated PBMC samples have a significantly higher proportion of macrophages compared to PBMCs from healthy, non-PCL donors.....	66

3.4.6 PCF has a significantly higher frequency of effector memory T cells compared to matched PCL-associated PBMC samples.....	68
3.4.7 The systemic immune system of PCL patients has a lower frequency of central memory and effector memory T cell subsets compared to PBMCs from healthy, non-PCL donors.....	71
3.4.8 PCF has a higher frequency of exhausted CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.	73
3.4.9 The systemic immune system of PCL patients has an increased frequency of exhausted T cells compared to PBMCs from healthy, non-PCL donors.....	75
3.4.10 PCF has higher frequencies of circulating mildly cytotoxic T cells and terminally exhausted resident T cells compared to matched PCL-associated PBMCs.....	77
3.4.11 The systemic immune system of PCL patients has significantly higher frequency of quiescent resident CD8 ⁺ T cells and significantly lower frequency of proliferative circulating CD8 ⁺ T cells.....	80
3.4.12 PCF has a significantly increased frequency of intracellular perforin in CD8 ⁺ T cells and a significantly decreased frequency of intracellular granzyme B in NK cells.	83
3.4.13 The systemic immune system of PCL patients has a significantly higher frequency of CD107a ⁺ effector immune cell degranulation and decreased perforin and IFN- γ cytokine production.	85
3.4.14 PCF has a significantly increased frequency of LAG-3 on CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.....	87
3.4.15 The systemic immune system of PCL patients has a significantly increased frequency of TIM-3 ⁺ T cells.	89
3.4.16 PCF has a significantly increased frequency of PD-L1 on CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.....	92
3.4.17 PBMCs from PCL patients have a significantly decreased frequency of PD-L1 and CD155 on CD8 ⁺ T cells.....	94
3.4.18 LAG-3 expression is significantly increased in intermediary neoplastic pancreatic cells and expression is increased in PC cells upon exposure to stress conditions.....	96
3.4.19 The expression of immune checkpoint ligands is altered in pancreatic cell lines following exposure to stress conditions.	100

3.4.20 Soluble adhesion molecules are differentially expressed across risk-stratified PCF.....	104
3.4.21 Soluble acute phase proteins are differentially expressed across risk-stratified PCF.....	106
3.4.22 Soluble cell-mediated immune response factors are differentially expressed across risk-stratified PCF.	108
3.4.23 Soluble humoral immune factors are differentially expressed across risk-stratified PCF.....	109
3.4.24 Soluble Th17-associated immune factors are differentially expressed across risk-stratified PCF.	112
3.4.25 Soluble lymphocyte development and differentiation factors are differentially expressed across risk-stratified PCF.....	114
3.4.26 Soluble immune checkpoint molecules are differentially expressed across risk-stratified PCF.	115
3.5 Discussion	117
3.6 Study limitations	122
3.7 Graphical summary	123

Chapter 4: The impact of pancreatic cyst fluid on T cell function and phenotype: implications for anti-tumour immunity 124

4.1 Introduction	125
4.2 Hypothesis.....	127
4.3 Specific aims	127
4.4 Results	128
4.4.1 Benign pancreatic pseudocyst fluid significantly decreases lymphocyte viability.....	128
4.4.2 Benign pancreatic pseudocyst fluid significantly decreases the expression of activation markers on CD4 ⁺ T cells and CD8 ⁺ T cells.	129
4.4.3 Benign pseudocyst fluid increases the frequency of central memory and effector memory T cells and NK cells.....	133
4.4.4 Benign pseudocyst fluid significantly decreases the frequency of CD103 expression on CD4 ⁺ and CD8 ⁺ T cell subsets.	136
4.4.5 Pre-malignant PCF maintains CD4 ⁺ T cells, CD8 ⁺ T cells and NK cells in an exhausted state.....	137

4.4.6 Activated T cells employ a predominant Th1 phenotype following co-culture with PCF, regardless of cancer risk stratification status.	141
4.4.7 The cytotoxic capacity of cytotoxic CD8 ⁺ T cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.	145
4.4.8 Changes in the cytotoxic capacity of CD8 ⁺ T cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.	148
4.4.9 The cytotoxic capacity of NK cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.	150
4.4.10 Changes in the cytotoxic capacity of NK cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.	153
4.4.11 Effector cell-mediated cytotoxicity is promoted upon exposure to pseudocyst fluid and low-risk PCF but remains unaffected by high-risk PCF.	155
4.5 Discussion.....	157
4.6 Study limitations.....	165
4.7 Graphical summary.....	166

Chapter 5: Chemotactic effects of risk-stratified pancreatic cyst fluid on effector immune cells 167

5.1 Introduction.....	168
5.2 Hypothesis	170
5.3 Specific aims.....	170
5.4 Results.....	171
5.4.1 Low-risk PCF exhibits higher effector immune cell migration compared to high-risk PCF and benign pseudocyst fluid.	171
5.4.2 Lower concentrations of high-risk PCF do not permit effector immune cell migration.	174
5.4.3 Effector immune cell migration is increased towards high-risk PCF following a spike-in of recombinant fractalkine/RANTES/I-TAC chemokine cocktail.	177
5.4.4 Soluble innate immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.	180

5.4.5 Soluble adaptive immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.	184
5.4.6 Soluble angiogenic and growth factors are differentially expressed across risk-stratified PCF.	187
5.4.7 Chemokine receptor expression is altered following co-culture with pre-malignant PCF.....	190
5.4.8 Under stress conditions, CD54 expression is increased in intermediary pancreatic cells but remains unaffected in normal pancreatic cells and PC cells.....	194
5.4.9 Under stress conditions, cell surface galectin-3 expression is decreased in intermediary pancreatic cells and PC cells but upregulated in normal pancreatic cells.....	197
5.4.10 Soluble galectin-3 is increased in high-risk PCF and predominantly localises in the cytoplasmic compartment of pancreatic cell lines.....	200
5.5 Discussion	204
5.6 Study limitations	211
5.7 Graphical summary	212
Chapter 6: Concluding discussion and future directions	213
6.1 Discussion	214
6.2 Future directions.....	221
References	225

List of figures

Chapter 1

Figure 1.1: Symptomology associated with advanced PC.....	3
Figure 1.2: Development of a desmoplastic microenvironment from normal epithelial tissue to pancreatic cystic lesion to pancreatic ductal adenocarcinoma tissue.	9
Figure 1.3: Presentation of PCLs within the pancreas.	11
Figure 1.4: Classification of pancreatic lesions.	13
Figure 1.5: The evolution of the immune T cell landscape during progression of pancreatic IMPN cysts to the development of invasive PDAC.	16

Chapter 2

Figure 2.1: PBMC isolation by density gradient centrifugation.....	37
Figure 2.2: Representative image of haemocytometer grid used in the process of cell counting	37

Chapter 3

Figure 3.1: PCF has a high proportion of CD4 ⁺ T cells and CD8 ⁺ T cells which is also reflected in matched PCL samples.....	59
Figure 3.2: PCF has a significantly lower proportion of adaptive immune cells compared to matched PCL-associated PBMC samples.....	61
Figure 3.3: PCL-associated PBMC samples have a significantly higher proportion of CD4 ⁺ T helper cells compared to PBMCs from healthy, non-PCL donors.	63
Figure 3.4: PCF has a significantly lower proportion of innate immune cell populations compared to matched PCL-associated PBMC samples.	65
Figure 3.5: PCL-associated PBMC samples have a significantly higher proportion of macrophages compared to PBMCs from healthy, non-PCL donors.	67
Figure 3.6: PCF has a significantly higher frequency of effector memory T cells compared to matched PCL-associated PBMC samples.....	70
Figure 3.7: PCL-associated PBMC samples have a lower frequency of central memory and effector memory T cell subsets compared to PBMCs from healthy, non-PCL donors.....	72

Figure 3.8: PCF has a higher frequency of exhausted CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.....	74
Figure 3.9: PCL-associated PBMC samples have an increased frequency of exhausted T cells compared to PBMCs from healthy, non-PCL donors.	76
Figure 3.10: PCF has higher frequencies of circulating mildly cytotoxic T cells and terminally exhausted resident T cells compared to matched PCL associated PBMC samples.	79
Figure 3.11: PCL-associated PBMC samples have a significantly higher frequency of quiescent resident CD8 ⁺ T cells and significantly lower frequency of proliferative circulating CD8 ⁺ T cells compared to PBMCs from healthy, non-PCL donors.....	82
Figure 3.12: PCF has a significantly increased frequency of intracellular perforin in CD8 ⁺ T cells and a significantly decreased frequency of intracellular granzyme B in NK cells compared to matched PCL-associated PBMC samples.	84
Figure 3.13: PCL-associated PBMC samples have a significantly higher frequency of CD107a ⁺ effector immune cell degranulation and decreased perforin and IFN- γ cytokine production compared to PBMCs from healthy, non-PCL donors.	86
Figure 3.14: PCF has a significantly increased frequency of LAG-3 on CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.	88
Figure 3.15: PCL-associated PBMC samples have a significantly increased frequency of TIM-3 ⁺ T cells compared to PBMCs from healthy, non-PCL donors.....	91
Figure 3.16: PCF has a significantly increased frequency of PD-L1 on CD8 ⁺ T cells compared to matched PCL-associated PBMC samples.	93
Figure 3.17: PCL-associated PBMC samples have a significantly decreased frequency of PD-L1 and CD155 on CD8 ⁺ T cells compared to PBMCs from healthy, non-PCL donors.	95
Figure 3.18: LAG-3 expression is significantly increased in intermediary pancreatic cells and expression is increased in PC cells upon exposure to stress conditions.....	99
Figure 3.19: The expression of immune checkpoint ligands is altered in pancreatic cell lines following exposure to stress conditions.....	103
Figure 3.20: Soluble adhesion molecules are differentially expressed across risk-stratified PCF.....	105
Figure 3.21: Soluble acute phase proteins are differentially expressed across risk-stratified PCF.....	107

Figure 3.22: Soluble cell-mediated immune response factors are differentially expressed across risk-stratified PCF.....	109
Figure 3.23: Soluble humoral immune factors are differentially expressed across risk-stratified PCF.	111
Figure 3.24: Soluble Th17-associated immune factors are differentially expressed across risk-stratified PCF.....	113
Figure 3.25: Soluble lymphocyte development and differentiation factors are differentially expressed across risk-stratified PCF.	114
Figure 3.26: Soluble immune checkpoint molecules are differentially expressed across risk-stratified PCF.....	116
Figure 3.27: Graphical summary of study findings from results chapter 3.	123

Chapter 4

Figure 4.1: Benign pancreatic pseudocyst fluid significantly decreases lymphocyte viability.	129
Figure 4.2: Benign pancreatic pseudocyst fluid significantly decreases the expression of activation markers on CD4 ⁺ T cells and CD8 ⁺ T cells.....	132
Figure 4.3: Benign pseudocyst fluid increases the frequency of central memory and effector memory T cells and NK cells.	135
Figure 4.4: Benign pseudocyst fluid decreases the frequency of CD103 expression on CD4 ⁺ and CD8 ⁺ T cell subsets.	137
Figure 4.5: Pre-malignant PCF maintains CD4 ⁺ T cells, CD8 ⁺ T cells and NK cells in an exhausted state.	140
Figure 4.6: Activated T cells employ a predominant Th1 phenotype following co-culture with pancreatic cyst fluid, regardless of cancer risk stratification status.....	144
Figure 4.7: The cytotoxic capacity of cytotoxic CD8 ⁺ T cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.	147
Figure 4.8: Changes in the cytotoxic capacity of CD8 ⁺ T cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.	149
Figure 4.9: The cytotoxic capacity of NK cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.	152

Figure 4.10: Changes in the cytotoxic capacity of NK cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.....	154
Figure 4.11: Effector cell-mediated cytotoxicity is promoted upon exposure to benign pseudocyst fluid and low-risk PCF but remains unaffected by high-risk PCF.	156
Figure 4.12: Graphical summary of study findings from results chapter 4.	166

Chapter 5

Figure 5.1: Low-risk PCF exhibits higher effector immune cell migration compared to high-risk PCF and benign pseudocyst fluid.	173
Figure 5.2: Lower concentrations of high-risk PCF do not permit effector immune cell migration.	176
Figure 5.3: Effector immune cell migration is increased towards high-risk PCF following a spike-in of recombinant fractalkine/RANTES/I-TAC chemokine cocktail.	179
Figure 5.4: Soluble innate immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.	182
Figure 5.5: Soluble adaptive immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.	186
Figure 5.6: Soluble angiogenic and growth factors are differentially expressed across risk-stratified PCF.	189
Figure 5.7: Chemokine receptor expression is altered following co-culture with pre-malignant PCF.....	193
Figure 5.8: Under stress conditions, cell surface and intracellular CD54 expression is increased in intermediary pancreatic cells but remains unaffected in normal pancreatic cells and PC cells.....	196
Figure 5.9: Under stress conditions, cell surface galectin-3 expression is decreased in intermediary pancreatic cells and PC cells but upregulated in normal pancreatic cells.....	199
Figure 5.10: Soluble galectin-3 is increased in high-risk PCF and predominantly localises in the cytoplasmic compartment of pancreatic cell lines.....	203
Figure 5.11: Graphical summary of study findings from results chapter 5.	212

List of tables

Chapter 1

Table 1.1: Pancreatic cancer staging for PDAC according to AJCC TNM staging.	4
Table 1.2: Approved chemotherapeutic agents currently used in the treatment of PC. ...	6
Table 1.3: Indications for the management of PCLs based on current clinical guidelines.	21
Table 1.4: Summary of immunobiological biomarkers under investigation in PCLs....	28

Chapter 2

Table 2.1: Patient demographic information for PCF samples used within this study. .	33
Table 2.2: Patient demographic information for stratification criteria for high-risk PCF samples used within this study.	35
Table 2.3: Required materials for buffer and media preparation.	36
Table 2.4: Fluorochrome-conjugated antibody panels used for extracellular and intracellular cytometric staining.	40
Table 2.5: Primary antibodies used for Western blotting to confirm fractionation efficiency.	52
Table 2.6: Secondary antibodies used for Western blotting to confirm fractionation efficiency.	53

Chapter 3

Table 3.1: Mean concentration of soluble adhesion molecules in PCF and patient-matched serum.	106
Table 3.2: Mean concentration of soluble acute phase proteins in PCF and patient-matched serum.	107
Table 3.3: Mean concentration of soluble cell-mediated immune factors in PCF and patient-matched serum.	110
Table 3.4: Mean concentration of soluble humoral immune factors in PCF and patient-matched serum.	111

Table 3.5: Mean concentration of soluble Th17-associated immune factors in PCF and patient-matched serum.	113
Table 3.6: Mean concentration of soluble lymphocyte development and differentiation factors in PCF and patient-matched serum.	115
Table 3.7: Mean concentration of soluble immune checkpoint molecules in PCF and patient-matched serum.	116

Chapter 5

Table 5.1: Mean concentration of soluble innate immune cell-associated chemokines in PCF and patient-matched serum.	183
Table 5.2: Mean concentration of soluble adaptive immune cell-associated chemokines in PCF and patient-matched serum.	187
Table 5.3: Mean concentration of soluble angiogenic and growth factors in PCF and patient-matched serum.	190

Abbreviations

ACG	American College of Gastroenterology
ACR	American College of Radiology
ADM	Acinar-to-ductal metaplasia
AGA	American Gastroenterological Association
ANOVA	Analysis of variance
AP	Acute pancreatitis
BCA assay	Bicinchoninic acid assay
BD	Branch-duct
BMI	Body mass index
BSA	Bovine serum albumin
CA19-9	Carbohydrate antigen 19-9
CAF	Cancer-associated fibroblast
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CEA	Carcinoembryonic antigen
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule 1
CER	Cytoplasmic extraction reagent
CP	Chronic pancreatitis
CRP	C-reactive protein
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
dH ₂ O	Deionised water
DM	Diabetes mellitus
dMMR	Deficient DNA mismatch repair
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEG	European evidence-based guidelines
ELISA	Enzyme-linked immunosorbent assay

EUS-FNA	Endoscope ultrasound-guided fine-needle aspiration
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HPDE cells	Human pancreatic duct epithelial cells
HPNE cells	Human pancreatic nestin-expressing cells
ICAM-1	Intracellular adhesion molecule 1
IFN- γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
IMP	Inflammatory mediator protein
IP-10	Interferon-gamma-induced protein 10
IPMN	Intraductal papillary mucinous neoplasm
I-TAC	Interferon-inducible T-cell alpha chemoattractant
LAG-3	Lymphocyte-activation gene 3
LAMP-1	Lysosomal-associated membrane protein 1
MCN	Mucinous cystic neoplasm
MCP-1	Monocyte chemoattractant protein 1
MCP-4	Monocyte chemoattractant protein 4
MD	Main-duct
MDC	Macrophage-derived chemokine
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MIF	Macrophage migration-inhibitory factor
MIP-1 α	Macrophage inflammatory protein 1 alpha
MIP-1 β	Macrophage inflammatory protein 1 beta
MIP-3 α	Macrophage inflammatory protein 3 alpha
MRI	Magnetic resonance imaging
MSI-H	Microsatellite instability-high
nCLE	Needle-based confocal laser endomicroscopy

NER	Nuclear extraction reagent
NK cell	Natural killer cell
PAI-1	Plasminogen activator inhibitor 1
PanIN	Pancreatic intraepithelial neoplasia
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Pancreatic cancer
PCF	Pancreatic cyst fluid
PCL	Pancreatic cystic lesion
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1 receptor
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PGE ₂	Prostaglandin E ₂
PIGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene fluoride
RANTES	Regulated on activation, normal T cell expressed and secreted
RIPA buffer	Radio-immunoprecipitation assay buffer
RT	Radiation therapy
SAA	Serum amyloid A
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERPIN	Serine protease inhibitor
SHH	Sonic hedgehog
SLAMF6	Signalling lymphocytic activation molecule family member 6
TARC	Thymus and activation-regulated chemokine
TBE	Tris-borate-EDTA
TGF- β	Transforming growth factor-beta
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains

TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TMB-H	Tumour mutational burden-high
TME	Tumour microenvironment
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TTNB	Though-the-needle biopsy
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau
VISTA	V-domain immunoglobulin suppressor of T cell activation

Chapter 1:

General Introduction

1.1 Pancreatic cancer

1.1.1 Overview

Pancreatic cancer (PC) is the third leading cause of cancer-related mortality globally, with incidence rates increasing annually^[1]. It is regarded as one of the most lethal malignant diseases in the world, with a 5-year survival rate for all PC stages combined at a dismal 13%^[1]. PC subtypes can be divided into two distinct categories: exocrine (nonendocrine) PC, which accounts for up to 95% of all PCs, and neuroendocrine PC. Pancreatic ductal adenocarcinoma (PDAC) is the predominant subtype of PC, representing approximately 90-95% of all PC cases^[2, 3]. PC also has several uncommon histological subtypes which vary in symptomology and prognosis. These exocrine subcategories include squamous cell carcinoma, adenosquamous carcinoma and colloid carcinoma^[4]. Pancreatic neuroendocrine tumour subtypes generally develop from the endocrine gland of the pancreas. These tumours are classified as endocrine or islet cell tumours and account for less than 5% of all PC diagnosed^[4].

1.1.2 Clinical presentation

PC is notoriously difficult to detect and to diagnose at early stages of disease due to the aggressive nature of the cancer and late stage of clinical presentation. The symptoms associated with PC are often vague and non-specific, such as fatigue, indigestion, loss of appetite, unintended weight loss, back pain, and nausea, with early stages of disease developing asymptotically^[5] (**Figure 1.1**). Frequent clinical presentation of locally advanced or incurable metastatic disease occurs in up to 80% of patients and is largely responsible for the poor 5-year survival rates in PC, which further highlights the importance of early detection and diagnosis^[6]. Up to 80.9% of patients diagnosed with PC, particularly late-stage disease, die within one year of diagnosis^[7].

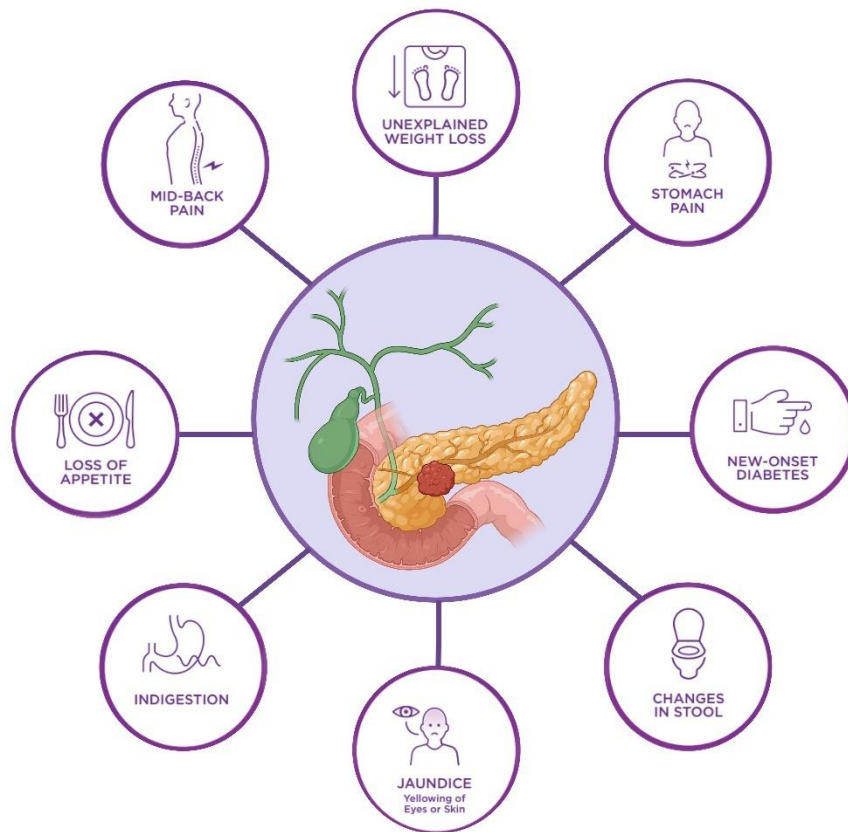


Figure 1.1: Symptomology associated with advanced PC. Early-stage PC typically develops asymptotically, while later stages of disease present with vague and non-specific symptoms such as abdominal or back pain, fatigue, indigestion, nausea, loss of appetite, unintended weight loss, and changes in bowel habits, often leading to delayed diagnosis and poor prognosis. Figure created with BioRender.com.

1.1.3 Tumour staging

The 8th edition of the American Joint Committee on Cancer (AJCC) TNM staging system is used in the staging of PC tumours (**Table 1.1**). This staging system is paramount to prognostic stratification and incorporates three assessment factors to optimise assessment. Tumour size and extent (T), lymph node involvement (N) and metastasis to distant sites (M) are taken into account^[8]. Early-stage PCs, although curable, are generally asymptomatic and as a direct result, advanced stage-III/IV disease are most commonly seen at clinical presentation^[6].

Table 1.1 Pancreatic cancer staging for PDAC according to AJCC TNM staging (8th ed.). This TNM staging system employs three independent factors relating to the tumour. The size and extent of the tumour (T) is classified as T1a, T1b, T1c, T2, T3, T4. Nodal involvement is another factor taken into consideration when staging PC tumours. Lymph node involvement (N) is classified as N0, N1, N2. The final factor taken into consideration is tumour metastasis (M) whereby M1 is an indicator that the tumour has invaded distal sites.

T	M	N0 No regional nodal involvement	N1 Involvement of 1-3 regional lymph nodes	N2 Involvement of 4 or more regional lymph nodes
T1a Tumour is confined to pancreas (<0.5 cm)	M0	IA	IIB	III
T1b Tumour is confined to pancreas (>0.5 cm and <1 cm)				
T1c Tumour is confined to pancreas (≥1 cm and ≤2cm)				
T2 Tumour is confined to pancreas (>2 cm to ≤4 cm)	M0	IB	IIB	III
T3 Tumour extends beyond the pancreas with no involvement of major vessels near pancreas (>4 cm)	M0	IIA	IIB	III
T4 Tumour involves the celiac axis, common hepatic artery or superior mesenteric artery	M0	III	III	III
Any T	M1 Metastasis to distant sites (liver, peritoneum, lungs, bone).	IV	IV	IV

1.1.4 Current treatments

1.1.4.1 Surgery

As a result of late-stage diagnosis, current treatment strategies primarily focus on improving quality of life and managing symptomology, as opposed to having curative intent. This approach of palliative care for PC patients has seen minimal improvement during recent decades. For early-stage PC, surgery with curative intent is used as the primary treatment option, with or without systemic therapy and/or radiotherapy^[9, 10]. Surgical resection may involve the selective removal of head of the pancreas (Whipple procedure), tail and body regions (distal pancreatectomy) or the pancreas in its entirety (total pancreatectomy)^[11, 12]. Furthermore, lymph nodes from different lymphatic stations

are also resected during the surgical resection to assess lymph node involvement, in addition to providing further information for tumour staging^[13, 14]. Currently, fewer than 20% of those newly diagnosed are eligible for surgery, which is potentially curative^[15]. However, most surgical patients ultimately relapse and succumb to PC within two years^[16].

1.1.4.2 Radiation therapy

External-beam radiation therapy (RT) is most frequently used in the treatment of PC. The most conventional form of RT offered to PC patients is standard fraction radiation therapy, whereby low doses of radiation (generally 1.8 Gy per fraction) is given for 25-30 fractions over a period of 5 to 6 weeks^[17].

1.1.4.3 Chemotherapy

Chemotherapy is often administered at the same time as RT due to its radiosensitisation effects^[18]. PC is generally poorly responsive to standard-of-care chemotherapy, with patients achieving little to no increases in overall survival, particularly due to the development of chemoresistance^[19]. With this knowledge it is surprising that there has been limited novelty regarding chemotherapy agents in the PC setting. A limited number of combinational chemotherapy regimens are currently in use for the treatment of PC and usually function to activate multiple routes of tumour cell death (**Table 1.2**). In the advanced setting of PC, the two standard-of-care chemotherapeutic options available to patients include gemcitabine-nanoparticle albumin-bound paclitaxel (nab-paclitaxel) and FOLFIRINOX (5-Fluorouracil, Leucovorin, Irinotecan, and Oxaliplatin)^[20, 21]. However, even with these combination therapies, the 2-year survival rate remains at 10% and median overall survival reaches 8-11 months^[22]. As a result of the limited response to chemotherapeutic agents, more effective therapeutic strategies are being explored in the form of combinational approaches using targeted therapies and immunotherapies.

Table 1.2: Approved chemotherapeutic agents currently used in the treatment of PC.

Chemotherapy	Drug Classification	Stage of Disease	Approved Use
Capecitabine (Xeloda®)	Anti-metabolite	Locally advanced PC	With RT after induction chemotherapy, Combination therapy with gemcitabine
Fluorouracil (5-FU®)	Antimetabolite	Locally advanced/metastatic PC	Adjuvant therapy (FOLFIRINOX), 2 nd line therapy (FOLFIRI)
Gemcitabine (Gemzar®)	Antimetabolite	Resectable PDAC, Locally advanced/metastatic PDAC	Adjuvant monotherapy or combination therapy with RT
Irinotecan (Camptosar®)	Topoisomerase I inhibitor	Locally advanced/metastatic PC	Adjuvant therapy (FOLFIRINOX), 2 nd line therapy (FOLFIRI)
Leucovorin (Wellcovorin®)	Folate analogue used to enhance the cytotoxicity of 5-FU	Locally advanced/metastatic PC	Adjuvant therapy (FOLFIRINOX), 2 nd line therapy (FOLFIRI)
Cisplatin (Platinol®)	Alkylating agent	Locally advanced/metastatic PC	Combination therapy with gemcitabine
Oxaliplatin (Eloxatin®)	Platinum salt	Locally advanced/metastatic PC	Adjuvant therapy (FOLFIRINOX)

PC=pancreatic cancer. RT=radiation therapy. 5-FU=5-fluorouracil. FOLFIRINOX=5-fluorouracil, leucovorin, irinotecan, and oxaliplatin. FOLFIRI=5-fluorouracil, leucovorin and irinotecan. PDAC=pancreatic ductal adenocarcinoma.

1.1.4.4 Immunotherapy

The incorporation of immunotherapy in the treatment of many solid tumours has marked an advance in cancer therapeutics. However, there is currently only one FDA approved immunotherapy for use in PC patients, which is the immune checkpoint inhibitor pembrolizumab (anti-programmed cell death 1 receptor (PD-1))^[23, 24]. Based on data from pembrolizumab clinical trials and the success of this immune checkpoint inhibitor in patients with refractory, metastatic cancers, pembrolizumab was initially approved for the treatment of solid tumours with high-level microsatellite instability (MSI-H) or deficient DNA mismatch repair (dMMR)^[24]. This immune checkpoint inhibitor was then later approved by the FDA for the treatment of solid tumours with high tumour mutational burden (TMB-H)^[25]. However, mismatch repair deficiency only occurs in approximately 1-3% of PC tumours, therefore highlighting the need for additional targets for immunotherapies for the vast majority of PC patients^[26].

1.1.5 Desmoplastic microenvironment of PC

A contributing factor to the poor outcomes for patients with PC is the lack of immune cell infiltrate, termed an “immunologically cold” tumour. “Immunologically cold” tumours are typically characterised by poor CD8⁺ T cell infiltration into the tumour microenvironment (TME). Additionally, these “immunologically cold” tumours often harbour immunosuppressive immune cell populations, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages (TAMs), which ultimately function to dampen the anti-tumour immune response^[27, 28]. Conversely, “immunologically hot” tumours are characterised by high levels of CD8⁺ T cell infiltration, increased programmed cell death ligand 1 (PD-L1) expression and high tumour mutational burden (TMB), features that are also associated with enhanced response to immune checkpoint blockade^[27, 28].

Extensive desmoplasia is a prominent pathological feature of the PC tumour microenvironment (TME)^[29] (**Figure 1.2**). Desmoplasia consists of a dense extracellular matrix (ECM) and a high level of α -smooth muscle actin-positive fibroblasts^[30]. Tumour cells are known to secrete factors including sonic hedgehog (SHH), fibroblast growth factor-2 (FGF2), transforming growth factor β 1 (TGF- β 1) and platelet-derived growth factor (PDGF) which ultimately result in the proliferation, differentiation and activation of cancer-associated fibroblasts (CAFs) and pancreatic stellate cells (PSCs)^[31-34]. TGF- β 1 is one such growth factor expressed by tumour cells that leads to ECM deposition and tissue fibrosis^[32]. Consequently, activated CAFs secrete immunosuppressive cytokines and chemokines, such as interleukin 1 β (IL-1 β), IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, CXCL12, CXCL13, chemokine (C-C motif) ligand 5 (CCL-5) and granulocyte-macrophage colony-stimulating factor (GM-CSF), in addition to ECM components, including collagen, fibronectin and matrix metalloproteinases, which promote the proliferation, invasion, migration and metastatic characteristics of PDAC cells and pancreatic stellate cells^[35]. Activated CAFs promote fibrosis via collagen deposition and ECM modulation, which ultimately perpetuates the development of the desmoplastic stroma^[36]. Dense ECM is a contributing factor of PDAC progression through the process of contact-mediated lymphocyte trapping and stimulation of integrin/focal adhesion kinase (FAK) signalling in the associated tumour cells^[37, 38]. Therefore, the desmoplastic stroma acts as a physical barrier preventing adequate

vascularisation, hindering anti-tumour immune cell infiltration, and perpetuating resistance to systemic treatments^[36].

As discussed, PC is widely classified as an “immunologically cold” tumour. The immunosuppressive desmoplastic microenvironment in the pancreas is heavily induced and facilitated by Tregs, MDSCs, and anti-inflammatory M2 macrophages. In an *in vivo* study carried out by Clark *et al.*, Tregs, MDSCs and macrophages were shown to dominate the immune infiltration in pre-malignant pancreatic cystic lesions (PCLs) and persist to invasive PC^[39].

PC cells secrete immunosuppressive, pro-tumourigenic cytokines including IL-6, IL-8, IL-10, IL-13, TGF- β and vascular endothelial growth factor (VEGF), in addition to chemokines, such as CXCL12 into the TME^[40]. The abundant secretion of these immune factors into the TME induces immune suppression by activating and recruiting Tregs, Th2 cells, MDSCs and M2 macrophages^[40, 41]. These immune cell subsets block the anti-tumour activity of cytotoxic CD8⁺ T cells, effector CD4⁺ T cells and NK cells^[41]. Significantly increased populations of Tregs have been identified in the peripheral blood and stromal areas of pancreatic tissue from PDAC patients^[42]. A study conducted by Hiraoka *et al.* examined clinical samples of precursor lesions and demonstrated that the accumulation of Treg cells and diminished cytotoxic CD8⁺ T cell infiltration correlated with the malignant progression of pre-neoplastic lesions^[43]. These results were corroborated by additional independent studies^[44, 45]. However, recent multiplex immunofluorescence studies investigating the immune microenvironment of resected tissue from precursor lesions of PC found that there was increased CD8⁺ T cell infiltration in pre-malignant lesions^[46, 47].

The hostile desmoplastic microenvironment plays a prominent role as a barrier to treatment and ultimately contributes to poor prognosis for PC patients. Since fewer than 20% of newly diagnosed PC patients are considered eligible for surgery, identifying patients at earlier stages of PC development is imperative to improved prognosis and survival^[15].

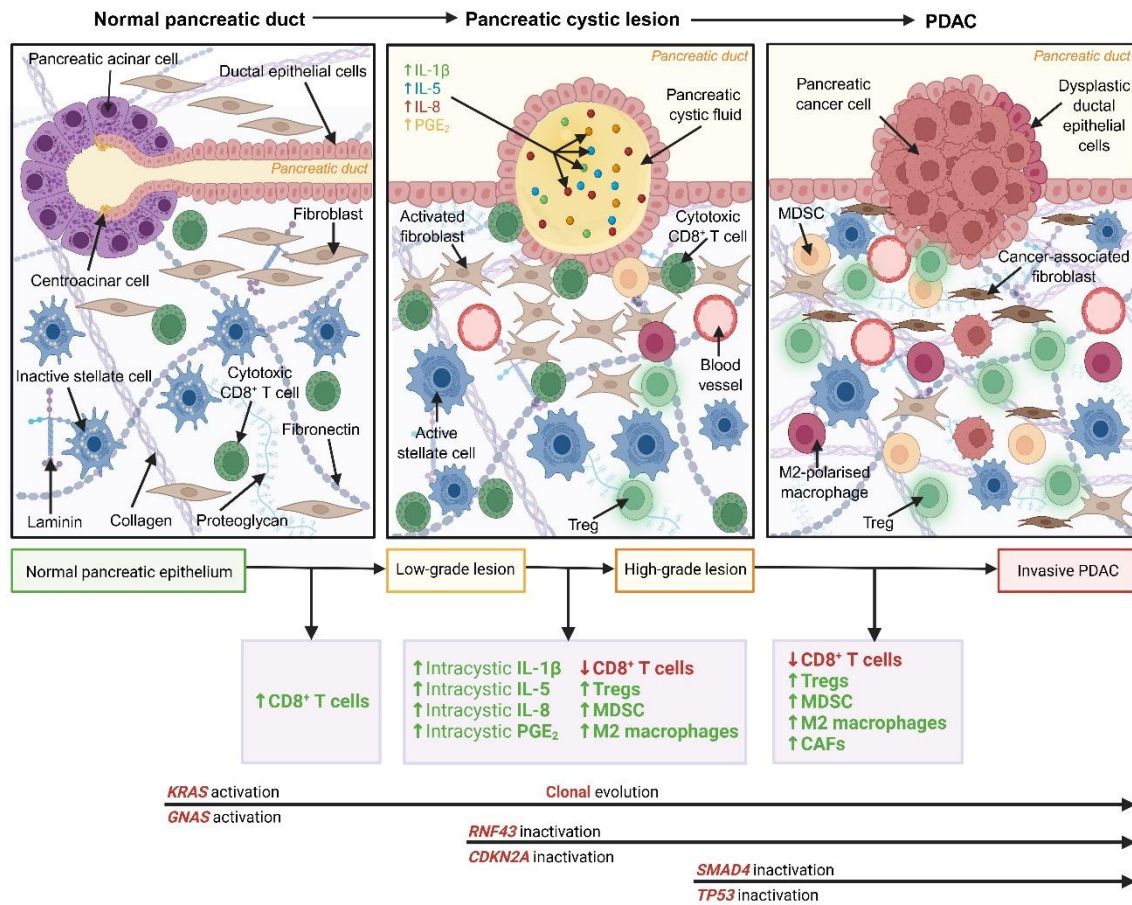


Figure 1.2: Development of a desmoplastic microenvironment from normal ductal epithelial tissue to pancreatic cystic lesion to pancreatic ductal adenocarcinoma tissue. Immune cell infiltration and therapeutic drug delivery is hindered by the dense desmoplastic microenvironment. With cancer progression, marked by increased dysplasia of ductal epithelial cells, ECM components and α -smooth muscle actin fibroblasts become more abundant. PCLs frequently accumulate genetic mutations in *KRAS*, *CDKN2A*, *TP53*, *SMAD4*, *GNAS*, and *RNF43* with progression of disease. Immune cell infiltration is reported to evolve from increased cytotoxic CD8⁺ T cells in low-grade dysplastic PCLs to a more immunosuppressive microenvironment in high-grade PCLs and invasive PDAC governed by Tregs, MDSCs and M2-polarised macrophages. Tumour cells secrete growth and survival factors which cause cancer-associated fibroblasts (CAFs) and Tregs to produce immunosuppressive cytokines and subsequently promote the proliferation, invasion, migration, and metastatic characteristics of pancreatic stellate cells and PC. ECM components, including collagen, fibronectin and laminin, are upregulated during the progression of disease, ultimately perpetuating a dense desmoplastic stromal environment. Figure created with BioRender.com.

1.1.6 Risk factors of PC

Incidence and mortality rates of PC have remained stable or slightly increased across many countries, likely reflective of the increasing prevalence of obesity, smoking and alcohol consumption, which are classified as modifiable risk factors of PC^[48, 49]. Age, gender, genetic risk factors such as von Hippel Lindau (VHL) disease and inflammation-driven risk factors including diabetes and pancreatitis also incur non-modifiable risk of PC development^[50]. A notable risk factor of PC development and progression is pre-malignant PCLs, which are often considered precursor lesions of PC^[51, 52].

1.2 PCLs as potential avenues for enhanced cancer risk stratification and earlier PC diagnosis

1.2.1 Pancreatic cystic lesions

PCLs are typically localised, fluid-filled structures that can be found within or on the surface of the pancreas. The vast majority of PCLs are detected incidentally during routine investigations, giving them their pseudonym “incidentalomas”^[53, 54]. While many PCLs are benign, others such as intraductal papillary mucinous neoplasms (IPMNs) or mucinous cystic neoplasms (MCNs), possess the ability to undergo malignant transformation and can be regarded as precursor lesions of PC^[51, 52, 55]. Understanding the mechanisms involved in cystogenesis and tumourigenesis may be the key to identifying avenues of earlier PC diagnosis and intervention.

1.2.1.1 Non-neoplastic lesions

Non-neoplastic cysts are benign lesions that can be further classified into epithelial or non-epithelial subtypes^[56]. Non-neoplastic cysts account for up to 6.3% of all resected cysts and include pseudocysts, retention cysts, benign epithelial cysts, lymphoepithelial cysts, squamous lined cysts, mucinous non-neoplastic cysts and lymphangiomas^[56].

1.2.1.1.1 Pseudocysts

Pseudocysts are generally considered the most common subtype of non-neoplastic cysts^[57]. Pseudocysts predominantly develop as a complication of alcohol-induced, biliary or traumatic acute pancreatitis (AP) and typically develop in the tail of the pancreas^[58] (**Figure 1.3**). Pseudocysts are not classified as true cysts given their presentation as amylase-rich fluid containing cellular debris, blood and inflammatory cells encased in a fibrous wall without an epithelial lining, as would otherwise typically

be a feature of true pancreatic cysts^[59, 60]. Pancreatic pseudocysts also have been shown to contain inflammatory cells, such as neutrophils and tissue-resident macrophages^[61]. However, further studies are required to establish a link between the expression of specific immune cells and factors in neoplastic PCLs and the risk of malignant transformation.

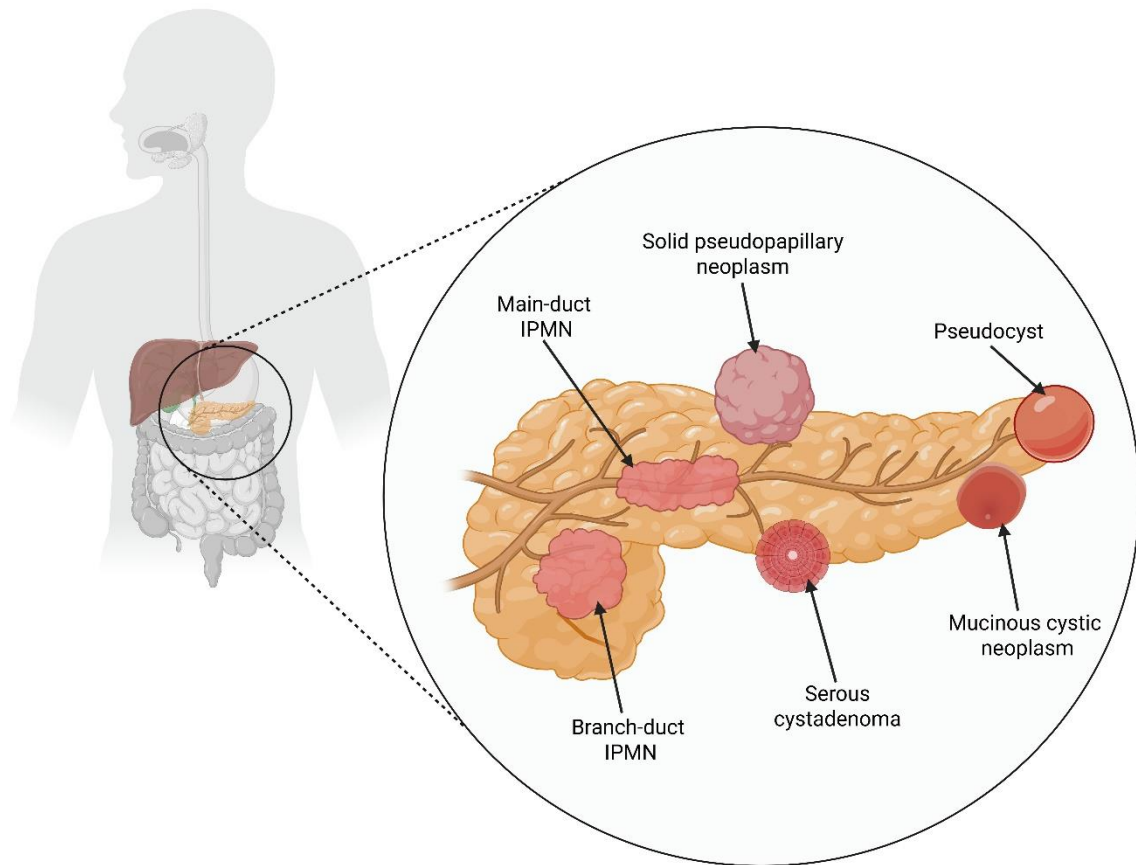


Figure 1.3: Presentation of PCLs within the pancreas. Intraductal papillary mucinous neoplasms (IPMNs) typically arise within the main pancreatic duct and its branches in the head and uncinate process of the pancreas. Mucinous cystic neoplasms (MCNs) present as unilocular or multilocular cysts typically located in the body and tail of the pancreas. Serous cystadenomas are microcystic, honeycomb-like lesions that may occur throughout the pancreas but are most commonly found within the body and tail of the pancreas. Solid pseudopapillary neoplasms are non-mucinous cystic neoplasms that can be located throughout the pancreas but more frequently in the body or tail in adults. Pseudocysts, which are non-neoplastic lesions without an epithelial lining that usually arise in a background of pancreatitis, are identified in variable locations within or around the pancreas, often near the head or tail. Figure created with BioRender.com

1.2.1.2 Neoplastic lesions

Neoplastic cysts are broadly categorised as serous, as in the case of serous cystic neoplasms, or mucinous cysts such as IPMNs and MCNs^[62]. Serous cystic or non-

mucinous neoplasms are generally classified as benign, whereas mucinous neoplasms are considered to have a malignant potential^[56] (**Figure 1.4**). There are a number of biomarkers currently used in clinical practice to differentiate mucinous from non-mucinous pancreatic cysts^[63, 64]. Among these, intra-cystic carcinoembryonic antigen (CEA) is one of the most extensively studied biomarkers for the diagnosis of neoplastic mucinous cysts^[63, 64]. A CEA concentration greater than 192 ng/mL demonstrates a sensitivity of 50-75%, specificity of 84-92%, and an overall diagnostic accuracy of approximately 79% for distinguishing mucinous from non-mucinous cysts^[64-66].

In parallel, a recent meta-analysis of PCF biomarkers reported that glucose concentrations below 50 mg/dL accurately identified IPMNs and MCNs with 93% sensitivity and 76% specificity^[63]. Mutational profiling of pancreatic lesions has proven valuable for distinguishing between different subtypes of precursor lesions^[67-69]. Low-grade pancreatic intraepithelial neoplasia (PanINs) commonly harbour mutations in *KRAS* and *p16/CDKN2A*, whereas high-grade PanINs and invasive PC exhibit additional alterations in *TP53* and *SMAD4*^[70, 71]. Similarly, IPMN lesions frequently harbour mutations in *KRAS*, *CDKN2A*, *TP53*, *SMAD4*, *GNAS*, and *RNF43*^[72-74]. Notably, next-generation sequencing (NGS) studies have demonstrated that the presence of *KRAS* or *GNAS* mutations is highly specific (98% specificity) for identifying mucinous cysts, providing valuable molecular markers for distinguishing these lesions from non-mucinous counterparts^[63, 64]. In contrast, MCNs share a comparable mutational profile with IPMNs, but typically lack *GNAS* mutations^[74]. It is yet to be elucidated whether these genetic alterations arise coincidentally and induce neoplastic changes within the cyst or otherwise arise as a consequence of exposure of cyst epithelial lining to PCF, which further perpetuates the neoplastic process. In a study conducted by Singhi *et al.*, DNA isolated from cyst epithelial lining that had shed into human pancreatic cystic fluid underwent next-generation sequencing^[67]. This study showed that *KRAS/GNAS* mutations in combination with alterations or deletions in *TP53*, *SMAD4*, *PIK3CA*, *CDKN2A*, and *PTEN* were associated with advanced neoplasia^[67].

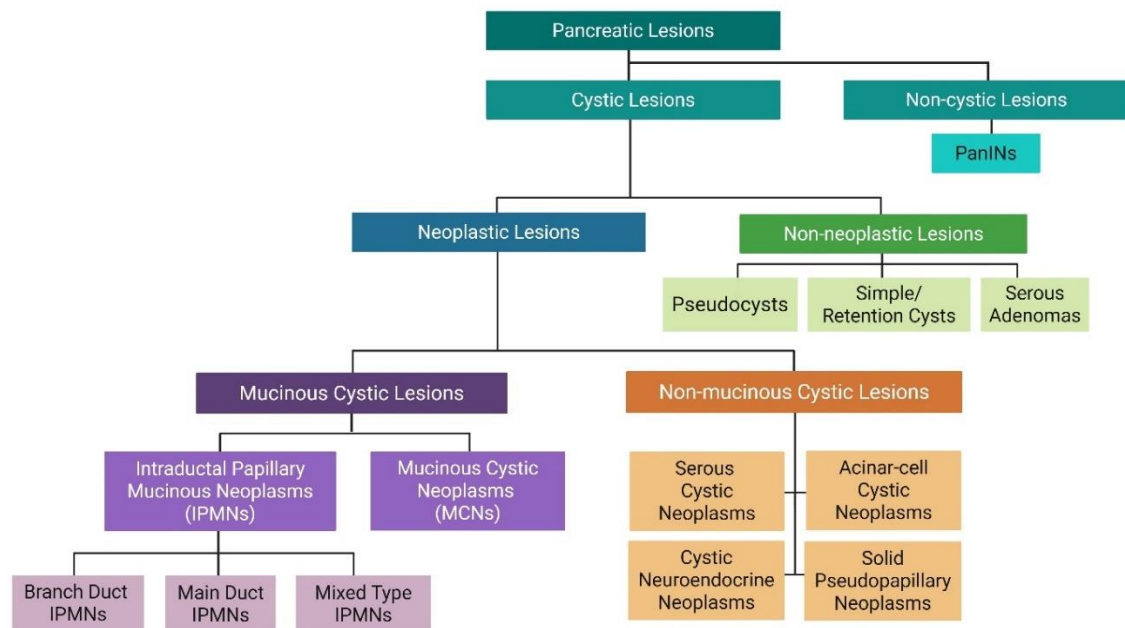


Figure 1.4: Classification of pancreatic lesions. PCLs that harbour malignant potential include mucinous and non-mucinous lesions. IPMNs are mucinous in nature and account for up to 38% of all neoplastic PCLs. MCNs represent up to 23% of all PCLs. Non-neoplastic cysts account for up to 6.3% of all resected cysts and include pseudocysts, simple/retention and serous adenomas. Figure created with BioRender.com

1.2.1.2.1 Pancreatic intraepithelial neoplasms

PanINs are non-cystic precursor lesions responsible for more than 90% of diagnosed pancreatic tumours^[75, 76]. However, despite their clinical significance, there is currently no established method for surveillance or early detection of PanINs^[75, 76]. These lesions are characteristically asymptomatic in nature and remain undetectable on standard radiological imaging due to their microscopic size^[75, 76]. The prevalence of these lesions increases with age and occur most commonly in small ducts in the head of the pancreas^[77]. PanIN lesions are graded based on levels of dysplasia, reflecting the progressive neoplastic morphological changes such as the structural alterations of cuboidal epithelium transitioning from flat, columnar shaped epithelial cells to papillary epithelial cells^[78]. Other morphological changes observed in PanIN progression include nuclear abnormalities, such loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism.^[78] Molecular alterations associated with PanINs have been shown to accumulate with increasing grades of dysplasia and eventually lead to uncontrolled cell proliferation and subsequent progression to invasive carcinoma^[79]. These alterations include activating point mutations to *KRAS*, an oncogene that acts as a mediator for cell cycle progression and proliferation^[80, 81]. Other molecular alterations identified in these precursor lesions include inactivation of point tumour suppressor genes

TP53, *p16* and *SMAD4*^[82, 83]. PanIN lesions have also been shown to exhibit telomere shortening in early stages of cyst development, which is also a mechanism implicated in acinar-to-ductal metaplasia (ADM), a key tumour initiation mechanism of PDAC^[84, 85].

1.2.1.2.2 Intraductal papillary mucinous neoplasms

IPMNs account for up to 38% of neoplastic PCLs^[86]. IPMNs possess malignant potential to progress to PC and are comprised of mucin-producing columnar cells arising in the main duct (MD), branch duct (BD) or a combination of both types (mixed type (MT)) in the head or uncinate of the pancreas^[87]. These precursor lesions develop papillary proliferation, cyst formation and varying degrees of cellular atypia^[72, 88]. Patients with MD-IPMNs and MT-IPMNs have been shown to be at an increased risk of malignant development, with approximately 45% having an associated invasive carcinoma compared to BD-IPMNs, where approximately 15% of diagnosed cases are associated with the development of invasive carcinoma^[89-91].

In contrast to PanINs, IPMN lesions are frequently detectable on radiological imaging, which makes clinical surveillance feasible^[92, 93]. Their detection has significant clinical implications, as IPMNs possess malignant potential to progress to invasive PC^[94, 95]. A key benefit of image-based surveillance is the detection of PC at an earlier, more treatable stage. In a large retrospective study of 450 patients, cancers detected in individuals undergoing surveillance were significantly more likely to be Stage I compared to those diagnosed in patients proceeding directly to surgery (69.2% vs. 37.3%), providing robust, data-driven justification for PCL surveillance^[96]. Furthermore, the prevalence of IPMNs in the adult population is estimated to be approximately 10-20%, underscoring their importance as a common and clinically relevant PCL^[92, 97]. IPMNs are graded based on the degree of dysplasia, which can be classified as low-grade dysplasia (adenoma) and high-grade dysplasia (carcinoma in situ)^[98, 99]. Additionally, in light of their high prevalence and malignant potential, several clinical guidelines have been established to stratify the risk of progression based on the presence of specific “worrisome features”^[100]. Reported estimates suggest that low-risk IPMNs carry a 0.02-7.77% probability of developing into invasive PC, whereas high-risk IPMNs have a markedly higher risk, ranging from 14.4-47.9%^[94, 95].

1.2.1.2.3 Mucinous cystic neoplasms

MCNs are a rarer subtype of neoplastic pancreatic lesion, accounting for up to 23% of PCLs^[86]. More than 95% of MCNs develop in the body and tail of the pancreas and histologically display characteristically tall, columnar, mucin-producing epithelial cells^[101]. MCNs generally arise in the middle-aged female population and can be differentiated from IPMNs due to the ovarian stromal involvement associated with MCNs^[102]. The preponderance of MCNs in females, in addition to the presence of oestrogen receptors has led to the hypothesis that as a result of ectopic ovarian stroma being incorporated during embryogenesis in the pancreas, the release of hormones and growth factors cause adjacent epithelium to proliferate and form cystic tumours^[103]. There are no distinguishable factors between MCNs that can progress to invasive carcinoma and those that remain benign and so resection of these cysts is recommended^[102].

1.3 Immunology of PCLs

Initial studies investigating the hypothesis that a proinflammatory environment exists in IPMN patients have found that there was significantly elevated IL-1 β levels in addition to overexpression of other inflammatory markers, such as IL-5 and IL-8, in high-risk IPMN cyst fluid in comparison to low-risk IPMN cyst fluid^[104]. IL-1 is primarily synthesised by monocytic phagocytes that have undergone stimulation in immunogenic and pro-inflammatory environments^[105]. IL-1 β is secreted into the extracellular space as opposed to intracellular, membrane-bound IL-1 α , which enables this cytokine to be measured in PCF^[105]. The presence of IL-1 β in these dysplastic cysts in the pancreatic setting may reflect how the inflammatory microenvironment plays a role in malignant progression. Some studies have found that elevated IL-1 β expression in tumour tissue is associated with increased cancer risk and poorer survival in a multitude of other neoplasms, including ovarian and cervical cancer, and mediated PC cell invasion^[106-108]. Cell mediated and humoral immune responses are facilitated through Th1 and Th2 CD4⁺ T cells, respectively, whereby immune activity can be measured through quantification of cytokines such as IL-1 β , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α)^[109]. IL-1 β signalling also assists in the activation of CD4⁺ T cells in an antigen dependent manner, whereby this process may be triggered when low-grade dysplastic IPMNs transform into highly dysplastic IPMNs^[110]. IL-5 is a key inflammatory marker produced by Th2 cells and mast cells, and functions by stimulating B cell growth and immunoglobulin secretion. Findings of increased serum

levels of IL-5 in PDAC patients in comparison to chronic pancreatitis supports the evidence of a systemic pro-tumour Th2 response in PC^[109].

A study carried out by Roth *et al.* assessed tissue samples that were obtained from IPMN resections using immunohistochemical analysis to examine the spatial distribution of immune cell infiltrates during IPMN progression^[111]. Findings from this analysis established that the immune microenvironment evolved from a diverse T cell population, which is comprised of CD8⁺ T cells, Th/c1, Th/c2, Th/c17, Th22, and Treg cells in low-grade IPMNs to a primarily Treg dominated immunosuppressive state during the carcinogenesis of IPMNs and invasive PC^[111]. This study also demonstrated the formation of tertiary lymphoid structures in the surrounding stroma of IPMNs, which accumulate immunosuppressive cell types and subsequently evolve during progression to invasive PC^[111]. However, the results of these studies further emphasise the need to examine the altered immune components in pancreatic cyst fluid to understand the mechanisms used by localised cells within and around these neoplastic cysts that ultimately promote malignant transformation (**Figure 1.5**). Nevertheless, this emerging evidence suggests that composition of the T cell repertoire in pre-malignant IPMN tissue may reflect changes in immune dynamics predictive of cyst progression and may have potential utility as an immunobiological biomarker for integration into PCL cancer risk stratification and management guidelines.

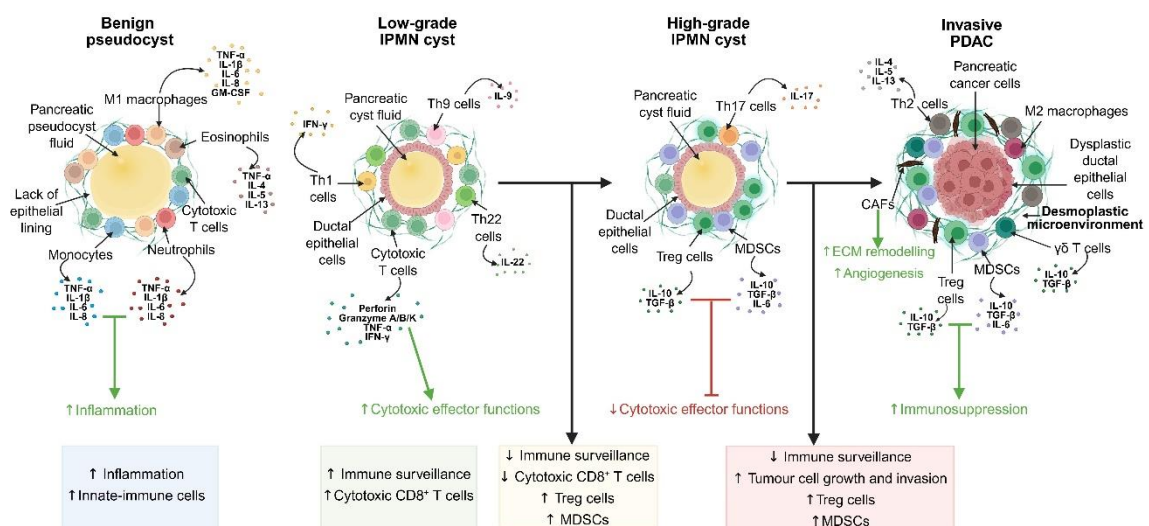


Figure 1.5: The evolution of the immune T cell landscape during progression of pancreatic IPMN cysts to the development of invasive PDAC. Benign pseudocysts are primarily governed by inflammatory innate immune cells, such as macrophages, monocytes and neutrophils. T cell phenotypes are primarily cytotoxic and pro-inflammatory in pre-malignant lesions, such as low-grade IPMN lesions. With

progression of pancreatic IPMN lesions from low-grade dysplasia to high-grade dysplasia, changes occur in the hierarchy of T cell phenotypes in the immune microenvironment as regulatory T cells, Th2 cells, Th17 cells, and $\gamma\delta$ T cell subsets work to suppress effector T cell infiltration and, in turn, hinder immune surveillance. With increased levels of dysplasia, IPMN lesions also develop an immunosuppressive landscape through an increased infiltration of Treg cells and MDSCs which are known to suppress cytotoxic effector functions. The TME of PDAC is also predominantly composed of immunosuppressive cell types (Tregs, MDSCs, M2-polarised macrophages), but also of cancer-associated fibroblasts which contribute to extracellular matrix (ECM) remodelling and increased angiogenesis. The immunosuppressive microenvironment of PDAC mediates tumour growth, immune evasion, and metastasis. Figure created with BioRender.com.

1.4 Co-morbidities associated with increased incidence of PCLs

1.4.1 Inflammation-driven co-morbidities

1.4.1.1 Pancreatitis

Inflammation of the pancreas, otherwise termed “pancreatitis”, is a significant risk factor for PC development and has been established as a critical factor in the initiation and maintenance of the pancreatic disease state^[39, 112-114]. The immunosuppressive environment cultivated by the pancreatic stroma in PC, has been hypothesised to promote tumour progression and invasion^[115]. Acute pancreatitis (AP) is one of the most commonly diagnosed gastrointestinal conditions with global incidence rates increasing annually^[116]. AP presents with increased serum concentrations of amylase and lipase^[117], in addition to a systemic decrease of circulating T cells, B cells and NK cells^[118-120]. Recurrent bouts of AP, often instigated through exposure to risk factors such as alcohol consumption, gallstones and smoking, leads to fibrosis and chronic pancreatitis (CP), which is an inherent risk factor for the development of PC^[121-123]. Although CP shares many of the same risk factors as AP, the clinical presentation differs dramatically. In contrast to the elevated serum amylase and lipase levels in AP, these pancreatic enzymes are generally normal or only slightly elevated in CP^[124]. CP can be characterised by extensive pancreatic fibrosis, acinar cell atrophy and immune cell infiltrate^[125]. Chronic inflammation of the pancreatic tissue, as in the case of CP, constitutively activates pro-inflammatory cytokines such as IL-6, IL-8, IFN- γ and TNF- α , which induce local tissue damage and are found to be increased in both CP and PDAC^[126, 127]. The longstanding inflammation of CP is characterised by macrophage, T cell and B cell infiltration into pancreatic tissue^[128-130], whilst the CD4⁺ and CD8⁺ T cell populations are reported to decrease in peripheral blood^[131, 132].

Uncontrolled activation of pancreatic acinar cells and release of digestive enzyme stores leads to the autodigestion of pancreatic cells^[133]. Autodigestion of these cells releases cellular contents that trigger recruitment of inflammatory cells, which ultimately release cytokines and immunomodulatory factors such as IL-1 β , IL-6, IL-8, IL-33, TNF- α , monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2)^[134-137].

In response to pancreatitis-driven inflammation, pancreatic acinar cells can undergo acinar-to-ductal metaplasia (ADM) transdifferentiation process^[138]. ADM is transient and reversible in patients with pancreatitis or pancreatic injury^[139]. However, the molecular mechanism driving metaplastic formation and malignant progression following persistent ADM is yet to be elucidated. An *in vivo* study carried out by Liou *et al.* found that macrophage-secreted chemokines and cytokines such as RANTES, which functions as a potent chemoattractant for a multitude of immune cells including T cells, and TNF- α promote ADM through activation of NF- κ B and matrix metalloproteinases^[113]. Constitutive NF- κ B activation has previously been described as an early distinguishing factor of both AP and CP, in addition to PDAC cells in comparison to normal pancreatic tissue^[140-143].

1.4.1.2 Diabetes mellitus

Diabetes mellitus (DM) is a global health problem caused by chronic high glucose levels in the blood as a result of dysfunctional β cells in the pancreas, which fail to produce adequate insulin or otherwise incur ineffective insulin functionality^[144]. In many patients, DM, particularly new-onset DM, often precedes the clinical diagnosis of PC^[145]. However, the mechanisms underlying this association are not yet fully understood.

DM has also been shown to be a predictive marker of malignant progression of pancreatic cysts, particularly so in the case of patients who develop new-onset diabetes subsequent to the development of a cystic lesion^[145, 146]. To date, only a minimal number of epidemiological studies have been published which aim to determine the frequency of PCL incidence in diabetic patients and vice versa^[147, 148]. Although these studies fail to indicate a specific factor responsible for cyst formation in patients with DM, an increased incidence of PCLs has been associated with the aging population, those with a high body mass index (BMI) and DM history^[148]. In addition to this, other studies have identified

an increased risk of PC in patients diagnosed with DM, particularly type 2 diabetes^[149, 150].

1.4.2 Genetically-driven co-morbidities

1.4.2.1 Von Hippel-Lindau disease

The formation of precursor lesions in the pancreas is often influenced by genetic factors as in the case of Von Hippel-Lindau (VHL) disease. VHL disease is a hereditary autosomal dominant genetic condition resulting from deletions or germline mutations of the *VHL* gene, an established tumour suppressor gene, on chromosome 3p^[151, 152]. Loss of heterozygosity and somatic inactivation of the wild-type *VHL* allele have been identified within VHL-associated lesions like PCLs^[153, 154]. Typically, the manifestation risk of pancreatic neuroendocrine tumours associated with VHL disease is estimated at 10-17%^[155, 156]. Mutations in the *VHL* gene are over 99% specific for serous cystadenomas, and their detection often provides clinicians confidence to discontinue surveillance due to their benign nature^[63]. Interestingly, up to 70% of patients with VHL disease will develop PCLs^[157, 158]. However, no association has been reported between the presence of PCLs in VHL disease and malignancy^[159].

A whole-exome sequencing study was performed on DNA from microdissected human neoplastic PCLs, whereby the results revealed that although loss of the chromosome 3p alleles in the *VHL* gene initiates cystogenesis in pancreatic cystadenomas, IPMNs did not show any alterations in 3p alleles^[73]. These lesions with malignant potential indicated that other genes encoding E3 ubiquitin ligases were involved^[73]. This suggests that protein turnover may be a major contributing factor in the underlying cystogenesis mechanism.

1.5 Biological framework for cancer risk stratification and management of pre-malignant PCLs

1.5.1 Clinical evaluation of PCLs

Due to the low accuracy of image-based tests and the invasive nature of surgical biopsy, endoscopic-ultrasound fine-needle aspiration (EUS-FNA) has gained popularity as an alternative technique to evaluate PCLs. This diagnostic modality is used clinically and has been shown to have low sensitivity and accuracy (45.4% and 57.5%, respectively) but high specificity (87.5%) for the detection of PCLs^[160]. EUS-FNA requires highly skilled gastroenterologists to perform the procedure, and studies have shown that PCLs

need a comprehensive approach to combine gastroenterologist expertise, cytological, radiological and cyst fluid analysis data to establish an accurate diagnosis^[161]. A major limitation of this technique is the low cellularity of cyst fluid samples, which often precludes definitive cytological diagnosis^[162]. To overcome these challenges, the field has evolved with the development of novel EUS-based tools that enhance tissue acquisition and real-time cyst characterisation. Through-the-needle biopsy (TTNB) employs micro-forceps passed through the EUS needle to obtain targeted tissue samples from the cyst wall or mural nodules, resulting in a significantly higher diagnostic yield compared to FNA alone^[163]. Meanwhile, needle-based confocal laser endomicroscopy (nCLE) enables real-time, high-resolution microscopic imaging of the cyst epithelium, allowing for the *in vivo* differentiation of PCL subtypes based on characteristic visual identifiers^[164]. In addition to diagnostic advances, EUS-guided ablation has emerged as a minimally invasive therapeutic alternative for patients who are poor surgical candidates or decline surgery^[165]. This technique involves the injection of ablative agents directly into the cyst cavity to induce epithelial necrosis and cyst resolution^[165]. EUS-guided ablation does not eliminate the need for ongoing surveillance, due to possible recurrence of the lesion or incomplete ablation. These advances to EUS technologies represent a substantial improvement in the endoscopic evaluation of PCLs, emphasising the need for a multimodal diagnostic approach that integrates gastroenterologist expertise with cytological, radiological, and cyst fluid analyses^[161].

1.5.2 Diagnostic guidelines

There are currently six major sets of guidelines presently in use to guide referrals for EUS-FNA or surgical resection procedures for patients with clinical presentation of asymptomatic PCLs^[100]. These include the 2024 International evidence-based Kyoto guidelines^[166], 2018 European evidence-based guidelines^[167], the 2018 American College of Gastroenterology (ACG) guidelines^[168], the 2017 International Association of Pancreatology Fukuoka guidelines^[95], the 2017 American College of Radiology (ACR) guidelines^[169], and the 2015 American Gastroenterological Association (AGA) guidelines^[170] (**Table 1.3**).

Table 1.3

Indications for the management of PCLs based on current clinical guidelines.

Guideline Publications	Indications for EUS* surveillance	Indications for surgical resection	Reference
2024 International evidence-based Kyoto guidelines	<i>Any of the following worrisome features;</i> Acute pancreatitis, Elevated serum CA19-9, New onset/exacerbation of diabetes mellitus, Cyst ≥ 30 mm, thickened/enhancing cyst walls, Main PD ≥ 5 mm and < 10 mm, enhancing mural nodule ≥ 5 mm Lymphadenopathy, rapid growth of cyst (≥ 2.5 mm/yr) during surveillance	Repeated acute pancreatitis to worsen patient's quality of life Multiple "worrisome features" Cytology suspicious or positive for malignancy or HG dysplasia Obstructive jaundice with lesion presenting in head of pancreas Enhancing mural nodule ≥ 5 mm or solid cystic component Main PD dilation ≥ 10 mm;	[166]
2018 European evidence-based guidelines (EEG)	Any clinical or radiological features of concern for malignancy, Results are expected to change clinical management.	<i>Relative indications:</i> Rapid growth of cyst (≥ 5 mm/yr) during surveillance, Elevated CA-19-9 levels (37 U/mL), Main PD dilation between 5–9mm/ cyst size ≥ 40 mm, Acute pancreatitis/ new-onset diabetes mellitus, Enhancing mural nodule ≥ 5 mm, <i>Absolute indications:</i> Cytology suspicious or positive for malignancy or HG dysplasia, Solid cystic component, main PD ≥ 10 mm, Patient presenting with symptomology.	[167]
2018 American College of Gastroenterology (ACG)	<i>Any of the following worrisome features;</i> Main PD ≥ 5 mm, IPMN or MCN ≥ 30 mm, Change in PD calibre with upstream atrophy, Rapid growth of cyst (≥ 3 mm/yr) during surveillance, Jaundice, pancreatitis, Mural nodule or solid cystic component.	IPMN or MCN ≥ 30 mm and/or cyst growth (≥ 3 mm/yr), Cytology with concerning features of HG dysplasia or invasive disease, Non-enhancing mural nodule/solid cystic component, New-onset diabetes, jaundice secondary to cyst, Acute pancreatitis secondary to cyst, Elevated CA-19-9 levels, Focal dilation of main PD concerning for obstructing lesion.	[168]
2017 International Association of Pancreatology Fukuoka	<i>Any of the following features;</i> Pancreatitis due to cyst, Cyst size (≥ 30 mm) with rapid growth (≥ 5 mm/2 yrs) during surveillance, Thickened/enhancing cyst walls, Abrupt change in main PD diameter with distal pancreatic atrophy, Lymphadenopathy, elevated CA-19-9 levels.	Obstructive jaundice with lesion presenting in head of pancreas, Enhanced mural nodule ≥ 5 mm, Main PD dilation ≥ 10 mm, Cytology suspicious or positive for HG dysplasia or invasive cancer.	[95]
2017 American College of Radiology (ACR)	Cyst size (≥ 30 mm), Thickened cyst wall, Non-enhancing mural nodule, Main PD diameter ≥ 7 mm.	Jaundice secondary to cyst, Enhancing solid cystic component, Main PD dilation ≥ 10 mm, Cytology suspicious or positive for HG dysplasia or invasive cancer.	[169]
2015 American Gastroenterological Association (AGA)	<i>Two or more high-risk features;</i> Cyst size (≥ 3 mm), Dilated main PD.	Cytology with HG dysplasia or invasive cancer, Dilated main PD, Solid cystic component.	[170]

EUS=endoscope ultrasound. PD=pancreatic duct. HG=high-grade. IPMN=intraductal papillary mucinous neoplasm. MCN=mucinous cystic neoplasm.

1.5.3 Lack of consensus in PCL management and stratification guidelines

These stratification guidelines consider a number of clinical factors at varying degrees of priority when establishing risk of malignancy. Clinical experience of PCLs, in coalition with the knowledge of current stratification guidelines, heavily influences PC risk stratification. Clinical factors considered during cancer risk stratification include cyst size, dilation of the main pancreatic duct, presence of a mural nodule, serum carbohydrate antigen 19-9 (CA19-9) levels, diagnosis of VHL disease, development of jaundice or newly diagnosed diabetes^[100]. These criteria and thresholds for intervention vary considerably among these guidelines. The 2015 AGA and 2017 ACR guidelines tend to adopt a more conservative approach, recommending EUS-FNA or surgical resection only when multiple high-risk features are present, thereby minimising unnecessary interventions^[169, 170]. In contrast, the 2017 Fukuoka, 2018 ACG and 2018 European evidence-based guidelines prioritise an extended evaluation by incorporating factors such as cyst growth rate, CA19-9 elevation, or new-onset DM as potential indications for intervention^[95, 167, 168]. The recently updated 2024 Kyoto evidence-based guidelines expand upon this framework by emphasising long-term surveillance intervals and patient-specific cancer risk stratification^[166]. Overall, these stratification guidelines evidently exhibit a vast lack of consensus between institutions and geographical locations, emphasising the need for more robust and standardised set of PCL characterisation guidelines to minimise surgical intervention and indicate for necessary surveillance.

With the absence of an established screening program for early-stage PC, late-stage disease remains to be at the forefront of PC diagnoses. The majority of screening tests use image-based methods such as computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS). However, the sensitivity or specificity of these image-based tests as screening tools for PC has yet to be reported. At present, PC screening is only reserved for genetic/familial high-risk individuals and uses a multimodal screening approach of image-based techniques and EUS-FNA^[171].

The prevalence of pre-malignant PCLs has vastly increased over recent years as a result of improved quality and frequency of imaging technology used in routine examinations. Over time, a small number of these PCLs will develop the ability to undergo malignant transformation and progress to invasive PC^[51, 52]. Stratification of patients diagnosed with PCLs who are at high-risk of malignant transformation provide a potential window for

the early detection and treatment of PC. As discussed, there are a number of clinical guidelines used for the stratification and management of PCLs, but there is a vast lack of consensus on the appropriate and ideal use of surveillance and surgical intervention^[95, 167-170].

Surgical resection of PCLs is associated with significant morbidity and mortality rates^[172, 173]. A post-operative recurrence rate of up to 20% has been recorded for non-invasive IPMN cystic lesions and up to 46% for invasive IPMN cystic lesion recurrences, following prophylactic surgical resection^[174, 175]. A number of studies have shown that multiple regions of dysplasia can be present within the pancreas, often with different mutational status of the same gene in adjacent regions with varying levels of dysplasia^[176, 177]. These results support the concept of the multifocal nature of the tumourigenic process of pre-malignant PCLs within the pancreas. With consideration of these findings, the preventative approach of prophylactic surgery for pre-malignant PCLs should be reserved for patients presenting with a high-risk of malignant transformation and all regions of dysplasia should be taken into account.

Despite the risk of tumourigenesis associated with PCLs, particularly in the case of MCNs and IPMNs, there are no existing screening programs to detect these pre-malignant lesions. Most PCLs are diagnosed incidentally once patients undergo routine image-based tests for other medical indications^[53, 178]. Accurately stratifying patients with high-risk pre-malignant PCLs will enable appropriate longitudinal surveillance to identify patients who are bona fide candidates for prophylactic surgical intervention and prevent unnecessary surgery and surveillance for patients at a lower risk of PC development. The current therapeutic landscape for PC has made minimal improvements to the 5-year survival of patients particularly due to late-stage diagnoses, development of therapeutic resistance and a lack of druggable targets.

Immune biomarkers have shown clear clinical utility in several established cancers, such as colon cancer and breast cancer, as prognostic and sometimes predictive tools^[179-183]. In colon cancer, the Immunoscore assay quantifies CD3⁺ and cytotoxic CD8⁺ T cell densities in tumour tissues by digital pathology^[179, 180]. This immune-based assay consistently outperformed or otherwise complemented traditional TNM staging in predicting recurrence-free and overall survival across large multicentre cohorts of patients with

colon cancer^[179, 180]. Similarly, tumour-infiltrating lymphocytes (TILs) have emerged as prognostic and sometimes predictive markers in breast cancer, particularly in triple-negative and HER2-positive subtypes^[181, 182]. High stromal TIL counts correlate with improved disease-free survival and enhanced benefit from chemotherapy and anti-HER2 therapy^[181, 182]. Indeed, the expression of immune checkpoint molecule PD-L1 and tumour mutational burden (TMB) also serve as independent predictive biomarkers guiding immune-checkpoint inhibitor therapy in a number of solid malignancies^[183]. These examples demonstrate that immune factors can directly inform therapeutic decisions for patients with cancer. In pre-malignant conditions, such as Barrett's oesophagus and cervical intraepithelial neoplasia, changes in immune cell composition and cytokine expression profiles within the immune microenvironment in the early stages of the neoplastic sequence can be associated with progression risk^[184, 185]. Although immune biomarkers have yet to be clinically validated for use in pre-malignant conditions, their demonstrated utility in established cancers highlights their potential to improve early detection and cancer risk stratification.

1.6 Potential immunobiological biomarkers for cancer risk stratification of PCLs

At present, the only biomarker approved by the FDA for the detection of PC, is serum CA19-9^[186]. However, use of CA19-9 is not recommended for use in early-stage PC screening approaches due to its limited specificity (75%) and sensitivity (80%)^[187]. CA19-9 is also known to perform poorly as a biomarker in patients with inflammation-driven co-morbidities already associated with PC, such as pancreatitis and diabetes^[186]. A number of studies have emerged to overcome the issue of limited sensitivity and specificity of CA19-9 by investigating the applicability of multi-biomarker panels^[188, 189].

CA19-9 is not elevated in pre-malignant PCLs, emphasising the need for the addition of novel biomarkers, in the pre-malignant setting, to current cancer risk stratification guidelines in order to accurately classify PCLs^[190]. To identify potential biomarkers that could guide early PC detection and intervention, some studies have employed liquid chromatography-tandem mass spectrometry to establish a comprehensive proteomic profile of human PCF^[191, 192]. Although these studies identified protein family members of mucins, carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), amylase and serine protease inhibitor (SERPIN) proteins, which show potential in providing a more accurate diagnosis of high-risk pre-malignant PCLs, differential

expression of immune-based factors between risk-stratified PCLs was not significant^[191, 192]. Interestingly, Th1 and Th2 immune profiles of cytokine markers have been shown to distinguish patients with PC from patients with CP and normal pancreatic tissue in both serum and pancreatic juice samples^[109, 193]. It is yet to be defined whether inflammation within PCLs leads to severe dysplasia or whether increased levels of PCL-associated dysplasia initiate an immune response. Under both circumstances, identification and quantification of specific immune response pathways in PCF may serve as a biomarker of dysplasia.

An exploratory study carried out by Lee *et al.* utilised microarray assays to investigate the expression of inflammatory mediator proteins (IMPs) in PCF obtained from patients with pre-malignant BD-IPMNs or benign pseudocysts, which arose from a background of inflammation, as is typically the case^[194]. Although this study was limited by sample size, the results showed differential IMP profiles for pre-malignant and benign PCLs^[194]. Eotaxin, hepatocyte growth factor (HGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were expressed at lower concentrations in high-risk pre-malignant PCLs compared to in benign pseudocysts^[194]. Eotaxin is a chemokine that promotes the selective recruitment of eosinophils, which are involved in the pathogenesis of several inflammatory diseases such as eosinophilic oesophagitis, gastroenteritis and pneumonia^[195]. In the pancreatic setting, murine models have demonstrated that the expression of GM-CSF plays a role in the recruitment of antigen-presenting immune cells, such as macrophages^[196].

Additionally, MDSCs have been observed to populate human PCLs with high-grade dysplasia but are almost absent in lesions with low-grade dysplasia^[197, 198]. MDSCs can suppress the anti-tumour immune response through programmed cell death 1 receptor (PD-1) expression and release of reactive oxygen species^[199, 200]. Furthermore, MDSCs can also differentiate into anti-inflammatory M2 macrophages, which contribute to the immunosuppressive environment through the release of chemokines such as IL-10 and TGF- β ^[201]. This mounting data seems to suggest there is a dysregulated chemotactic mechanism in high-risk pre-malignant lesions, such as BD-IPMNs.

Prostaglandins play a critical role in the generation and resolution of inflammation. Differential expression levels of prostaglandin E₂ (PGE₂) in human pancreatic cyst fluid

was observed between PCLs with high-grade dysplasia and invasive carcinoma^[202]. Similarly, Yip-Schneider *et al.* found that expression levels of PGE₂ in low-grade, high-grade, and invasive human IPMN cyst fluid increased in a stepwise manner^[203]. PGE₂ has been shown to influence the differentiation of human effector T cell populations such as Th1, Th2, Th17, Treg, T follicular helper cells, and circulating T cells^[204]. Interestingly, this *in vitro* study highlighted the capacity of PGE₂ to suppress Treg differentiation, particularly via the G-coupled protein receptor, EP2^[204]. This data contrasts previous findings of increased human Treg accumulation associated with the malignant progression of pre-neoplastic lesions^[43, 45]. With increased expression of both Treg cell subsets and PGE₂ recorded in PCLs with high-grade dysplasia, perhaps dysregulation of EP2 receptor expression promotes Treg accumulation, permitting the immunosuppression in PCLs with higher risk of PC progression.

Interestingly, a number of studies have emerged demonstrating that mutant-specific KRAS inhibition can significantly transform the immune microenvironment of PC and PanIN lesions^[205-207]. In a study by Mahadevan *et al.* using murine models, non-covalent small molecule KRAS inhibitors were shown to remodel the immune microenvironment of PanIN lesions and advanced PC from an "immunologically cold" state to an "immunologically hot" state, marked by increased immune cell infiltration and enhanced sensitivity to immunotherapies^[207]. The effect of KRAS inhibition on the immune microenvironment of PCLs has not yet been elucidated. However, given that *KRAS* mutations are also identified in pre-malignant PCLs, KRAS inhibitors may be a promising avenue to investigate.

Distinct patterns of T cell responses have been shown to be associated with varying grades of dysplasia^[197]. A pro-inflammatory immune response, comprised of cytotoxic T cells, activated T-helper cells, and dendritic cells, was readily observed in human PCLs with low-grade dysplasia^[197]. In a study conducted by Enzler *et al.* where multiplex immunohistochemical staining was used to characterise the cellular immune component of surgically resected tissue from PCLs and PC, a high CD8⁺ T cell infiltration was identified in pre-malignant lesions^[46, 47]. Populations of T cells progressively decreased during progressive dysplasia in human PCLs, which suggests a diminished immune response or otherwise, a tumour escape mechanism during the process of tumour progression^[197]. These findings were consistent with a study conducted by Maker *et al.*

who investigated the expression of cytokines in human IPMN cyst fluid to determine anti-tumour immune response^[104]. The results from this evaluation showed significantly higher concentrations of pro-inflammatory cytokines IL-1 β , IL-5 and IL-8 in the presence of high-grade dysplasia or invasive carcinoma compared to low-risk PCLs with low-grade or moderate dysplasia^[104]. Although IL-8 concentration was the most highly expressed among the assessed analytes and differentially expressed between low-risk and high-risk PCLs, when multivariate analysis was performed, only IL-1 β remained a significant immunobiological biomarker of high-risk PCLs for the prediction of PC development^[104].

There is an urgent unmet need for improved biological characterisation of pre-malignant PCLs to implicate appropriate management strategies for patients with high-risk pre-malignant PCLs. Previous studies have demonstrated the value of integrating immunobiological biomarkers into cancer staging, prognosis, and therapeutic decision-making^[179-183]. As such, immunobiological parameters of pre-malignant PCLs, as in the case of immune cell infiltrates of CD8⁺ T cells, Tregs and MDSCs and/or the soluble immune mediators including IL-1 β , IL-5 and IL-8, could be incorporated to further strengthen the accuracy of current PCL cancer risk stratification guidelines and inform therapeutic intervention strategies to prevent late-stage PC development (**Table 1.4**).

Table 1.4

Summary of immunobiological biomarkers under investigation in PCLs.

Biomarker	Study type	Study Size	Key findings	Reference
<i>Differences in immune cell profiles</i>				
CD8 ⁺ T cell infiltration	Single-cell transcriptomics of the stromal compartment of PC and precursor lesions Spatial and phenotypic profiling of surgically resected tissue from PC and precursor lesions	n=6 (n=2 PC, n=2 LGD IPMN, n=2 HGD IPMN) n=265 (n=36 normal pancreatic tissue, n=17 MCN, n=71 IPMN, n=29 PanIN, n=8 IPMN-associated PDAC, n=104 PDAC)	CD8 ⁺ T cell population increased in HGD-IPMN lesions vs LGD-IPMN lesions Increased CD8 ⁺ T cell infiltration was observed in the microenvironment of MCN and even more so in IPMN lesions	[197] [46, 47]
Treg infiltration	Immunohistochemical analysis of surgically resected tissue from PC and precursor lesions Spatial and phenotypic profiling of surgically resected tissue from PC and precursor lesions	n=264 (n=15 non-neoplastic lesion, n=51 IPMN, n=198 PDAC) n=214 (n=21 normal pancreatic tissue, n=16 MCN, n=49 IPMN, n=20 PanIN, n=8 IPMN-associated PDAC, n=100 PDAC)	CD4 ⁺ Treg frequency increased with increased dysplasia (non-neoplastic tissue from PDAC patients 7.9%, non-neoplastic lesions 10.1%, PDAC 34.6%) Infiltration of Tregs was low in the microenvironment of cystic lesions MCN and IPMN and elevated in IPMN-associated PDAC	[43] [46, 47]
MDSC infiltration	Single-cell transcriptomics of the stromal compartment of PC and precursor lesions	n=6 (n=2 PC, n=2 LGD IPMN, n=2 HGD IPMN)	MDSC population enrichment in IPMN lesions with high-grade dysplasia (3.5%) vs IPMN lesions with low-grade dysplasia (2.3%)	[197]
<i>Differences in immune factor expression</i>				
Eotaxin, HGF, GM-CSF	Multiplex inflammatory mediator protein-targeted microarray	n=10 (n=5 BD-IPMN, n=5 inflammatory cysts)	Concentration of eotaxin, HGF, and GM-CSF was significantly decreased in BD-IPMNs vs. inflammatory cysts	[194]
PGE ₂	PGE ₂ concentration assessed by ELISA in PCF	n=101 (n=47 LGD/moderate dysplasia IPMN, n=34 HGD IPMN, n=20 invasive IPMN)	Concentration of PGE ₂ increased with increased dysplasia (LGD-IPMN 1.2 pg/μL, HGD-IPMN 3.5 pg/μL, IPMN with invasive carcinoma 4.4 pg/μL)	[203]
IL-1β, IL-5, IL-8	Cytokine concentration assessed by multiplex ELISA in PCF	n=40 (n=21 low-risk cysts defined by low-grade or moderate dysplasia, n=19 high-risk cysts defined by high-grade dysplasia or invasive PC)	Concentration of pro-inflammatory cytokines was significantly increased in high-risk cysts vs. low-risk cysts (IL-1β: 539 pg/μL vs. 0.2 pg/μL, IL-5: 0.2 pg/μL vs. 0.05 pg/μL, IL-8: 8089 pg/μL vs. 2758 pg/μL).	[104]

LGD=low-grade dysplasia. HGD=high-grade dysplasia. IPMN=intraductal papillary mucinous neoplasm. MCN=mucinous cystic neoplasm. PC=pancreatic cancer. PDAC=pancreatic ductal adenocarcinoma. MDSC=myeloid-derived suppressor cell. HGF=hepatocyte growth factor. GM-CSF=granulocyte-macrophage colony-stimulating factor. BD-IPMN=branch duct-IPMN. PGE₂=prostaglandin E2. PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay.

1.7 Summary

Given the 5-year survival rate of PC reaching only 13%, in conjunction with the vast majority of late-stage clinical disease and resistance to mainstay therapies, this highlights the crucial need for earlier diagnosis and intervention of PC. At present, a number of recognised risk-factors are associated with the biological establishment of PC, one of which includes pre-malignant PCLs. Some PCLs possess the ability to undergo malignant transformation and are regarded as precursor lesions of PC. However, there is a notable geographical and institutional lack of consensus on current cancer risk stratification guidelines for the classification and management of pre-malignant PCLs. Further refining the knowledge of crosstalk between the immune landscape and pancreatic microenvironment across different PCL subtypes is crucial to develop more in-depth and well-informed cancer risk stratification guidelines for the management of PCLs. Incorporating differential immunobiological features of pre-malignant PCLs into current PC risk stratification guidelines will provide a more accurate identification of patients with high-risk PCLs and reduce late-stage PC diagnosis.

1.8 Hypothesis and Aims

1.8.1 Overall hypothesis

Low- and high-risk PCF has differential effects on anti-tumour immunity which may be used for diagnostic and therapeutic purposes.

1.8.2 Overall aim

The overall aim of this thesis was to determine if risk-stratified PCF differentially promotes immunosuppression in the pre-malignant pancreatic setting and subsequent risk of malignant progression.

1.8.3 Specific aims

1. Characterise the immune cell infiltrate and immunomodulatory secretome of risk-stratified PCL cyst fluid for improved cancer risk stratification.
2. Investigate the influence of the soluble immunomodulatory secretome of risk-stratified PCF on anti-tumour effector immune cell function.
3. Elucidate key chemokine profiles in risk-stratified PCF to identify potential chemokine-based therapeutic targets that may enhance effector immune cell infiltration into the TME.

Chapter 2:

Materials and Methods

2.1 Ethical approval

Prior to the commencement of this study, ethical approval was obtained from the HSE Research Ethics Committee for the collection of patient samples in three designated sites: St. James's Hospital, St. Vincent's University Hospital and Tallaght University Hospital. Informed, written consent was provided by all participating patients for sample and data acquisition. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki ethical guidelines for medical research involving human subjects. All patient samples were pseudonymised in accordance with GDPR and data protection policies to protect the privacy and rights of all involved.

2.2 Patient sample acquisition

All patients aged 18 years or older who were diagnosed with a PCL at any of the three designated study sites and referred for EUS-FNA as part of their standard clinical care, in accordance with current best practice, were invited to participate in the study. Written informed consent was obtained from all participants prior to sample collection. All PCF samples were collected via EUS-FNA by gastroenterologists at Tallaght University Hospital and St. James's Hospital Dublin, and risk-stratified with the aid of the 2018 European evidence-based guidelines. Patient-matched blood was collected prior to the EUS-FNA procedure.

Non-cancer blood was taken fresh from consenting healthy donors at Trinity Translational Cancer Institute, St. James's Hospital, Dublin.

2.3 Patient PCF collection and storage

PCF was obtained via EUS-FNA of the PCL. Samples were transported on ice from the endoscopy suite to the laboratory and centrifuged at 2,500 g for 15 minutes. The upper PCF layer was collected and aliquoted into 250 μ L volumes in cryovials. These cryovials were labelled with an anonymous patient identifier before being snap-frozen in liquid nitrogen and stored at -80°C. Sample locations were documented in a dedicated biobank logbook. The PCF cell pellet was used immediately for phenotypic analysis by flow cytometry.

For selected assays, sonication was performed on PCF samples to disrupt large mucin proteins and reduce sample viscosity. Indirect sonication was carried out using the Fisherbrand™ Model 120 Sonic Dismembrator (Fisher Scientific, Ireland) with a 1.5-

inch Fisherbrand™ Cup Horn (Fisher Scientific, Ireland). PCF samples in Eppendorf tubes were positioned in the holder above the cup horn, which was filled with ice-cold deionised water (dH₂O) until the meniscus matched that of the sample inside the tube. Samples were sonicated for two 3 second bursts at 25% amplification, with a 5 second pause in between bursts. The dH₂O inside the apparatus was replaced between samples to ensure samples were kept cold and to prevent heat build-up.

2.4 Patient blood collection and storage

Peripheral venous blood samples were collected from patients with PCLs at the time of EUS-FNA procedure. For serum preparation, blood was drawn into Vacuette CAT Serum Sep Clot Activator tubes (Greiner Bio-One, UK), allowed to clot at room temperature for 30 minutes, and centrifuged at 2,500 g for 15 minutes at room temperature. The upper serum layer was aliquoted into 1 mL volumes, labelled with an anonymised patient identifier, snap-frozen in liquid nitrogen, and stored at -80°C. Sample locations were recorded in a dedicated biobank logbook.

For peripheral blood mononuclear cell (PBMC) isolation, blood was collected into BD Vacutainer® EDTA tubes (Greiner Bio-One, UK) from PCL patients at the time of the EUS-FNA procedure and processed as per the protocol for PBMC isolation by density gradient centrifugation.

2.5 Patient demographics

PCF was obtained from PCL patients undergoing an EUS-FNA procedure at Tallaght University Hospital and St. James's Hospital Dublin. Samples used within this study were obtained from August 2019 to August 2025. The cohort consisted of 22 males and 31 females, with a median age of 67 years. Patient demographics for PCF samples used within this research project are detailed in **Table 2.1**.

Table 2.1: Patient demographic information for PCF samples used within this study.

Patient Demographics		
Characteristics	Number of Patients (n=53)	Percentage (%)
Age at EUS, Years		
Median (Range)	67 (18-83)	
Unknown	3	
Sex		
Male (M)	22	41.5
Female (F)	31	58.5
Risk Stratification		
Pseudocyst (M:F)	14 (5:9)	26.4
Low-risk PCL (M:F)	23 (9:14)	43.4
High-risk PCL (M:F)	16 (8:8)	30.2
Clinical Presentation		
Non-symptomatic (incidental)	24	45.3
Abdominal pain	8	15.0
Pancreatitis	11	20.8
Unknown	10	18.9
Smoking Status		
Never-smokers	26	49.1
Ex-smokers	10	18.9
Active smokers	4	7.5
Unknown	13	24.5
Alcohol Consumption		
No alcohol	29	54.7
Regular alcohol consumption	11	20.8
Unknown	13	24.5
Diabetes Status		
Diabetic	4	7.5
Non-diabetic	33	62.3
Unknown	16	30.2
Presence of Jaundice		
Jaundice	2	3.8
No jaundice	45	84.9
Unknown	6	11.3
History of Pancreatitis		
Prior Pancreatitis	9 (8 pseudocyst; 1 low-risk PCL)	17.0
No prior pancreatitis	31	58.5
Unknown	13	24.5
History of Prior Malignancy		
Prior malignancy	3 (1 Breast Ca; 1 Prostate Ca)	5.7
No prior malignancy	36	67.9
Unknown	14	26.4
PCF Appearance		
Serous	13	24.5
Mucinous	24	45.3
Serosanguineous	5	9.4
Turbid	11	20.8
Location of PCL		
Head	24	45.3
Neck	5	9.4
Body	9	17.0
Tail	14	26.4
Uncinate	1	1.9

EUS-FNA=endoscope ultrasound-guided fine-needle aspiration. PCL=pancreatic cystic lesion.

Cancer risk stratification of PCLs was determined by gastroenterologists at Tallaght University Hospital and St. James's Hospital Dublin using the 2018 European evidence-based guidelines upon assessment of EUS and imaging or biochemical findings.

Patients were specifically stratified into the “high-risk” cohort if they fulfilled one or more of the following “worrisome” criteria:

- Cyst size (largest diameter of the PCL) ≥ 30 mm
- Positive cytology for high grade dysplasia or malignancy
- Main duct IPMN
- Presence of solid nodule
- Mural nodule ≥ 5 mm
- Main pancreatic duct ≥ 5 mm
- CEA ≥ 192 ng/mL
- Clinical jaundice as a result of PCL

Patient demographics and cancer risk stratification criteria recorded for high-risk PCF samples used within this research project are detailed in **Table 2.2**.

Table 2.2: Patient demographic information for stratification criteria for high-risk PCF samples used within this study.

Patient Demographics		
Characteristics	Number of High-risk Patients (n=16)	Percentage (%)
Age at EUS-FNA, Years		
Median (Range)	70 (39-83)	
Sex		
Male (M)	8	50
Female (F)	8	50
PCF Appearance		
Serous	1	6.25
Mucinous	14	87.50
Serosanguineous	1	6.25
Turbid	0	0.0
Location of PCL		
Head	9	56.25
Neck	0	0.0
Body	3	18.75
Tail	4	25.00
Uncinate	0	0.0
PCL Subtype		
IPMN	10 (6 BD; 1 MD)	62.50
MCN	1	6.25
Serous cystic adenoma	1	6.25
Undefined	4	25.00
PCL Size (Largest Diameter)		
Largest diameter $\geq$ 30 mm	9	56.25
Largest diameter $<$ 30 mm	7	43.75
Presence of Dysplasia		
Dysplasia	2	12.50
No dysplasia	14	87.50
Presence of Solid Nodule		
Solid nodule	3	18.75
No solid nodule	13	81.25
Enhancing Mural Nodule		
Enhancing mural nodule $\geq$ 5 mm	1	6.25
No enhancing mural nodule	15	93.75
Main Pancreatic Duct Dilation		
Pancreatic duct dilation $\geq$ 5 mm	4	25.0
Pancreatic duct dilation $<$ 5 mm	12	75.0
CEA Measurement		
CEA $\geq$ 192 ng/mL	10	62.50
CEA $<$ 192 ng/mL	3	18.75
Undefined (due to PCF viscosity)	3	18.75

EUS-FNA=endoscopy ultrasound-guided fine-needle aspiration. PCL=pancreatic cystic lesion. IPMN=intraductal papillary mucinous neoplasm. BD=branch duct. MD=main duct. MCN=mucinous cystic neoplasm. CEA=carcinoembryonic antigen. PCF=pancreatic cyst fluid.

2.6 Materials

2.6.1 Preparation of buffers and media

Table 2.3: Required materials for buffer and media preparation

Media	Materials required
Complete Roswell Park Memorial Institute (cRPMI) Media	500 mL RPMI 1640 (Gibco, UK) supplemented with; 10% (v/v) foetal bovine serum (FBS) (Gibco, UK), 1% (v/v) pen-strep (50 U/mL penicillin and 50 µg/mL streptomycin) (Lonza Group, Switzerland)
Complete Dulbecco's Modified Eagle Media (cDMEM)	500 mL DMEM (Gibco, UK) supplemented with; 10% (v/v) FBS 1% (v/v) pen-strep 10 ng/mL human recombinant EGF (Biosciences, Ireland)
Complete Keratinocyte Serum-Free Media (cKSFM)	500 mL Keratinocyte serum-free medium with L-glutamine (Biosciences, Ireland) supplemented with; 5 ng/mL human recombinant EGF (Biosciences, Ireland), 50 mg/mL bovine pituitary extract (Biosciences, Ireland), 1% (v/v) pen-strep
Buffers	
FACS Buffer	500 mL phosphate buffered saline (PBS) (Corning, Poland) 2% FBS, 0.01% sodium azide (Sigma-Aldrich, USA).
Blocking Buffer	50% FACS Buffer 50% FBS

2.7 Methods

2.7.1 PBMC isolation by density gradient centrifugation

Peripheral blood mononuclear cell (PBMC) isolation was carried out by density gradient centrifugation using Ficoll® Paque (Cytiva, Sweden). Blood was diluted 1:2 with room-temperature, sterile PBS (Corning, Poland). Diluted blood was carefully layered onto Ficoll® Paque in a 4:3 (diluted blood: density gradient medium) ratio so as not to disturb the interface between the blood and the density gradient medium. The layered blood was centrifuged at 400 g for 25 minutes with the brake off. Following centrifugation, high density cells (erythrocytes/granulocytes) are sedimented below the density gradient medium, whereas low density PBMCs (lymphocytes/monocytes) sit at the interface between the density gradient medium and the plasma layer (**Figure 2.1**). The white, cloudy layer containing the PBMCs (buffy layer) was transferred to a new centrifuge tube and washed twice in PBS with a centrifugation of 800 g for 5 minutes. The PBMC pellet was resuspended in 5mL of cRPMI (**Table 2.3**) and cells were counted.

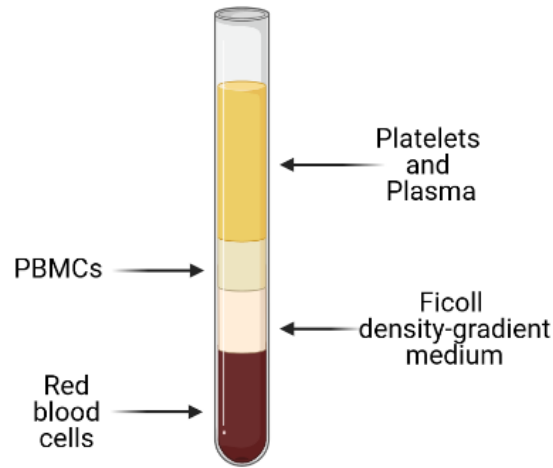


Figure 2.1: PBMC isolation by density gradient centrifugation. Density gradient separation of cell types facilitating the isolation of peripheral blood mononuclear cells by exploiting the density differential between lymphocytes/monocyte and erythrocytes/granulocytes.

2.7.2 Cell counting

Cells were pelleted by centrifugation and resuspended in 5 mL of culture media. A 1:10 dilution of cell suspension and trypan blue (Sigma-Aldrich, USA) was prepared (20 μ L cells, 180 μ L trypan blue), and 10 μ L was added to the haemocytometer (Neubauer glass haemocytometer 0.01 mm depth, Marienfeld-Superior). Viable, unstained cells were visualised at 20X magnification under a light microscope and counted in each of the four corner sections of the haemocytometer grid (**Figure 2.2**). The average number of cells per mL was obtained using the formula as follows:

$$\text{Average number of cells/mL} = \text{Average number of cells counted} \times 10^4 \times 10(\text{dilution factor})$$

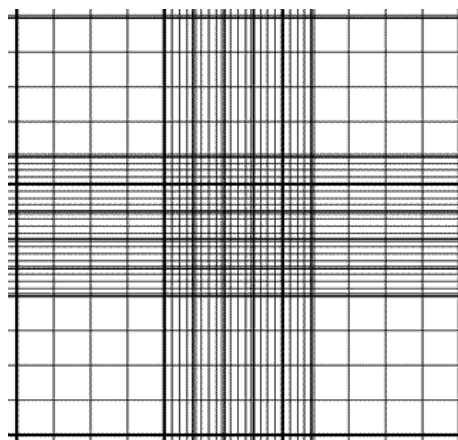


Figure 2.2: Representative image of haemocytometer grid used in the process of cell counting. Four outermost corner 4x4 squares were used to gain an average number of cells.

2.7.3 Anti-CD3 and anti-CD28 antibody T cell activation

The base of a 96-well flat-bottomed plate was coated with 50 μ L of goat anti-mouse immunoglobulin G (IgG) antibody (Sigma-Aldrich, USA; prepared to: 5 μ g/mL in sterile PBS) and incubated for 2 hours at 37°C. Following incubation, unbound IgG antibody was removed, and wells were washed twice with PBS. The base of each well was coated with 50 μ L solution of anti-CD3 antibody (1 μ g/mL, Biolegend, USA) and anti-CD28 antibody (1 μ g/mL, Biolegend, USA) and incubated for 2-hours at 37°C. Unbound antibody solution was carefully removed so as not to disrupt plate-bound anti-CD3/anti-CD28 antibodies. Wells were washed with PBS and cells were plated at a seeding density of 1×10^6 cells/mL. Cells were incubated for 24 hours at 37°C, 5% CO₂.

2.7.4 Co-culture of PBMCs with risk-stratified PCF

To assess the impact of PCF on T cell phenotype and function, PBMCs were co-cultured with risk-stratified PCF samples for 24 hours. PBMCs were isolated from whole blood using density gradient centrifugation from three healthy donors and activated for 24 hours in a 96 well plate, pre-coated with anti-CD3/anti-CD28 antibodies. Pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) (0% v/v, 20% v/v, 50% v/v) were added to the appropriate wells at the time of activation and left to incubate for 24/48 hours at 37°C, 5% CO₂. PBMCs were then harvested and stained for flow cytometric analysis.

2.7.5 Flow cytometric staining

2.7.5.1 Extracellular flow cytometric staining

PBMCs isolated from whole blood were washed twice with PBS using a centrifugation of 1300 RPM for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in prepared Zombie-near infrared (NIR)/Zombie-aqua viability dye (BioLegend, USA; overall 1 in 1000 titrated dilution with PBS) and left to incubate in the dark for 15 minutes at room temperature. The cells were washed twice in FACS buffer at 1300 RPM for 3 minutes. The PBMC cell pellet was resuspended in 100 μ L of blocking buffer and incubated in the dark for 10 minutes at room temperature. Cells were washed twice in FACS buffer. Following washes, the cells were resuspended in 100 μ L of FACS buffer and extracellular antibodies were left to incubate in the dark for 30 minutes at 4°C (**Table 2.4**). Stained PBMCs were washed twice in FACS buffer and resuspended in 300 μ L of FACS buffer for flow cytometric acquisition using the BD FACs Diva Software

(BD, USA) on the BD FACS CANTO II flow cytometer or BD LSR Fortessa flow cytometer. Data was analysed using FlowJo software (v10.10.0) (BD Biosciences, USA).

2.7.5.2 Intracellular flow cytometric staining

For intracellular cytokine staining, PBMCs were stimulated with a protein kinase C activator phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, USA; 10 ng/ml) and calcium ionophore ionomycin (Sigma Aldrich, USA; 1 µg/ml) for the last 4 hours of the incubation. Anti-CD107a-PE (BioLegend, USA) was added at the time of stimulation. For the last 3 hours of the incubation, brefeldin A (eBiosciences, USA; 10µg/ml) was added to each sample to inhibit protein transport from the endoplasmic reticulum to the golgi complex. Cells were harvested, washed in FACS buffer and subjected to viability staining and blocking of non-specific antibody binding. Cells were stained with cell surface antibodies first, washed, permeabilised, and then stained for intracellular cytokines (**Table 2.4**). Intracellular cytokines were assessed using a fixation/permeabilisation kit (Nordic-MUBio, NL) as per manufacturer's recommendations. Where transcription factors were incorporated into flow cytometric panels, the FoxP3/transcription factor staining buffer kit (eBioscience, USA) was prepared and used as per manufacturer's recommendations. Stained PBMCs were washed twice in FACS buffer and resuspended in 300 µL of FACS buffer for flow cytometric acquisition the BD FACS Diva Software (BD, USA) on the BD FACS CANTO II flow cytometer or BD LSR Fortessa flow cytometer. Data was analysed using FlowJo software (v10.10.0) (BD Biosciences, USA).

Table 2.4: Fluorochrome-conjugated antibody panels used for extracellular and intracellular cytometric staining.

Antibody	Antibody Clone	Manufacturer	Staining Protocol	Volume/ test (μL)	Catalogue No.
<i>Viability</i>					
Viability – Zombie-NIR		BioLegend	Extracellular	Dilution 1:1000	423105
Viability – Zombie-Aqua		BioLegend	Extracellular	Dilution 1:1000	423101
<i>Phenotypic Markers</i>					
CD45 – FITC	HI30	BioLegend	Extracellular	1	304005
CD3 – PerCP	HIT3a	BioLegend	Extracellular	1	300325
CD3 – PerCP/Cy5.5	HIT3a	BioLegend	Extracellular	1	300327
CD3 – PE/Cy7	HIT3a	BioLegend	Extracellular	1	300316
CD3 – AF700	HIT3a	BioLegend	Extracellular	1	300323
CD4 – BV510	OKT4	BioLegend	Extracellular	1	317443
CD4 – AF700	OKT4	BioLegend	Extracellular	1	317425
CD8 – Pacific Blue	SK1	BioLegend	Extracellular	1	344717
CD8 – BV510	SK1	BioLegend	Extracellular	1	344731
CD56 – BV510	5.1H11	BioLegend	Extracellular	1	362533
CD56 – APC	5.1H11	BioLegend	Extracellular	1	362503
CD56 – FITC	5.1H11	BioLegend	Extracellular	1	362545
CD19 – PE	H1B19	BioLegend	Extracellular	1	302207
CD38 – PE-Cy7	HB-7	BioLegend	Extracellular	1	356607
CD11b – APC	ICRF44	BioLegend	Extracellular	1	301309
CD11c – Pacific Blue	3.9	BioLegend	Extracellular	1	301625
CD14 – BV510	M5E2	BioLegend	Extracellular	1	301841
CD16 – PE-Cy7	3G8	BioLegend	Extracellular	1	302015
CD33 – PE	WM53	BioLegend	Extracellular	1	303403
CD68 – PerCP-Cy5.5	Y1/82A	BioLegend	Extracellular	1	333813
<i>Degranulation</i>					
CD107a – PE	H4A3	BioLegend	Extracellular	1	328607
<i>Cytotoxicity</i>					
Granzyme B – APC	QA16A02	BioLegend	Intracellular	2	372203
Perforin – FITC	dG9	BioLegend	Intracellular	2	308103
IFN-γ – PE-Cy7	4S.B3	BioLegend	Intracellular	2	502527
<i>Cytokines</i>					
IFN-γ – PE	4S.BS	BioLegend	Intracellular	2	502508
IL-4 – BV605	MP4-25D2	BioLegend	Intracellular	2	500827
IL-17 – BV785	BL168	BioLegend	Intracellular	2	512337
IL-10 – PE/Dazzle 594	JES3-9D7	BioLegend	Intracellular	2	501425
<i>Transcription Factors</i>					
T-bet – BV711	4B10	BioLegend	Intracellular	2	644819
GATA-3 – PE/Cy7	16E10A23	BioLegend	Intracellular	2	653819
RORγT – APC	600380	R&D Systems	Intracellular	2	IC6006A
FoxP3 – BV421	206D	BioLegend	Intracellular	2	320123
<i>Activation and Exhaustion Markers</i>					
CD27 – PE/Cy5	O323	BioLegend	Extracellular	1	302857
CD45RA – BV605	HI100	BioLegend	Extracellular	1	304133
CD62L – BV785	DREG-56	BioLegend	Extracellular	1	304829

CD62L – PE/Dazzle 594	DREG-56	BioLegend	Extracellular	1	304841
CD69 – PE/Cy7	FN50	BioLegend	Extracellular	1	310911
SLAMF6 – PE	W19035D	BioLegend	Extracellular	1	332303
<i>Inhibitory Proteins and Immune Checkpoint Receptors</i>					
PD-1 – PE/Cy7	EH12.2H7	BioLegend	Extracellular	1	329918
PD-1 – BV785	EH12.2H7	BioLegend	Extracellular	1	329929
LAG-3 – PE/Dazzle 594	11C3C65	BioLegend	Extracellular	1	369331
LAG-3 – BV421	11C3C65	BioLegend	Extracellular	1	369313
TIM-3 – PE	F38-2E2	BioLegend	Extracellular	1	345005
TIM-3 – BV711	F38-2E2	BioLegend	Extracellular	1	345023
TIGIT – PE/Cy5	A15153G	BioLegend	Extracellular	1	372747
VISTA – AF700	730804	R&D Systems	Extracellular	1	FAB71261N
B7-H3 – PE/Dazzle594	MIH42	BioLegend	Extracellular	1	351011
<i>Immune Checkpoint Ligands</i>					
PD-L1 – APC	MIH2	BioLegend	Extracellular	1	393609
PD-L2 – PE	MIH18	BioLegend	Extracellular	1	345505
Galectin-9– PerCP/Cy5.5	9M1-3	BioLegend	Extracellular	1	348909
CEACAM-1 – BV605	ASL-32	BioLegend	Extracellular	1	342323
CD112 – PE/Cy7	TX31	BioLegend	Extracellular	1	337413
CD155 – FITC	SKII.4	BioLegend	Extracellular	1	337627
<i>Adhesion Molecules</i>					
CD103 – APC	Ber-ACT8	BioLegend	Extracellular	1	350215
CD54 (ICAM-1) – APC	HA58	BioLegend	Extracellular	1	353111
Galectin-3 – PE	M3/38	BioLegend	Extracellular	1	125405
<i>Chemokine Receptors</i>					
CCR1 – PerCP/Cy5.5	5F10B29	BioLegend	Extracellular	1	362912
CCR2 – BV711	K036C2	BioLegend	Extracellular	1	357231
CCR6 – APC	G034E3	BioLegend	Extracellular	1	353416
CXCR3 – PE	G025H7	BioLegend	Extracellular	1	353706
CXCR4–VioBright FITC	REA649	Miltenyi	Extracellular	1	130-117-370
CX3CR1 – BV421	2A9-1	BioLegend	Extracellular	1	341619

2.7.5.3 Flow cytometry acquisition and analysis

Cells were acquired using the BD FACs Diva Software (BD, USA) on the BD FACS CANTO II flow cytometer or BD LSR Fortessa flow cytometer. FCS (v3.0) files were analysed using FlowJo software (v10.10.0) (BD Biosciences, USA).

2.7.6 Boyden chamber chemotaxis assay

PBMCs were isolated from non-cancer healthy controls (n=3) by density gradient centrifugation and seeded at a density of 1×10^6 cells/mL RPMI 1640 (Gibco, UK) supplemented 1% (v/v) penicillin-streptomycin (50 U/mL penicillin and 50 µg/mL streptomycin) and 10% (v/v) foetal bovine serum (FBS) (ThermoFisher Scientific, USA). T cells were activated using the T cell activation protocol which included plate bound anti-CD3 antibody (1 µg/mL, Biolegend, USA), anti-CD28 antibody (1 µg/mL,

Biologend, USA) for 24 hours. The bottom chamber of the Boyden chamber system (24-well plate) was prepared with 10% risk-stratified PCF (pseudocyst fluid (n=3), low-risk PCF (n=3), high-risk PCF (n=3)) diluted with serum-free RPMI. Where high-risk PCF was titrated, samples were prepared to 10% PCF, 1% PCF and 0.1% PCF using serum-free RPMI. Additionally, when investigating if immune cell migration could be reinstated towards high-risk PCF, a chemokine cocktail of recombinant human fractalkine (100 ng/mL), recombinant human RANTES (100 ng/mL) and recombinant human I-TAC (100 ng/mL) (PeproTech, UK) was spiked into the bottom chamber with 10% high-risk PCF. Serum-free RPMI was used as a negative control, and RPMI supplemented with 20% FBS was used as a positive control. Activated cells were subsequently washed twice in serum-free RPMI and added at a density of 0.2×10^6 cells/100 μ L RPMI medium to a 5 μ m pore Transwell filter system (Corning, Poland). This system was incubated for 2 hours at 37°C, 5% CO₂. Migrated cells were collected from the bottom chamber and stained for flow cytometric analysis. Stained PBMCs were washed twice in FACS buffer and resuspended in 200 μ L of FACS buffer for flow cytometric acquisition. CountBright™ absolute counting beads (ThermoFisher Scientific, USA) were used to enumerate the migrated cell populations. The total volume of each stained cell suspension was acquired using the FACS Diva software (BD, USA) on the BD FACS CANTO II flow cytometer. Data was analysed using FlowJo software (v10.10.0) (BD Biosciences, USA).

2.7.7 Quantification of soluble immune factors by flow cytometry-based AssayGenie multiplex ELISA

A custom bead-based multiplex enzyme-linked immunosorbent assay (ELISA) (Assay Genie, Ireland) was carried out as per manufacturer's instructions to assess the soluble pro-inflammatory cytokine, chemokine, adhesion, growth and survival factor secretions from pseudocyst fluid (n=5), low-risk PCF (n=6), high-risk PCF (n=7) and patient-matched serum samples. PCF samples were subjected to two 3 second indirect sonications at 25% amplification to break up large mucin proteins. Samples were diluted 1:4 with assay buffer. This multiplex ELISA was used to quantify the secretions of eotaxin, eotaxin-3, interleukin-8 (IL-8), interferon- γ -induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), monocyte chemoattractant protein 4 (MCP-4), macrophage-derived chemokine (MDC), macrophage inflammatory protein 1 α (MIP-1 α), macrophage inflammatory protein 1 β (MIP-1 β), thymus and activation-regulated chemokine (TARC), macrophage migration-inhibitory factor (MIF), fractalkine,

interferon-inducible T-cell alpha chemoattractant (I-TAC), regulated on activation, normal T cell expressed and secreted (RANTES), stromal cell-derived factor 1 (SDF-1), interleukin-6 (IL-6), hepatocyte growth factor (HGF), platelet-derived growth factor-AB subunits (PDGF-AB), vascular endothelial growth factor A (VEGF-A), granulocyte-macrophage colony stimulating factor (GM-CSF), soluble intracellular adhesion molecule 1 (sICAM-1) and thymic stromal lymphopoietin (TLSP). Due to sample pre-treatment, the TGF- β assay was isolated from the multiplex assays. Risk-stratified PCF samples and patient-matched sera were pretreated with 1N HCl (Sigma-Aldrich, USA) and then neutralised with 1.2N NaOH (Fisher Scientific, Germany)/0.5M HEPES (Sigma-Aldrich, USA) to activate latent TGF- β 1 to the immunoreactive configuration for accurate measurement. Samples were acquired using the CANTO II (BD Biosciences, USA) flow cytometer. FCS files were obtained and processed using FCAP Array software (v3). Data was graphed and statistically analysed using GraphPad Prism™ software (v10.6.1).

2.7.8 Quantification of soluble immune factors by MSD multiplex ELISA

To assess angiogenic, vascular injury, pro-inflammatory, cytokine, chemokine and soluble checkpoint secretions from the serum, a 54-plex ELISA kit was used (Meso Scale Diagnostics, USA). The multiplex kit was used to quantify the secretions of C-reactive protein (CRP), eotaxin, eotaxin-3, FGF(basic), Flt-1, GM-CSF, ICAM-1, IFN- γ , IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17A/F, IL-17B, IL-17C, IL-17D, IL-1RA, IL-1 α , IL-1 β , IL-2, IL-21, IL-22, IL-23, IL-27, IL-3, IL-31, IL-4, IL-5, IL-6, IL-7, IL-8, IL-8, IL-9, IP-10, MCP-1, MCP-4, MDC, MIP-1 α , MIP-1 β , MIP-3 α , placental growth factor (PlGF), serum amyloid A (SAA), TARC, Tie-2, TNF- α , TNF- β , TSLP, vascular cell adhesion molecule 1 (VCAM-1), VEGF-A, VEGF-C and VEGF-D and immune checkpoints PD-1 epitope 1, PD-1 epitope 2, programmed cell death ligand 1 (PD-L1) epitope 1, PD-L1 epitope 2, PD-L2, and CD40L. All PCF samples were diluted 1:4 in the appropriate diluent solutions provided with the ELISA kits. All assays were run as per manufacturer's instructions, with an overnight supernatant incubation protocol used for all assays except Angiogenesis Panel 1 and Vascular Injury Panel 2, which were run according to the same day protocol.

2.7.9 Quantification of soluble immune factors by sandwich ELISA

Analytes not compatible with the bead-based multiplex panel were quantified in risk-stratified PCF samples and patient-matched sera using standard, single-analyte sandwich ELISAs (CD147, Plasminogen activator inhibitor 1 (PAI-1) (Assay Genie, Ireland) and galectin-3 (BioTechne, USA)). PCF samples were processed via indirect sonications at 25% amplification to break up large mucin proteins. All reagents were prepared as per the manufacturer's instructions. Samples were diluted 1:4 in sample dilution buffer. Fluorometric data at 450 nm was acquired using the GloMax Explorer microplate reader (Promega, USA) and statistically analysed using GraphPad Prism™ software (v10.6.1).

2.7.10 Lymphocyte-mediated cytotoxicity assay

PBMCs were isolated from healthy, consenting donors using density gradient centrifugation and prepared to a seeding density of 1×10^6 PBMCs/mL. These lymphocytes were subjected to plate-bound T cell activation with anti-CD3 antibody (10 µg/mL, Biolegend, USA), anti-CD28 antibody (10 µg/mL, Biolegend, USA) for 24 hours. Following this activation, lymphocytes were co-cultured with 20% (v/v) pseudocyst fluid, low-risk PCF or high-risk PCF for 24 hours at 37°C, 5% CO₂. After this pre-treatment with risk-stratified PCF, the activated effector lymphocytes were centrifuged at 1300 RPM for 3 minutes to remove the co-culture supernatant and the cells were resuspended in cRPMI, maintaining the 1×10^6 PBMCs/mL seeding density.

The target PC cells, BxPC-3 cells and Panc-1 cells, were plated at a seeding density of 5×10^3 in 100 µL of cRPMI in a 96-well flat-bottomed plate and were left to adhere overnight at 37°C, 5% CO₂. Activated lymphocytes, exposed to risk-stratified PCF, were added at a density of 2.5×10^4 PBMCs to 5×10^3 PC cells to achieve an effector cell to target cell (E:T) ratio of 5:1. This co-culture was left to incubate for 48 hours at 37°C, 5% CO₂. Activated lymphocytes and PC cell lines were also cultured alone to account for the absorbance of lymphocytes only and PC cells only. After 48 hours, 5 µL of cell counting kit-8 (CCK-8) assay solution (Sigma-Aldrich, USA) was added to each well. Cells were incubated at 37°C, 5% CO₂ in foil for 2 hours until an appropriate pink to orange colour development was observed. Absorbance was measured at 450 nm using the GloMax Explorer microplate reader (Promega, USA).

The proportion of cell survival was calculated using the formula as follows:

$$\% \text{ Live Cells} = \frac{(\text{Absorbance PC cells \& PBMC coculture}) - (\text{Absorbance untreated PBMCs alone})}{(\text{Absorbance untreated PC cells alone})}$$

2.7.11 Cell culture

Aseptic technique was adopted for all cell culture procedures. Cell culture work was performed within a sterile grade II laminar flow hood, which was disinfected with 70% (v/v) ethanol prior to use. All reagents and equipment were sterilised prior to being introduced into the laminar flow hood.

2.7.11.1 Pancreatic cell line maintenance

A normal human pancreatic duct epithelial cell line, H6c7 cells (H6c7-HPDE), was obtained from Kerafast Inc. (Boston, MA, USA). These cells were maintained in Keratinocyte Serum-Free Medium containing L-glutamine and supplemented with 5 ng/mL human recombinant EGF (Biosciences, Dublin, Ireland), 50 mg/mL bovine pituitary extract (Biosciences, Dublin, Ireland) and 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza Group, Basel, Switzerland) (hereafter referred to as cKSFM). H6c7-HPDE cells were maintained as monolayers in either 25 cm² or 75 cm² sterile vented culture flasks, in an incubator at 37°C in 95% humidified air containing 5% CO₂.

An intermediary human pancreatic nestin-expressing cell line formed during acinar-to-ductal metaplasia, hTERT cells (TERT-HPNE), was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The hTERT-HPNE cell line was originally isolated from the pancreas of a 52-year-old, male donor. These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher, UK) with high glucose supplemented with 10% (v/v) FBS, 10 ng/mL human recombinant EGF (Biosciences, Ireland) and 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza Group, Switzerland) (hereafter referred to as cDMEM). HPNE-intermediary cells were maintained as monolayers in either 25 cm² or 75 cm² sterile vented culture flasks, in an incubator at 37°C in 95% humidified air containing 5% CO₂.

The human pancreatic cancer cell lines BxPC-3 and Panc-1, were also obtained from the ATCC (Manassas, VA, USA). BxPC-3 cells were isolated from the pancreas of a 61-year-old female patient with adenocarcinoma while Panc-1 cells were isolated from the

pancreatic duct of a 56-year-old male patient with epithelioid carcinoma. Both cell lines were maintained in RPMI 1640 (Gibco, UK) supplemented with 10% (v/v) FBS (Gibco, UK) and 50 U/mL penicillin and 50 µg/mL streptomycin (Lonza Group, Switzerland) (hereafter referred to as cRPMI). BxPC-3 and Panc-1 cells were both maintained as monolayers in either 25 cm² or 75 cm² sterile vented culture flasks, in an incubator at 37°C in 95% humidified air containing 5% CO₂.

2.7.11.2 Cell line sub-culture

Phosphate-buffered saline (PBS), complete growth medium, and trypsin-EDTA (Lonza Group, Switzerland) were pre-warmed to 37°C in a water bath before sub-culturing.

Cell cultures were maintained in an exponential growth phase in vented T75 cm² tissue culture flasks and subcultured at 70%-80% confluency. Cell culture supernatants were harvested and stored at -80°C for later use. Otherwise, spent medium was discarded and all cell waste decontaminated by treatment for 24 hours with Haz-Tab chlorine tablets (Guest Medical, UK).

Cells were briefly washed with sterile PBS prior to detachment. In the case of hTERT-HPNE cells, calcium/magnesium-free Dulbecco's PBS (ThermoFisher, London) was used in place of PBS to aid trypsinisation. Subsequently, adherent cells were detached from the surface of the flask using trypsin-EDTA (Sigma-Aldrich, USA). To promote enzyme activity and cell detachment, cells were incubated with trypsin-EDTA for 3-5 minutes at 37°C. Light microscopy was used to ensure all cells had successfully detached from the bottom of the flask before an equal volume of complete growth medium was added to neutralise the trypsin-EDTA. In the case of H6c7-HPDE cells, an equal volume of defined trypsin inhibitor (Gibco, UK) and an added 2 mL of cKSFM was used to neutralise trypsin-EDTA. Cell suspensions were transferred to sterile 15 mL tubes and centrifuged at 1300 RPM for 3 minutes at room temperature. Supernatants were discarded, and cell pellets were resuspended in the appropriate complete growth medium. H6c7-normal cells were passaged with a sub-cultivation ratio between 1:2 and 1:4 ratio. HPNE-intermediary cells were passaged with a sub-cultivation ratio between 1:8 and 1:12 ratio. BxPC-3 cells and Panc-1 cells were passaged with a sub-cultivation ratio between 1:6 and 1:8 ratio. Passaged cells were then stored in an incubator at 37°C in 95% humidified air containing 5% CO₂.

2.7.11.3 Cryopreservation and reconstitution of frozen cell stocks

Frozen cell stocks were prepared from cells in the exponential growth phase. Adherent cells were detached from the flask by trypsinisation as previously described. Trypsin-EDTA was neutralised, and the cell suspension was transferred to a sterile 15 mL tube. Cells were pelleted by centrifugation at 1300 RPM for 3 minutes. The cell pellet was resuspended in cryopreservation medium consisting of complete growth medium supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). This cell suspension was added in 1 mL aliquots to sterile, labelled 2 mL cryovials. Cryovials were added to a Mr Frosty™ Freezing Container (ThermoFisher Scientific, Massachusetts, United States) containing isopropyl alcohol, to gradually lower the temperature of the cell solution by 1°C per minute. Cell stocks were stored at -80°C overnight and transferred to a liquid nitrogen container for long-term storage.

For the resuscitation of cells from cryopreservation, cell line stocks were retrieved from the -80°C freezer or liquid nitrogen storage and thawed rapidly at 37°C in a water bath. The cell solution was transferred dropwise to a 15 mL tube containing 10 mL of pre-warmed complete growth medium to avoid osmotic shock. Cells were pelleted by centrifugation at 1300 RPM for 3 minutes. The supernatant was discarded to waste, and the cell pellet was resuspended in 1 mL of warm complete growth medium. This cell suspension was then added to a 25 cm² flask containing 7 mL of complete growth medium. Cells were then stored in an incubator at 37°C in 95% humidified air containing 5% CO₂.

2.7.11.4 Mycoplasma testing

Cell lines were routinely screened for mycoplasma contamination. For each assay, 1 mL of culture supernatant was collected from a confluent 75 cm² culture flask and stored at 4°C until analysis. Supernatants were centrifuged at 2000 RPM for 1 minute to pellet cellular debris. A polymerase chain reaction (PCR) for each sample was set up in 0.2 mL PCR tubes to contain:

- 12.5 µL of Green GoTaq (Promega, USA)
- 0.5 µL of forward primer (10 uM) (ThermoFisher, UK)
- 0.5 µL of reverse primer (10 uM) (ThermoFisher, UK)

- 10.5 µL of molecular biology grade sterile water (ThermoFisher, UK)
- 1 µL of cell culture supernatant.

Positive and negative controls were prepared using mycoplasma-positive supernatant and sterile water, respectively. PCR was performed on a thermal cycler with the following program: 95°C for 5 minutes before being subjected to 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. After the 40 cycles, PCR tubes were kept at 72°C for 10 minutes and finally allowed to cool to 4°C.

While the PCR tubes were on the thermal cycler, 2% agarose gel was prepared and allowed to set. 10X Tris-Borate-EDTA (TBE) buffer was made by dissolving 165 g Tris base, 27.5 g boric acid, and 9.3 g EDTA in 800 mL of dH₂O, adjusting the pH to 8.3 using acetic acid and making up the final volume to 1 L with dH₂O. Working solution (1X TBE) was prepared by diluting the stock 1:10 with dH₂O. Agarose gels were prepared by adding 4 g agarose to 200 mL 1X TBE, melting by gentle microwaving with frequent stirring until the agarose was melted and the solution was a clear liquid. The liquid agarose gel was allowed to cool for 5 minutes before being transferred to 50 mL tubes. SYBR Safe DNA gel stain (3 µL; ThermoFisher, UK) was added to each 50 mL tube and mixed by inversion. The gel solution was steadily poured into the gel electrophoresis rig with a 20-well comb inserted. Any bubbles present were promptly removed with a pipette tip before allowing the gel to set for 1 hour at room temperature. Once set, the comb was removed and the gel was submerged in 1X TBE buffer, with the wells being positioned at the anode. After being cooled to 4°C on the thermal cycler, samples were transferred on ice to the gel rig. A 100 bp ladder was loaded into each side of the gel at a volume of 3 µL per well. Samples and controls were then loaded into the gel at a volume of 10 µL per well. Electrophoresis was then carried out using at a voltage of 80 V for 1 hour.

After electrophoresis, the PCR products were then imaged using a BioRad ChemiDoc System (Bio-Rad Laboratories, USA) under the SYBR Green setting with the expected band size for mycoplasma-positive samples at 270 bp.

2.7.12 Preparation of whole cell lysates from pancreatic cell lines

Confluent T75 cm² flasks of pancreatic cells (H6c7-HPDE, hTERT-HPNE, BxPC-3, Panc-1) were washed with 6 mL of ice-cold PBS. Adherent cells were lifted from the flask using cell scrapers. These cells were transferred to a 15 mL centrifuge tube and

centrifuged at 500 g for 3 minutes. The supernatants were discarded, and the cell pellets were resuspended in 100 μ L of ice-cold radio-immunoprecipitation assay buffer (RIPA) buffer (50 mM Tris-HCl pH 8, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, with the addition of protease and phosphatase inhibitor tablets (Sigma-Aldrich, Arklow, Ireland)). The cell suspensions were then vortexed vigorously for 30 seconds at the highest setting and incubated on ice for 30 minutes. Following this incubation, the cell suspensions were centrifuged at 14,000 RPM for 10 minutes in a microcentrifuge pre-cooled to 4°C. The supernatants (whole cell lysates) were carefully and immediately transferred to pre-chilled microcentrifuge tubes for storage at -80°C, avoiding disturbance of the pellets.

2.7.13 Cytoplasmic and nuclear extraction from pancreatic cell lines

Pancreatic cell lines were harvested with trypsin, washed in PBS and neutralised as per standard protocol. The cells were resuspended in PBS and counted. 2×10^6 cells were transferred to a 1.5 mL microcentrifuge tube and centrifuged at 500 g for 3 minutes. The supernatant was gently removed, leaving the pellet as dry as possible. Cytoplasmic and nuclear extracts were isolated using the NE-PER Nuclear and Cytoplasmic Extraction kit (ThermoFisher Scientific, USA). All reagents were prepared as per manufacturer's recommendations. A protease inhibitor cocktail (ThermoFisher Scientific, USA) was added to cytoplasmic extraction reagent I (CER I) and nuclear extraction reagent (NER). The cell pellet was resuspended in 200 μ L ice-cold CER I, vortexed vigorously for 30 seconds and incubated on ice for 20 minutes. Following this incubation, 11 μ L of ice-cold CER II was added and the tube was vortexed at the highest setting for 30 seconds. The cells were then incubated on ice for a further 10 minutes. The tube was vortexed again at the highest setting for 30 seconds and centrifuged at 14,000 g for 30 seconds in a microcentrifuge pre-cooled to 4°C. The supernatant (cytoplasmic extract) was immediately transferred to pre-chilled microcentrifuge tubes for storage at -80°C.

The insoluble fraction (pellet) was used for nuclear fraction collection. This pellet was resuspended in 100 μ L of ice-cold NER and vortexed vigorously for 30 seconds at the highest setting. This suspension was then left to incubate on ice for 10 minutes. The sample was vortexed for 30 seconds every 10 minutes for a total of 40 minutes, incubating the tube on ice in between. The tube was centrifuged at 16,000 g for 10 minutes in a microcentrifuge pre-cooled to 4°C. The supernatant (nuclear extract) was immediately

transferred to pre-chilled microcentrifuge tubes for storage at -80°C. These cytoplasmic and nuclear extracts were then used to confirm cellular fractionation by Western blot and to quantify galectin-3 in the specific cellular compartments of pancreatic cells by ELISA.

2.7.14 Total protein quantification by BCA protein assay

The protein concentration of whole cell lysates, cytoplasmic extracts and nuclear extracts from pancreatic cell lines was assessed by PierceTM bicinchoninic acid (BCA) protein assay (Fisher Scientific, Ireland) as per the manufacturer's instructions. The bovine serum albumin standard was serially diluted from the provided stock solution (2000 µg/mL) at the recommended concentrations to create a standard curve, with the final standard containing no bovine serum albumin (blank). Cell line supernatants, whole cell lysates, cytoplasmic extracts and nuclear extracts were diluted 1:10 with RIPA buffer (50 mM Tris-HCl pH 8, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl (Sigma-Aldrich, Arklow, Ireland)). A volume of 25 µL of each standard and sample dilution was plated in a clear, flat-bottomed 96-well microplate in triplicate. Working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B. A volume of 200 µL of working reagent was added to each well and the plate was mixed on a plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. Once cooled to room temperature, the absorbance at 562 nm was measured using the GloMax Explorer microplate reader (Promega,USA).

2.7.15 SDS-PAGE

Protein samples were quantified and adjusted to a concentration of 10 µg per well. Samples were prepared in RIPA buffer to a final volume of 30 µL in 1.5 mL microcentrifuge tubes. To each sample, 7.5 µL of loading buffer (Invitrogen NuPAGE LDS Sample Loading Buffer: 3.3% sodium dodecyl sulphate (SDS), 6 M urea, 17 mM Tris-HCl pH 7.5, 0.01% bromophenol blue, and 0.07 M β-mercaptoethanol: ThermoFisher Scientific, USA) was added. Samples were briefly centrifuged to pool contents, denatured at 95 °C for 8 minutes, cooled to room temperature, and centrifuged again prior to electrophoresis.

Proteins were resolved by SDS-PAGE using pre-cast polyacrylamide gradient gels (4-20%) (Mini-Protean® TGXTM Precast Gels, Bio-Rad Laboratories, USA) and electrophoresed to separate proteins according to the size within the gel matrix. Gels were

assembled into a gasket and mounted into the electrophoresis tank with short plates oriented toward the central buffer reservoir. The integrity of the gel system was confirmed by adding a small volume of 1X running buffer (prepared from a 10X stock: 30.3 g Tris base, 144 g glycine, 100 mL 10% SDS, and dH₂O to 1 L) between the glass plates to check for leakage. Both inner and outer buffer reservoirs were then filled with 1X running buffer. After comb removal, gel wells were flushed with 1X running buffer to remove residual polymerised stacking gel and ensure unobstructed loading.

For electrophoresis, 5 µL of molecular weight marker (1 kDa NeoPRO 10 Prestained Protein Ladder; CliniSciences, Ireland) was loaded into the first well, followed by 30 µL of each protein sample in subsequent wells using gel loading tips (ThermoFisher Scientific, USA). Gels were then electrophoresed (PowerPac Universal, Bio-Rad, UK) for an initial 25 minutes at 80 V to prevent the samples being pulled down the gel too quickly. Once the dye front reached past the bottom of the well, the voltage was altered to 120 V for 90 minutes. Gel electrophoresis was stopped when the dye front became close to the bottom of the gel. Glass plates were carefully separated after electrophoresis was completed, and the wells were excised from the gel. Gels were then transferred to a box tray containing 1X transfer buffer (diluted from 10X stock: 30.2 g Tris base, 144 g glycine, dH₂O up to 1 L) for 5 minutes while transfer apparatus and reagents were prepared.

2.7.16 Western blot analysis to determine fractionation efficiency

Proteins separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (0.2 µm; ThermoFisher Scientific, USA). Prior to assembly, PVDF membranes were activated in methanol for 10 seconds. Transfer cassettes were assembled appropriately, and all components were thoroughly soaked in 1X transfer buffer, ensuring the clear side was facing down. Firstly, a sponge was placed onto the clear side of the cassette, followed by two filter papers, the polyacrylamide gel, PVDF membrane, two filter papers, and finally, another sponge. Subsequently, a roller was used to ensure no air bubbles were present. The sandwich cassette was then carefully closed, fastened, and inserted into the transfer gasket, with the black side of the cassette facing the black side of the gasket (negative electrode) and the red side of the cassette facing the red side of the gasket (positive electrode). The transfer gasket was slotted into the tank and filled to the marked level with 1X transfer buffer. A magnetic stir bar and a cooling unit (Bio-Rad,

UK) was added to the tank to control temperature and circulate buffer during transfer. The tank was then placed on a magnetic stirrer and run at 100 V for 90 minutes.

Following the transfer, the cassette was removed and carefully disassembled using forceps to minimise contact with the PVDF membranes. Used filter papers and gels were discarded to waste. The orientation of the membrane was noted and using a scalpel, the top left-hand corner of the membrane was cut. Subsequently, the PVDF membrane was transferred into a 50 mL tube to block the membrane, thus preventing non-specific binding of antibody, which contained 15 mL 5% bovine serum albumin (BSA) in 1X TBST (TBS diluted from 10X: 24 g Tris HCl, 5.6 g Tris base, 88 g NaCl, up to 1 L dH₂O, with the addition of 0.1% Tween 20) solution. Membranes were incubated at room temperature on a gyro rocker (Stuart 3D gyratory rocker, Bibby Scientific, UK) set at 40 RPM for 1 hour. After blocking, the blocking solution was discarded, and membranes were incubated in primary antibody overnight at 4 °C on a tube roller. Primary antibodies (**Table 2.5**) were prepared in 50 mL tubes, with 5% BSA solution prepared with TBST.

Table 2.5: Primary antibodies used for Western blotting to confirm fractionation efficiency.

Target protein	Molecular Weight	Manufacturer	Catalogue No.	Species	Dilution used
<i>Cytoplasmic Marker</i>					
GAPDH	37 kDa	Santa Cruz Biotechnology	sc-32233	Mouse	1:1000 (5% BSA)
<i>Nuclear Marker</i>					
Histone H3	17 kDa	Cell Signaling	9715S	Rabbit	1:1000 (5% BSA)

Following primary antibody incubation, membranes were washed in 1X TBST for 30 minutes, with at least three buffer changes during that period. Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse/anti-rabbit) were prepared in fresh 50 mL tubes in a solution of 5% BSA prepared with TBST (**Table 2.6**). Membranes were incubated in HRP-conjugated secondary antibody in a box tray at room temperature on a gyrorocker set at 40 RPM for 1 hour. Membranes were subsequently washed in 1X TBST for 30 minutes, with at least three buffer changes during that period.

Table 2.6: Secondary antibodies used for Western blotting to confirm fractionation efficiency.

Target protein	Manufacturer	Catalogue No.	Species	Dilution used
Anti-mouse (HRP-conjugated)	Cell Signaling	7076S	Rabbit	1:2000 (5% BSA)
Anti-rabbit (HRP-conjugated)	Cell Signaling	7074P2	Goat	1:2000 (5% BSA)

Imaging of the membranes was completed through a chemiluminescent imaging system (BioRad ChemiDoc System, Bio-Rad Laboratories, USA). Firstly, SuperSignal™ West Pico PLUS chemiluminescence substrate (ThermoFisher Scientific, USA) was prepared by combining the two reagents in a 1:1 ratio. Membranes were put onto a sheet of clean acetate and 1.5 mL chemiluminescence substrate solution was poured onto each membrane, ensuring the entirety of the surface was covered. Membranes were incubated in the prepared chemiluminescence substrate solution for 2 minutes, after which this solution was carefully removed. Membranes were then inputted into the imaging system and imaged for 5 minutes under the “Chemiluminescence” setting. ImageJ software (v1.54p) was used to isolate images of samples at specific sizes of proteins of interest (GAPDH and Histone H3) based on the molecular weight ladder.

2.7.17 Statistical analysis

Graphing of results and statistical analysis was performed using GraphPad Prism™ software (v10.6.1). All data are presented as the mean $\pm$ standard error of the mean (SEM), unless otherwise stated. Experimental data was assessed using non-parametric tests, such as a Mann-Whitney test or Kruskal-Wallis test, as appropriate based on the number of groups to be assessed. In cases where the data was paired (i.e. PCF vs Serum/ PCF vs. PBMC), a Wilcoxon test or Friedman test were employed. Where the analysis involved 3 or more groups (Pseudo vs. Low-risk vs. High-risk), Dunn’s multiple comparison test was implemented to determine the statistical significance between the groups. Statistical significance was defined as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$.

Chapter 3:
**Immunophenotyping the immune cell infiltrate of
pancreatic cystic lesions and the associated role
these cellular immune profiles play in malignant
progression**

3.1 Introduction

Pancreatic cancer (PC) is among the most aggressive and lethal malignancies, characterised by an immunologically cold tumour phenotype, dismal survival rates, and limited therapeutic options^[1, 208, 209]. Despite significant advances in cancer detection and treatment over recent decades, PC continues to have one of the lowest five-year survival rates of all solid tumours, reaching only 13%^[1]. This poor prognosis is attributable to the asymptomatic nature of early stages of disease, which often results in late clinical presentation, limited eligibility for surgical resection and the intrinsic resistance of PC tumours to conventional chemoradiotherapy^[210, 211]. Current standard-of-care approaches rely primarily on surgical resection followed by adjuvant chemotherapy for early-stage disease, while systemic chemotherapy and palliative care remain the mainstays for advanced or metastatic stages^[9, 10]. Immunotherapy has revolutionised the treatment of many cancers^[212]. However, the success of immunotherapies in PC has been minimal^[213, 214]. To date, the anti-PD-1 immune checkpoint inhibitor pembrolizumab has been approved for use only in small subsets of PC patients with unresectable or metastatic microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) solid tumours that progressed following previous treatment^[215, 216]. Notably, only 1-2% of PC tumours exhibit MSI-H or dMMR phenotypes for which pembrolizumab is a valid option as a second line treatment and beyond, meaning that immunotherapy remains a viable option for only a very limited proportion of PC patients^[217]. Consequently, treatment strategies for late-stage PC predominantly focus on improving quality of life and managing symptomology, as opposed to having curative intent^[218]. These limited successes highlight the urgent need to elucidate the biological processes underpinning pancreatic tumourigenesis and therapeutic resistance.

The TME plays a pivotal role in driving tumour progression, immune evasion, and therapeutic resistance in cancer^[219, 220]. In PC, the desmoplastic TME is exceptionally dense and immunosuppressive, forming a physical and biochemical barrier that limits drug penetration and dampens antitumour immunity^[29]. The TME is a dynamic and complex landscape composed of malignant epithelial cells, CAFs, stromal cells, immune cells, ECM and soluble mediators, all of which interact to shape tumour growth^[221-224]. The desmoplastic stroma characteristic of PC contributes to hypoxia, metabolic stress, and altered immune cell trafficking^[36]. A deeper understanding of the immune microenvironment of pre-malignant lesions and the specific immune constituents is

therefore critical for identifying key drivers of PC progression which may ultimately aid in the prevention of late-stage clinical presentation.

While most PCLs remain benign, a subset possesses the potential to undergo malignant transformation and progress to invasive PC^[225]. The biological mechanisms driving this transition are not completely understood, and it remains unclear how the immune landscape of these lesions contributes to malignant potential. Immunophenotyping, through methods like flow cytometry, provides an opportunity to identify and phenotype the immune cell populations within these lesions, providing insight into their role in disease progression. Moreover, it is unknown whether the immune landscape within PCLs reflects that observed in peripheral circulation. Understanding this relationship may identify opportunities to leverage minimally invasive sampling techniques for immune profiling of pre-malignant lesions.

To date, a limited number of studies have examined the expression of inflammatory mediator proteins (IMPs) in IPMN cyst fluid or otherwise have profiled immune populations within the surrounding tissue of pancreatic cysts^[39, 111, 194]. However, the specific immune cell subsets present in pre-malignant PCF across risk-stratified lesions remain poorly characterised and may be used to improve stratification of lesions that are at a higher risk of PC transformation.

A number of studies have investigated the cytokine and growth factor milieu within PCF to evaluate its potential as a source of diagnostic and prognostic biomarkers^[104, 194, 226]. Elevated levels of inflammatory mediators such as IL-1 β , IL-5, IL-8, IL-10, eotaxin, HGF and GM-CSF have been reported in PCF from precursor lesions, with certain cytokines correlating with histopathological grade or malignant potential^[104, 194, 226]. Maker *et al.* demonstrated that IL-1 β levels in PCF can discriminate between high-risk and low-risk IPMNs, while Lee *et al.* and others have shown that multiplex cytokine profiling can differentiate neoplastic from non-neoplastic cysts^[104, 194, 226]. However, these investigations primarily focused on the soluble inflammatory mediator profile of PCF, without assessment of the accompanying cellular immune composition or functional phenotypes of infiltrating immune populations. As a result, it remains unclear whether the observed cytokine signatures reflect active immune surveillance, chronic inflammation, or immune exhaustion within these lesions. A comprehensive assessment

that integrates both soluble mediators and cellular immune profiling is therefore essential to fully delineate the immunobiology of pre-malignant PCLs.

This chapter aims to profile the immune cell infiltrate of benign pseudocysts, pre-malignant low-risk PCF and pre-malignant high-risk PCF. This study specifically aims to identify and quantify populations of innate and adaptive immune cells by flow cytometric analysis to provide a detailed map of the immune composition across risk-stratified PCLs. This analysis was also carried out on PCL patient-matched PBMCs and age-matched, healthy PBMC control samples to discern whether the immune landscape in circulation was reflective of the local immune microenvironment of pre-malignant PCLs.

Furthermore, this chapter assesses the phenotypic profiles of the immune cellular compartment of risk-stratified PCLs with a focus on effector immune cell degranulation and cytotoxicity, activation, exhaustion and immune checkpoint expression. By comparing the immune infiltrates within these risk-stratified lesions and the associated phenotypes, this study aims to define specific immunological signatures associated with malignant progression. Characterising the immune landscape of these PCLs offers novel insight into early stages of PC tumourigenesis but may also reveal biomarkers for improved stratification of patients at risk of PC development and identify new immune targets for therapeutic intervention.

3.2 Hypothesis

The immune cell infiltrate in PCLs is associated with a differential risk of malignant progression.

3.3 Specific aims

1. Study the innate and adaptive immune cellular component of benign pseudocyst fluid, pre-malignant low-risk PCF and pre-malignant high-risk PCF.
2. Perform a phenotypic analysis on cell populations identified in the immune cell infiltrate of risk-stratified PCF.
3. Investigate the expression of immune checkpoint receptors and ligands across disease progression in pancreatic cells lines, both basally and under stress conditions to understand their potential involvement in PC progression.

3.4 Results

3.4.1 PCF has a high proportion of CD4⁺ T cells and CD8⁺ T cells.

Immunophenotyping the soluble immune microenvironment of PCLs is crucial for elucidating differences between lesions that are at a higher risk of PC progression and those at a lower risk. Importantly, identifying key immune cell subsets within the immune cell infiltrate of PCF will play a key role in understanding how the immune system plays a role in the progression of pre-malignant lesions to PC. This study used flow cytometry to identify and phenotype key immune cell subsets in patient-derived PCF and matched PBMC samples. Cells within PCF were isolated from the soluble component using centrifugation and PBMCs were isolated from whole blood using density gradient centrifugation. The proportion of adaptive and immune cells such as T cells, NK cells, B cells, macrophages, monocytes and dendritic cells were assessed by flow cytometry using immunophenotypic markers including CD45, CD3, CD4, CD8, CD56, CD19, CD38 CD11b, CD11c, CD14, CD16, CD33 and CD68.

Although not significant, data from this study showed that PCF from pre-malignant lesions contains a higher proportion of T cells compared to other immune cell subsets. This was particularly the case for CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells and NK cells (**Figure 3.1A**). There was also a higher prevalence of macrophages within the pre-malignant PCF, which was heavily influenced by the total macrophage count in the benign pseudocyst sample (**Figure 3.1A**). Despite limited numbers of high-risk PCL samples within this analysis, it is interesting to note that the cellular immune profile of some high-risk PCF samples was found to have a higher proportion of CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, NK cells, B cells and non-classical monocytes compared to low-risk PCF and pseudocyst fluid. Benign pseudocyst fluid also had higher proportions of macrophages compared to low-risk PCF and high-risk PCF (**Figure 3.1A**).

The most prevalent immune cell populations, though not significant, identified in patient-matched PBMC samples that were isolated from PCL patients was found to be CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells (**Figure 3.1B**). This study also found a notable, but non-significant number of classical monocytes in the matched PBMC samples from PCL patients (**Figure 3.1B**).

Overall, this data suggests that adaptive immune cell populations such as T cells and NK cells predominantly compose the local immune microenvironment within pre-malignant PCLs.

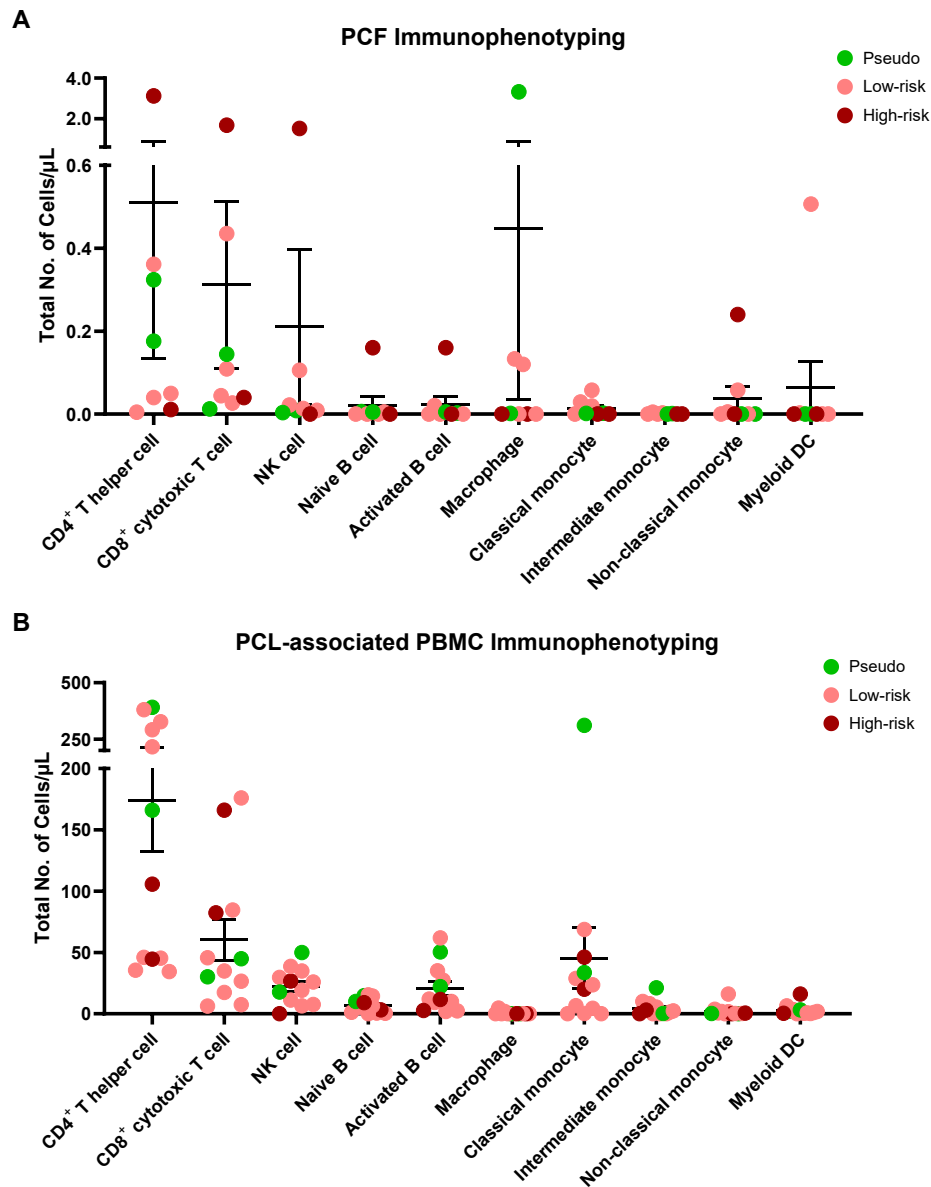


Figure 3.1: PCF has a high proportion of CD4⁺ T cells and CD8⁺ T cells which is also reflected in matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=2, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=2, low-risk n=8, high-risk n=2). Flow cytometric staining was carried out to characterise viable immune cell populations. Graphical data are representative of mean total number of cells/ μ L $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test.

3.4.2 PCF has a significantly lower proportion of adaptive immune cells compared to matched PCL-associated PBMCs.

Within this study, when the total number of adaptive immune cells within pre-malignant PCF was compared to that found within patient-matched PBMC samples, it was identified that there was a significantly higher proportion of adaptive immune cells in circulation as to be expected.

Specifically, pre-malignant PCF had a significantly lower number of CD3⁺CD4⁺ T helper cells compared to that found in PBMC samples isolated from PCL patients (Mean no. of cells/ μ L $\pm$ SEM; 0.51 ± 0.38 Total no. of cells/ μ L vs. 139.30 ± 47.72 Total no. of cells/ μ L, $p=0.0078$) (**Figure 3.2A**). Similarly, there was a significantly lower number of CD3⁺CD8⁺ cytotoxic T cells identified within pre-malignant PCF compared to PBMCs from PCL patients (0.31 ± 0.20 Total no. of cells/ μ L vs. 52.59 ± 18.10 Total no. of cells/ μ L, $p=0.0078$) (**Figure 3.2B**). The total number of NK cells within a defined volume was significantly lower in pre-malignant PCF compared to that found within patient-matched PBMCs (0.21 ± 0.19 Total no. of cells/ μ L vs. 19.79 ± 6.14 Total no. of cells/ μ L, $p=0.0078$) (**Figure 3.2C**). Naïve B cells, which play a key role in patrolling secondary lymphoid tissues to identify specific foreign antigens, also had significantly higher cell numbers within PBMC samples isolated from PCL patients compared to pre-malignant PCF (5.67 ± 1.89 Total no. of cells/ μ L vs. 0.02 ± 0.01 Total no. of cells/ μ L, $p=0.0078$) (**Figure 3.2D**). Activated B cells, which are responsible for the production of antigen-specific antibodies, were also found to have significantly higher numbers within PBMC samples isolated from PCL patients compared to pre-malignant PCF (20.37 ± 8.22 Total no. of cells/ μ L vs. 0.02 ± 0.02 Total no. of cells/ μ L, $p=0.0078$) (**Figure 3.2E**).

T cell and NK cell subsets dominated the local immune microenvironment of both pre-malignant PCF and patient-matched PBMCs. However, this analysis demonstrates that circulating PBMCs contained a greater overall proportion of adaptive immune cells compared to that identified within patient-matched cyst fluid from pre-malignant pancreatic lesions.

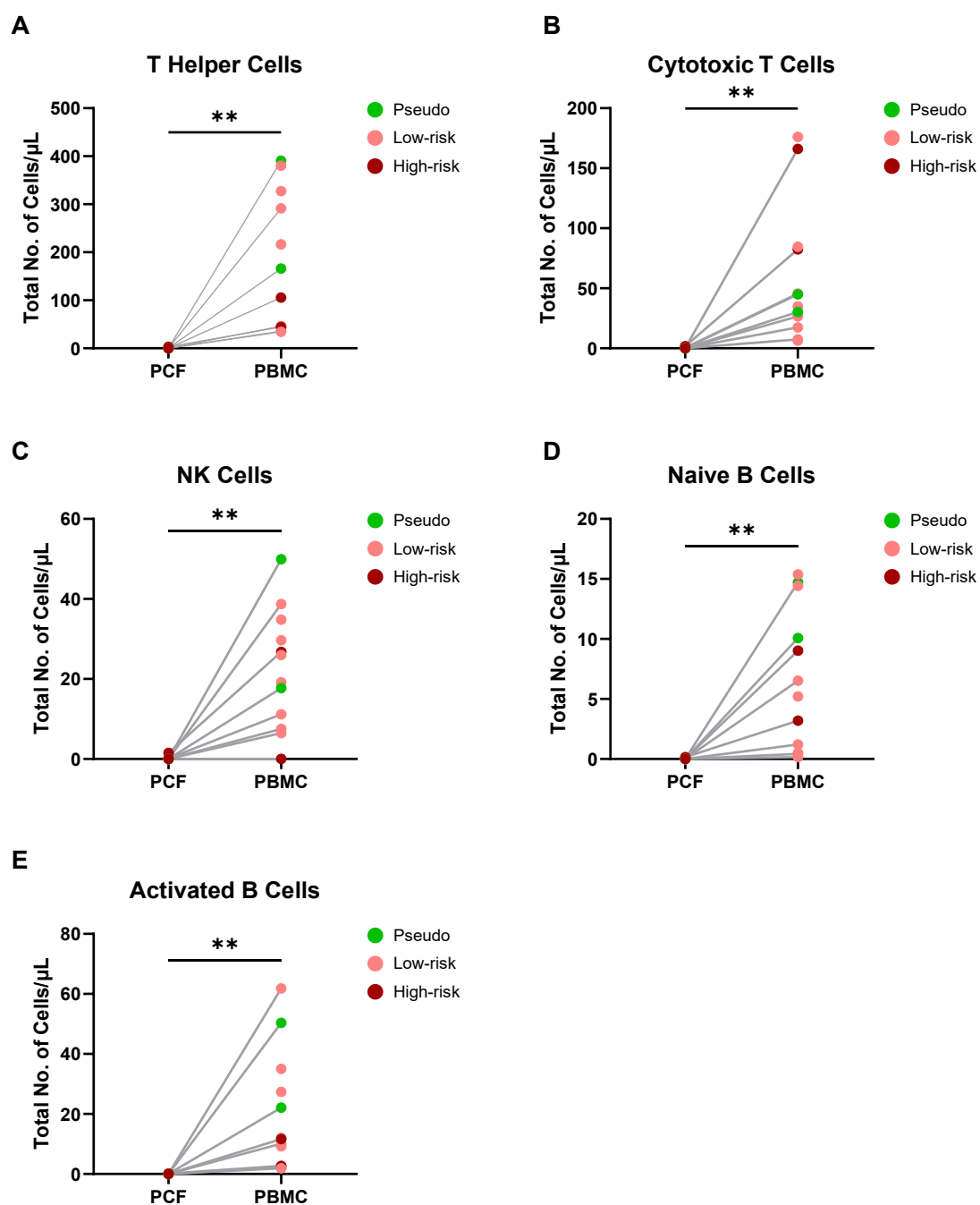


Figure 3.2: PCF has a significantly lower proportion of adaptive immune cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=2, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=2, low-risk n=8, high-risk n=2). Flow cytometric staining was carried out to characterise viable adaptive immune cell populations using cell surface markers CD45, CD3, CD4, CD8, CD56, CD19, CD38. Graphical data are representative of mean total number of cells/µL $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as **p $\leq$ 0.01].

3.4.3 PCL-associated PBMC samples have a significantly higher proportion of CD4⁺ T helper cells compared to PBMCs from healthy, non-PCL donors.

When the total number of adaptive immune cells within PCL patient-derived PBMC samples was compared to that found within healthy, non-PLC PBMC donors, this analysis found that PBMCs from patients with PCLs had a significantly higher number of adaptive immune cells compared to that of healthy controls.

PBMC samples isolated from patients with PCLs had a significantly higher number of CD3⁺CD4⁺ T helper cells compared to PBMCs isolated from healthy, non-PCL donors (173.60 ± 40.96 Total no. of cells/ μ L vs. 34.06 ± 5.86 Total no. of cells/ μ L, $p=0.0136$) (**Figure 3.3A**).

Although not significant, a higher number of CD3⁺CD8⁺ cytotoxic T cells, NK cells, naïve B cells and active B cells were identified in PBMC samples isolated from pre-malignant PCL patients compared to that found within healthy, non-PCL PBMC samples (**Figure 3.3B-E**).

Collectively, these results suggest that the expanded adaptive immune landscape within PCF and patient-matched PBMCs may be indicative of enhanced immune surveillance or dysregulated T cell activity in patients with pre-malignant pancreatic lesions.

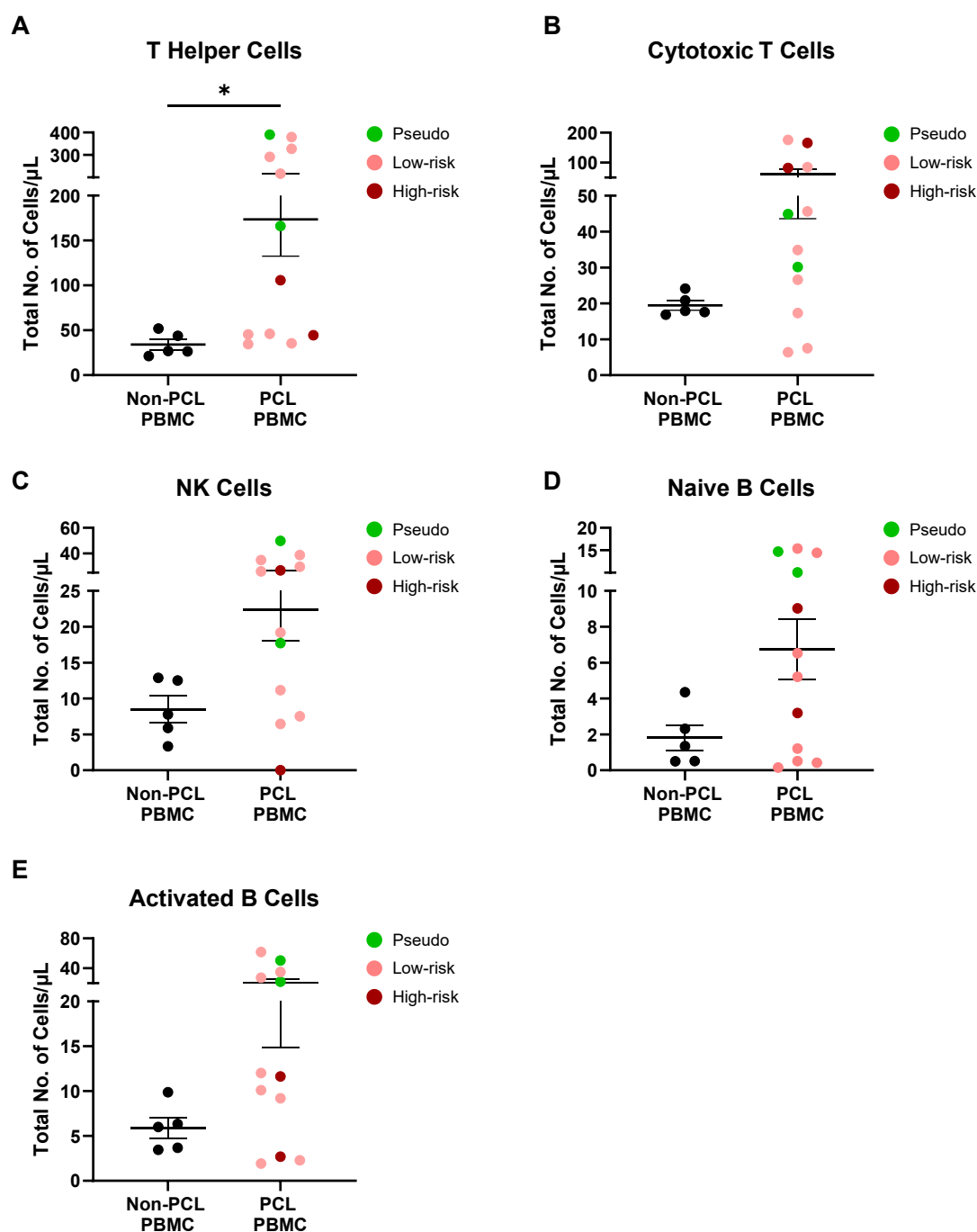


Figure 3.3: PCL-associated PBMC samples have a significantly higher proportion of CD4⁺ T helper cells compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=2, low-risk n=8, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to characterise viable adaptive immune cell populations using cell surface markers CD45, CD3, CD4, CD8, CD56, CD19, and CD38. Graphical data are representative of mean total number of cells/ μ L $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as * $p \leq 0.05$].

3.4.4 PCF has a significantly lower proportion of innate immune cell populations compared to matched PCL-associated PBMC samples.

Within this study, flow cytometric immunophenotyping was used to identify the total number of innate immune cells, using cell surface markers CD45, CD33, CD11b, CD11c, CD14, CD16, and CD68, within pre-malignant PCF and patient-matched PBMC samples.

Pre-malignant PCF had a significantly lower number of classical monocytes, which are crucial players in initiating immune responses against pathogens and injury, compared to that found in PBMC samples isolated from PCL patients (0.01 ± 0.007 Total no. of cells/ μL vs. 54.95 ± 37.09 Total no. of cells/ μL , $p=0.0078$) (**Figure 3.4B**). Similarly, there was a significantly lower number of intermediate monocytes identified within pre-malignant PCF compared to PBMCs from matched PCL patients (0.0010 ± 0.0005 Total no. of cells/ μL vs. 5.37 ± 2.67 Total no. of cells/ μL , $p=0.0156$) (**Figure 3.4C**). The total number of non-classical monocytes within a defined volume was significantly lower in pre-malignant PCF compared to that found within patient-matched PBMCs (0.04 ± 0.03 Total no. of cells/ μL vs. 1.11 ± 0.44 Total no. of cells/ μL , $p=0.0391$) (**Figure 3.4D**). Myeloid dendritic cells, which play a key role in linking the innate and adaptive immune systems through antigen presentation, also had a significantly lower total cell number within pre-malignant PCF compared to PBMCs isolated from PCL patients (0.06 ± 0.03 Total no. of cells/ μL vs. 4.15 ± 1.84 Total no. of cells/ μL , $p=0.0078$) (**Figure 3.4E**). This study showed that there were no significant differences in the total number of macrophages within pre-malignant PCF and patient-matched PBMC samples (**Figure 3.4A**).

These observations may indicate that the local immune microenvironment within pre-malignant PCF may be relatively deficient in key innate immune cells which are crucial for pathogen recognition, antigen presentation, and initiation of adaptive immune responses.

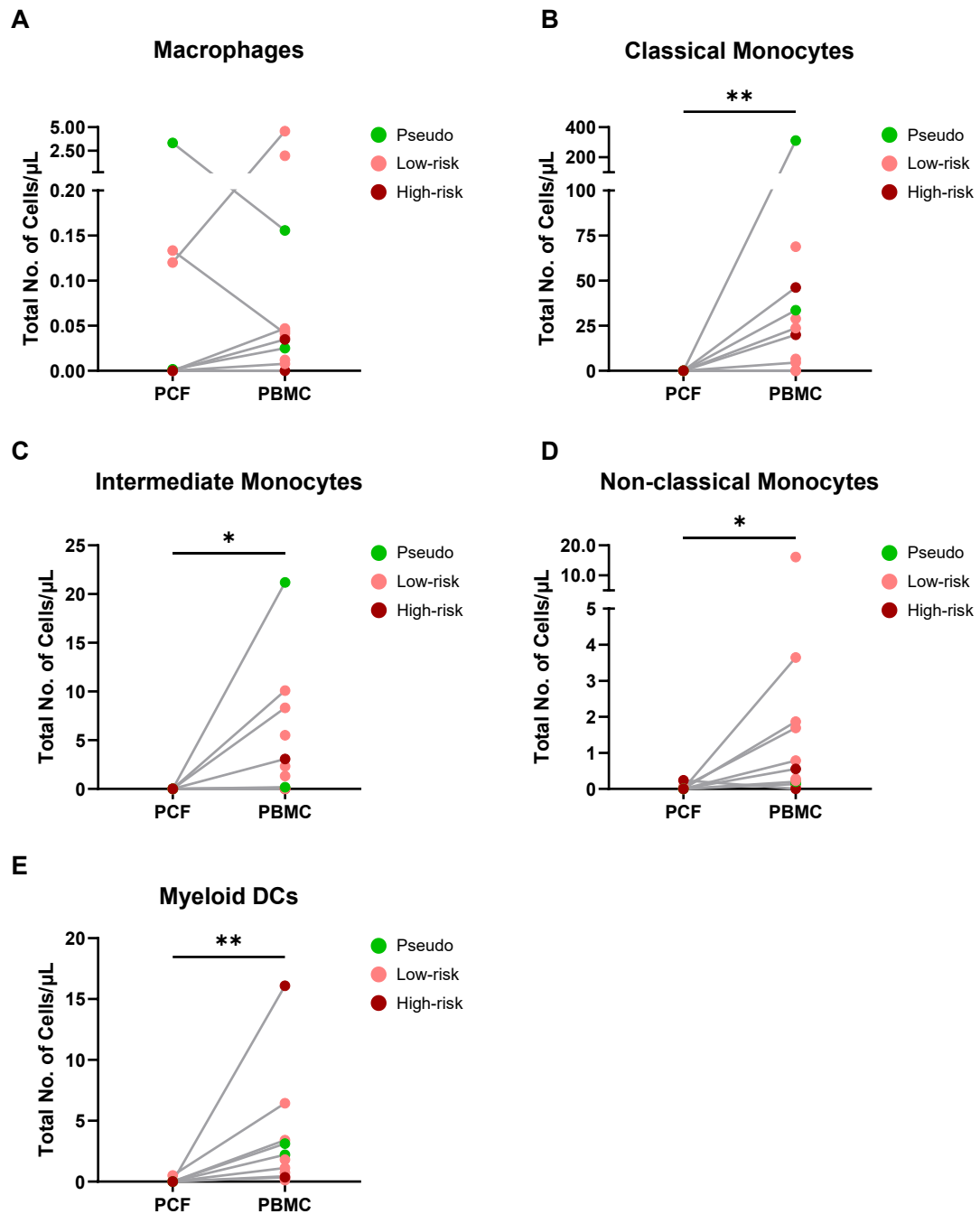


Figure 3.4: PCF has a significantly lower proportion of innate immune cell populations compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=2, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=2, low-risk n=8, high-risk n=2). Flow cytometric staining was carried out to characterise viable innate immune cell populations using cell surface markers CD45, CD33, CD11b, CD11c, CD14, CD16, and CD68. Graphical data are representative of mean total number of cells/μL ± SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as *p≤0.05, and **p≤0.01].

3.4.5 PCL-associated PBMC samples have a significantly higher proportion of macrophages compared to PBMCs from healthy, non-PCL donors.

When the total number of innate immune cells within PCL patient-derived PBMC samples was compared to that found within healthy, non-PLC PBMC donors, this analysis found that PBMCs from patients with PCLs had a significantly higher number of innate immune cells compared to that of healthy controls.

PBMC samples isolated from patients with PCLs had a significantly decreased number of macrophages compared to PBMCs isolated from healthy, non-PCL donors (0.58 ± 0.39 Total no. of cells/ μ L vs. 0.71 ± 0.12 Total no. of cells/ μ L, $p=0.0365$) (**Figure 3.5A**).

However, although not significant, an increased number of classical monocytes, intermediate monocytes, non-classical monocytes and myeloid dendritic cells were found in PBMC samples isolated from pre-malignant PCL patients compared to that found within healthy, non-PCL PBMC samples (**Figure 3.5B-E**).

Overall, this data suggests that there is a dysregulation of the innate immune response in PCL patients.

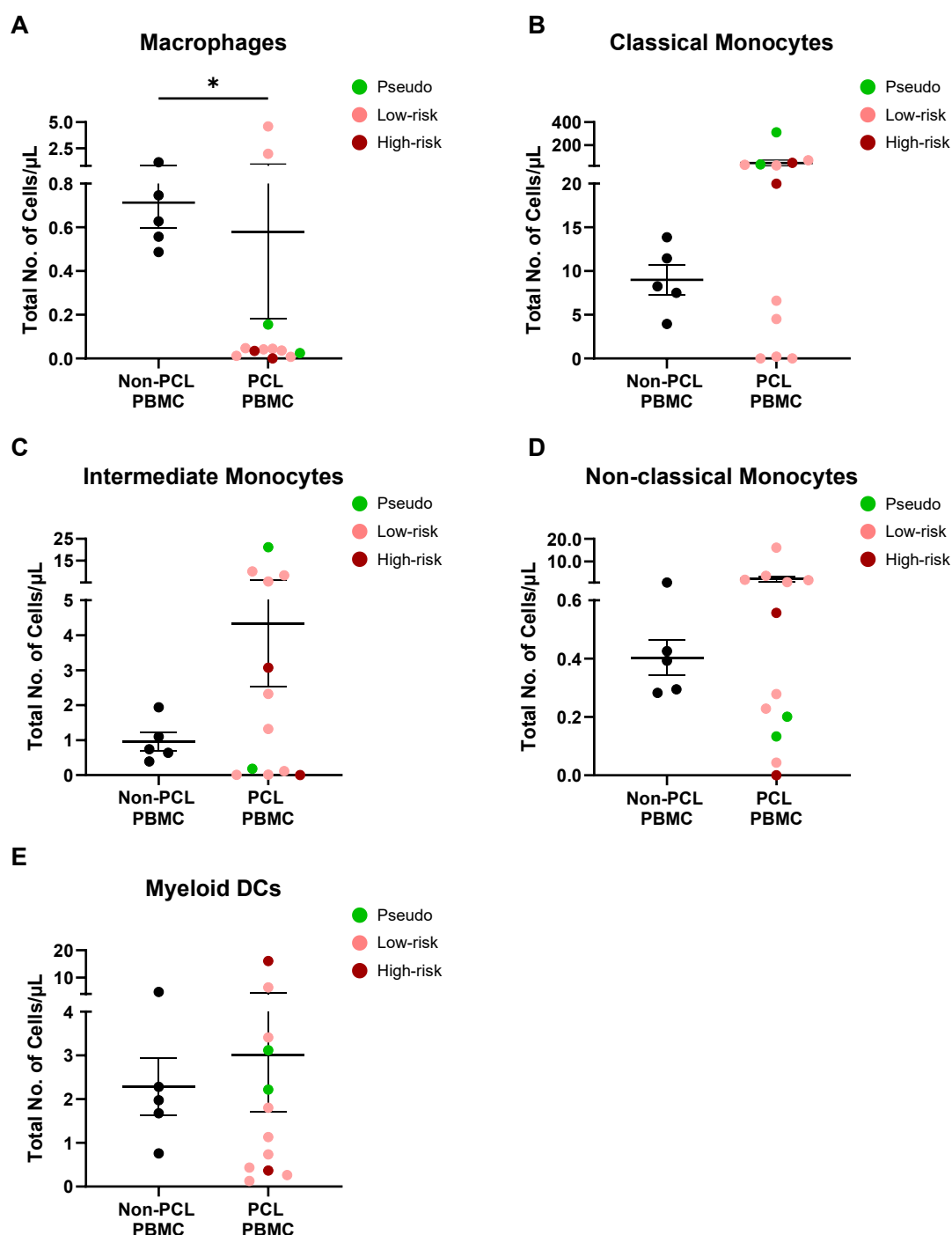


Figure 3.5: PCL-associated PBMC samples have a significantly higher proportion of macrophages compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=2, low-risk n=8, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to characterise viable adaptive immune cell populations using cell surface markers CD45, CD33, CD11b, CD11c, CD14, CD16, and CD68. Graphical data are representative of mean total number of cells/ μ L $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as * $p \leq 0.05$].

3.4.6 PCF has a significantly higher frequency of effector memory T cells compared to matched PCL-associated PBMC samples.

As part of this study, the T cell population within pre-malignant PCF, patient-matched PBMC samples and PBMCs isolated from healthy non-PCL donors underwent immunophenotyping analysis by flow cytometry to assess the expression of CD45RA and CD62L, markers of activation and memory function. Understanding the activation status and memory subsets within pre-malignant PCLs may reveal whether the local immune microenvironment within these lesions have the capacity to recognise and induce an appropriate immune response to abnormal, tumourigenic cells. Additionally, analysing the memory T cell component of the immune cell infiltration within these pre-malignant lesions may indicate whether these cells have the capacity to elicit a targeted and sustained immune response.

This study found that there was a non-significant decrease of naïve ($CD45RA^+CD62L^+$) $CD8^+$ T cells and $CD8^+$ T cells within pre-malignant PCF compared to matched PBMCs isolated from PCL patients (**Figure 3.6A, B**).

T cell differentiation was found to be significantly altered between pre-malignant PCF and patient-matched PBMC samples. This immunophenotyping analysis identified a significant decrease of central memory ($CD45RA^-CD62L^+$) $CD8^+$ T cells in pre-malignant PCF samples compared to that of matched PBMCs (Mean frequency (%) $\pm$ SEM; $14.50 \pm 3.80\%$ vs. $23.83 \pm 6.31\%$, $p=0.0156$) (**Figure 3.6C**). This decrease in central memory T cell phenotype was also observed in $CD8^+$ T cells in pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.6D**).

Furthermore, there was a significant increase in the frequency of effector memory ($CD45RA^-CD62L^-$) $CD8^+$ T cells in pre-malignant PCF compared to matched PCL patient-derived PBMCs ($56.83 \pm 13.92\%$ vs. $21.81 \pm 7.10\%$, $p=0.0312$) (**Figure 3.6F**). Although not significant, this trend was also seen in effector memory subsets of $CD8^+$ T cells (**Figure 3.6E**).

The frequency of terminally differentiated effector memory ($CD45RA^+CD62L^-$) $CD8^+$ T cells was not significantly impacted by exposure to risk-stratified PCF. Although, there does appear to be a non-significant decrease of terminally differentiated effector memory $CD8^+$ T cells compared to PBMCs isolated from matched PCL patients (**Figure 3.6G, H**).

These findings suggest that the local immune microenvironment in pre-malignant pancreatic lesions may be skewed towards a more differentiated effector memory state, potentially reflecting an environment with ongoing antigen exposure.

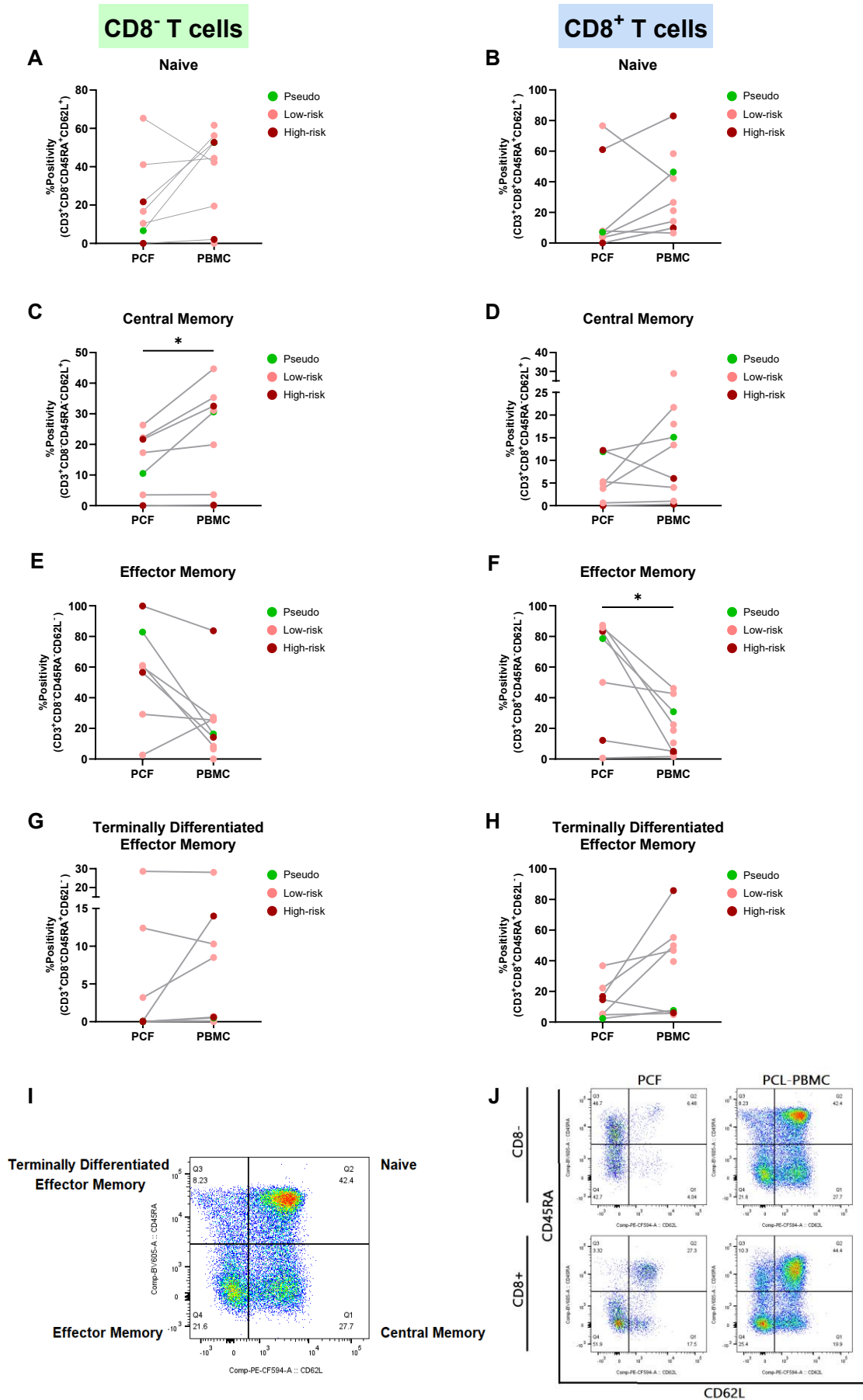


Figure 3.6: PCF has a significantly higher frequency of effector memory T cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess naïve (CD45RA⁺CD62L⁺) (A, B), central memory (CD45RA⁻CD62L⁺) (C, D), effector memory (CD45RA⁻CD62L⁻) (E, F) and terminally differentiated effector memory (CD45RA⁺CD62L⁻) (G, H) subsets of viable CD3⁺CD8⁻ T cells (A, C, E, G) and viable CD3⁺CD8⁺ T cells (B, D, F, H). Graphical data are representative of mean percentage positivity ± SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as *p≤0.05].

3.4.7 The systemic immune system of PCL patients has a lower frequency of central memory and effector memory T cell subsets compared to PBMCs from healthy, non-PCL donors.

The frequency of T cell differentiation into effector and memory subsets was investigated in PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors. The frequency of naïve T cells, which are cells that have not yet encountered antigen, was not significantly altered in PCL patient-derived PBMCs compared to healthy control PBMCs (**Figure 3.7A, B**).

Central memory T cells retain memory of previous encounters with antigens, allowing for a rapid adaptive immune response upon subsequent antigen exposures. These central memory T cells were found to be non-significantly decreased in PBMCs isolated from PCL patients compared to that of healthy, non-PCL PBMCs (**Figure 3.7C, D**).

Similarly, T cells with an effector memory status, which are responsible for providing rapid and targeted effector functions such as cytotoxic killing and cytokine production, were non-significantly decreased in PBMC samples isolated from PCL patients compared to the healthy, non-PCL PBMC control samples (**Figure 3.7E, F**).

Although not significant, terminally differentiated effector memory T cells which have both memory and effector characteristics, were increased in PCL patient-derived PBMCs compared to PBMCs from healthy, non-PCL donors (**Figure 3.7G, H**).

Overall, this data suggests that there is a dysregulation of systemic T cell differentiation into central memory, effector memory and terminally differentiated effector memory subsets in patients with pre-malignant pancreatic lesions.

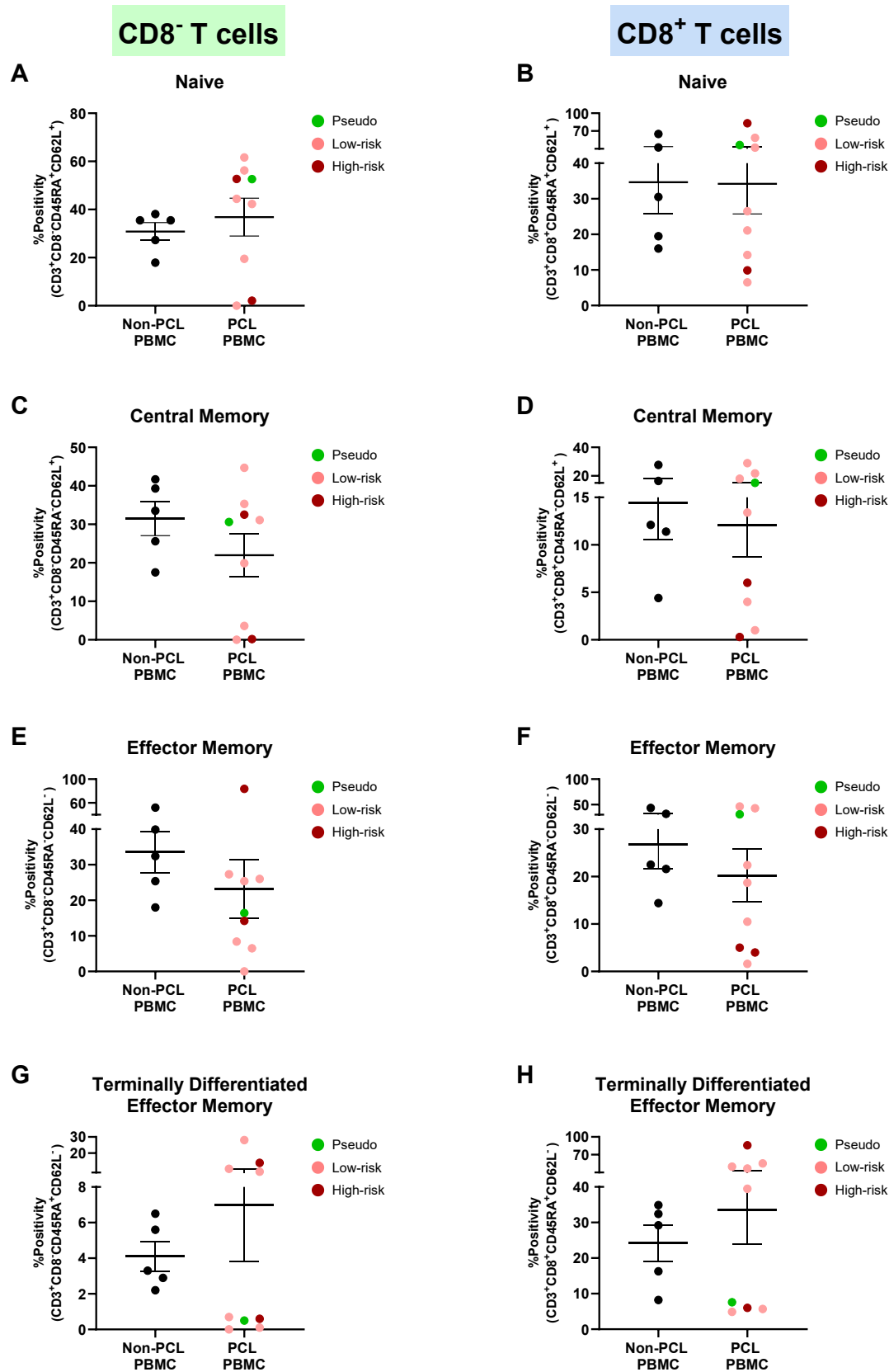


Figure 3.7: PCL-associated PBMC samples have a lower frequency of central memory and effector memory T cell subsets compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-

PCL donors (n=5). Flow cytometric staining was carried out to assess naïve ($CD45RA^+CD62L^+$) (A, B), central memory ($CD45RA^+CD62L^+$) (C, D), effector memory ($CD45RA^+CD62L^-$) (E, F) and terminally differentiated effector memory ($CD45RA^+CD62L^-$) (G, H) subsets of viable $CD3^+CD8^-$ T cells (A, C, E, G) and viable $CD3^+CD8^+$ T cells (B, D, F, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test).

3.4.8 PCF has a higher frequency of exhausted $CD8^+$ T cells compared to matched PCL-associated PBMC samples.

T cell exhaustion represents a major barrier to effective anti-tumour immunity. However, the exhaustion status of T cells within pre-malignant PCLs and systemic circulation of affected patients remains poorly defined. Exhausted T cells can be characterised by an upregulated co-expression of inhibitory checkpoint proteins including PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3)^[227, 228]. Findings from this study provide more insight into the immune landscape, specifically the T cell response, within the local microenvironment of PCLs.

There was a non-significant increase in the frequency of $PD-1^+CD8^-$ T cells within pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.8A**). Similarly, the frequency of $PD-1^+CD8^+$ T cells was non-significantly increased in pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.8B**).

This study also found that there was a non-significant decrease of the co-expression of $PD-1^+TIM-3^+$ on $CD8^-$ T cells in the immune cell infiltrate of pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.8C**). On the contrary, the frequency of $PD-1^+TIM-3^+$ co-expression was non-significantly increased in $CD8^+$ T cells found within pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.8D**). No significant differences were found in the co-expression levels of $PD-1^+TIM-3^+LAG-3^+$ on $CD8^-$ T cells or $CD8^+$ T cells in pre-malignant PCF or patient-matched PBMC samples (**Figure 3.8E, F**).

Although there is little to no expression of LAG-3 found within these PCL-associated immune compartments, the increased co-expression of PD-1 and TIM-3 inhibitory receptors in $CD8^+$ T cells suggest that these cells are in a state of T cell exhaustion which may result in dampened effector T cell functions.

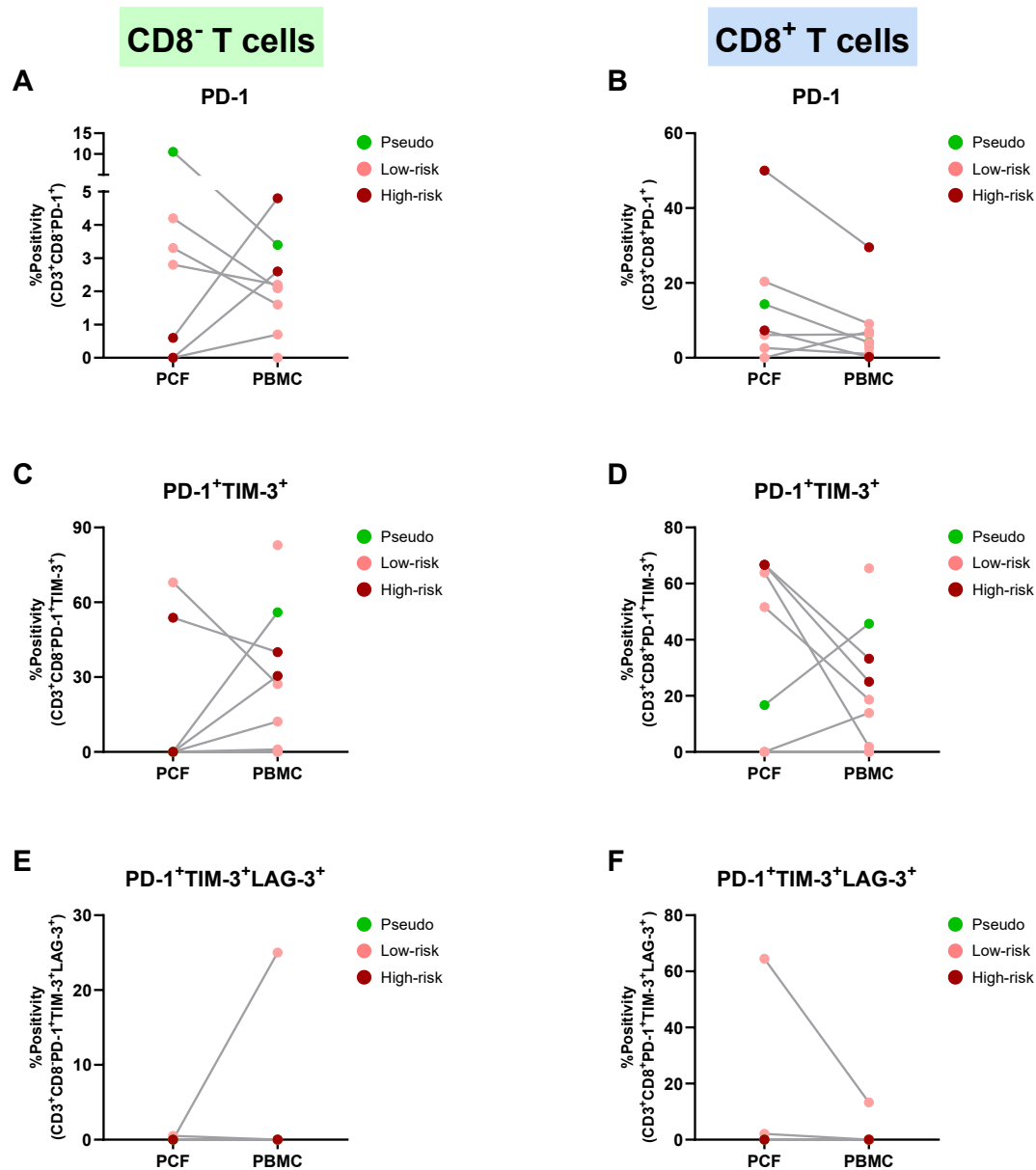


Figure 3.8: PCF has a higher frequency of exhausted CD8⁺ T cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess PD-1⁺ (A, B), PD-1⁺LAG-3⁺ (C, D) and PD-1⁺LAG-3⁺TIM-3⁺ (E, F) expression on viable CD3⁺CD8⁻ T cells (A, C, E) and viable CD3⁺CD8⁺ T cells (B, D, F). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test).

3.4.9 The systemic immune system of PCL patients has an increased frequency of exhausted T cells compared to PBMCs from healthy, non-PCL donors.

The frequency of PD-1⁺ CD8⁻ T cells within PBMCs isolated from PCL patients was non-significantly increased compared to healthy, non-PCL PBMC donor samples (**Figure 3.9A**). Similarly, there was a trend towards significance in the increased frequency of PD-1⁺ CD8⁺ T cells in PCL patient-derived PBMCs compared to healthy, non-PCL PBMCs ($1.60 \pm 0.43\%$ vs. $7.09 \pm 2.96\%$, $p=0.0639$) (**Figure 3.9B**).

The results from this study also found that there was a non-significant increase of the co-expression of PD-1⁺TIM-3⁺ on CD8⁻ T cells in PBMCs isolated from PCL patients compared to healthy, non-PCL PBMC samples (**Figure 3.9C**). Furthermore, there was a trend towards a significant increase in the frequency of PD-1⁺TIM-3⁺ co-expression on CD8⁺ T cells from PBMCs isolated from PCL patients compared to healthy, non-PCL PBMC donor samples ($0.32 \pm 0.15\%$ vs. $22.62 \pm 7.48\%$, $p=0.0539$) (**Figure 3.9D**).

There was little to no co-expression of PD-1⁺TIM-3⁺LAG-3⁺ inhibitory receptors on CD8⁻ T cells or CD8⁺ T cells isolated from PCL patients or healthy, non-PCL donors (**Figure 3.9E, F**).

Collectively, these results suggest that there is a systemic T cell dysfunction via immune cell exhaustion in the systemic immune system of patients with pre-malignant PCLs compared to healthy, non-PCL controls.

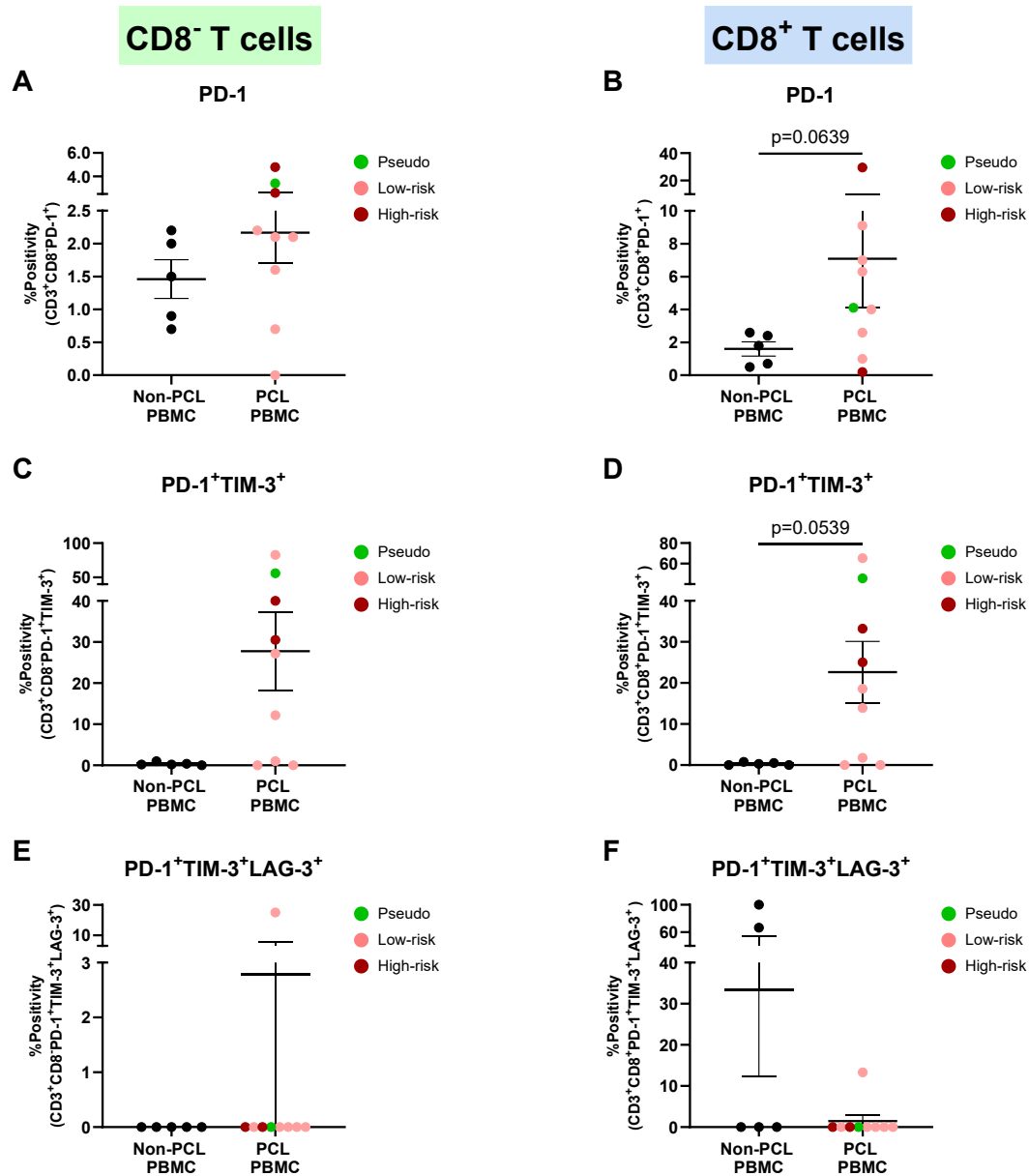


Figure 3.9: PCL-associated PBMC samples have an increased frequency of exhausted T cells compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to assess PD-1⁺ (A, B), PD-1⁺LAG-3⁺ (C, D) and PD-1⁺LAG-3⁺TIM-3⁺ (E, F) expression on viable CD3⁺CD8⁻ T cells (A, C, E) and viable CD3⁺CD8⁺ T cells (B, D, F). Graphical data are representative of mean percentage positivity ± SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test).

3.4.10 PCF has higher frequencies of circulating mildly cytotoxic T cells and terminally exhausted resident T cells compared to matched PCL-associated PBMCs.

T cell exhaustion is conventionally defined by an upregulated accumulation of inhibitory checkpoint proteins^[229]. However, a recent study conducted by Beltra *et al.*, defined a hierarchical developmental pathway for T cell exhaustion consisting of four stages defined by various expression levels of CD69, an early-stage activation marker, and signalling lymphocytic activation molecule family member 6 (SLAMF6), an immune checkpoint receptor otherwise known as Ly108^[230]. The frequency of these defined four states of T cell exhaustion was assessed by flow cytometry in the immune cell infiltrate of PCF and patient-matched PBMCs.

This study found that there was a non-significant decrease of quiescent resident (CD69⁺SLAMF6⁺) CD8⁻ T cells within pre-malignant PCF compared to matched PBMCs isolated from PCL patients (**Figure 3.10A**). However, no significant differences were found in the quiescent resident CD8⁺ T cells within pre-malignant PCF or patient-matched PBMCs (**Figure 3.10B**).

This immunophenotyping analysis identified a non-significant decrease of proliferative circulating (CD69⁻SLAMF6⁺) CD8⁻ T cells in pre-malignant PCF samples compared to that of matched PBMCs (**Figure 3.10C**). This non-significant decrease in the proliferative circulating phenotype was also observed in CD8⁺ T cells derived from pre-malignant PCF compared to that of patient-matched PBMCs (**Figure 3.10D**).

This study also revealed that there was a non-significant increase in the frequency of circulating mildly cytotoxic (CD69⁻SLAMF6⁻) CD8⁻ T cells in pre-malignant PCF compared to matched PCL patient-derived PBMCs (**Figure 3.10E**). Although not significant, this trend was also seen in the frequency of circulating mildly cytotoxic subsets of CD8⁺ T cells (**Figure 3.10F**).

There was a significant increase of terminally exhausted resident (CD69⁺SLAMF6⁻) CD8⁺ T cells in pre-malignant PCF compared to patient-matched PBMCs ($7.49 \pm 5.74\%$ vs. $0.10 \pm 0.06\%$, $p=0.0312$) (**Figure 3.10H**). Similarly, although not significant, there does appear to be an increased frequency of terminally exhausted resident CD8⁻ T cells in pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.10G**).

Overall, this data shows that pre-malignant PCF exhibits a shift in T cell states characterised by reduced frequencies of quiescent and proliferative circulating subsets

and an increased prevalence of terminally exhausted resident T cells. These changes suggest an enrichment of exhausted T cell phenotypes within the local immune microenvironment of PCLs, potentially impairing effective T cell immune responses in the pre-malignant setting.

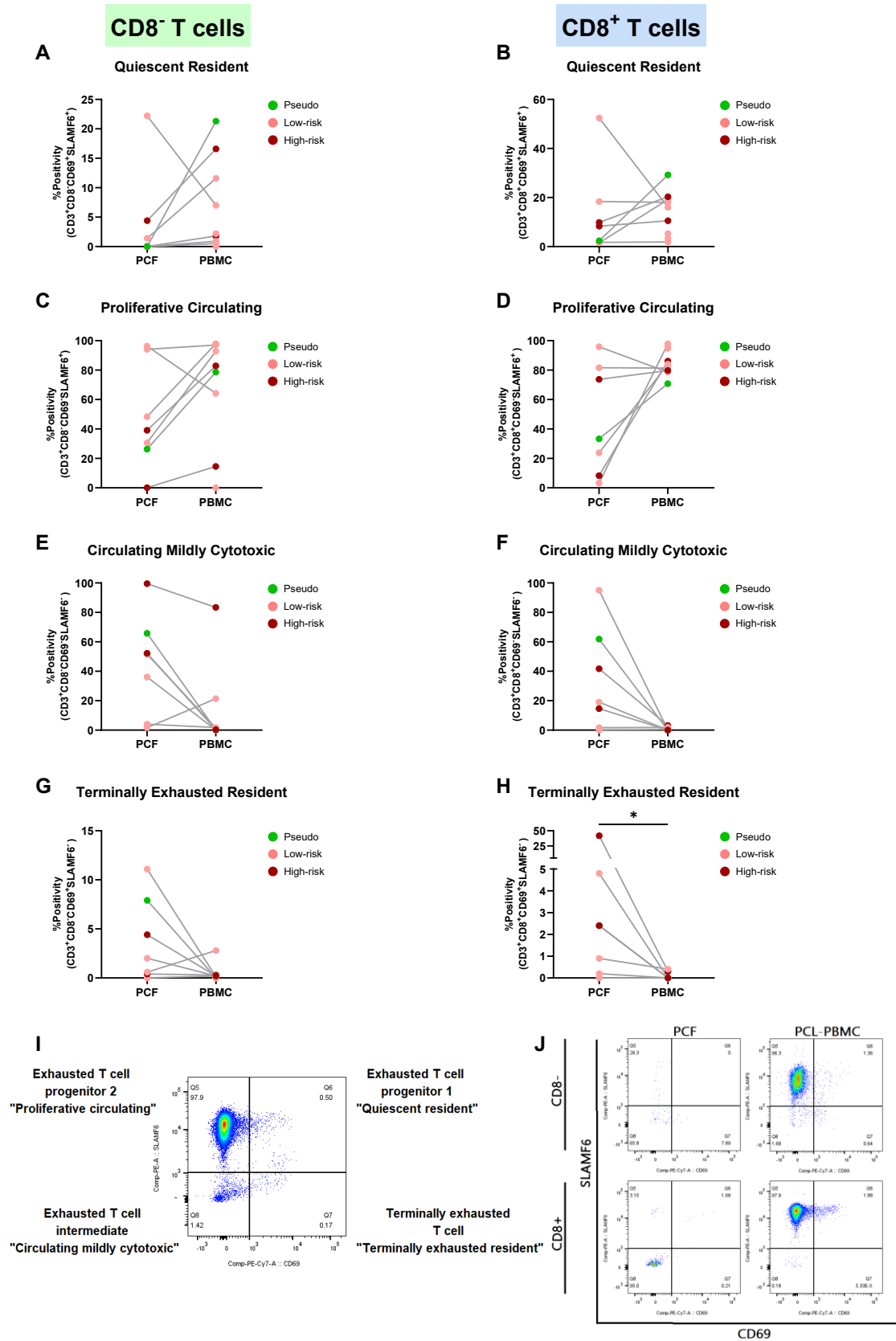


Figure 3.10: PCF has higher frequencies of circulating mildly cytotoxic T cells and terminally exhausted resident T cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole

blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess quiescent resident (CD69⁺SLAMF6⁺) (A, B), proliferative circulating (CD69⁻SLAMF6⁺) (C, D), circulating mildly cytotoxic (CD69⁻SLAMF6⁻) (E, F) and terminally exhausted (CD69⁺SLAMF6⁻) (G, H) subsets of viable CD3⁺CD8⁻ T cells (A, C, E, G) and viable CD3⁺CD8⁺ T cells (B, D, F, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as *p $\leq$ 0.05].

3.4.11 The systemic immune system of PCL patients has significantly higher frequency of quiescent resident CD8⁺ T cells and significantly lower frequency of proliferative circulating CD8⁺ T cells.

The frequency of T cell exhaustion states was investigated in PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors.

The frequency of quiescent resident T cells, which are cells that remain in a dormant state and are poised for activation upon local immune challenges, was significantly increased in CD8⁺ T cells in PCL patient-derived PBMCs compared to healthy non-PCL PBMCs ($13.74 \pm 3.04\%$ vs. $3.36 \pm 1.25\%$, p=0.0275) (**Figure 3.11B**). Although not significant, this trend was also seen in the frequency of quiescent resident subsets of CD8⁻ T cells (**Figure 3.11A**).

Proliferative circulating T cells function to rapidly increase the number of antigen-specific T cells in circulation. These proliferative circulating CD8⁺ T cells were found to be significantly decreased in PBMCs isolated from PCL patients compared to healthy, non-PCL PBMCs ($85.53 \pm 3.07\%$ vs. $96.54 \pm 1.25\%$, p=0.0180) (**Figure 3.11D**). Similarly, although not significant, the frequency of proliferative circulating CD8⁻ T cells was decreased in PBMCs from PCL patients compared to PBMCs from healthy, non-PCL donors (**Figure 3.11C**).

CD8⁻ T cells defined with a circulating mildly cytotoxic phenotype, which are responsible for inducing an adaptive immune response by releasing cytotoxic molecules such as granzymes, perforin and cytokines, were non-significantly decreased in PBMC samples isolated from PCL patients compared to the healthy, non-PCL PBMC control samples (**Figure 3.11E**). This study identified groups within the data indicating groups of samples with distinct low and high frequencies of circulating mildly cytotoxic CD8⁻ T cells which requires further investigation to establish any clinical significance (**Figure 3.11E**). Moreover, the frequency of circulating mildly cytotoxic CD8⁺ T cells, was non-

significantly increased in PCL patient-derived PBMCs compared to healthy non-PCL PBMCs (**Figure 3.11F**).

This study also found that there was a significant decrease in the frequency of terminally exhausted resident CD8⁺ T cells in PBMCs from PCL patients compared to PBMCs from healthy, non-PCL donors ($0.42 \pm 0.29\%$ vs. $0.52 \pm 0.16\%$, $p=0.0305$) (**Figure 3.11G**). However, the frequency of terminally exhausted resident CD8⁺ T cells, was non-significantly increased in PCL patient-derived PBMCs compared to healthy, non-PCL PBMCs (**Figure 3.11H**).

These findings suggest that there is disrupted immune regulation of T cell exhaustion in the systemic immune microenvironment of patients with pre-malignant PCLs.

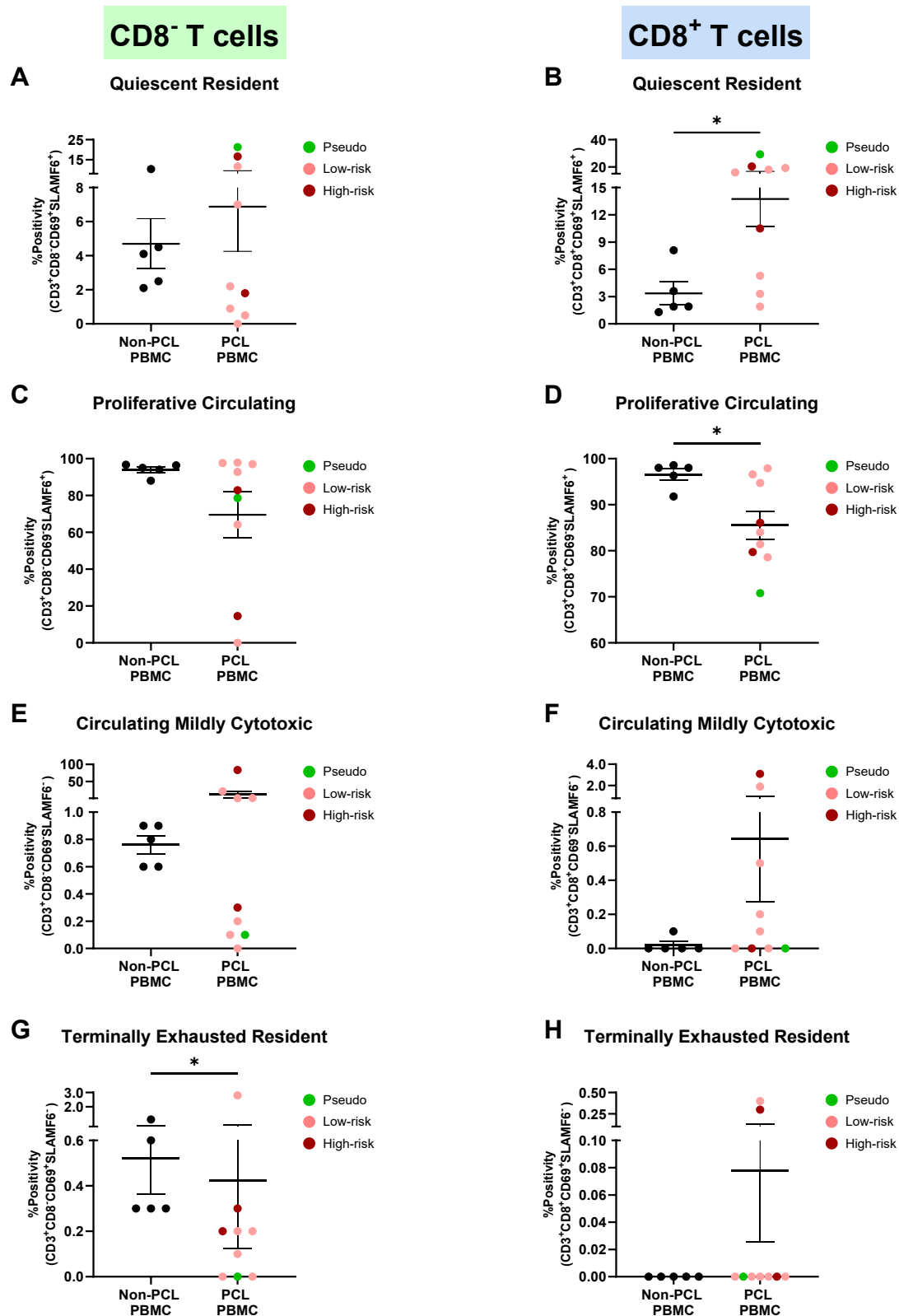


Figure 3.11: PCL-associated PBMC samples have a significantly higher frequency of quiescent resident CD8⁺ T cells and significantly lower frequency of proliferative circulating CD8⁺ T cells compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-PCL donors (n=5). Flow

cytometric staining was carried out to assess quiescent resident (CD69⁺SLAMF6⁺) (A, B), proliferative circulating (CD69⁺SLAMF6⁺) (C, D), circulating mildly cytotoxic (CD69⁺SLAMF6⁻) (E, F) and terminally exhausted (CD69⁺SLAMF6⁻) (G, H) subsets of viable CD3⁺CD8⁻ T cells (A, C, E, G) and viable CD3⁺CD8⁺ T cells (B, D, F, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as * $p \leq 0.05$].

3.4.12 PCF has a significantly increased frequency of intracellular perforin in CD8⁺ T cells and a significantly decreased frequency of intracellular granzyme B in NK cells.

This study investigated the cytotoxic function of effector immune cell populations found within the local immune microenvironment of pre-malignant PCLs and also systemically in patient-matched PBMC samples.

This study found that there was a non-significant increase of CD107a degranulation of CD8⁺ cytotoxic T cells within pre-malignant PCF compared to matched PBMCs isolated from PCL patients (**Figure 3.12A**). On the contrary, the frequency of CD107a degranulation in NK cells was non-significantly decreased in pre-malignant PCF compared to patient-matched PBMC samples (**Figure 3.12B**).

The frequency of perforin, a cytotoxic molecule released from effector immune cells that creates pores in target cells, was significantly increased in CD8⁺ T cells isolated from pre-malignant PCF compared to PBMCs isolated from matched PCL patients ($69.59 \pm 12.11\%$ vs. $11.47 \pm 5.04\%$, $p=0.0312$) (**Figure 3.12D**). However, there was a non-significant decrease of perforin in NK cells found in pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.12E**).

This immunophenotyping analysis also identified a significant decrease in the frequency of granzyme B, a cytotoxic molecule involved in the activation of caspase-induced cell death, in NK cells found within pre-malignant PCF compared to matched PCL patient-derived PBMCs ($8.28 \pm 2.17\%$ vs. $34.90 \pm 4.48\%$, $p=0.0156$) (**Figure 3.12H**). Although not significant, this trend was also seen in the frequency of intracellular granzyme B in CD8⁺ cells (**Figure 3.12G**).

Furthermore, there was a non-significant decrease of intracellular cytokine IFN- γ in CD8⁺ T cells and NK cells within pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.12J, K**).

The data from this immunophenotyping analysis suggests that the effector immune cell population within the local immune microenvironment of pre-malignant PCLs may have dysfunctional cytotoxic activity.

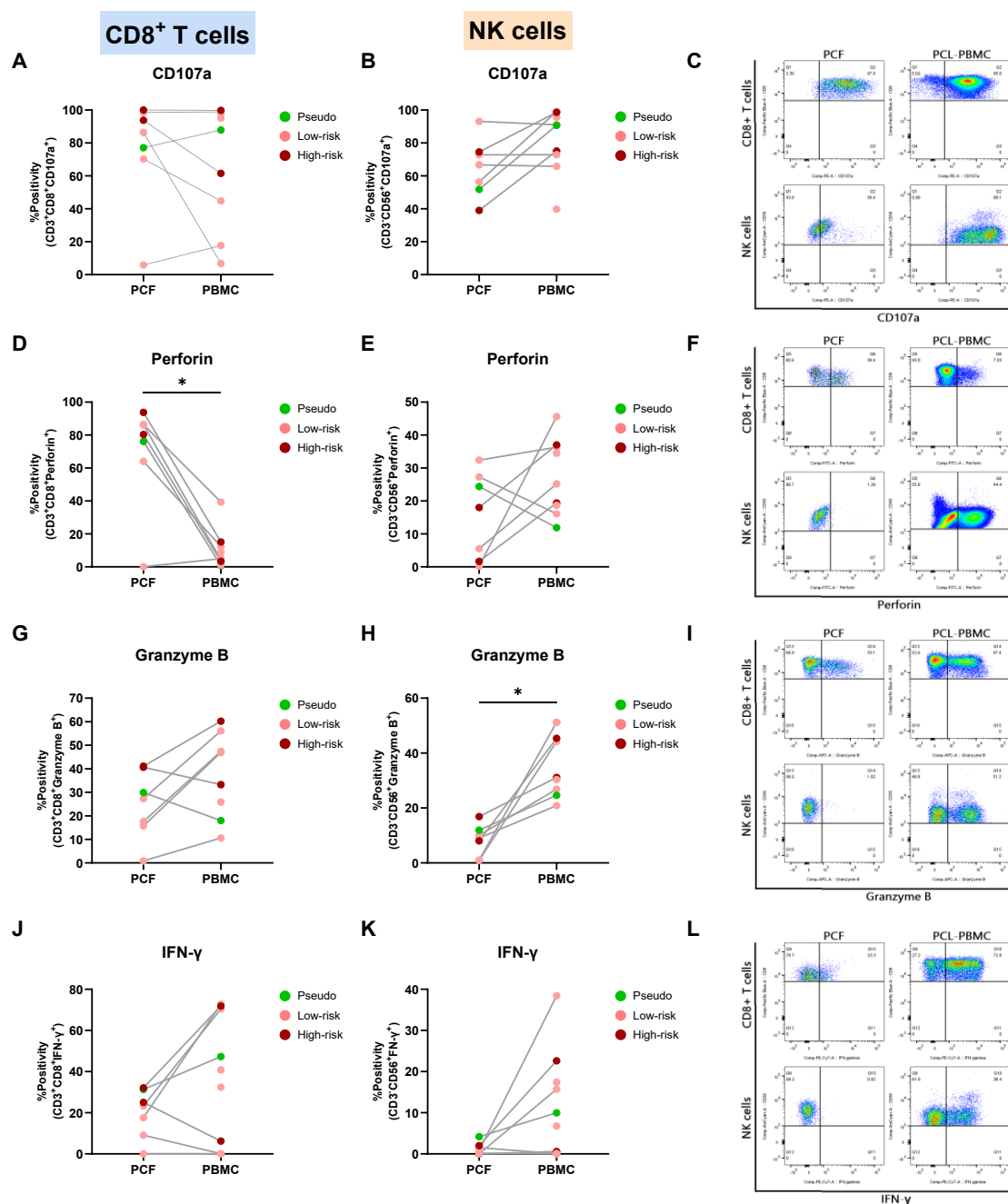


Figure 3.12: PCF has a significantly increased frequency of intracellular perforin in CD8⁺ T cells and a significantly decreased frequency of intracellular granzyme B in NK cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess CD107a⁺ degranulation (A, B, C), perforin⁺

(D, E, F), granzyme B⁺ (G, H, I) and IFN- γ ⁺ (J, K, L) in viable CD3⁺CD8⁺ T cells (A, D, G, J) and viable CD3⁺CD56⁺ NK cells (B, E, H, K). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as * $p \leq 0.05$].

3.4.13 The systemic immune system of PCL patients has a significantly higher frequency of CD107a⁺ effector immune cell degranulation and decreased perforin and IFN- γ cytokine production.

The frequency of effector immune cell cytotoxic molecules was investigated in PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors.

The frequency of CD107a⁺ CD8⁺ T cell degranulation was significantly increased in PCL patient derived PBMCs compared to healthy, non-PCL control PBMCs ($67.78 \pm 12.26\%$ vs. $4.78 \pm 0.38\%$, $p=0.0010$) (**Figure 3.13A**). Similarly, CD107a NK cell degranulation was significantly increased in PCL patient-derived PBMCs compared to healthy non-PCL control PBMCs ($80.94 \pm 6.53\%$ vs. $6.39 \pm 0.18\%$, $p=0.0010$) (**Figure 3.13B**).

Intracellular perforin was seen to be significantly decreased in NK cells found within PBMCs isolated from PCL patients compared to healthy, non-PCL PBMCs ($27.19 \pm 3.85\%$ vs. $56.12 \pm 2.38\%$, $p=0.0010$) (**Figure 3.13D**). Although not significant, this trend was also recorded for the frequency of intracellular perforin in CD8⁺ cells (**Figure 3.13C**).

Although not significant, the frequency of granzyme B was found to be increased in CD8⁺ T cells within the PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors (**Figure 3.13E**). On the contrary, there was a non-significant decrease of granzyme B in NK cells from PCL patient PBMCs compared to healthy, non-PCL control PBMCs (**Figure 3.13F**).

However, the frequency of intracellular IFN- γ was significantly decreased in NK cells from PBMCs isolated from PCL patients and compared to that of healthy, non-PCL PBMC control samples ($12.39 \pm 4.25\%$ vs. $41.80 \pm 1.43\%$, $p=0.0020$) (**Figure 3.13H**). However, there was no difference in the expression of intracellular IFN- γ in CD8⁺ T cells from PBMCs from PCL-patients compared to healthy, non-PCL PBMCs (**Figure 3.13G**).

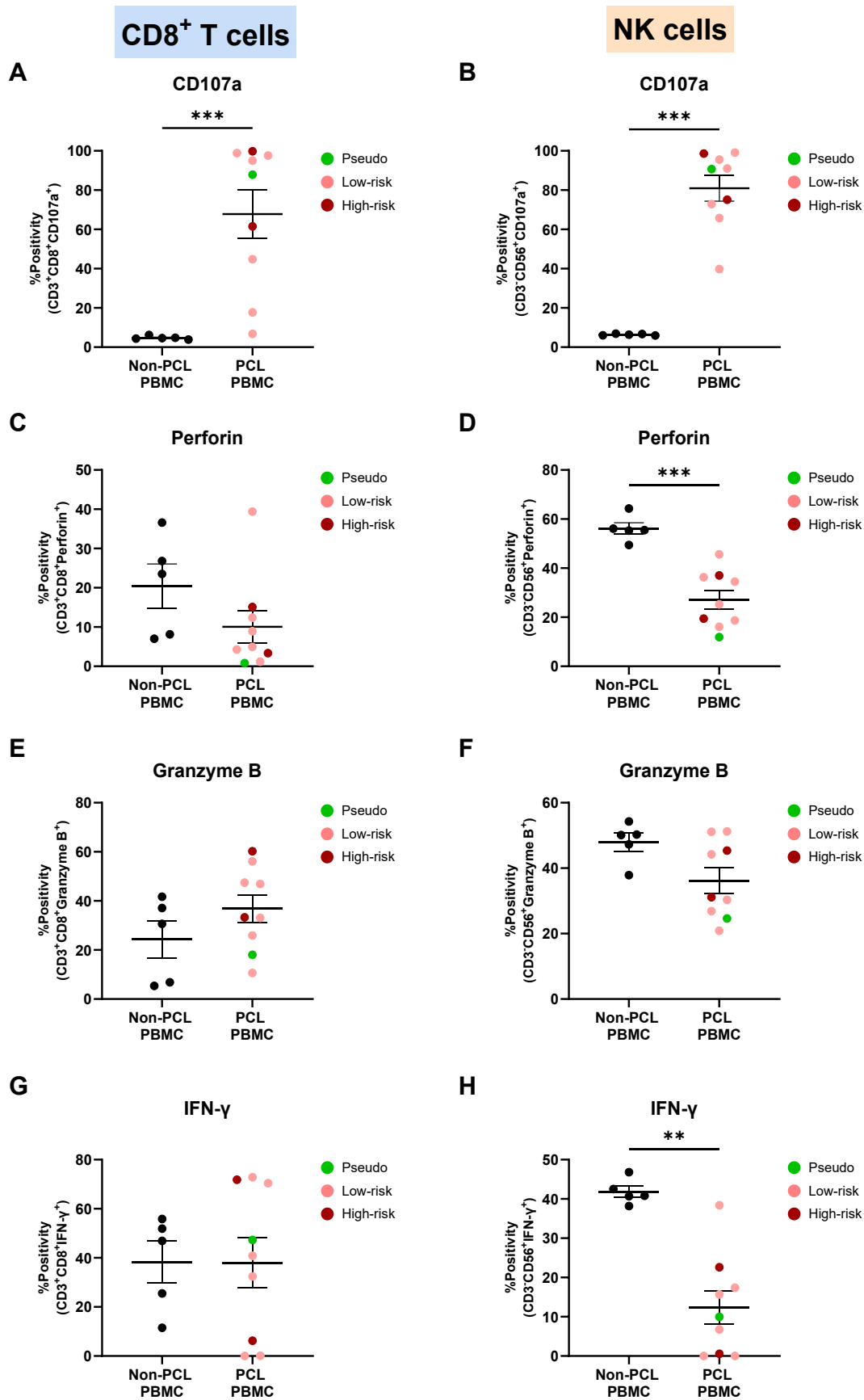


Figure 3.13: PCL-associated PBMC samples have a significantly higher frequency of CD107a⁺ effector immune cell degranulation and decreased perforin and IFN- γ cytokine production compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to assess CD107a⁺ degranulation (A, B), perforin⁺ (C, D), granzyme B⁺ (E, F) and IFN- γ ⁺ (G, H) in viable CD3⁺CD8⁺ T cells (A, C, E, F) and viable CD3⁺CD56⁺ NK cells (B, D, F, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as **p $\leq$ 0.01, and ***p $\leq$ 0.001].

3.4.14 PCF has a significantly increased frequency of LAG-3 on CD8⁺ T cells compared to matched PCL-associated PBMC samples.

At present, the local immune microenvironment of pre-malignant PCLs is poorly characterised. To provide insight into the immune infiltrate of these lesions, the expression of a range of immune checkpoint molecules was assessed on the surface of CD8⁺ T cells and CD8⁻ T cells isolated from pre-malignant PCF and in circulation.

There was a non-significant increase in the frequency of PD-1⁺ CD8⁻ T cells within pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.14A**). Similarly, the frequency of PD-1⁺ CD8⁺ T cells was non-significantly increased in pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.14B**).

This study also found that there was a non-significant decrease of TIM-3 on CD8⁻ T cells and CD8⁺ T cells within the immune cell infiltrate of pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.14D, E**).

On the contrary, the frequency of LAG-3 expression was significantly increased in CD8⁺ T cells found within pre-malignant PCF compared to PBMCs isolated from matched PCL patients ($14.84 \pm 7.47\%$ vs. $0.17 \pm 0.16\%$, p=0.0312) (**Figure 3.14H**). Although not significant, the expression of the immune checkpoint receptor LAG-3 was also increased in the CD8⁻ T cell population within pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.14G**).

Additionally, this immunophenotyping analysis identified a non-significant decrease in the frequency of T cell immunoreceptor with Ig and ITIM domains (TIGIT) expression in CD8⁻ T cells and CD8⁺ T cells within pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.14J, K**).

Elevated expression of the immune checkpoint receptors PD-1 and LAG-3 on T cells within pre-malignant pancreatic lesions suggests impaired effector function and an actively suppressed local immune surveillance, potentially facilitating progression towards PC.

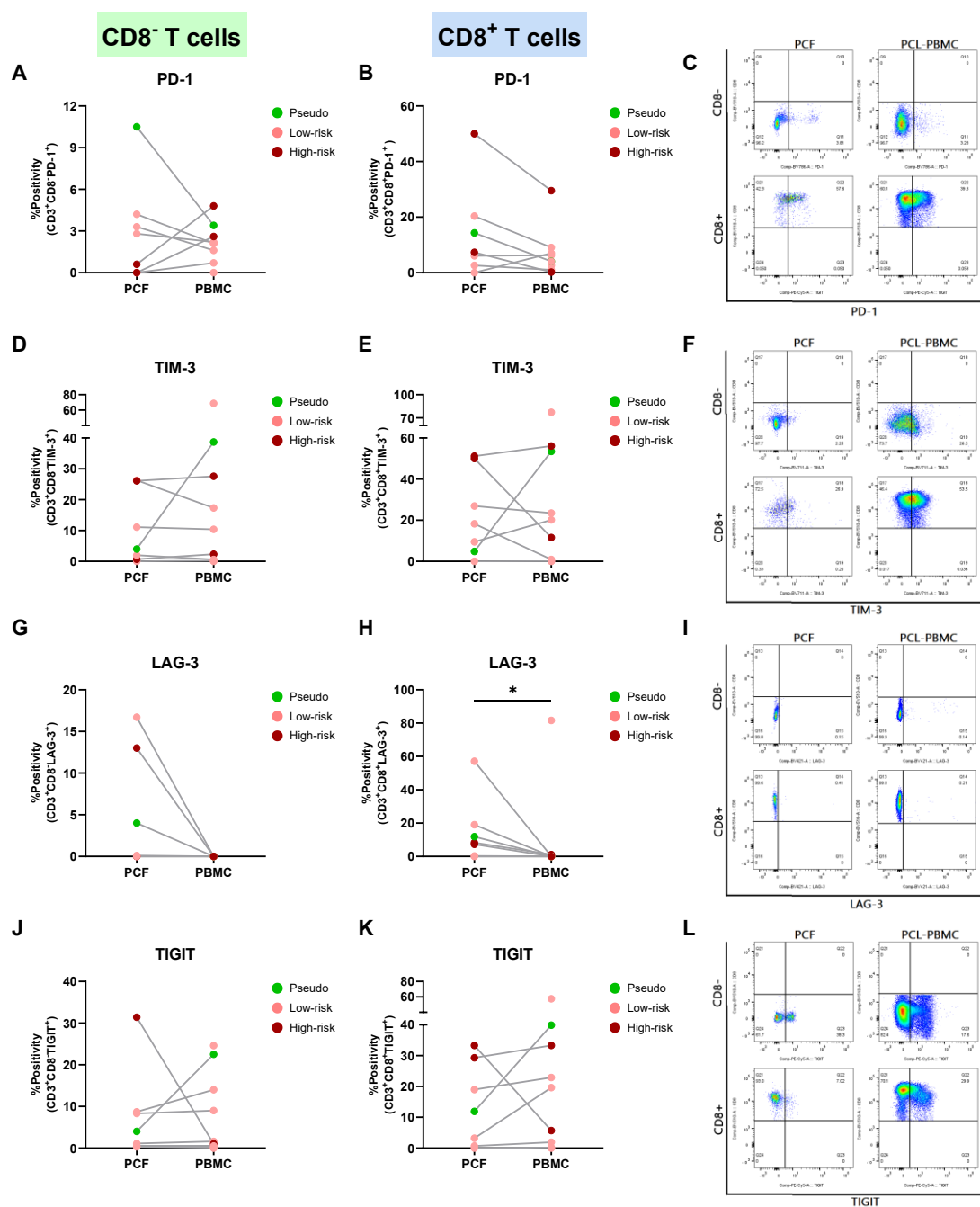


Figure 3.14: PCF has a significantly increased frequency of LAG-3 on CD8⁺ T cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess expression of immune checkpoint

receptors PD-1⁺ (A, B, C), TIM-3⁺ (D, E, F), LAG-3⁺ (G, H, I) and TIGIT⁺ (J, K, L) on viable CD3⁺CD8⁻ T cells (A, D, G, J) and viable CD3⁺CD8⁺ T cells (B, E, H, K). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as * $p \leq 0.05$].

3.4.15 The systemic immune system of PCL patients has a significantly increased frequency of TIM-3⁺ T cells.

The frequency of immune checkpoint receptor molecules was investigated by flow cytometry in PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors.

This study found that there was a non-significant increase in the frequency of PD-1 on CD8⁻ T cells from PBMCs isolated from PCL patients compared to healthy, non-PCL control PBMCs (**Figure 3.15A**). Similarly, there was a trend towards a significant increase in the expression frequency of PD-1 on CD8⁺ T cells from PCL-patient PBMCs compared to healthy, non-PCL control PBMCs ($7.09 \pm 2.96\%$ vs. $1.60 \pm 0.43\%$, $p=0.0639$) (**Figure 3.15B**).

The frequency of TIM-3 was significantly higher in CD8⁺ T cells in PCL patient-derived PBMCs compared to healthy, non-PCL PBMCs ($18.43 \pm 7.77\%$ vs. $0.16 \pm 0.02\%$, $p=0.0305$) (**Figure 3.15D**). Additionally, TIM-3 expression was significantly increased in CD8⁻ T cells from PCL patients compared to those isolated from healthy, non-PCL donors ($27.01 \pm 9.50\%$ vs. $0.10 \pm 0.04\%$, $p=0.0410$) (**Figure 3.15C**).

There were no significant differences in the expression of immune checkpoint receptor LAG-3 in CD8⁻ T cells or CD8⁺ T cells isolated from PCL patients or healthy, non-PCL donors (**Figure 3.15E, F**).

Furthermore, there was a non-significant increase in the frequency of TIGIT in CD8⁻ T cells from PBMCs isolated from PCL patients compared to healthy, non-PCL control PBMCs (**Figure 3.15G**). Although not significant, the expression of the immune checkpoint receptor TIGIT was also increased in the CD8⁺ T cell population from PCL patient-derived PBMCs compared to PBMCs isolated from healthy, non-PCL donors (**Figure 3.15H**).

Increased PD-1, TIM-3, and LAG-3 immune checkpoint receptor expression suggests systemic T cell exhaustion and impaired anti-tumour immunity, potentially permitting the progression of pre-malignant pancreatic lesions.

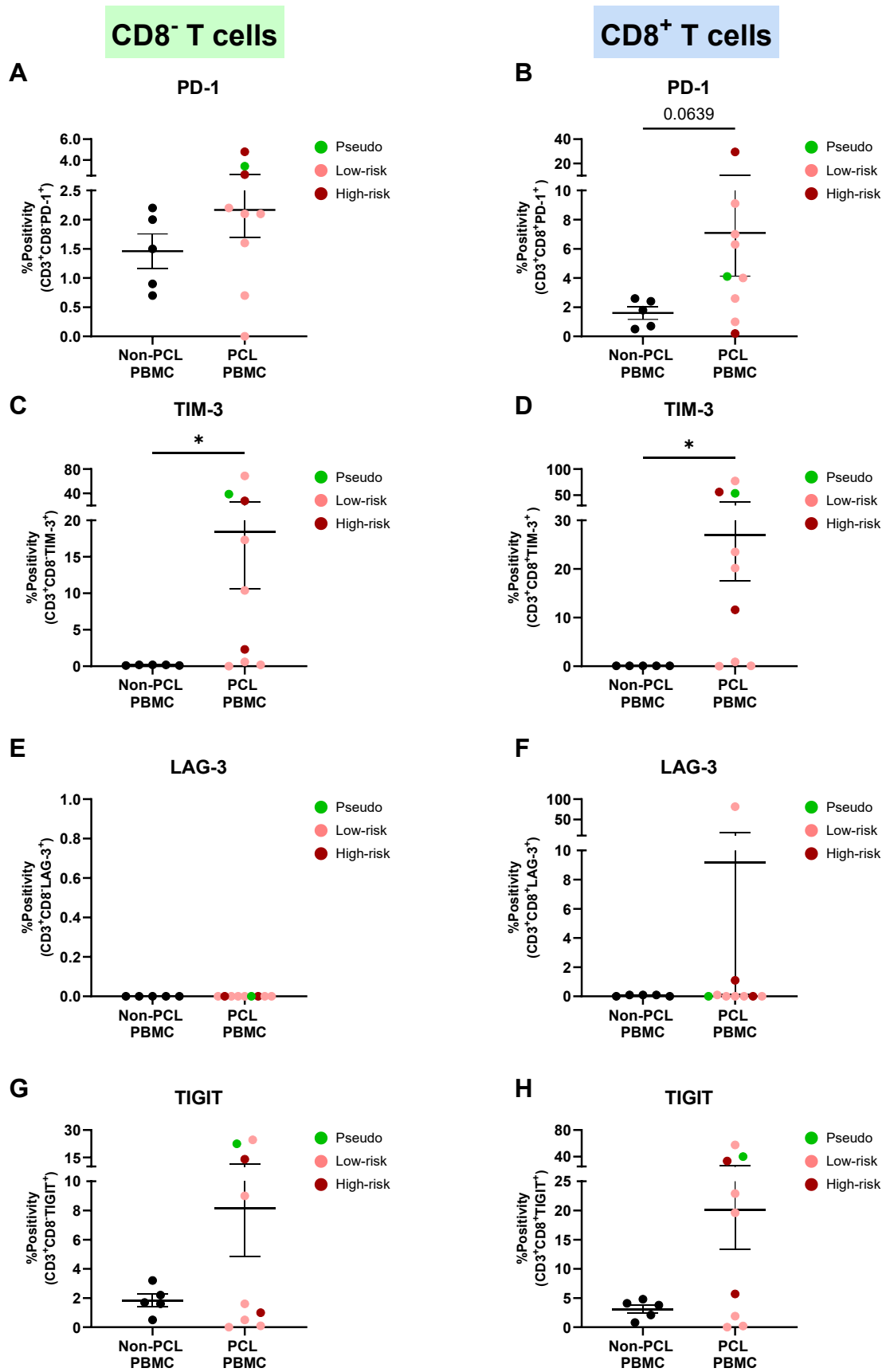


Figure 3.15: PCL-associated PBMC samples have a significantly increased frequency of TIM-3⁺ T cells compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to assess expression of immune checkpoint receptors PD-1⁺ (A, B), TIM-3⁺ (C, D), LAG-3⁺ (E, F) and TIGIT⁺ (G, H) on viable CD3⁺CD8⁻ T cells (A, C, E, G) and viable CD3⁺CD8⁺ T cells (B, D, F, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as * $p \leq 0.05$].

3.4.16 PCF has a significantly increased frequency of PD-L1 on CD8⁻ T cells compared to matched PCL-associated PBMC samples.

This study found that there was a significantly higher frequency of programmed cell death ligand 1 (PD-L1) on CD8⁻ T cells within the immune cell infiltrate of pre-malignant PCF compared to patient-matched PBMCs ($2.71 \pm 1.75\%$ vs. $0.13 \pm 0.05\%$, $p=0.0469$) (**Figure 3.16A**). Although not significant, the expression of PD-L1 was also increased in the CD8⁺ T cell population within pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.16B**).

Furthermore, the frequency of immune checkpoint receptors CD155 and galectin-9 were found to be non-significantly increased on CD8⁻ T cells within pre-malignant PCF compared to PBMCs isolated from matched PCL patients (**Figure 3.16D, G**).

This immunophenotyping analysis also identified a trend towards a significant increase in the frequency of CD155 expression on CD8⁺ T cells within pre-malignant PCF compared to PBMCs isolated from matched PCL patients ($4.40 \pm 2.18\%$ vs. $0.36 \pm 0.32\%$, $p=0.0625$) (**Figure 3.16E**). Indeed, there was a non-significant increase in the frequency of galectin-9 expression on CD8⁺ T cells found within pre-malignant PCF compared to patient-matched PBMCs (**Figure 3.16H**).

Overall, this data may suggest that T cells within pre-malignant PCF may contribute to an immunosuppressive microenvironment through elevated immune checkpoint ligand expression, which may potentially dampen effective anti-tumour immunity and permit PCL persistence or malignant progression.

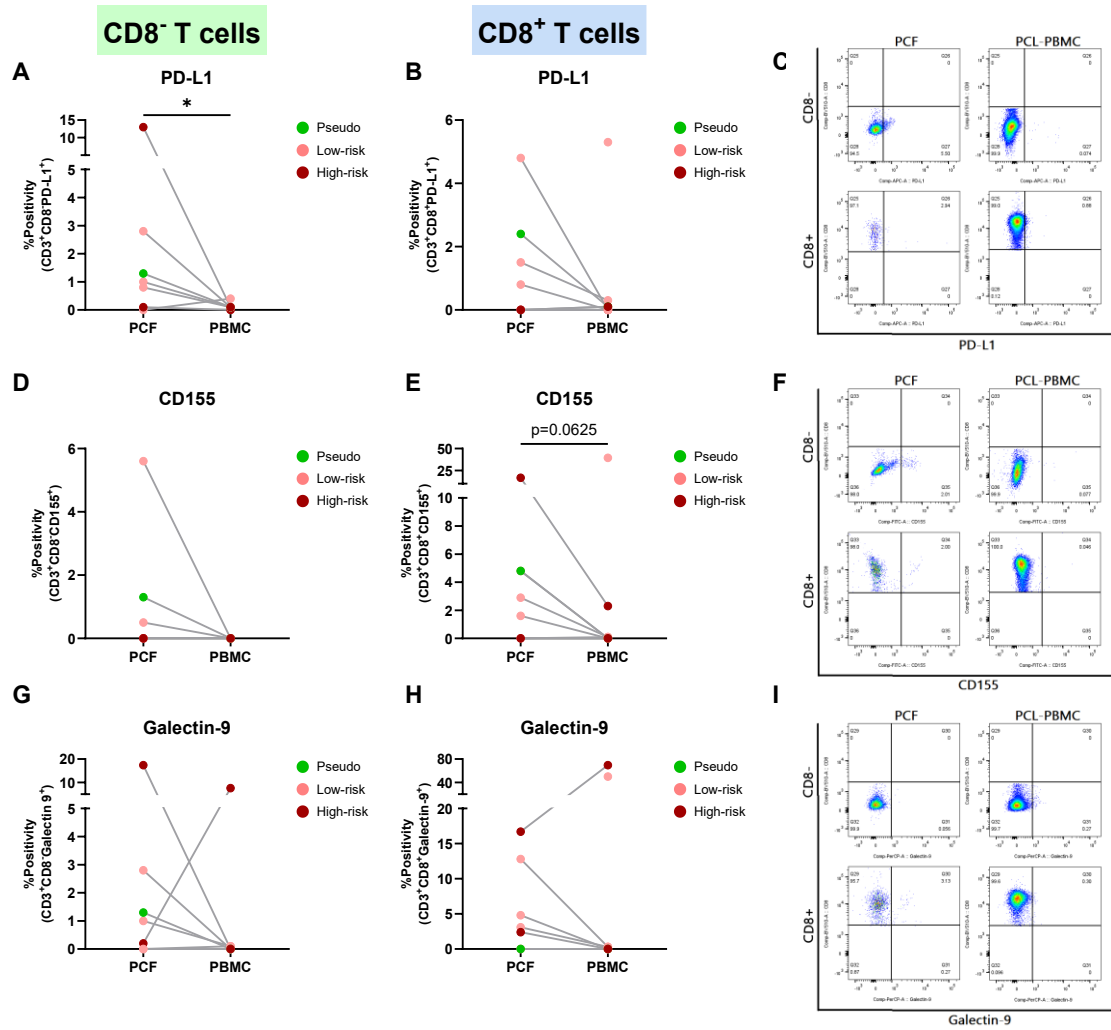


Figure 3.16: PCF has a significantly increased frequency of PD-L1 on CD8⁺ T cells compared to matched PCL-associated PBMC samples. Cells were isolated from PCF (pseudo n=1, low-risk n=4, high-risk n=2) and PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2). Flow cytometric staining was carried out to assess expression of immune checkpoint ligands PD-L1⁺ (A, B, C), CD155⁺ (D, E, F), galectin-9⁺ (G, H, I) on viable CD3⁺CD8⁺ T cells (A, D, G) and viable CD3⁺CD8⁺ T cells (B, E, H). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) [significance defined as *p $\leq$ 0.05].

3.4.17 PBMCs from PCL patients have a significantly decreased frequency of PD-L1 and CD155 on CD8⁻ T cells.

The frequency of immune checkpoint ligands was investigated by flow cytometry, in PBMCs isolated from PCL patients and compared to that of healthy, non-PCL donors.

The frequency of PD-L1 expression on CD8⁻ T cells within PBMCs isolated from PCL patients was significantly decreased compared to healthy, non-PCL PBMC donor samples ($0.11 \pm 0.04\%$ vs. $1.04 \pm 0.21\%$, $p=0.0005$) (**Figure 3.17A**). However, the frequency of PD-L1 expression on CD8⁺ T cells was non-significantly increased in PCL patient-derived PBMCs compared to healthy, non-PCL PBMCs (**Figure 3.17B**).

The results from this study also found that there was a significant increase of CD155 expression on CD8⁻ T cells in PBMCs isolated from PCL patients compared to healthy, non-PCL PBMC samples ($0.26 \pm 0.17\%$ vs. $0.00 \pm 0.00\%$, $p=0.0275$) (**Figure 3.17C**). On the contrary, CD155 expression was non-significantly increased in CD8⁺ T cells found in PBMCs isolated from PCL patients compared to healthy, non-PCL PBMC donor samples (**Figure 3.17D**).

This study also showed that the frequency of galectin-9 was non-significantly increased in CD8⁻ T cells and CD8⁺ T cells isolated from PCL patients compared to PBMCs from healthy, non-PCL donors (**Figure 3.17E, F**).

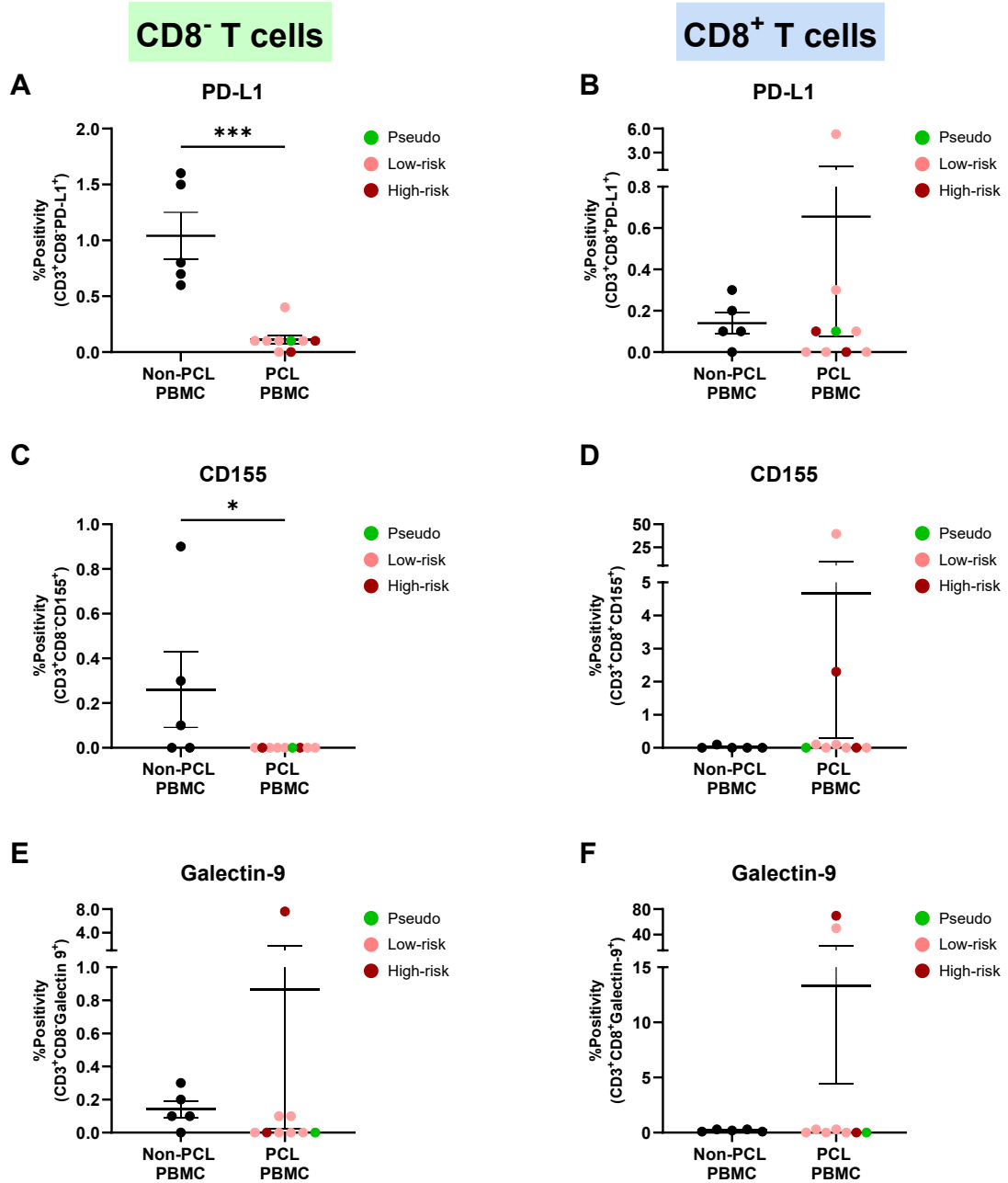


Figure 3.17: PCL-associated PBMC samples have a significantly decreased frequency of PD-L1 and CD155 on CD8⁺ T cells compared to PBMCs from healthy, non-PCL donors. PBMCs were isolated from peripheral whole blood of PCF donors (pseudo n=1, low-risk n=6, high-risk n=2) and healthy, non-PCL donors (n=5). Flow cytometric staining was carried out to assess expression of immune checkpoint ligands PD-L1⁺ (A, B), CD155⁺ (C, D), galectin-9⁺ (E, F) on viable CD3⁺CD8⁺ T cells (A, C, E) and viable CD3⁺CD8⁺ T cells (B, D, F). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric unpaired t-test (Mann-Whitney test) [significance defined as *p $\leq$ 0.05, and ***p $\leq$ 0.001].

3.4.18 LAG-3 expression is significantly increased in intermediary neoplastic pancreatic cells and expression is increased in PC cells upon exposure to stress conditions.

Immune checkpoint molecules are key regulators of immune responses, mediating stimulatory and inhibitory interactions between antigen-presenting cells and immune cells^[231]. Hostile features of the pancreatic TME including hypoxia, acidosis and nutrient deprivation are reported to have profound effects on immune checkpoint expression^[232-234]. The interaction between cancer cells and immune cells through immune checkpoints in the TME is known to suppress immunosurveillance and mediate immune evasion. Little is known of changes to the immune checkpoint landscape during malignant progression of PC, particularly when exposed to a hostile microenvironment^[235, 236]. Serum-free culture media of normal pancreatic cell lines used within this study did not allow for nutrient deprivation. However, PC is commonly characterised by numerous and severe hypoxic regions, with a median tissue partial oxygen pressure (pO₂) of 0-5.3 mmHg (0-0.7% O₂) compared to the adjacent normal pancreas pO₂ at 24.3-92.7 mmHg (3.2-12.3% O₂)^[237]. In this study, the expression of immune checkpoint receptors was assessed by flow cytometry in normal pancreatic cells (H6c7-HPDE), intermediary pancreatic cells (hTERT-HPNE) and pancreatic cancer cells (BxPC-3, Panc-1) under normal conditions (normoxia: 21% O₂) and under stress conditions (severe hypoxia: 0.5% O₂).

The mean fluorescence intensity (MFI) of immune checkpoint receptor PD-1 was significantly increased in hTERT-HPNE intermediary pancreatic cells, which are classified as human pancreatic neoplastic epithelium, compared to normal pancreatic H6c7-HPDE cells (Mean MFI $\pm$ SEM; 366.00 $\pm$ 36.72 MFI vs. 130.00 $\pm$ 23.94 MFI, $p=0.0361$) (**Figure 3.18A**). Similarly, the MFI of PD-1 was significantly increased in intermediary hTERT-HPNE pancreatic cells compared to PC cell lines BxPC-3 and Panc-1 (hTERT-HPNE vs. BxPC-3: 366.00 $\pm$ 36.72 MFI vs. 94.81 $\pm$ 60.89 MFI, $p=0.0261$ and hTERT-HPNE vs. Panc-1: 366.00 $\pm$ 36.72 MFI vs. 112.20 $\pm$ 62.62 MFI, $p=0.0310$) (**Figure 3.18A**). Although not significant, this increase in PD-1 MFI was also evident in hTERT-HPNE cells cultured under hypoxic stress conditions (**Figure 3.18A**). Compared to cells cultured under normal conditions, the MFI of PD-1 was non-significantly decreased when cultured under hypoxic stress conditions in H6c7-HPDE cells, hTERT-HPNE cells, BxPC-3 cells and Panc-1 cells (**Figure 3.18A**).

This study also found that the MFI of LAG-3 was significantly increased in hTERT-HPNE cells cultured under severe hypoxic stress conditions compared to normal pancreatic H6c7-HPDE cells (1665.00 ± 176.90 MFI vs. $724.30.00 \pm 18.56$ MFI, $p=0.0034$). Similarly, hypoxia-cultured hTERT-HPNE cells had significantly increased LAG-3 MFI compared to hypoxia-cultured BxPC-3 pancreatic cancer cells (1665.00 ± 176.90 MFI vs. 752.00 ± 124.20 MFI, $p=0.0034$) (**Figure 3.18C**). The MFI of LAG-3 was also significantly increased in hTERT-HPNE cells cultured under hypoxic stress conditions compared to Panc-1 pancreatic cancer cells cultured under severe hypoxia (1665.00 ± 176.90 MFI vs. 806.30 ± 107.10 MFI, $p=0.0041$) (**Figure 3.18C**). Although not significant, intermediary pancreatic hTERT-HPNE cells cultured under normal culture conditions also showed increased LAG-3 MFI compared to normal pancreatic cells and PC cells (**Figure 3.18C**). Similar to that of PD-1, the MFI of LAG-3 was non-significantly increased in H6c7-HPDE, hTERT-HPNE cells, BxPC-3 cells and Panc-1 cells when cultured under hypoxic stress conditions compared to cells cultured under normal conditions (**Figure 3.18C**).

The MFI of TIM-3 was non-significantly increased in hTERT-HPNE intermediary pancreatic cells compared to all other cell lines, regardless of cell culture conditions (**Figure 3.18E**). The MFI of TIM-3 was also found to be non-significantly decreased when cultured under hypoxic stress conditions in hTERT-HPNE cells, BxPC-3 cells and Panc-1 cells (**Figure 3.18E**). However, TIM-3 MFI was non-significantly increased in normal H6c7-HPDE pancreatic cells when cultured under hypoxic stress conditions compared to normal culture conditions (**Figure 3.18E**).

This study showed that the MFI of immune checkpoint receptor TIGIT was non-significantly increased in the intermediary hTERT-HPNE pancreatic cells, BxPC-3 cells and Panc-1 pancreatic cancer cells compared to the normal H6c7-HPDE pancreatic cells, regardless of cell culture conditions (**Figure 3.18G**). TIGIT MFI was non-significantly decreased when cultured under hypoxic stress conditions in BxPC-3 cells and Panc-1 pancreatic cancer cell lines (**Figure 3.18G**). Conversely, the MFI of TIGIT was non-significantly increased in normal H6c7-HPDE pancreatic cells when cultured under hypoxic stress conditions compared to normal culture conditions (**Figure 3.18G**).

Similar to the expression of PD-1, TIM-3, and TIGIT, the MFI of V-domain immunoglobulin suppressor of T cell activation (VISTA) was non-significantly increased in hTERT-HPNE intermediary pancreatic cells compared to all other cell lines, regardless of cell culture conditions (**Figure 3.18I**). Similar to that of TIGIT, the MFI of immune

checkpoint receptor VISTA was also found to be non-significantly decreased when cultured under hypoxic stress conditions in hTERT-HPNE cells, BxPC-3 cells and Panc-1 cells (**Figure 3.18I**). On the contrary, the MFI of VISTA was non-significantly increased in normal H6c7-HPDE pancreatic cells when cultured under hypoxic stress conditions compared to normal culture conditions (**Figure 3.18I**).

Overall, intermediary hTERT-HPNE pancreatic cells consistently had increased expression of multiple immune checkpoint receptors compared to normal pancreatic cells and cancer cells. These findings suggest that intermediary hTERT-HPNE pancreatic cells may adopt an immune-evasive phenotype early in malignant transformation.

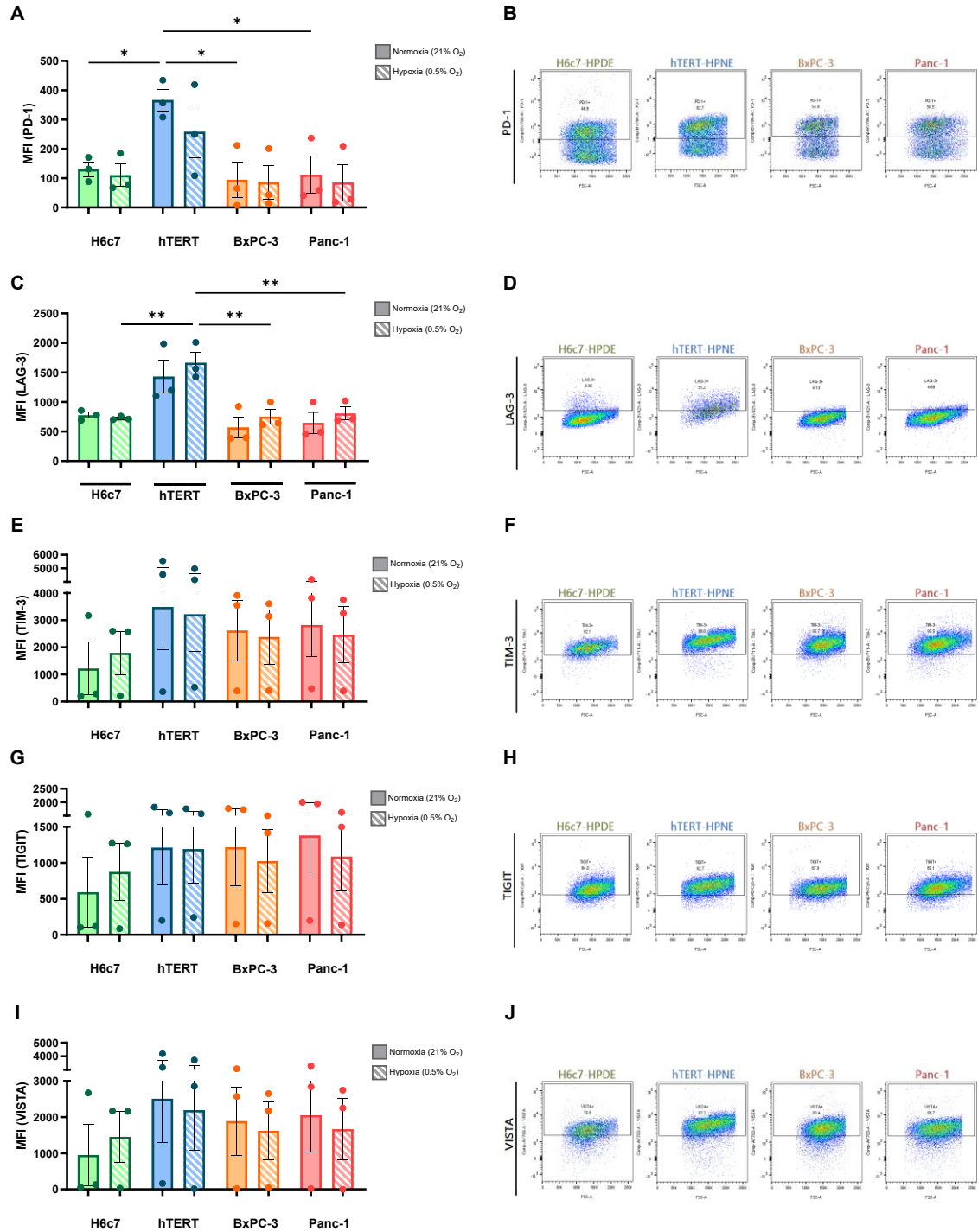


Figure 3.18: LAG-3 expression is significantly increased in intermediary pancreatic cells and expression is increased in PC cells upon exposure to stress conditions. Normal pancreatic cells (H6c7-HPDE n=3), intermediary pancreatic cells (hTERT-HPNE n=3), and pancreatic cancer cells (BxPC-3 n=3, Panc-1 n=3) were screened for the expression of immune checkpoint receptor expression under basal conditions (normoxia; 21% O₂, 37°C) and under stress conditions (severe hypoxia; 0.5% O₂, 37°C). Flow cytometric staining was carried out to assess expression of immune checkpoint receptors PD-1⁺ (A, B), LAG-3⁺ (C, D), TIM-3⁺ (E, F), TIGIT⁺ (G, H), VISTA⁺ (I, J) on viable cell populations. Graphical data are representative of mean MFI ± SEM. Statistical significance was assessed using parametric ordinary one-

way ANOVA (Normoxia vs. normoxia between cell lines/ Hypoxia vs. hypoxia between cell lines; significance defined as * $p \leq 0.05$ and ** $p \leq 0.01$) and parametric paired t-test (Normoxia vs. Hypoxia within cell lines).

3.4.19 The expression of immune checkpoint ligands is altered in pancreatic cell lines following exposure to stress conditions.

In this study, the expression of immune checkpoint ligands was also assessed by flow cytometry in normal pancreatic cells (H6c7-HPDE), intermediary pancreatic cells (hTERT-HPNE) and pancreatic cancer cells (BxPC-3, Panc-1) under normal culture conditions (normoxia: 21% O₂) and under stress conditions (severe hypoxia: 0.5% O₂).

Although not significant, the MFI of immune checkpoint ligand PD-L1 was increased in hTERT-HPNE intermediary pancreatic cells cultured under hypoxic stress conditions compared to hTERT-HPNE cells cultured under normal culture conditions (**Figure 3.19A**). Conversely, the MFI of PD-L1 was non-significantly decreased when cultured under hypoxic stress conditions in BxPC-3 and Panc-1 pancreatic cancer cells (**Figure 3.19A**).

The MFI of PD-L2 was found to be significantly decreased in Panc-1 pancreatic cancer cells cultured under hypoxic stress compared to normal culture conditions (1901.00 ± 138.10 MFI vs. 3385.00 ± 433.90 MFI, $p=0.0378$) (**Figure 3.19C**). This study also found that the MFI of PD-L2 was significantly increased in hTERT-HPNE cells cultured under normal conditions compared to H6c7-HPDE pancreatic cells cultured under these same conditions (44713.00 ± 4423.00 MFI vs. 1989.00 ± 1024.00 MFI, $p=0.0001$) (**Figure 3.19C**). Similarly, the MFI of PD-L2 was significantly increased in hTERT-HPNE pancreatic cells cultured under normoxia compared to normoxia-cultured BxPC-3 pancreatic cells (44713.00 ± 4423.00 MFI vs. 3042.00 ± 356.40 MFI, $p=0.0001$) (**Figure 3.19C**). This significant increase in PD-L2 MFI was also evident in hTERT-HPNE cells cultured under normal conditions compared to Panc-1 cells cultured under these same conditions (44713.00 ± 4423.00 MFI vs. 3385.00 ± 433.90 MFI, $p=0.0001$) (**Figure 3.19C**). Intermediary pancreatic hTERT-HPNE cells cultured under hypoxic stress conditions also showed a trend towards a significant increase of PD-L2 MFI compared to normal H6c7-HPDE pancreatic cells and BxPC-3 and Panc-1 PC cells (hTERT-HPNE vs. H6c7-HPDE; 24801.00 ± 9871.00 MFI vs. 1993.00 ± 315.30 MFI, $p=0.0696$, hTERT-HPNE vs. BxPC-3; 24801.00 ± 9871.00 MFI vs. 3519.00 ± 1621.00 MFI, $p=0.0696$ and

hTERT-HPNE vs. Panc-1; 24801.00 ± 9871.00 MFI vs. 1901.00 ± 138.10 MFI, $p=0.0696$) (**Figure 3.19C**).

Although not significant, the MFI of immune checkpoint ligand CD112 was increased in H6c7-HPDE normal pancreatic cells cultured under hypoxic stress conditions compared to those cultured under normal culture conditions (**Figure 3.19E**). However, the MFI of CD112 was significantly increased in hTERT-HPNE intermediary pancreatic cells cultured under hypoxic stress compared to normal culture conditions (857.00 ± 228.00 MFI vs. 668.00 ± 207.20 MFI, $p=0.0230$) (**Figure 3.19E**). Furthermore, hypoxia-cultured BxPC-3 pancreatic cells also had significantly increased MFI of CD112 compared to BxPC-3 cells cultured under normal conditions (514.00 ± 152.40 MFI vs. 467.30 ± 154.70 MFI, $p=0.0153$) (**Figure 3.19E**). This significant increase in CD112 MFI was also evident in Panc-1 pancreatic cancer cells cultured under hypoxic stress compared to Panc-1 cells cultured under normal conditions (541.00 ± 166.60 MFI vs. 514.70 ± 169.30 MFI, $p=0.0229$) (**Figure 3.19E**).

This study found that the MFI of galectin-9 was non-significantly increased in normal H6c7-HPDE cells and intermediary hTERT-HPNE cells compared to BxPC-3 and Panc-1 pancreatic cancer cells (**Figure 3.19G**). There was little to no expression of galectin-9 found on BxPC-3 and Panc-1 pancreatic cancer cell lines (**Figure 3.19G**). Galectin-9 MFI was non-significantly increased in normal H6c7-HPDE pancreatic cells when cultured under hypoxic stress conditions compared to normal culture conditions, an effect not seen in hTERT-HPNE cells (**Figure 3.19G**).

The MFI of CEACAM-1, was non-significantly increased when cultured under hypoxic stress conditions in H6c7-HPDE cells, BxPC-3 cells and Panc-1 cells (**Figure 3.19I**). However, CEACAM-1 MFI was non-significantly decreased in intermediary hTERT-HPNE pancreatic cells when cultured under hypoxic stress conditions compared to normal culture conditions (**Figure 3.19I**).

The MFI of B7-H3 was significantly decreased when cultured under hypoxic stress conditions in hTERT-HPNE intermediary pancreatic cells compared to those cultured under normoxia (23059.00 ± 4568.00 MFI vs. 26750.00 ± 5106.00 MFI, $p=0.0226$) (**Figure 3.19K**). On the contrary, H6c7-HPDE normal pancreatic cells were found to have a non-significantly increased B7-H3 MFI when cultured under hypoxic stress conditions compared to normal culture conditions (**Figure 3.19K**). There were no significant differences in the expression of B7-H3 in BxPC-3 and Panc-1 pancreatic cancer cell lines, regardless of culture conditions (**Figure 3.19K**).

The MFI of CD155 was found to be significantly decreased in BxPC-3 pancreatic cancer cells cultured under hypoxic stress compared to normal culture conditions (58371.00 ± 623.60 MFI vs. 86063.00 ± 525.6 MFI, $p=0.0016$) (**Figure 3.19M**). Similarly, CD155 MFI was significantly decreased in hypoxia cultured Panc-1 cells compared to cells cultured under normal conditions (58129.00 ± 1812.00 MFI vs. 89862.00 ± 2587.00 MFI, $p=0.0008$) (**Figure 3.19M**). This study also found that the MFI of CD155 was significantly increased in BxPC-3 pancreatic cancer cells cultured under normal conditions compared to H6c7-HPDE normal pancreatic cells cultured under these same conditions (86063.00 ± 525.60 MFI vs. 17187.00 ± 6978.00 MFI, $p=0.0001$) (**Figure 3.19M**). The MFI of CD155 was also significantly increased in BxPC-3 pancreatic cancer cells cultured under normal conditions compared to hTERT-HPNE intermediary pancreatic cells cultured under normoxia (86063.00 ± 525.60 MFI vs. 29190.00 ± 1294.00 MFI, $p=0.0001$) (**Figure 3.19M**). Similarly, the MFI of CD155 was significantly increased in Panc-1 pancreatic cancer cells cultured under normoxia compared to normoxia-cultured H6c7-HPDE pancreatic cells (89862.00 ± 2587.00 MFI vs. 17187.00 ± 6978.00 MFI, $p=0.0001$) (**Figure 3.19M**). This significant increase in CD155 MFI was also evident in Panc-1 cells cultured under normal conditions compared to hTERT-HPNE cells cultured under these same conditions (89862.00 ± 2587.00 MFI vs. 29190.00 ± 1294.00 MFI, $p=0.0001$) (**Figure 3.19M**). BxPC-3 pancreatic cancer cells cultured under hypoxic stress conditions had significantly increased CD155 MFI compared to normal H6c7-HPDE and intermediary hTERT-HPNE cells cultured under hypoxic stress conditions (BxPC-3 vs. H6c7-HPDE: 58371.00 ± 623.60 MFI vs. 18674.00 ± 691.40 MFI, $p=0.0001$ and BxPC-3 vs. hTERT-HPNE: 58371.00 ± 623.60 MFI vs. 26545.00 ± 457.40 MFI, $p=0.0001$) (**Figure 3.19M**). The MFI of CD155 was also significantly increased in Panc-1 pancreatic cancer cells cultured under hypoxic stress conditions compared to H6c7-HPDE cells and intermediary hTERT-HPNE cells cultured under severe hypoxia (BxPC-3 vs. H6c7-HPDE: 58129.00 ± 1812.00 MFI vs. 18674.00 ± 691.40 MFI, $p=0.0001$ and BxPC-3 vs. hTERT-HPNE: 58371.00 ± 623.60 MFI vs. 26545.00 ± 457.40 MFI, $p=0.0001$) (**Figure 3.19M**).

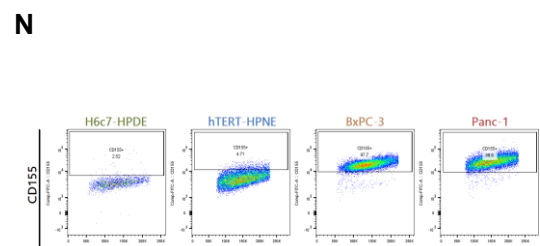
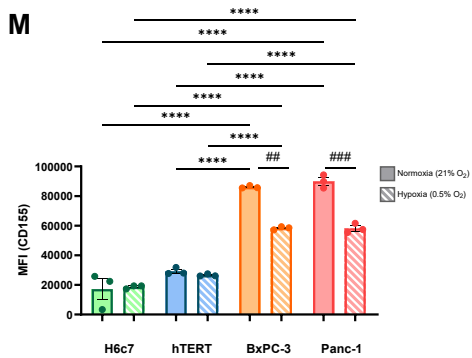
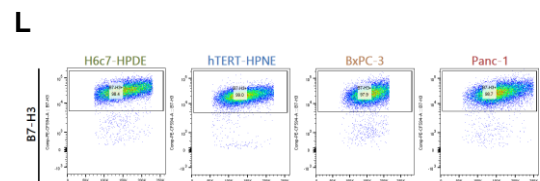
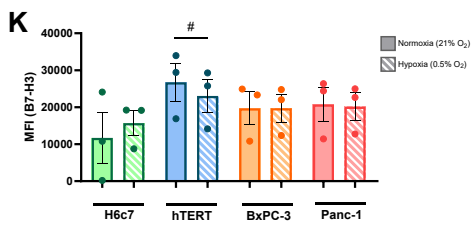
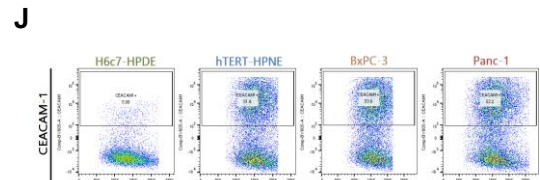
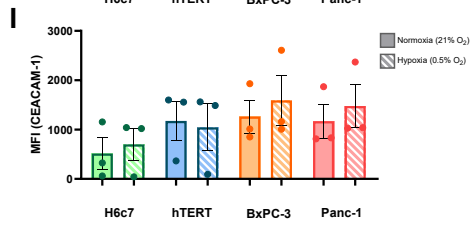
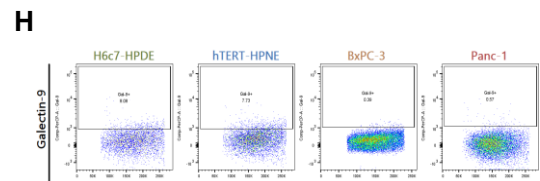
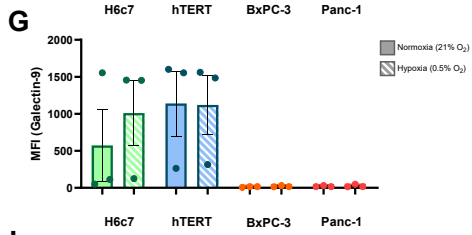
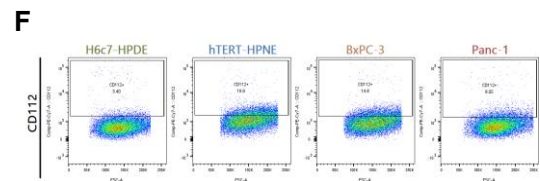
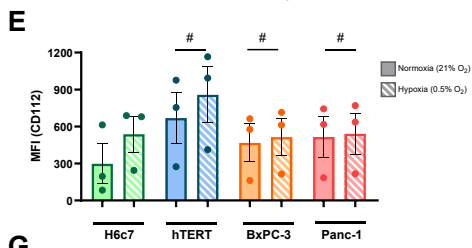
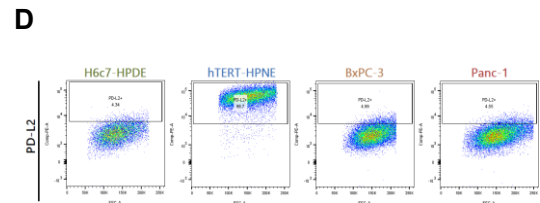
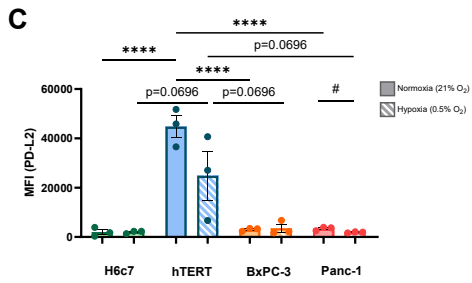
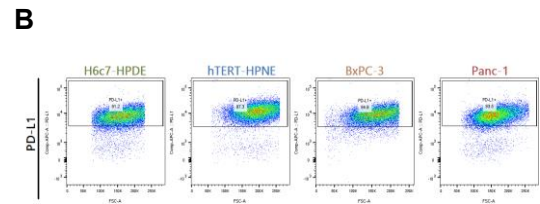
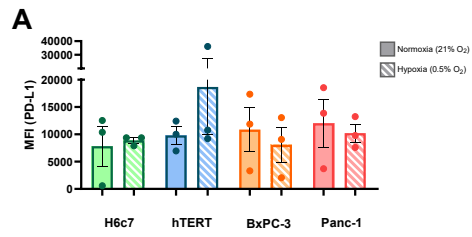


Figure 3.19: The expression of immune checkpoint ligands is altered in pancreatic cell lines following exposure to stress conditions. Normal pancreatic cells (H6c7-HPDE n=3), intermediary pancreatic cells (hTERT-HPNE n=3), and pancreatic cancer cells (BXPC-3 n=3, Panc-1 n=3) were screened for the expression of immune checkpoint ligand expression under basal conditions (normoxia; 21% O₂, 37°C) and under stress conditions (severe hypoxia; 0.5% O₂, 37°C). Flow cytometric staining was carried out to assess expression of immune checkpoint receptors PD-L1⁺ (A, B), PD-L2⁺ (C, D), CD112⁺ (E, F), galectin-9⁺ (G, H), CEACAM-1⁺ (I, J), B7-H3⁺ (K, L), and CD155⁺ (M, N) on viable cell populations. Graphical data are representative of mean MFI ± SEM. Statistical significance was assessed using parametric ordinary one-way ANOVA (Normoxia vs. normoxia between cell lines/ Hypoxia vs. hypoxia between cell lines; significance defined as ****p≤0.0001) and parametric paired t-test (Normoxia vs. Hypoxia within cell lines; significance defines as #p≤0.05, ##p≤0.01, and ###p≤0.001).

3.4.20 Soluble adhesion molecules are differentially expressed across risk-stratified PCF.

Alongside integrins, adhesion molecules are cell surface proteins involved in mediating immune cell trafficking to sites of infection, inflammation, or tissue damage. The membrane-bound adhesion molecules can be shed from cell surfaces and released into the local immune microenvironment. Multiplex ELISAs were used to assess the concentration of soluble adhesion molecules in risk-stratified PCF and patient-matched sera.

In an initial screen, carried out using a custom AssayGenie flow cytometry-based multiplex ELISA, the concentration of soluble ICAM-1 was found to be significantly increased in high-risk serum compared to patient-matched high-risk PCF (Mean concentration ± SEM; 476867.00 ± 27304.00 pg/mL vs. 4599.00 ± 2562.00 pg/mL, p=0.0156) (**Figure 3.20A**). The concentration of ICAM-1 was also significantly higher in low-risk serum compared to low-risk PCF (429698.00 ± 48264.00 pg/mL vs. 94773.00 ± 54307.00 pg/mL, p=0.0312) (**Figure 3.20A**). CD147, otherwise recognised as EMMPRIN or basigin, is an adhesion molecule involved in cell-cell and cell-matrix adhesion. This study found that CD147 concentration was significantly higher in serum from patients with high-risk PCLs compared to the matched high-risk PCF samples (2252.00 ± 381.80 pg/mL vs. 416.20 ± 60.10 pg/mL, p=0.0156) (**Figure 3.20B**).

The independent screen using the MSD multiplex ELISA showed that there were significantly higher concentrations of adhesion molecules in PCF from patients with a low-risk of PC progression compared to PCF from high-risk patients. Soluble ICAM-1

exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (3124227.00 ± 166744.00 pg/mL vs. 20168.00 ± 12967.00 pg/mL, $p=0.0219$) (**Figure 3.20C**). There was also a significantly higher concentration of soluble VCAM-1 in low-risk PCF compared to high-risk PCF (1787014.00 ± 513010.00 pg/mL vs. 7184.00 ± 4422.00 pg/mL, $p=0.0219$) (**Figure 3.20D**). **Table 3.1** also details the non-significant data for the concentration of soluble adhesion molecules in risk-stratified PCF and patient-matched sera.

Increased concentrations of soluble adhesion molecules in PCLs with a low-risk of PC progression may play a role in promoting cell migration to the pre-malignant lesion by reducing or altering cell adhesion to surrounding matrix components or other cells.

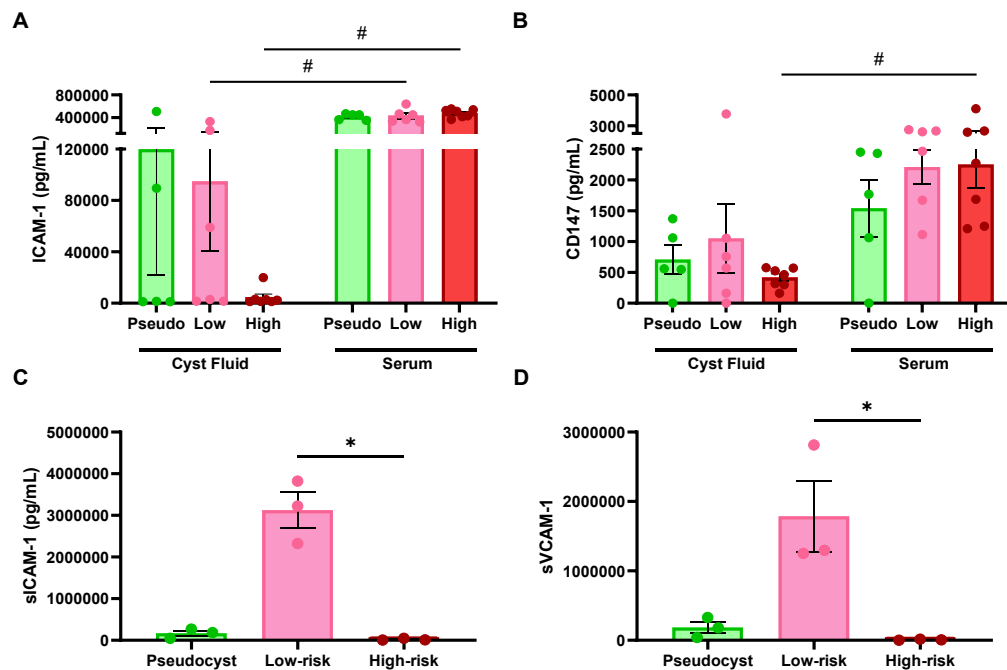


Figure 3.20: Soluble adhesion molecules are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=5), low-risk PCF (n=6) and high-risk PCF (n=7) and patient matched sera were screened for the expression of soluble adhesion molecules using a custom flow cytometry-based multiplex ELISA; ICAM-1 (**A**) and CD147 (**B**). Further screening was carried out in a cohort of pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) using an MSD multiplex ELISA; soluble ICAM-1 (**C**) and soluble VCAM-1 (**D**). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as * $p \leq 0.05$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum) [significance defined as # $p \leq 0.05$].

Table 3.1: Mean concentration of soluble adhesion molecules in PCF and patient-matched serum.

		PCF				Serum				PCF vs. Serum
ELISA Platform	Analyte	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	<i>p-value Non-parametric paired t-test</i>
<i>Adhesion molecules</i>										
Assay Genie	ICAM-1 (pg/mL)	119934	94773	4599	NS	415873	429686	476867	NS	0.0312 (Lv.L) 0.0156 (Hv.H)
	CD147 (pg/mL)	706.90	1051.00	416.20	NS	1541	2209	2252	NS	0.0156 (Hv.H)
MSD	ICAM-1 (pg/mL)	166744	3124227	20168	0.0219 (Lv.H)					
	VCAM-1 (pg/mL)	183244	1787014	7184	0.0219 (Lv.H)					

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. ICAM-1=intercellular adhesion molecule-1. VCAM-1=vascular cell adhesion molecule-1. NS=non-significant. Lv.H=low-risk vs. high-risk. Lv.L=low-risk vs. low-risk. Hv.H=high-risk vs. high-risk.

3.4.21 Soluble acute phase proteins are differentially expressed across risk-stratified PCF

The concentration of soluble acute phase proteins was also assessed in the multiplex ELISA screening of risk-stratified PCF and patient-matched sera. Acute phase proteins are key components of the innate immune response and function to regulate the expression of inflammatory mediators and to further activate the acute phase response to resolve inflammation or tissue damage.

The concentration of IL-6 was found to be significantly increased in serum from high-risk PCL patients compared to serum from low-risk PCL patients (753.80 ± 596.50 pg/mL vs. 23.08 ± 1.47 pg/mL, $p=0.0066$) (**Figure 3.21A**). Plasminogen activator inhibitor-1 (PAI-1), had a significantly lower concentration in high-risk PCF compared to benign pseudocyst fluid (1817.00 ± 1047.00 pg/mL vs. 23252.00 ± 5826.00 pg/mL, $p=0.0061$) (**Figure 3.21B**). There was also a significantly higher concentration of PAI-1 in high-risk serum compared to patient-matched high-risk PCF (12360.00 ± 2532.00 pg/mL vs. 1817.00 ± 1047.00 pg/mL, $p=0.0078$) (**Figure 3.21B**).

In an independent screening study using MSD 54-plex multiplex ELISA, serum amyloid A (SAA) exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (15257493 ± 7047613 pg/mL vs. 0.00 ± 0.00 pg/mL, $p=0.0190$) (**Figure 3.21D**). No significant differences were recorded for the concentration of CRP or IL-6 between risk-stratified PCF samples (**Figure 3.21C, E**). Non-significant data for the concentration of soluble acute phase proteins in risk-stratified PCF and patient-matched sera are detailed in **Table 3.2**.

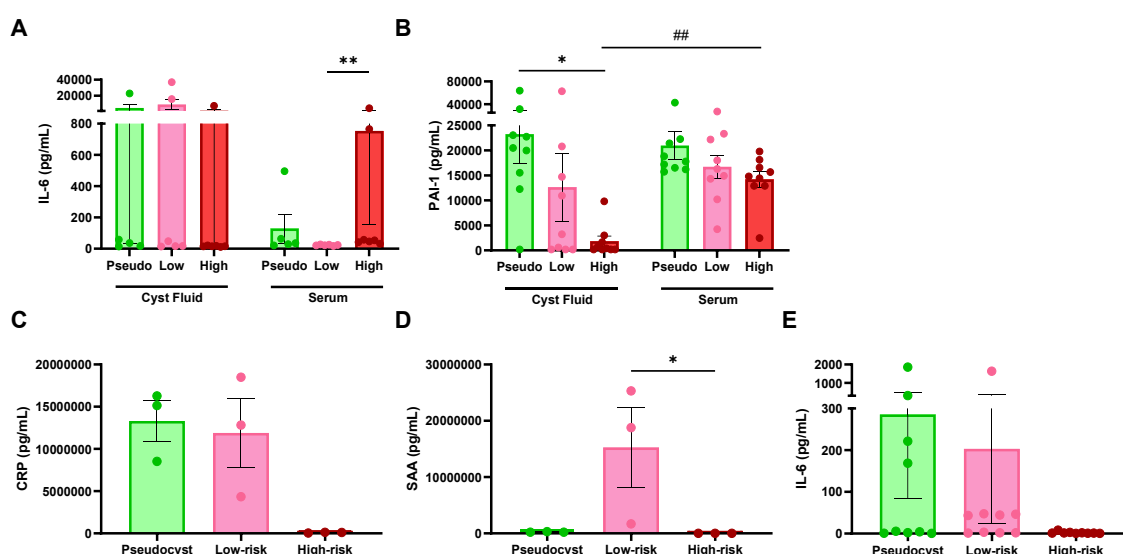


Figure 3.21: Soluble acute phase proteins are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=5), low-risk PCF (n=6) and high-risk PCF (n=7) and patient matched sera were screened for the expression of soluble acute phase proteins using a custom flow cytometry-based multiplex ELISA; IL-6 (A) and PAI-1 (B). Further screening was carried out in a cohort of pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) using an MSD multiplex ELISA (where additional samples were screened via MSD multiplex ELISA; Sample numbers: pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9)); CRP (C), SAA (D) and IL-6 (E). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as * $p \leq 0.05$, and ** $p \leq 0.01$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum) significance defined as ## $p \leq 0.01$].

Table 3.2: Mean concentration of soluble acute phase proteins in PCF and patient-matched serum.

		PCF				Serum				PCF vs. Serum
ELISA Platform	Analyte	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	<i>p-value Non-parametric paired t-test</i>
<i>Acute phase proteins</i>										
Assay Genie	IL-6 (pg/mL)	4572	8797	1060	NS	128.80	23.08	753.80	0.0066 (Lv.H)	NS
	PAI-1 (pg/mL)	23252	12623	1817	0.0061 (Pv.H)	20950	16722	14176	NS	0.0078 (Hv.H)
MSD	CRP (pg/mL)	13316426	11885251	94050	NS					
	SAA (pg/mL)	249177	15257493	0.00	0.0190 (Lv.H)					
	IL-6 (ng/mL)	285.60	203.10	2.09	NS					

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. IL-6=interleukin-6. PAI-1=plasminogen activator 1. CRP=C-reactive protein. SAA=serum amyloid A. NS=non-significant. Lv.H=low-risk vs. high-risk. Pv.H=pseudo vs. high-risk. Hv.H=high-risk vs. high-risk.

3.4.22 Soluble cell-mediated immune response factors are differentially expressed across risk-stratified PCF.

To elucidate how the soluble microenvironment of PCLs influences progression from pre-malignant lesions to PC, the MSD 54-plex multiplex ELISA was used to quantify the concentration of soluble cell-mediated immune response factors across a cohort of risk-stratified PCF samples.

This study found that the concentration of IL-21 was significantly higher in benign pseudocyst fluid compared to low-risk PCF (596.00 ± 448.30 pg/mL vs. 1.18 ± 1.12 pg/mL, $p=0.0498$) (**Figure 3.22G**). The concentration of IL-12/IL-23p40, a shared subunit between cytokines IL-12 and IL-23 involved in the promotion of Th1 and Th17 immune responses, was shown to be significantly increased in low-risk PCF compared to high-risk PCF (31.01 ± 9.46 pg/mL vs. 0.65 ± 0.57 pg/mL, $p=0.0265$) (**Figure 3.22K**).

Data from this analysis showed a trend towards a significantly higher concentration of IL-31 in benign pseudocyst fluid compared to low-risk PCF (6943.00 ± 1834.00 pg/mL vs. 264.70 ± 134.30 pg/mL, $p=0.0512$) (**Figure 3.22H**). Furthermore, there was also a trend towards a significant increase of IL-12p40 concentration in pre-malignant low-risk PCF compared to high-risk PCF (173.50 ± 100.20 pg/mL vs. 0.61 ± 0.16 pg/mL, $p=0.0688$) (**Figure 3.22I**).

However, although benign pseudocyst fluid demonstrated higher concentrations of a number of cell-mediated immune response factors, there were no significant differences in the concentration of IL-1 α , IL-1 β , IL-2, IFN- γ , TNF- α , TNF- β and IL-12p70 between risk-stratified PCF (**Figure 3.22A-F, J**). **Table 3.3** details the non-significant data for the concentration of soluble cell-mediated immune response factors in risk-stratified PCF and patient-matched sera.

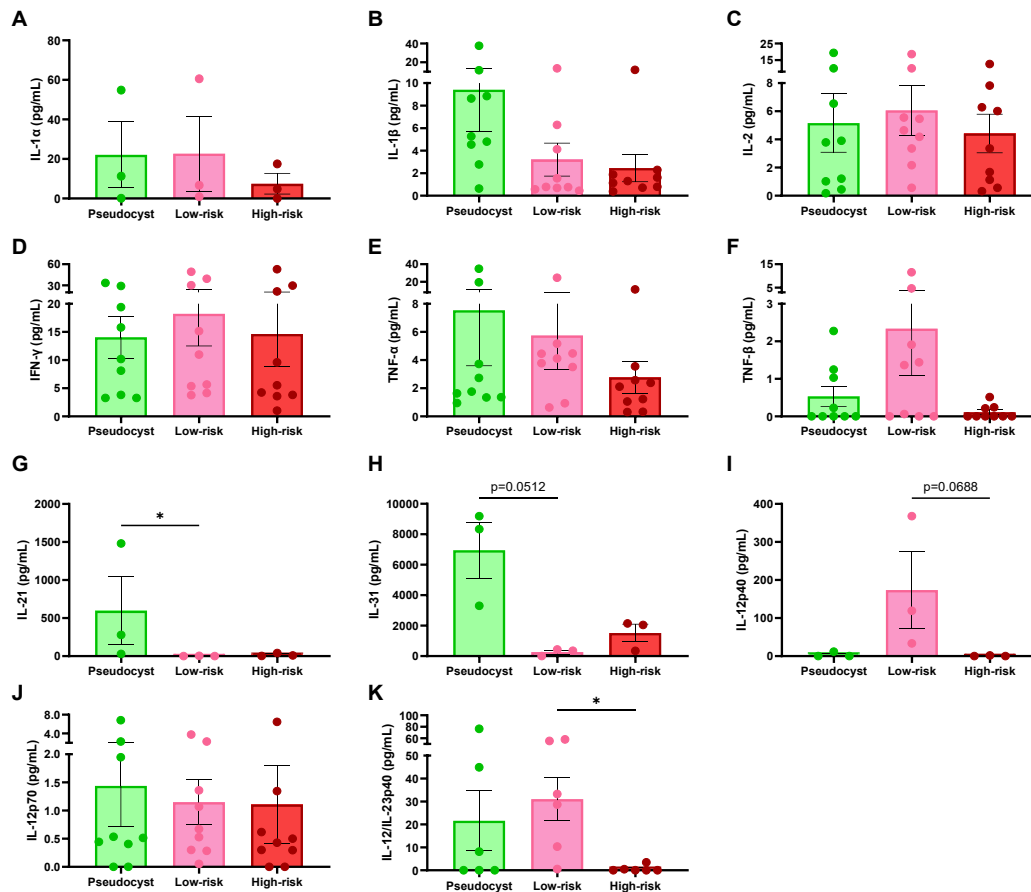


Figure 3.22: Soluble cell-mediated immune response factors are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) were screened for the expression of soluble cell-mediated immune response factors using an MSD multiplex ELISA (where additional samples were screened via MSD multiplex ELISA; Sample numbers: pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9)); IL-1α (A), IL-1β (B), IL-2 (C), IFN-γ (D), TNF-α (E), TNF-β (F), IL-21 (G), IL-31 (H), IL-12p40 (I), IL-12p70 (J), and IL-12/IL-23p40 (K). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF) [significance defined as $*p \leq 0.05$].

Table 3.3: Mean concentration of soluble cell-mediated immune factors in PCF and patient-matched serum.

		PCF			
ELISA Platform	Analyte	Pseudocyst	Low-risk	High-risk	<i>p-value</i> <i>Non-parametric one-way ANOVA</i>
<i>Cell-mediated immune response factors</i>					
MSD	IL-1 α (pg/mL)	22.09	22.72	7.45	NS
	IL-1 β (pg/mL)	9.41	3.20	2.45	NS
	IL-2 (pg/mL)	5.16	6.06	4.42	NS
	IFN- γ (pg/mL)	14.03	18.21	14.63	NS
	TNF- α (pg/mL)	7.54	5.75	2.77	NS
	TNF- β (pg/mL)	0.53	2.34	0.11	NS
	IL-21 (pg/mL)	596.00	1.18	16.99	0.0498 (Pv.L)
	IL-31 (pg/mL)	6943.00	264.70	1511.00	0.0512 (Pv.L)
	IL-12p40 (pg/mL)	4.03	173.50	0.61	0.0688 (Lv.H)
	IL-12p70 (pg/mL)	1.44	1.15	1.11	NS
	IL-12/IL-23p40 (pg/mL)	21.57	31.01	0.65	0.0265 (Lv.H)

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. IL=interleukin. IFN- γ =interferon gamma. TNF=tumour necrosis factor. NS=non-significant. Pv.L=pseudo vs. low-risk. Lv.H=low-risk vs. high-risk.

3.4.23 Soluble humoral immune factors are differentially expressed across risk-stratified PCF.

The concentration of soluble humoral immune factors was also quantified across risk-stratified PCF samples using the MSD 54-plex multiplex ELISA.

There were no significant differences in the concentration of humoral immune factors IL-1 receptor antagonist (IL-1Ra), IL-4, IL-5, IL-9, IL-10 and IL-13 between risk-stratified PCF (**Figure 3.23A-F**). However, this study revealed a trend towards a significant increase in the concentration of IL-27 in benign pseudocyst fluid compared to low-risk PCF (5239.00 ± 2267.00 pg/mL vs. 74.47 ± 38.12 pg/mL, $p=0.0610$) (**Figure 3.23G**).

Similarly, a non-significant trend was observed in this analysis showing that benign pseudocyst fluid had increased concentrations of humoral immune factors including IL-1Ra, IL-5, IL-10 and IL-13 compared to pre-malignant low-risk and high-risk PCF (**Figure 3.23A, C, E, F**). Non-significant data for the concentration of soluble humoral immune factors in risk-stratified PCF and patient-matched sera are detailed in **Table 3.4**.

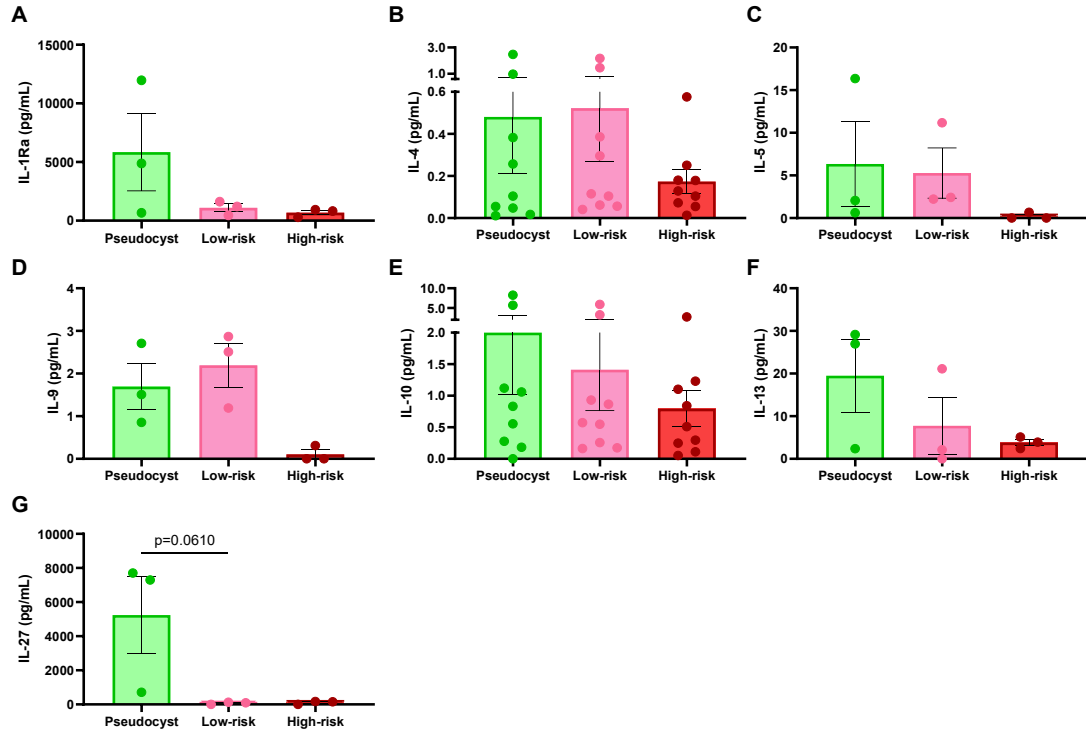


Figure 3.23: Soluble humoral immune factors are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) were screened for the expression of soluble humoral immune factors using an MSD multiplex ELISA (where additional samples were screened via MSD multiplex ELISA; Sample numbers: pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9)); IL-1Ra (A), IL-4 (B), IL-5 (C), IL-9 (D), IL-10 (E), IL-13 (F) and IL-27 (G). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF).

Table 3.4: Mean concentration of soluble humoral immune factors in PCF and patient-matched serum.

		PCF			
ELISA Platform	Analyte	Pseudocyst	Low-risk	High-risk	<i>p-value</i> <i>Non-parametric one-way ANOVA</i>
Humoral immune factors					
MSD	IL-1Ra (pg/mL)	5842.00	1096.00	684.70	NS
	IL-4 (pg/mL)	0.48	0.52	0.17	NS
	IL-5 (pg/mL)	6.35	5.27	0.22	NS
	IL-9 (pg/mL)	1.69	2.19	0.10	NS
	IL-10 (pg/mL)	2.00	1.41	0.79	NS
	IL-13 (pg/mL)	19.48	7.74	3.82	NS
	IL-27 (pg/mL)	5239.00	74.47	108.80	0.0610 (Pv.L)

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. IL=interleukin. NS=non-significant. Pv.L=pseudo vs. low-risk.

3.4.24 Soluble Th17-associated immune factors are differentially expressed across risk-stratified PCF.

The concentration of soluble Th17-associated immune factors was quantified across risk-stratified PCF samples. The Th17-associated immune response is particularly important in mounting a defence against extracellular pathogens at mucosal barriers, but also for recruiting and activating neutrophils to sites of inflammation.

This investigation found that the concentration of IL-22, a cytokine produced by Th17 cells and often associated with tissue repair, was significantly decreased in low-risk PCF compared to benign pseudocyst fluid (10.64 ± 5.88 pg/mL vs. 1345.00 ± 372.50 pg/mL, $p=0.0338$) (**Figure 3.24F**). Although not significant, this effect was also recorded for the concentration of IL-23 in risk-stratified PCF (**Figure 3.24G**). Additionally, this screening analysis also showed a non-significant increase in IL-17A and IL-17D concentration in low-risk PCF compared to high-risk PCF and benign pseudocyst fluid (**Figure 3.24A, E**). Moreover, there was a trend towards a significant increase in the concentration of IL-17C in benign pseudocyst fluid compared to high-risk PCF (9.31 ± 0.94 pg/mL vs. 3.17 ± 1.37 pg/mL, $p=0.0512$) (**Figure 3.24D**). A non-significant stepwise decrease in the concentration of Th17-associated immune factors such as IL-17A/F and IL-17B was observed across PCF samples in accordance with increased risk of PC progression (**Figure 3.24B, C**). **Table 3.5** details the non-significant data for the concentration of soluble Th17-associated immune factors in risk-stratified PCF and patient-matched sera.

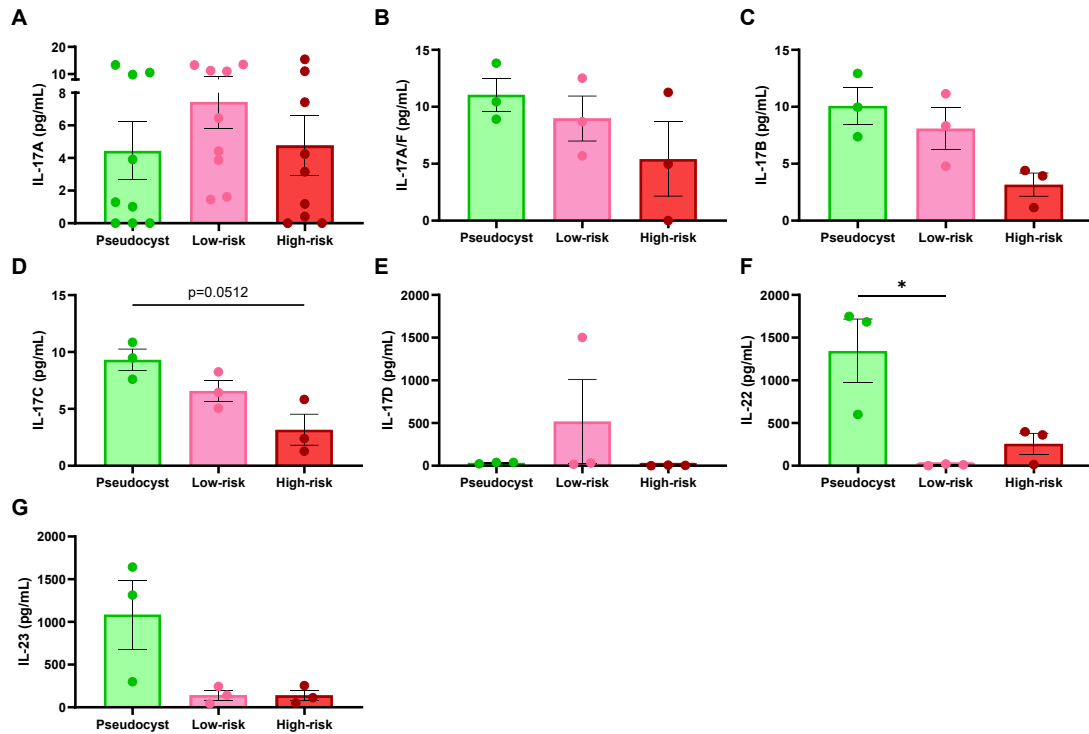


Figure 3.24: Soluble Th17-associated immune factors are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) were screened for the expression of soluble Th17-associated immune factors using an MSD multiplex ELISA (where additional samples were screened via MSD multiplex ELISA; Sample numbers: pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9)); IL-17A (A), IL-17A/F (B), IL-17B (C), IL-17C (D), IL-17D (E), IL-22 (F), and IL-23 (G). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF) [significance defined as * $p \leq 0.05$].

Table 3.5: Mean concentration of soluble Th17-associated immune factors in PCF and patient-matched serum.

		PCF			
ELISA Platform	Analyte	Pseudocyst	Low-risk	High-risk	<i>p-value</i> <i>Non-parametric one-way ANOVA</i>
<i>Th17-associated immune factors</i>					
MSD	IL-17A (pg/mL)	4.44	7.44	4.77	NS
	IL-17A/F (pg/mL)	11.06	8.97	5.41	NS
	IL-17B (pg/mL)	10.09	8.08	3.16	NS
	IL-17C (pg/mL)	9.31	6.59	3.17	0.0512 (Pv.H)
	IL-17D (pg/mL)	33.64	517.20	4.17	NS
	IL-22 (pg/mL)	1354.00	10.64	257.40	0.0338 (Pv.L)
	IL-23 (pg/mL)	1085.00	142.50	139.00	NS

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. IL=interleukin. NS=non-significant. Pv.H=pseudo vs. high-risk. Pv.L=pseudo vs. low-risk.

3.4.25 Soluble lymphocyte development and differentiation factors are differentially expressed across risk-stratified PCF.

The development and activation of lymphocytes is particularly important for establishing effective adaptive immune responses, including the recognition and elimination of pathogens, the generation of immunological memory, and the maintenance of long-term protective immunity.

When screening risk-stratified PCF to identify and quantify the concentration of soluble lymphocyte development and differentiation factors, soluble IL-15, a pro-inflammatory cytokine involved in the development, activation and proliferation of NK cells and T cells, was found to be significantly increased in low-risk PCF compared to high-risk PCF (11.57 ± 0.510 pg/mL vs. 0.69 ± 0.17 pg/mL, $p=0.0328$) (**Figure 3.25C**). Indeed, a trend towards a significant increase in the concentration of IL-7 was recorded in low-risk PCF compared to benign pseudocyst fluid (23.44 ± 19.26 pg/mL vs. 0.21 ± 0.21 pg/mL, $p=0.0688$) (**Figure 3.25B**). Although not significant, a stepwise decrease in IL-3 concentration was observed across PCF samples in accordance with increased risk of PC progression (**Figure 3.25A**). Non-significant data for the concentration of soluble lymphocyte development and differentiation factors in risk-stratified PCF and patient-matched sera are detailed in **Table 3.6**.

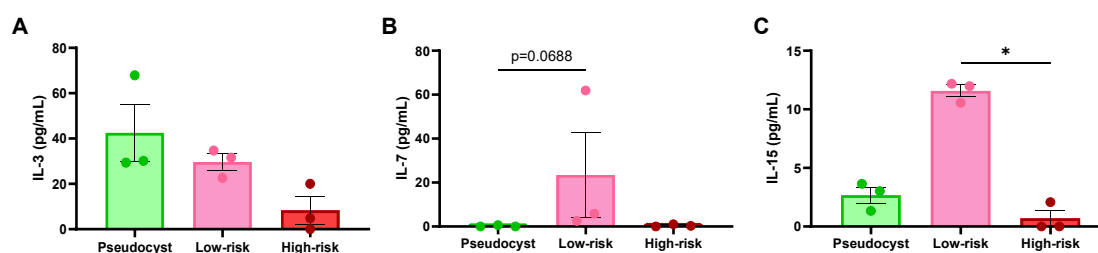


Figure 3.25: Soluble lymphocyte development and differentiation factors are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) were screened for the expression of soluble lymphocyte development and differentiation factors using an MSD multiplex ELISA; IL-3 (A), IL-7 (B) and IL-15 (C). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF) [significance defined as $*p \leq 0.05$].

Table 3.6: Mean concentration of soluble lymphocyte development and differentiation factors in PCF and patient-matched serum.

patient-matched serum.					
		PCF			
ELISA Platform	Analyte	Pseudocyst	Low-risk	High-risk	<i>p-value</i> <i>Non-parametric one-way ANOVA</i>
<i>Lymphocyte development and differentiation factors</i>					
MSD	IL-3 (pg/mL)	42.49	29.61	8.29	<i>NS</i>
	IL-7 (pg/mL)	0.21	23.44	0.43	<i>0.0688</i> <i>(Pv.L)</i>
	IL-15 (pg/mL)	2.67	11.57	0.69	<i>0.0328</i> <i>(Lv.H)</i>

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. IL=interleukin. NS=non-significant. Pv.L=pseudo vs. low-risk. Lv.H=low-risk vs. high-risk.

3.4.26 Soluble immune checkpoint molecules are differentially expressed across risk-stratified PCF.

Immune checkpoint molecules are regulatory proteins, primarily expressed on T cells, that act to prevent excessive immune activation and maintain immune homeostasis. Using a 54-plex MSD multiplex ELISA, a small cohort of risk-stratified PCF was screened to investigate the expression of soluble immune checkpoint molecules.

The immune checkpoint ligand for PD-1, PD-L1 epitope 2, exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (51.19 ± 34.96 pg/mL vs. 0.49 ± 0.23 pg/mL, $p=0.0461$) (**Figure 3.26D**). PD-L2, another ligand for the immune checkpoint receptor PD-1, was also shown to have a significantly increased concentration in low-risk PCF compared to high-risk PCF (746.80 ± 324.60 pg/mL vs. 9.78 ± 4.97 pg/mL, $p=0.0467$) (**Figure 3.26E**). Moreover, this study also observed a trend towards a significant increase in the concentration of PD-1 epitope 1 in pre-malignant low-risk PCF compared to high-risk PCF (61.31 ± 31.23 pg/mL vs. 2.48 ± 1.06 pg/mL, $p=0.0602$) (**Figure 3.26A**). Similarly, there was also a trend towards a significant increase in the concentration of PD-L1 epitope 1 in low-risk PCF compared to high-risk PCF (40.44 ± 24.08 pg/mL vs. 0.49 ± 0.21 pg/mL, $p=0.0519$) (**Figure 3.26C**). Although not significant, this effect was also recorded for the concentration of PD-1 epitope 2 and CD40L (**Figure 3.26B, F**). **Table 3.7** details the non-significant data for the concentration of soluble immune checkpoint molecules in risk-stratified PCF and patient-matched sera.

Increased concentrations of soluble immune checkpoint molecules in PCLs with a low-risk of PC progression may suggest that these lesions have a more active local

immunoregulatory environment which may be involved in preventing the progression of PCLs to PC.

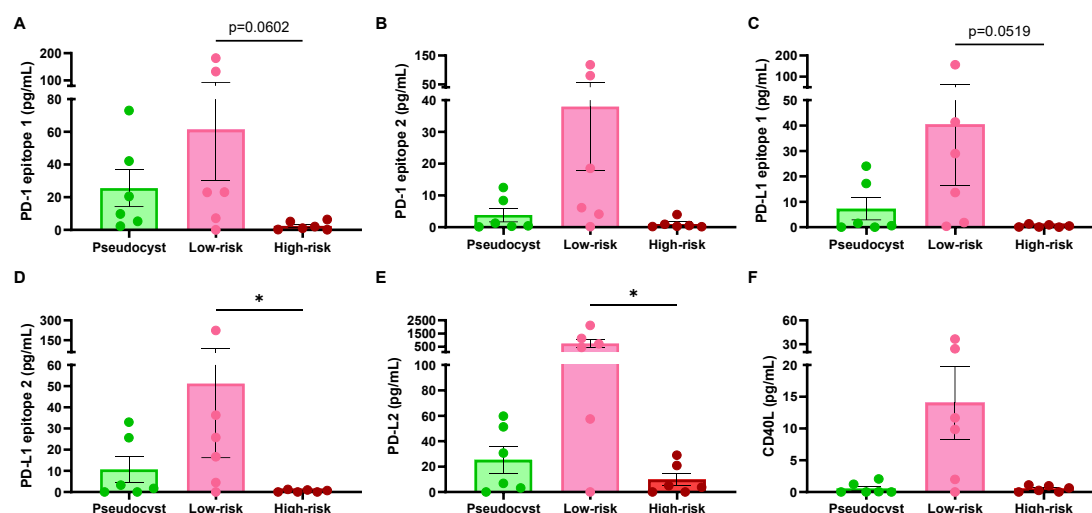


Figure 3.26: Soluble immune checkpoint molecules are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9) were screened for the expression of soluble immune checkpoint molecules using an MSD multiplex ELISA; PD-1 epitope 1 (A), PD-1 epitope 2 (B), PD-L1 epitope 1 (C), PD-L1 epitope 2 (D), PD-L2 (E) and CD40L (F). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF) [significance defined as * $p \leq 0.05$].

Table 3.7: Mean concentration of soluble immune checkpoint molecules in PCF and patient-matched serum.

		PCF			
ELISA Platform	Analyte	Pseudocyst	Low-risk	High-risk	p-value Non-parametric one-way ANOVA
Immune checkpoint molecules					
MSD	PD-1 epitope 1 (pg/mL)	25.50	61.31	2.48	0.0602 (Lv.H)
	PD-1 epitope 2 (pg/mL)	3.85	37.95	1.02	NS
	PD-L1 epitope 1 (pg/mL)	7.24	40.44	0.49	0.0519 (Lv.H)
	PD-L1 epitope 2 (pg/mL)	10.62	51.19	0.49	0.0461 (Lv.H)
	PD-L2 (pg/mL)	25.30	746.80	9.78	0.0467 (Lv.H)
	CD40L (pg/mL)	0.56	14.04	0.50	NS

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. PD-1=programmed cell death 1 receptor. PD-L1=programmed cell death ligand 1. PD-L2=programmed cell death ligand 2. NS=non-significant. Lv.H=low-risk vs. high-risk.

3.5 Discussion

Within this study, the local and systemic cellular immune microenvironment of pre-malignant PCLs was characterised to better define the immune cell populations that may influence malignant progression. By immunophenotyping pre-malignant PCF and patient-matched PBMCs, this analysis aimed to identify immune-related features that could contribute to the identification of immunobiological biomarkers for patients at a high risk of malignant progression. Additionally, this analysis provides important insights into the immune contexture within the dysregulated pancreas.

The findings from this thesis demonstrate that PCF contains an increased frequency of adaptive immune cell populations, including CD4⁺ T cells, CD8⁺ T cells and NK cells. These results are consistent with recent studies employing single-cell RNA sequencing of IPMNs, which revealed that low-grade IPMNs are infiltrated by cytotoxic CD8⁺ T cells, activated CD4⁺ T helper cells and dendritic cells, whereas higher-grade dysplastic lesions become dominated by immune subsets with immunosuppressive phenotypes^[197]. Similarly, Eckhoff *et al.* used multidimensional immunophenotyping to identify that the infiltrate of IPMN lesions was primarily composed of T cell and macrophage populations^[238]. Furthermore, previous investigations in PC have reported that CD8⁺ T cells tend to accumulate in peritumoral regions, while Treg cells preferentially infiltrate intraepithelial areas of neoplastic tissue during progression from pre-malignant to malignant disease^[43, 239-241]. Comparable patterns were observed in the cohort of PCL samples used in this project, where alterations in the frequency of T cell subsets were detected in both PCF and matched PBMCs from patients with pre-malignant PCLs. These findings may reflect either enhanced immune surveillance or, conversely, dysregulated T cell activity during early pancreatic tumourigenesis. Notably, CD4⁺ T cells represented the predominant immune population within pre-malignant PCF, highlighting the need for further in-depth characterisation of the CD4⁺ T cell compartment, both locally and systemically. Such analyses could elucidate the contribution of CD4⁺ T cell subsets to immune regulation in pre-malignant disease.

Indeed, tumour immune surveillance is critically shaped by helper T cell polarisation, as T helper cell subsets often orchestrate the activity of other immune cells^[242-245]. In PC, an imbalance between Th1-polarised and Th2-polarised CD4⁺ T cells has been described, with reduced intratumoral Th1 cells relative to Th2 cells, correlating with poorer survival

outcomes^[246]. Investigating whether similar shifts in Th subset composition occur in pre-malignant PCLs could therefore provide valuable insight into early immune dysregulation and its potential role in driving malignant transformation.

This immunophenotypic analysis further revealed that both pre-malignant PCF and patient-matched PBMCs exhibited a high frequency of CD8⁺ T cells. CD8⁺ T cells are central mediators of anti-tumour immunity, exerting cytotoxic effects through CD107a-associated degranulation and the release of effector molecules such as perforin, granzymes and pro-inflammatory cytokines^[247, 248]. These mechanisms are central to the elimination of transformed cells and under normal circumstances, act as a barrier to malignant progression^[249]. Data generated from this analysis to elucidate the cytotoxic capacity of effector immune cells showed that the frequency of intracellular perforin in CD8⁺ T cells was significantly higher than that found in circulating PBMCs suggesting its increased capacity to carry out pore-forming functions and mediating granzyme delivery into target cells. However, the frequency of granzyme B, a serine protease that activates apoptosis through caspase activation, was reduced in pre-malignant PCF, which may indicate that these CD8⁺ T cells and NK cells within pre-malignant PCF may not effectively be able to kill target cells through granzyme B-mediated caspase activation. In a study by Hernandez *et al.*, multiplex immunofluorescence of IPMN lesions showed higher densities of granzyme B-positive cytotoxic T cells in low-grade lesions compared to high-grade lesions^[250]. Although this finding contrasts with data generated from this study, it is worth highlighting that Hernandez *et al.* focused their analysis on the epithelial compartment of IPMN lesions whereas this study focuses on the immune cell infiltrate of pre-malignant PCF^[250]. Alternatively, other granzymes, such as granzyme A which mediates caspase-independent cell death, may be playing a role in the effector immune cell infiltrate of pre-malignant PCF and would be a worthwhile avenue to investigate^[251].

In a study conducted by Enzler *et al.*, multiplex immunohistochemical staining was used to characterise the cellular immune component of surgically resected tissue from PCLs and PC^[46]. This study also found a high CD8⁺ T cell infiltration in pre-malignant lesions^[46]. Importantly, while Enzler *et al.* assessed immune cells within tissue samples, the analysis performed in this thesis uniquely focuses on the immune cell compartment within pre-malignant PCF, which directly interacts with the epithelial lining of the lesion where malignant transformation occurs. This distinction highlights the relevance of the

data from this study, as the immune profile of PCF may provide a more dynamic representation of the local immune microenvironment and the interactions between PCF-associated immune cell populations and neoplastic cells in the epithelium at the earliest stages of malignant progression.

Furthermore, when the functional state of T cells was examined, data from this analysis revealed that CD8⁺ T cells found in pre-malignant PCF exhibited a pronounced exhausted phenotype. This was characterised by the upregulated accumulation and co-expression of inhibitory immune checkpoint receptors, including PD-1, TIM-3, and LAG-3. This finding was also consistent based on the analysis conducted within this study, in line with the hierarchical developmental pathway for T cell exhaustion defined by Beltra *et al.*^[230], whereby there was a higher frequency of terminally exhausted resident CD8⁺ T cells within PCF and patient-matched PBMCs. Such terminally exhausted CD8⁺ T cells have a limited capacity to mount effective cytotoxic responses, suggesting that their presence within pre-malignant PCLs may contribute to the establishment of an immunosuppressive microenvironment. This, in turn, may facilitate immune evasion and support the progression of neoplastic lesions towards malignancy.

Immune checkpoints are inhibitory receptors expressed on the surface of T cells and other immune subsets that regulate immune activation and prevent excessive or autoreactive immune responses^[252-254]. While these mechanisms are essential for maintaining peripheral tolerance and limiting tissue damage, malignant and pre-malignant cells can exploit them to evade immune-mediated cell death^[23, 220, 255]. Engagement of immune checkpoint receptors effectively acts as a brake on anti-tumour activity, which then subsequently creates a permissive immune microenvironment for malignant transformation and progression^[23, 220, 255, 256].

In this study, the phenotypic analysis of cells isolated from pre-malignant PCF and matched PBMCs demonstrated a significant upregulation of immune checkpoint receptors PD-1 and LAG-3 on CD8⁺ T cells. Furthermore, this finding was also found in intermediary hTERT-HPNE pancreatic cells which also exhibited high expression of these inhibitory molecules. Additionally, PD-L2, the immune checkpoint ligand of PD-1, was observed to be upregulated in these neoplastic epithelial cells suggesting that the PD-1 pathway may represent a key mechanism of immune suppression within the pre-malignant PCL microenvironment. These findings are in line with multiplex and spatial

analysis studies that report an increased expression of PD-1 on T cells in IPMN tissue and other precursor lesions, while PD-L1 expression is often low or heterogeneous on neoplastic epithelial cells^[46, 250, 257]. These findings suggest that engagement of the PD-1/PD-L2 immune checkpoint pathway occurs early in pancreatic tumourigenesis and may facilitate the establishment of a more immune tolerant microenvironment.

From a translational perspective, these findings highlight the potential therapeutic relevance of immune checkpoint inhibitors in high-risk pancreatic precursor lesions. Targeting PD-1, for example with pembrolizumab, may restore effector immune function in exhausted CD8⁺ T cells and enhance cytotoxic activity against dysplastic epithelial cells which may prevent malignant progression. However, it is important to recognise that terminally exhausted CD8⁺ T cells, characterised by sustained checkpoint expression, may not fully recover effector immune function upon PD-1 blockade. This raises the possibility that intervention at an earlier stage of exhaustion or through combination immune checkpoint inhibition, may be required to achieve robust immune reinvigoration in pre-malignant PCLs.

The cytokine and immune mediator profile detected in the soluble compartment of low-risk PCF suggests a microenvironment that is immunologically active yet already exhibiting early regulatory features. Compared with pseudocysts and high-risk lesions, low-risk PCF showed elevated levels of adhesion molecules (ICAM-1, VCAM-1, CD147), acute-phase and inflammatory mediators (SAA, IL-6), and a number of effector immune cell-associated cytokines (IFN- γ , IL-2, IL-12p40, IL-15). This profile suggests that there may be ongoing immune cell recruitment and activation in low-risk PCLs, consistent with prior studies of IPMNs that demonstrate increased immune infiltration and Th1-mediated cytotoxic signalling in early-stage lesions^[111, 197, 250]. The presence of adhesion molecules such as ICAM-1 and VCAM-1 may indicate that endothelial activation accompanies leukocyte trafficking into pre-malignant lesions, while enrichment of IL-6 and SAA may reflect a localised inflammatory response and stromal remodelling^[258-262].

Notably, this immune activation signature co-exists with elevated levels of regulatory and checkpoint molecules, including PD-1, PD-L1, PD-L2, and CD40L. Soluble checkpoint molecules can be proteolytically released from immune cells or epithelial cells, and they may indicate an early engagement of inhibitory pathways that dampen cytotoxic

activity^[263]. Findings from this study suggest that immune surveillance within low-risk PCLs is counterbalanced by inhibitory checkpoint pathways which typically function to limit tissue damage, but which may facilitate immune evasion in pre-malignant lesions^[257, 264]. The detection of IL-9, IL-17 and TNF- β in pre-malignant PCF further supports a complex cytokine milieu incorporating Th9 and Th17-type responses, which have been implicated in inflammatory regulation and epithelial dysplasia^[265-267]. Similar mixed pro-inflammatory and immunoregulatory profiles have been observed in immunohistochemical analyses of IPMNs, in which Th1, Th2, Th9, Th17 and Th22 T cell subsets were found to infiltrate surgically resected tissue from low-grade lesions but decline with progression to high-grade dysplasia or invasive PC^[111].

Collectively, these findings support the concept that low-risk PCLs are represented by an immune active local microenvironment but regulated intermediate state, characterised by concurrent upregulation of pro-inflammatory cytokines and regulatory immune checkpoint signalling pathways. This dynamic is consistent with the broader literature describing the immunologic remodelling that accompanies pancreatic neoplastic progression from inflammation and immune surveillance in precursor cysts to the immunosuppressive microenvironment typical of invasive PC^[46, 111, 250].

In conclusion, experiments conducted in this chapter sought to immunophenotype the immune cell infiltrate of pre-malignant PCF and patient-matched PBMCs. Data generated for the purpose of this study highlights how adaptive immune cell populations may play a role in early pancreatic tumourigenesis. While increased frequencies of CD4⁺ and CD8⁺ T cells were identified within PCF, functional analysis revealed that a substantial proportion of these CD8⁺ T cells adopt a terminally exhausted resident phenotype and upregulate the expression of inhibitory immune checkpoint receptors PD-1 and LAG-3. This study, therefore, provides an in-depth characterisation of the local and systemic immune microenvironment of pre-malignant PCLs.

3.6 Study limitations

This study provides novel insights into both the cellular and soluble immune microenvironment of PCLs. Notably, it demonstrates that a measurable and characterisable cellular immune component is present within benign pseudocysts and pre-malignant PCLs, supporting the feasibility of immune profiling of PCF.

However, the limitations of any study should be acknowledged. The principal limitation of this study relates to the relatively small sample size available for immunophenotyping analyses, particularly for high-risk PCF and benign pseudocyst fluid samples. This restricted cohort limited statistical power and prevented robust comparisons across clinically relevant PCL cancer risk stratification groups. Consequently, meaningful evaluation of differences in immune composition between benign pseudocyst fluid, low-risk PCLs and high-risk PCLs could not be performed.

In addition, the immunophenotyping panel used within this study focused primarily on broad immune cell populations. Future studies could expand upon this by incorporating more detailed characterisation of immunoregulatory subsets, such as Tregs, as well as assessing activation status, exhaustion markers, and functional phenotypes of both innate and adaptive immune populations. These additional analyses would provide greater mechanistic insight into the immunological landscape associated with cyst progression and malignant transformation.

3.7 Graphical summary

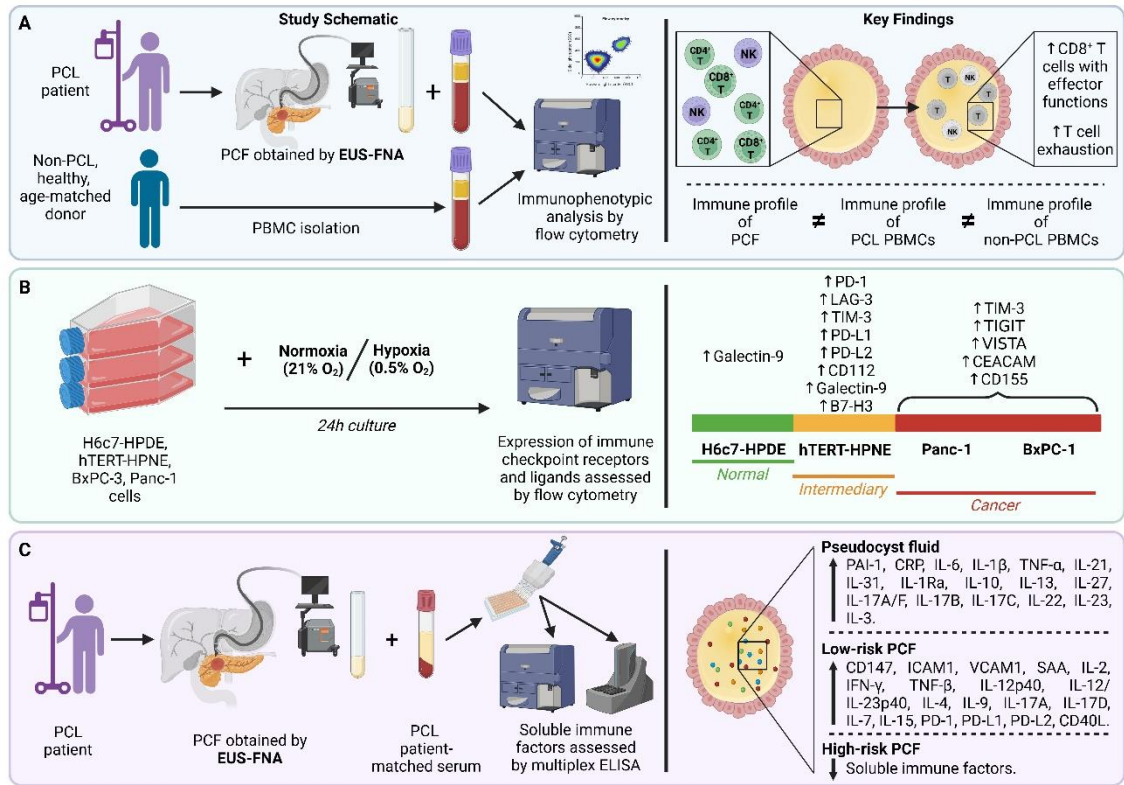


Figure 3.27: Graphical summary of study findings from results chapter 3. (A) PCF and patient-matched PBMCs were isolated from PCL patients. Immunophenotyping by flow cytometry was carried out to characterise the immune cell populations within the cellular infiltrate of pancreatic lesions. This analysis was also performed on non-PCL, age-matched healthy donor PBMCs. The immune landscape of PCF was highly dominated by CD4⁺ T cells, CD8⁺ T cells and NK cells. Further analysis showed that T cells within PCF were mostly effector memory subsets and exhibited increased (↑) levels of T cell exhaustion. (B) Pancreatic cell lines were cultured under normal (21% O₂) and severe hypoxic stress (0.5% O₂) conditions. Immune checkpoint expression was then assessed by flow cytometry. Intermediary pancreatic cells (hTERT-HPNE) were found to have higher (↑) levels of several immune checkpoint receptors and ligands. (C) The concentration of soluble immune factors in PCF and patient-matched serum was assessed by multiplex ELISA. Pseudocyst fluid, low-risk PCF and high-risk PCF were characterised by distinct soluble immune microenvironments.

Chapter 4:

**The impact of pancreatic cyst fluid on T cell
function and phenotype: implications for anti-
tumour immunity**

4.1 Introduction

It is well understood that the immunosuppressive desmoplastic stromal microenvironment of PC has profound effects on tumour progression and response to treatments^[29, 268]. The dense fibrotic stroma of PC, known as desmoplasia, is composed of cancer-associated fibroblasts (CAFs), ECM proteins and infiltrating immune cells^[269]. This stroma functions not only as a physical barrier that restricts drug perfusion and immune infiltration, but also as a dynamic immunoregulatory compartment that actively promotes tumour immune evasion^[36].

The immunosuppressive desmoplastic microenvironment is driven and maintained by both stromal and immune cell populations, including Tregs, MDSCs, and anti-inflammatory M2 macrophages^[40, 41]. These immune subsets are enriched in PC and are recruited and sustained by cytokines such as IL-10, TGF- β , and CXCL12^[40]. Tregs, marked by high expression of CD25 and the transcription factor FoxP3, suppress effector T cell activation through IL-10 secretion and cell-contact dependent mechanisms, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) engagement^[270]. MDSCs, a heterogeneous population of immature myeloid cells, inhibit T cell function by depleting amino acids critical for T cell metabolism, such as L-arginine, and through production of reactive oxygen and nitrogen species^[200]. M2 macrophages further perpetuate immune suppression by releasing IL-10 and TGF- β and by polarising naïve T cells away from effector phenotypes^[201, 271]. Collectively, these immunosuppressive mechanisms block the anti-tumour activity of CD8⁺ cytotoxic T cells, effector CD4⁺ T helper cells and NK cells^[41].

T cells are the primary mediators of adaptive anti-tumour immunity, and their presence in many solid tumours correlates with improved prognosis and response to therapy. In PC, however, the T cell infiltrate is characteristically sparse, and when present, often exhibits features of dysfunction or suppression^[42, 272]. CD8⁺ T cells are often excluded from tumour epithelial regions and trapped within peritumoral stroma^[239]. CD8⁺ T cells in pancreatic tumours frequently display an exhausted phenotype, with high expression of inhibitory receptors, including CTLA-4, PD-1, LAG-3 and TIM-3, and reduced effector cytokine production^[227, 228].

Effector CD4⁺ T cells, particularly Th1 cells producing IFN- γ , are also dysfunctional in PC, while immunosuppressive subsets such as Tregs and Th2 cells are enriched^[246, 273]. A high prevalence of CD4⁺CD25⁺FoxP3⁺ Treg cells within the TME has been associated with poorer survival, underscoring the importance of T cell balance in disease progression^[42, 274]. A number of studies examining clinical samples of PC precursor lesions have also shown that the accumulation of Treg cells and diminished cytotoxic CD8⁺ T cell infiltration correlates with the malignant progression of pre-neoplastic lesions^[43-45]. The data from these immune phenotyping studies suggest that the balance between T cell subsets is often disrupted early, potentially even before the development of pancreatic malignancy.

Recent evidence supports the idea that immune suppression in PC begins at the pre-invasive stage^[111]. A study carried out by Roth *et al.* assessed resected tissue samples from IPMNs, a common precursor lesion to PC, using immunohistochemical analysis to examine the spatial distribution of immune cell infiltrates during IPMN progression to PC^[111]. Findings from this analysis established that the immune microenvironment evolved from a diverse T cell arena, which is comprised of CD8⁺ T cells, Th/c1, Th/c2, Th/c17, Th22 and Treg cells in low-grade IPMNs to a primarily Treg-dominated immunosuppressive state during the progression of IPMNs and invasive PC^[111]. Notably, immune profiling of resected human IPMN lesions has demonstrated that low-grade dysplastic lesions are associated with a pro-inflammatory immune cell infiltrate, dominated by CD4⁺ and CD8⁺ effector T cells, while high-grade dysplastic lesions show a significant enrichment of immunosuppressive Tregs and M2 macrophages, similar to invasive PC^[39]. This phenotypic switch suggests that immune evasion is an early and progressive event in PC development.

PCF, derived from pre-malignant PCLs, harbours a diverse array of soluble mediators, cellular debris, and immune-related factors^[104, 194, 275]. It provides a unique opportunity to investigate the evolution of immune suppression in pre-malignant PCLs, and higher levels of certain inflammatory mediators have been associated with high-grade dysplasia^[104]. However, little is known about the direct effect of pre-malignant PCF on effector immune cell function.

Several studies have shown that soluble factors from cancer-associated fluid, as in the case of malignant ascites for ovarian cancer and pleural effusions from lung cancer, can suppress T cell activation, reduce cytokine production and skew differentiation towards regulatory phenotypes^[276, 277]. Malignant ascites and pleural effusions have been shown to impair antigen-specific T cell responses, suggesting that local soluble microenvironments are key modulators of immunity^[278-280]. Whether similar immunosuppressive properties exist in pre-malignant PCF is unknown but may have important implications for immune evasion and the subsequent development of PC.

In this chapter, the impact of soluble factors within risk-stratified PCF on T cell function and phenotype is assessed. Using co-culture assays to expose healthy, activated T cells and NK cells to benign pseudocyst fluid and pre-malignant PCF, the capacity of PCF to suppress CD8⁺ and CD4⁺ T cell responses was evaluated. Changes in T cell activation markers, exhaustion markers and markers of effector immune cell cytotoxicity were also assessed. By comparing the impact of risk-stratified PCF on T cells and NK cells, this study aimed to identify whether high-risk PCLs are associated with a poorer capacity to illicit an anti-tumour immune response. This study provides important information on the ability of pre-malignant PCF to impair an adaptive immune response and evade killing from effector immune cells. It also sheds light on the immune landscape of PCLs and may inform future strategies for early immune-based interventions in PC prevention.

4.2 Hypothesis

Risk-stratified PCF differentially impacts T cell function and phenotype, potentially contributing to mechanisms of immune suppression and tumourigenesis in some patients.

4.3 Specific Aims

1. Elucidate whether exposure to risk-stratified PCF alters the activation of T cells derived from healthy PBMC donors.
2. Examine whether exposure to benign pseudocyst fluid or pre-malignant PCF specifically induces exhaustion in T cells derived from healthy PBMC donors.
3. Investigate whether exposure to risk-stratified PCF induces a phenotypic switch towards a pro-tumour phenotype in healthy T cells.

4.4 Results

4.4.1 Benign pancreatic pseudocyst fluid significantly decreases lymphocyte viability.

Immune cell viability is critical for effective anti-tumour immune responses. In this study, T cells and NK cells were isolated from healthy donor PBMCs, activated for 24 hours and co-cultured with 20% (v/v) risk-stratified PCF for 24 hours. Viability was then assessed by flow cytometry using Zombie viability staining. Zombie viability stains are amine-reactive fluorescent dyes that are permeant to cells with compromised membranes but impermeable to live cells. Findings from this study provide further insight into how risk-stratified PCF differentially influences the survival of effector immune cells.

This study found that there was a significant decrease in the frequency of viable NK cells that were exposed for 24 hours to benign pseudocyst fluid compared to NK cells co-cultured with pre-malignant PCF (Mean frequency (%) $\pm$ SEM; $47.34 \pm 12.29\%$ vs. $91.22 \pm 1.08\%$, $p=0.0022$) (**Figure 4.1C**). Although not significant, this effect was also recorded for T cells where benign pseudocyst fluid induced a decreased frequency of viable $CD4^+$ T cells and $CD8^+$ T cells compared to cells co-cultured with the non-treated cRPMI control or pre-malignant PCF (**Figure 4.1A, B**).

Overall, this data suggests that factors within benign pseudocyst fluid, such as digestive enzymes, may play a role in influencing the local immune microenvironment, potentially through alterations in pH, thereby impairing T cell and NK cell viability. Importantly, exposure to pre-malignant PCF does not adversely impact the viability of these effector immune cell populations.

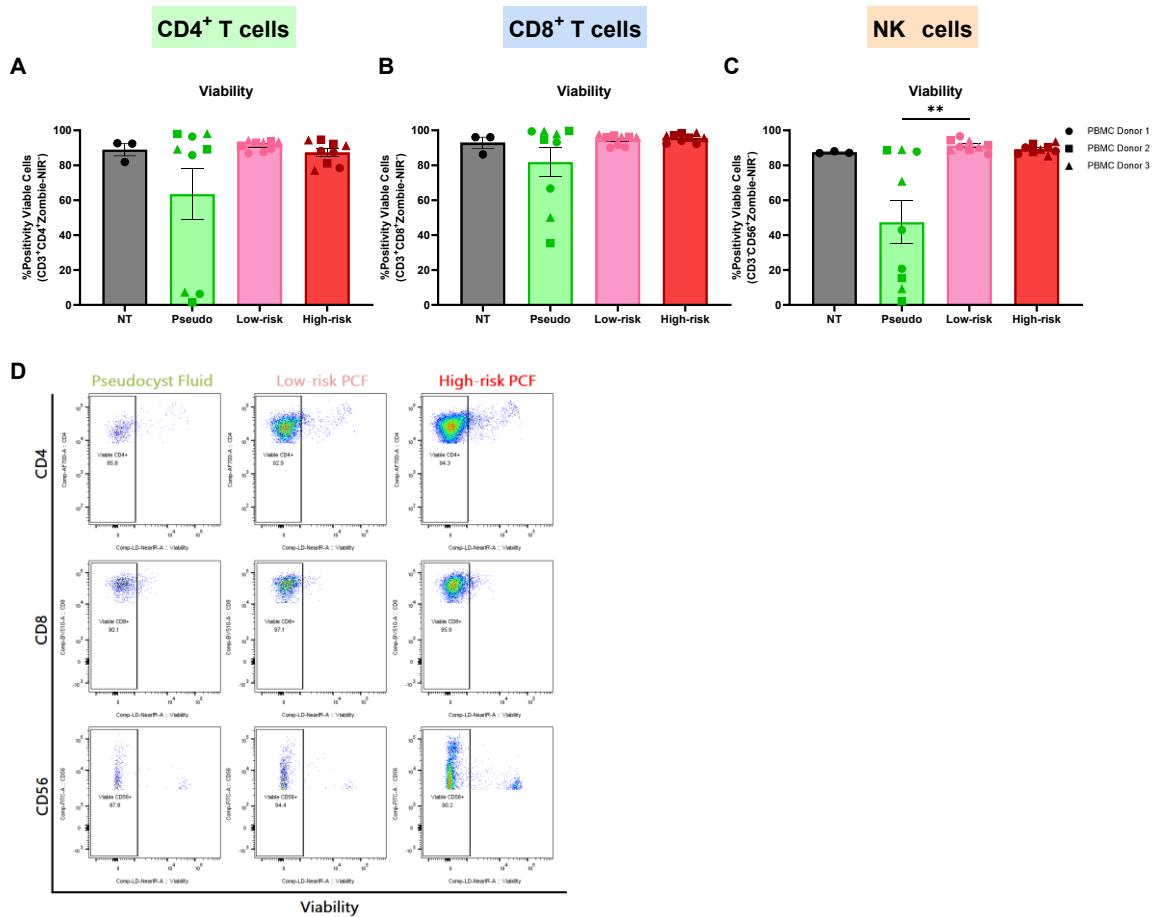


Figure 4.1: Benign pancreatic pseudocyst fluid significantly decreases lymphocyte viability. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess viability (A, B, C, D) of CD3⁺CD4⁺ T cells (A), CD3⁺CD8⁺ T cells (B) and CD3⁺CD56⁺ NK cells (C). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as $**p \leq 0.01$].

4.4.2 Benign pancreatic pseudocyst fluid significantly decreases the expression of activation markers on CD4⁺ T cells and CD8⁺ T cells.

Effector immune cell activation is crucial for initiating the differentiation of naive effector immune cells into various effector and memory subsets, which are essential for carrying out specific immune functions. The impact of soluble immune factors within risk-stratified PCF on T cell and NK cell function is currently unknown. This study investigated the impact of benign pseudocyst fluid, low-risk PCF and high-risk PCF on the activation status of CD4⁺ T cells, CD8⁺ T cells and NK cells. T cells and NK cells

were isolated from healthy PBMCs, activated for 24 hours and co-cultured with 20% (v/v) risk-stratified PCF for 24 hours. The expression of activation markers, CD27, CD45RA and CD62L, was assessed by flow cytometry. The results from this study provide insight into the differential impact of risk-stratified PCF on the activation of T helper cells and cytotoxic T cells.

After a 24-hour co-culture with risk-stratified PCF, CD27 expression, expressed in percentage positivity, was significantly decreased in CD8⁺ T cells exposed to benign pseudocyst fluid compared to CD8⁺ T cells exposed to low-risk PCF ($43.37 \pm 7.31\%$ vs. $83.84 \pm 4.31\%$, $p=0.0015$) (**Figure 4.2B**). Moreover, there was a trend towards a significant decrease of the frequency of CD27 on CD8⁺ T cells exposed to benign pseudocyst fluid compared to high-risk PCF ($43.37 \pm 7.31\%$ vs. $76.82 \pm 4.28\%$, $p=0.0563$) (**Figure 4.2B**). Similarly, there was also a trend towards a significant decrease in the expression of CD27 on the surface of CD4⁺ T cells co-cultured with benign pseudocyst fluid compared to pre-malignant low-risk PCF ($54.39 \pm 8.91\%$ vs. $83.23 \pm 2.12\%$, $p=0.0610$) (**Figure 4.2A**). Although not significant, CD4⁺ T cells and CD8⁺ T cells exposed to benign pseudocyst fluid had diminished CD27 expression compared to the non-treated cRPMI control (PBMCs co-cultured with cRPMI) (**Figure 4.2A, B**). Conversely, the frequency of CD27 was non-significantly increased in NK cells following a 24-hour co-culture with risk-stratified PCF, particularly in the case of NK cells co-cultured with benign pseudocyst fluid (**Figure 4.2C**).

CD45RA expression, expressed in percentage positivity, was significantly increased in CD4⁺ T cells exposed to low-risk PCF compared to CD4⁺ T cells exposed to benign pseudocyst fluid ($60.08 \pm 3.37\%$ vs. $23.45 \pm 6.11\%$, $p=0.0062$) (**Figure 4.2D**). This study also identified a trend towards a significant decrease in CD45RA expression on CD4⁺ T cells co-cultured with benign pseudocyst fluid compared to pre-malignant high-risk PCF ($23.45 \pm 6.11\%$ vs. $53.82 \pm 4.95\%$, $p=0.0541$) (**Figure 4.2D**). Similarly, CD45RA expression was significantly decreased in CD8⁺ T cells exposed to benign pseudocyst fluid compared to the non-treated cRPMI control co-cultured with cRPMI, low-risk PCF and high-risk PCF (Pseudocyst fluid vs. Control: $25.15 \pm 6.46\%$ vs. $76.53 \pm 4.66\%$, $p=0.0200$; Pseudocyst fluid vs Low-risk PCF: $25.15 \pm 6.46\%$ vs. $75.21 \pm 2.84\%$, $p=0.0006$; Pseudocyst fluid vs. High-risk PCF: $25.15 \pm 6.46\%$ vs. $65.78 \pm 5.65\%$, $p=0.0296$) (**Figure 4.2E**). Although not significant, NK cells exposed to benign

pseudocyst fluid had diminished CD45RA expression compared to the non-treated cRPMI control, low-risk PCF and high-risk PCF. (**Figure 4.2F**).

This study found that there was a trend towards a significant decrease in CD62L expression on the surface of CD4⁺ T cells exposed to benign pseudocyst fluid compared to pre-malignant low-risk PCF ($73.63 \pm 4.54\%$ vs. $87.01 \pm 1.51\%$, $p=0.0586$) (**Figure 4.2G**). Although not significant, these trends were also observed in CD62L expression on CD8⁺ T cells and NK cells exposed to benign pseudocyst fluid and low-risk PCF (**Figure 4.2H, I**).

Overall, this data suggests that soluble factors within benign pseudocysts and pre-malignant lesions have differential effects on T cell activation which may have important biological and clinical implications.

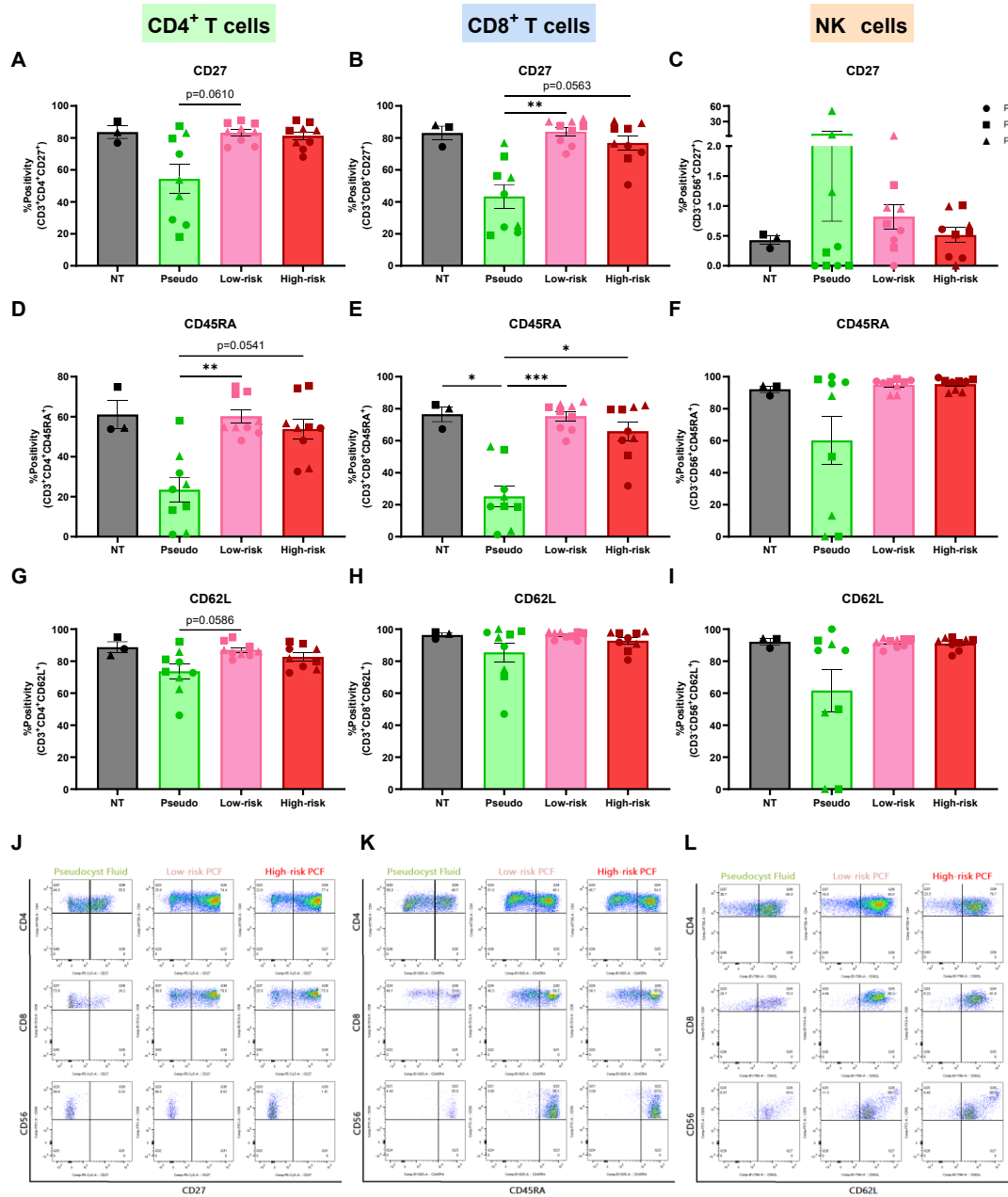


Figure 4.2: Benign pancreatic pseudocyst fluid significantly decreases the expression of activation markers on CD4⁺ T cells and CD8⁺ T cells. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess CD27⁺ (A, B, C, J), CD45RA⁺ (D, E, F, K) and CD62L⁺ (G, H, I, L) expression on viable CD3⁺CD4⁺ T cells (A, D, G), viable CD3⁺CD8⁺ T cells (B, E, H) and viable CD3⁺CD56⁺ NK cells (C, F, I). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$].

4.4.3 Benign pseudocyst fluid increases the frequency of central memory and effector memory T cells and NK cells.

Interestingly, changes in T cell differentiation state were also observed following co-culture with risk-stratified PCF (**Figure 4.3**). There was a significant decrease in the frequency of naïve memory CD4⁺ T cells and CD8⁺ T cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with low-risk PCF for 24 hours (naïve CD4⁺ T cells: $20.63 \pm 5.87\%$ vs. $56.07 \pm 3.73\%$, $p=0.0083$ and naïve CD8⁺ T cells: $24.78 \pm 6.30\%$ vs. $73.97 \pm 4.30\%$, $p=0.0007$) (**Figure 4.3A, B**). Additionally, there was a trend towards a significant decrease in the frequency of naïve CD4⁺ T cells co-cultured with benign pseudocyst fluid compared to the non-treated cRPMI control ($20.63 \pm 5.87\%$ vs. $58.30 \pm 7.45\%$, $p=0.0539$) (**Figure 4.3A**). Pseudocyst fluid also induced a decreased frequency of naïve CD8⁺ T cells compared to high-risk PCF and the non-treated cRPMI control (Pseudocyst fluid vs. High-risk PCF: $24.78 \pm 6.30\%$ vs. $62.84 \pm 6.22\%$, $p=0.0007$ and Pseudocyst fluid vs. Control: $24.78 \pm 6.30\%$ vs. $75.30 \pm 4.30\%$, $p=0.0323$) (**Figure 4.3B**). Similarly, there was a non-significant decrease in the frequency of naïve memory NK cells that were exposed for 24 hours to benign pseudocyst fluid compared to the non-treated cRPMI control, low-risk PCF and high-risk PCF. (**Figure 4.3C**).

Pseudocyst fluid induced a significant increase in the frequency of central memory CD8⁺ T cells compared with low-risk PCF, high-risk PCF and the non-treated cRPMI control (Pseudocyst fluid vs. Low-risk PCF: $41.42 \pm 4.66\%$ vs. $14.99 \pm 1.45\%$, $p=0.0011$, Pseudocyst fluid vs. High-risk PCF: $41.42 \pm 4.66\%$ vs. $17.73 \pm 2.00\%$, $p=0.0136$ and Pseudocyst fluid vs. Control: $41.42 \pm 4.66\%$ vs. $14.13 \pm 1.70\%$, $p=0.0226$) (**Figure 4.3E**). These effects are also present in CD4⁺ T cells exposed to risk-stratified PCF, although not significant (**Figure 4.3D**). Additionally, there was a non-significant increase in the frequency of central memory NK cells after a 24-hour co-culture with pre-malignant PCF, regardless of cancer risk stratification, compared to the non-treated cRPMI control (**Figure 4.3F**).

Furthermore, there was a significant increase in the percentage of effector memory CD4⁺ T cells and CD8⁺ T cells following co-culture with pseudocyst fluid compared with low-risk PCF (effector memory CD4⁺ T cells: $38.14 \pm 6.58\%$ vs. $15.07 \pm 2.02\%$, $p=0.0379$ and effector memory CD8⁺ T cells: $32.63 \pm 6.79\%$ vs. $8.77 \pm 1.38\%$, $p=0.0040$) (**Figure 4.3G, H**). There was also an increased frequency of effector memory NK cells following a 24-hour co-culture with benign pseudocyst fluid, although this finding was not significant (**Figure 4.3I**).

The frequency of terminally differentiated effector memory T cells was not significantly impacted by exposure to risk-stratified PCF. Although, there does appear to be a trend of increased frequency of terminally differentiated effector memory CD4⁺ T cells and CD8⁺ T cells with exposure to PCF with increased risk of PC progression (**Figure 4.3J, K**). Indeed, this study identified a trend towards a significant increase in the frequency of terminally differentiated effector memory CD8⁺ T cells co-cultured with pre-malignant high-risk PCF compared to benign pseudocyst fluid ($4.35 \pm 1.07\%$ vs. $1.18 \pm 0.42\%$, $p=0.0540$) (**Figure 4.3K**). This effect was also found in NK cells co-cultured with pre-malignant PCF (**Figure 4.3L**). However, little to no difference in the frequency of terminally differentiated effector memory NK cells was seen between cells that were exposed to low-risk PCF or high-risk PCF (**Figure 4.3L**).

Overall, this data suggests that low-risk and high-risk pre-malignant PCF maintains T cells in a more naïve state whereas benign pseudocyst fluid permits activation and differentiation of T cells to become central memory and effector memory phenotypes, both of which are important for eliciting an immune response.

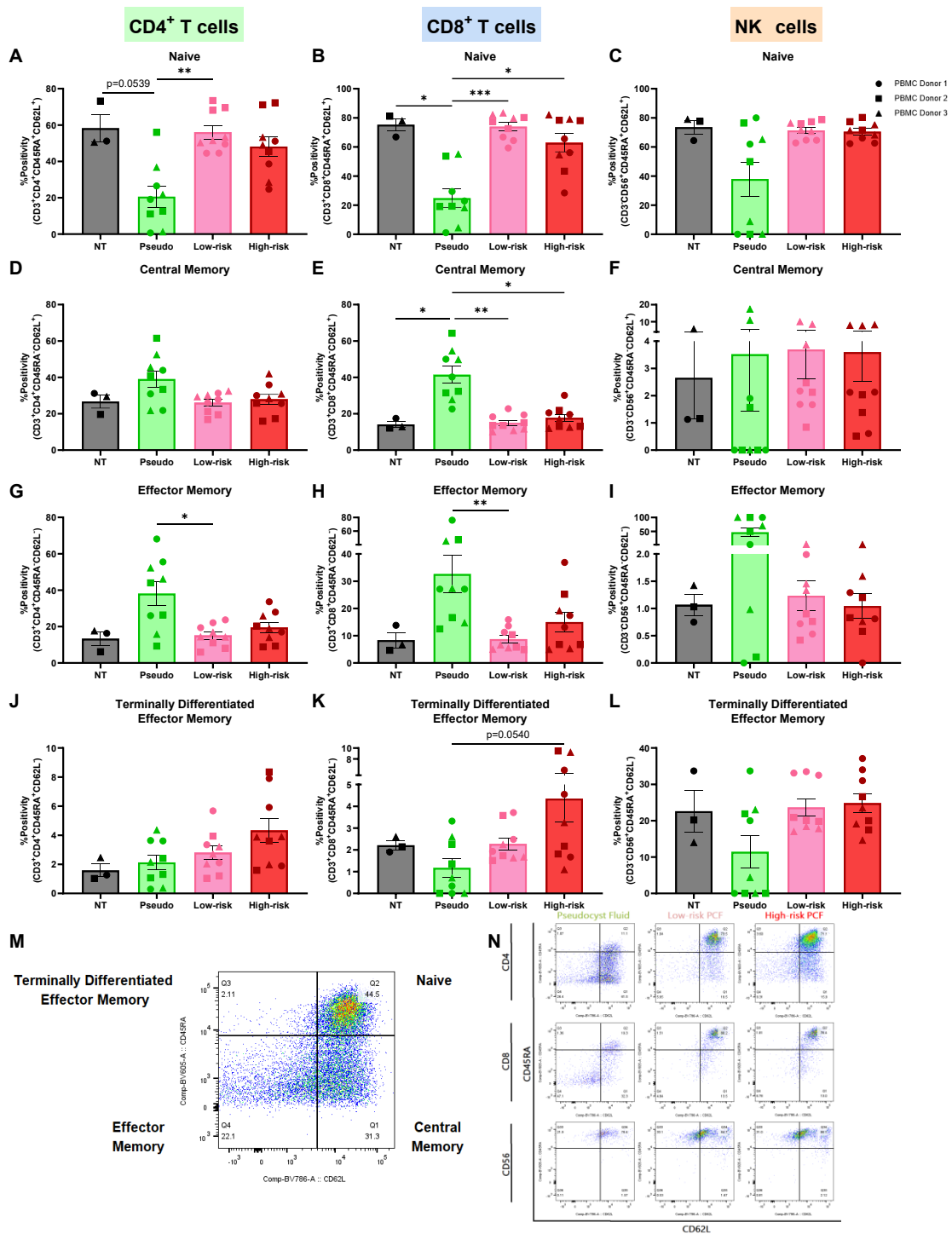


Figure 4.3: Benign pseudocyst fluid increases the frequency of central memory and effector memory T cells and NK cells. PBMCs were isolated from peripheral whole blood of healthy donors ($n=3$) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid ($n=3$), low-risk PCF ($n=3$) and high-risk PCF ($n=3$) for 24 hours after activation. Flow cytometric staining was carried out to assess naïve ($CD45RA^+CD62L^+$) (A, B, C), central memory ($CD45RA^-CD62L^+$) (D, E, F), effector memory ($CD45RA^-CD62L^-$) (G, H, I) and terminally differentiated effector memory ($CD45RA^+CD62L^-$) (J, K, L) subsets in viable $CD3^+CD4^+$ T cells (A, D, G, J), viable $CD3^+CD8^+$ T cells (B, E, H, K) and viable $CD3^+CD56^+$ NK cells (C, F, I, L). Graphical

data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$].

4.4.4 Benign pseudocyst fluid significantly decreases the frequency of CD103 expression on CD4⁺ and CD8⁺ T cell subsets.

CD103, also recognised as $\alpha E\beta 7$ integrin, is a cell surface marker involved in mediating cell adhesion, migration, and lymphocyte homing through its interaction with E-cadherin, which is broadly expressed on epithelial cells^[281]. In this study, T cells and NK cells were isolated from healthy donor PBMCs, activated for 24 hours and co-cultured with 20% (v/v) risk-stratified PCF for 24 hours. CD103 expression was then assessed by flow cytometry. Findings from this study provide further insight into how risk-stratified PCF differentially influences the expression of the integrin molecule CD103, and consequently, the homing and migratory potential of effector immune cells exposed to pre-malignant PCF.

This study found that there was a significant decrease in the frequency of CD103⁺ expression on CD4⁺ T cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with low-risk PCF for 24 hours ($43.79 \pm 8.28\%$ vs. $75.44 \pm 2.53\%$, $p=0.0230$) (**Figure 4.4A**). Similarly, benign pseudocyst fluid also induced a decreased frequency of CD103⁺ on CD8⁺ T cells compared to CD8⁺ T cells co-cultured with low-risk PCF ($36.92 \pm 6.51\%$ vs. $78.27 \pm 2.96\%$, $p=0.0005$) (**Figure 4.4B**). Furthermore, there was a non-significant decrease in the frequency of CD103⁺ expression on NK cells that were exposed for 24 hours to benign pseudocyst fluid compared to the non-treated cRPMI control, low-risk PCF and high-risk PCF (**Figure 4.4C**).

Although not significant, there is diminished CD103 expression in CD4⁺ T cells, CD8⁺ T cells and NK cells exposed to high-risk PCF compared to low-risk PCF (**Figure 4.4A, B, C**).

Overall, this data suggests that soluble factors within benign pseudocyst fluid and high-risk PCF, to some extent, are suppressing CD103 integrin molecule expression which may have implications for integrin-mediated adhesion and homing mechanisms.

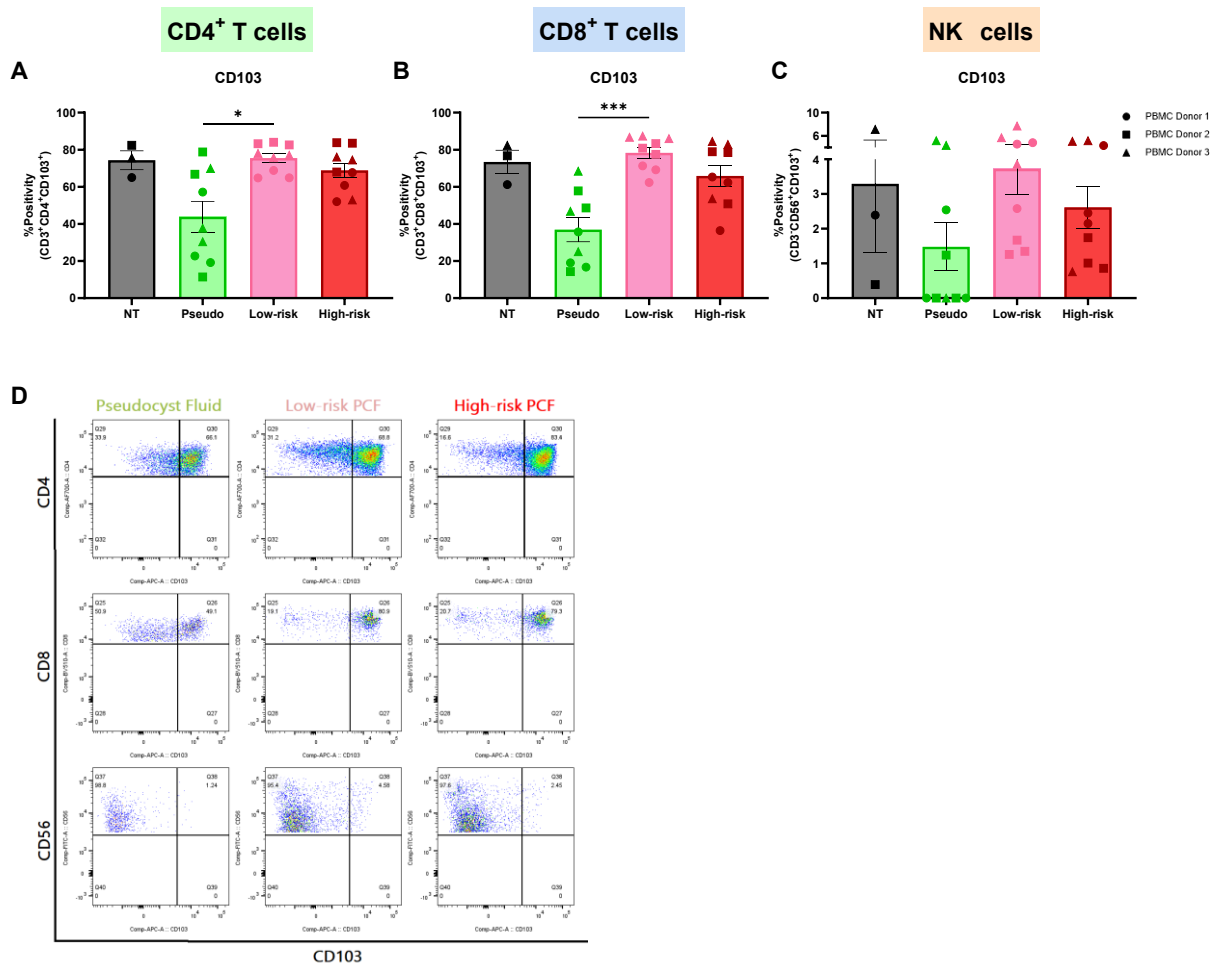


Figure 4.4: Benign pseudocyst fluid decreases the frequency of CD103 expression on CD4⁺ and CD8⁺ T cell subsets. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess CD103⁺ expression (A, B, C, D) on viable CD3⁺CD4⁺ T cells (A), viable CD3⁺CD8⁺ T cells (B) and viable CD3⁺CD56⁺ NK cells (C). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, and *** $p \leq 0.001$].

4.4.5 Pre-malignant PCF maintains CD4⁺ T cells, CD8⁺ T cells and NK cells in an exhausted state.

T cell exhaustion is a state of T cell dysfunction characterised by reduced effector function, impaired self-renewal and increased expression of inhibitory receptors, frequently seen in cancer. Exhausted T cells are often identified by an upregulated accumulation of inhibitory checkpoint proteins including PD-1, LAG-3 and TIM-3 production^[227, 228]. An exhausted T cell phenotype represents a significant barrier to

effective immune responses, particularly in the context of immunotherapy. However, the impact of soluble immune factors within risk-stratified PCF on T cell exhaustion is currently unknown. The results from this study provide more insight into the differential impact of risk-stratified PCF on the exhaustion of T helper cells and cytotoxic T cells.

Results from this study showed that there was a significant decrease in the expression of PD-1 on CD4⁺ T cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with low-risk PCF and high-risk PCF, which could indicate activation or the onset of exhaustion (Pseudocyst fluid vs. Low-risk PCF: $52.28 \pm 7.15\%$ vs. $78.98 \pm 1.56\%$, $p=0.0095$ and Pseudocyst fluid vs. High-risk PCF: $52.28 \pm 7.15\%$ vs. $78.67 \pm 3.05\%$, $p=0.0037$) (**Figure 4.5A**). Similarly, PD-1 expression was significantly decreased on CD8⁺ T cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with low-risk PCF and high-risk PCF (Pseudocyst fluid vs. Low-risk PCF: $49.12 \pm 7.31\%$ vs. $81.14 \pm 0.99\%$, $p=0.0015$ and Pseudocyst fluid vs. High-risk PCF: $49.12 \pm 7.31\%$ vs. $76.82 \pm 4.27\%$, $p=0.0057$) (**Figure 4.5B**). There was also a significant decrease in the expression of PD-1 on NK cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with high-risk PCF ($0.13 \pm 0.10\%$ vs. $0.51 \pm 0.09\%$, $p=0.0167$) (**Figure 4.5C**).

Furthermore, pseudocyst fluid induced a significant decrease on the co-expression of PD-1⁺LAG-3⁺ on CD4⁺ T cells compared to low-risk PCF ($41.49 \pm 7.56\%$ vs. $68.74 \pm 1.94\%$, $p=0.0284$) (**Figure 4.5D**). This study also revealed a trend towards a significant decrease of PD-1⁺LAG-3⁺ co-expression on CD4⁺ T cells co-cultured with benign pseudocyst fluid compared to CD4⁺ T cells co-cultured with pre-malignant high-risk PCF ($41.49 \pm 7.56\%$ vs. $65.74 \pm 3.64\%$, $p=0.0658$) (**Figure 4.5D**). Similarly, the co-expression of PD-1⁺LAG-3⁺ on CD8⁺ T was significantly decreased when exposed to benign pseudocyst fluid compared to pre-malignant low-risk PCF and high-risk PCF (Pseudocyst fluid vs. Low-risk PCF: $67.49 \pm 6.86\%$ vs. $91.49 \pm 0.62\%$, $p=0.0022$ and Pseudocyst fluid vs. High-risk PCF: $67.49 \pm 6.86\%$ vs. $87.46 \pm 3.03\%$, $p=0.0363$) (**Figure 4.5E**). Additionally, there was a trend towards a significant decrease of PD-1⁺LAG-3⁺ co-expression on CD4⁺ T cells co-cultured with benign pseudocyst fluid compared to the non-treated cRPMI control ($67.49 \pm 6.86\%$ vs. $91.27 \pm 0.23\%$, $p=0.0523$) (**Figure 4.5E**). The frequency of PD-1⁺LAG-3⁺ co-expression was also decreased in NK cells following a 24-hour co-culture with risk-stratified PCF compared to non-treated cRPMI control, particularly in the case of NK cells co-cultured with benign pseudocyst fluid (**Figure 4.5F**).

No significant differences were found in the co-expression levels of PD-1⁺LAG-3⁺TIM-3⁺ on CD4⁺ T cells or CD8⁺ T cells co-cultured with benign pseudocyst fluid, low-risk PCF or high-risk PCF. However, these cells did have a higher frequency of PD-1⁺LAG-3⁺TIM-3⁺ co-expression on T cells when exposed to pre-malignant PCF compared to benign pseudocyst fluid (**Figure 4.5G, H**). There was also a non-significant decrease in the frequency of PD-1⁺LAG-3⁺TIM-3⁺ co-expression on NK cells that were exposed to benign pseudocyst fluid compared to cells co-cultured with non-treated cRPMI control, low-risk PCF and high-risk PCF (**Figure 4.5I**).

These findings suggest that exposure to pre-malignant PCF promotes an exhausted phenotype in both T helper and cytotoxic T cells. This T cell dysfunction may facilitate early immune evasion mechanisms within the pancreatic cystic microenvironment.

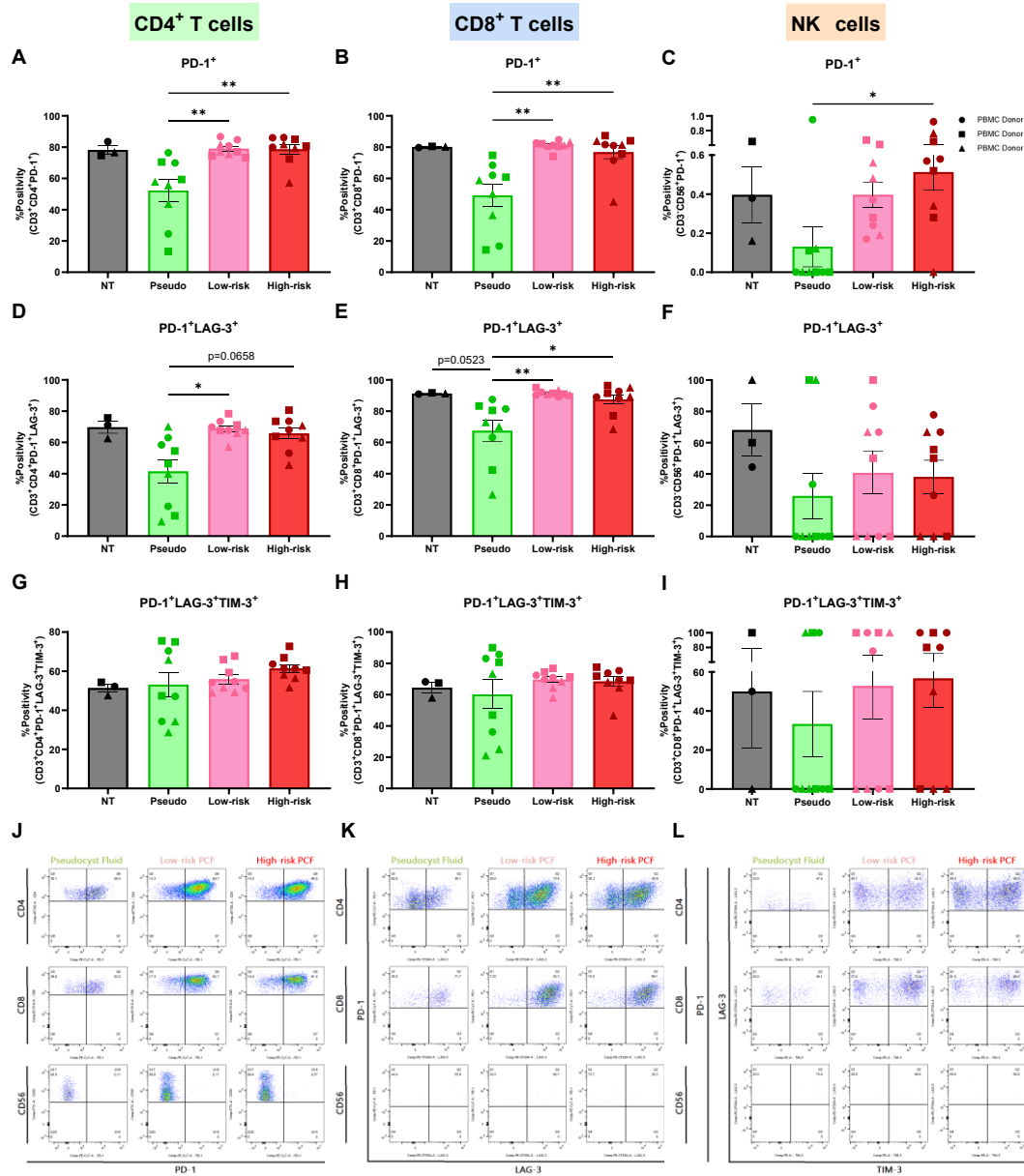


Figure 4.5: Pre-malignant PCF maintains CD4⁺ T cells, CD8⁺ T cells and NK cells in an exhausted state. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess PD-1⁺ (A, B, C, J), PD-1⁺LAG-3⁺ (D, E, F, K) and PD-1⁺LAG-3⁺TIM-3⁺ (G, H, I, L) expression on viable CD3⁺CD4⁺ T cells (A, D, G), viable CD3⁺CD8⁺ T cells (B, E, H) and viable CD3⁺CD56⁺ NK cells (C, F, I). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as *p $\leq$ 0.05, and **p $\leq$ 0.01].

4.4.6 Activated T cells employ a predominant Th1 phenotype following co-culture with PCF, regardless of cancer risk stratification status.

T cells are important players in eliminating neoplastic cells^[282, 283]. Different T cell subsets exhibit varying phenotypes, impacting their ability to recognise, target and eliminate cancer cells. T cell differentiation is governed by lineage-defining transcription factors that drive specialised T helper phenotypes with distinct immunological functions^[284, 285]. T-bet is the master transcription factor of Th1 cells and promotes production of IFN- γ , enhancing cytotoxic immunity and anti-tumour responses^[286]. In contrast, GATA-3 directs differentiation towards a Th2 phenotype characterised by secretion of IL-4, IL-5 and IL-13 cytokines, which are associated with humoral immunity and have been linked to tumour-permissive immune microenvironments^[287]. ROR γ t defines Th17 cell populations and drives the production of IL-17 family cytokines, which can exert either pro-tumour or anti-tumour effects depending on the inflammatory context but are frequently associated with chronic inflammation and tumour progression^[288]. FoxP3 is the master transcription factor of Tregs which drive production of IL-10 and suppress effector T cell responses to facilitate immune tolerance, thereby promoting tumour immune evasion^[289]. Currently, the influence of soluble immune factors within benign pseudocyst fluid, low-risk PCF and high-risk PCF on the differentiation of T helper cell phenotypes remains unknown. Data from this study provides more insight into whether risk-stratified PCF is inducing a phenotypic switch in T cells towards a pro-tumour phenotype.

Data from this study showed that benign pseudocyst fluid had a significantly higher frequency of the Th1 transcription factor T-bet compared to FoxP3, the transcription factor for Treg subsets ($45.90 \pm 7.10\%$ vs. $0.72 \pm 0.17\%$, $p=0.0004$) (**Figure 4.6A**). Additionally, when T cells were exposed to pseudocyst fluid, the Treg-associated transcription factor, FoxP3, was significantly decreased compared to the Th2 transcription factor, GATA-3 ($0.72 \pm 0.17\%$ vs. $36.23 \pm 1.83\%$, $p=0.0002$) (**Figure 4.6A**). Similarly, this analysis demonstrated that when T cells were co-cultured with benign pseudocyst fluid, there was a trend towards a significant decrease of the frequency of ROR γ t-expressing T cells compared to GATA-3-expressing T cells ($10.77 \pm 1.28\%$ vs. $36.23 \pm 1.83\%$, $p=0.0635$) (**Figure 4.6A**).

When the cytokines associated with T cell phenotypes were assessed, this study found that the Th1-associated cytokine, IFN- γ , was significantly increased compared to Th17-

associated IL-17 and Treg-associated IL-10 cytokines (IFN- γ vs. IL-17; $26.31 \pm 6.46\%$ vs. $0.50 \pm 0.18\%$, $p=0.0011$ and IFN- γ vs. IL-10; $26.31 \pm 6.46\%$ vs. $1.91 \pm 1.30\%$, $p=0.0002$) (**Figure 4.6B**).

When this analysis was performed to investigate the impact of pre-malignant PCF on T cell differentiation, this study revealed that T cells exposed to low-risk PCF had a significantly higher frequency of the Th1-associated transcription factor, T-bet, compared to ROR γ t and FoxP3, the transcription factors associated with Th17 and Treg subsets respectively (T-bet vs. ROR γ t; $52.79 \pm 4.54\%$ vs. $7.83 \pm 1.14\%$, $p=0.0061$ and T-bet vs. FoxP3; $52.79 \pm 4.54\%$ vs. $0.90 \pm 0.16\%$, $p=0.0001$) (**Figure 4.6D**). Additionally, when T cells were co-cultured with low-risk PCF, GATA-3, the transcription factor for Th2 cell subsets, was shown to be significantly increased compared to FoxP3, the transcription factor associated with Treg cell subsets ($29.09 \pm 1.58\%$ vs. $0.90 \pm 0.16\%$, $p=0.0061$) (**Figure 4.6D**).

The cytokine profile of T cells co-cultured with pre-malignant low-risk PCF showed that the Th1-associated cytokine IFN- γ was significantly increased compared to that of the Th2-associated cytokine, IL-4 ($24.52 \pm 4.86\%$ vs. $0.89 \pm 0.08\%$, $p=0.0031$) (**Figure 4.6E**). Indeed, when T cells were exposed to low-risk PCF, IFN- γ was also found to be significantly increased compared to that of the Th17-associated cytokine, IL-17 ($24.52 \pm 4.86\%$ vs. $1.14 \pm 0.40\%$, $p=0.0011$) (**Figure 4.6E**).

Similar to results recorded for T cells co-cultured with low-risk PCF, this study showed that T cells exposed to pre-malignant high-risk PCF had a significantly higher frequency of the Th1-associated transcription factor, T-bet, compared to the Th17-associated transcription factor, ROR γ t ($53.63 \pm 2.10\%$ vs. $7.56 \pm 0.87\%$, $p=0.0061$) (**Figure 4.6G**). Similarly, T cells co-cultured with high-risk PCF were also shown to have a significantly higher frequency of T-bet compared to the Treg-associated transcription factor, FoxP3 ($53.63 \pm 2.10\%$ vs. $1.09 \pm 0.12\%$, $p=0.0001$) (**Figure 4.6G**). Additionally, when T cells were co-cultured with high-risk PCF, the Th2-associated transcription factor, GATA-3, was significantly increased compared to FoxP3, the transcription factor associated with Treg cell subsets ($29.12 \pm 2.09\%$ vs. $1.09 \pm 0.12\%$, $p=0.0061$) (**Figure 4.6G**).

The cytokine profile of T cells co-cultured with pre-malignant high-risk PCF showed that the Th1-associated cytokine IFN- γ was significantly increased compared to that of the Th2-associated cytokine, IL-4 ($24.51 \pm 4.57\%$ vs. $1.11 \pm 0.14\%$, $p=0.0008$) (**Figure**

4.6H). Similarly, when T cells were exposed to low-risk PCF, IFN- γ was also found to be significantly increased compared to that of the Th17-associated cytokine, IL-17 ($24.51 \pm 4.57\%$ vs. $1.34 \pm 0.45\%$, $p=0.0016$) (**Figure 4.6H**).

Overall, this data demonstrates that T cells employed a predominant Th1 phenotype, indicated by the intracellular expression of T-bet⁺ and IFN- γ ⁺, in CD4⁺ T cells, regardless of co-culture with pseudocyst fluid, low-risk PCF or high-risk PCF (**Figure 4.6A-B, D-E, G-H**).

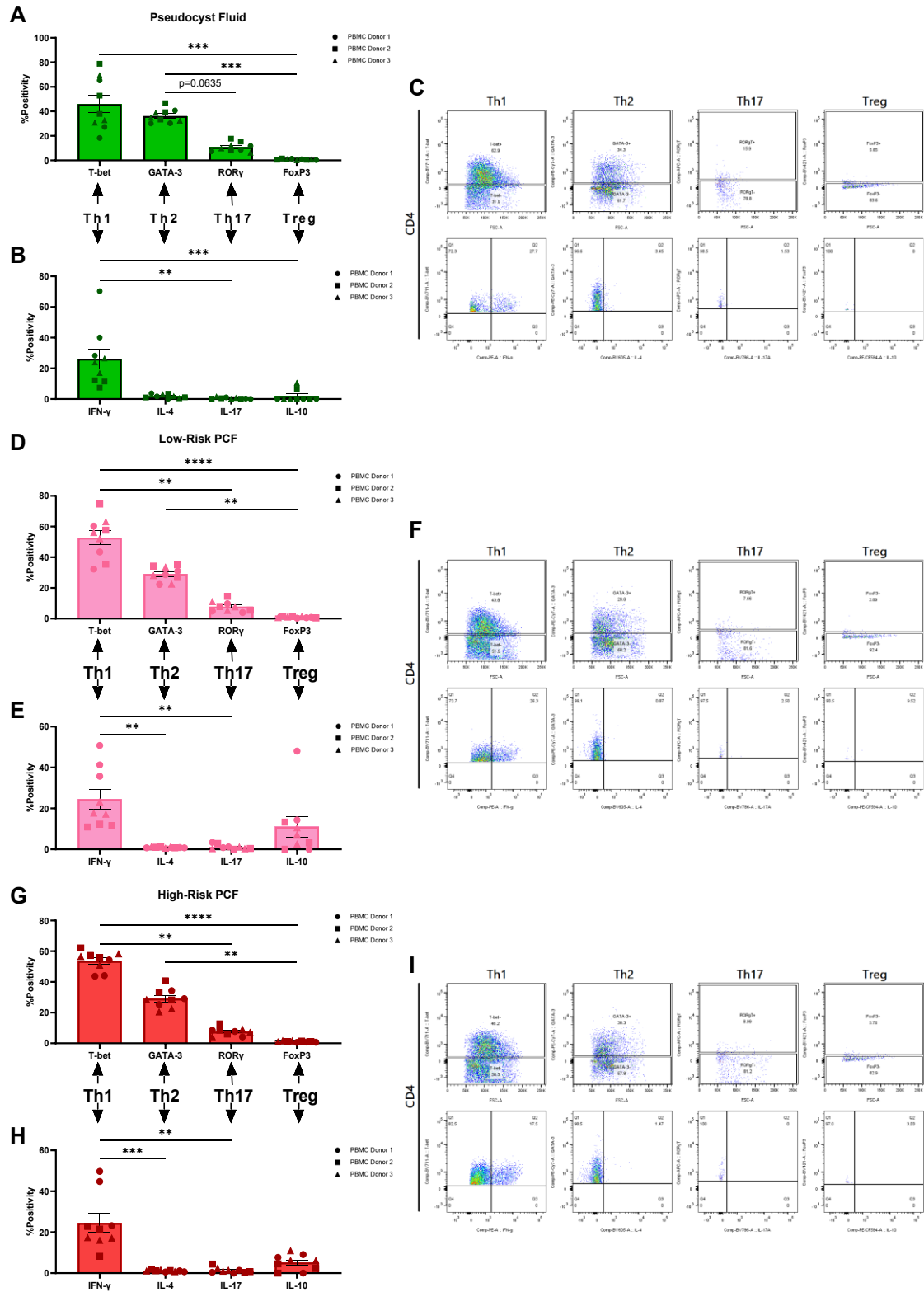


Figure 4.6: Activated T cells employ a predominant Th1 phenotype following co-culture with PCF, regardless of cancer risk stratification status. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess T

cell subset-specific transcription factors T-bet⁺, GATA-3⁺, RORγT⁺, FoxP3⁺ (A, D, G) and co-expression of associated cytokines IFN-γ⁺ (T-bet⁺IFN-γ⁺), IL-4⁺ (GATA-3⁺IL-4⁺), IL-17⁺ (RORγT⁺IL-17⁺) and IL-10⁺ (FoxP3⁺IL-10⁺) (B, E, H) in viable CD3⁺CD4⁺ T cells co-cultured with benign pseudocyst fluid (A, B, C), low-risk PCF (D, E, F) and high-risk PCF (G, H, I). Graphical data are representative of mean percentage positivity ± SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Friedman) with a Dunn's post-hoc test [significance defined as **p≤0.01, ***p≤0.001, and ****p≤0.0001].

4.4.7 The cytotoxic capacity of cytotoxic CD8⁺ T cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.

T cells and NK cells play a pivotal role in the anti-tumour immune response. Their cytotoxic effector function is crucial to eliminate tumour growth and progression. However, the immune response in PC is largely ineffective, as effector T cells are excluded, via the desmoplastic microenvironment, or otherwise skewed towards immunosuppressive Treg cells. The impact of soluble chemokines and growth factors within PCF on T cell function is currently unknown. This study investigated the impact of benign pseudocyst fluid, low-risk PCF and high-risk PCF on the cytotoxic ability of CD8⁺ T cells and NK cells. The results from this study provide more insight into the differential impact of risk-stratified PCF on the cytotoxic function of effector immune cells.

Cytotoxic lymphocytes such as CD8⁺ T cells and NK cells recognise and kill target cells, as in the case of tumour cells, through the secretion of cytolytic granules. Upon activation, cytolytic vesicles migrate to the surface of the effector immune cell and fuse to the cytoplasmic membrane, resulting in the surface exposure of lysosomal-associated proteins and cytolytic granules. CD107a, otherwise known as lysosomal-associated membrane protein 1 (LAMP-1), is a glycosylated transmembrane protein typically present on the lipid bilayer of cytolytic granules of PBMCs. Cell surface expression of CD107a, therefore, is a marker of immune cell activation and importantly, cytotoxic degranulation. After a 24-hour T cell activation and co-culture with risk-stratified PCF, CD107a expression, expressed in percentage positivity, was significantly decreased in CD8⁺ T cells after 50% (v/v) co-culture with low-risk PCF in comparison to the non-treated cRPMI control (32.54 ± 7.36% vs. 84.89 ± 1.62%, p=0.0004) (**Figure 4.7A**). Despite the increased frequency of CD107a⁺ cytotoxic CD8⁺ T cells co-cultured with high-risk PCF compared to low-risk PCF, the overall trend shows that treatment with PCF,

irrespective of cancer risk stratification, elicits a downregulation of the cell surface expression of the degranulation marker, CD107a, compared to non-treated cells (**Figure 4.7A**).

Perforin is a glycoprotein released from the cytolytic vesicle upon formation of the cytolytic synapse between an effector immune cell and a target cell. Upon release, perforin localises to the target cell and forms a pore in the cell membrane to disrupt the protective barrier and hence destroy the integrity of the target cell. After a 24-hour T cell activation and co-culture with risk-stratified PCF, perforin expression was only detected at levels below 10% in CD8⁺ T cells. There was no significant difference in the perforin expression of these effector immune cell populations following co-culture of low-risk PCF, high-risk PCF and benign pseudocyst fluid (**Figure 4.7C**).

Granzyme B is a serine protease that is commonly expressed in the cytolytic granules of effector immune cells such as CD8⁺ T cells and NK cells. Granzyme B is released from cytotoxic effector immune cells and accesses the target cell by taking advantage of pores created by perforin. Once inside the target cell, granzyme B mediates programmed cell death by cleaving caspase enzymes, which trigger apoptosis. Granzyme B expression was not significantly altered in CD8⁺ T cells following a 24-hour co-culture with PCF, irrespective of cancer risk stratification. However, the overall trend shows that treatment with PCF, elicits a downregulation of the intracellular expression of granzyme B compared to CD8⁺ T cells co-cultured with the non-treated cRPMI control (**Figure 4.7E**). Cytokines such as IFN- γ are also released upon degranulation of cytotoxic vesicles. IFN- γ is the only member of the type-II class interferons and primarily contributes to the cytotoxic function of effector immune cells by promoting an inflammatory microenvironment through the recruitment and activation of other immune cells in order to induce programmed cell death in target cells. After a 24-hour T cell activation and co-culture with risk-stratified PCF, intracellular IFN- γ expression, was recorded to be significantly increased in CD8⁺ T cell populations after 20% (v/v) co-culture with high-risk PCF in comparison to 20% (v/v) co-culture with benign pseudocyst fluid ($67.30 \pm 4.12\%$ vs. $44.40 \pm 3.44\%$, $p=0.0074$) (**Figure 4.7G**). Interestingly, CD8⁺ T cells had an overall increased expression of IFN- γ following a 24-hour co-culture with low-risk PCF and high-risk PCF compared to the control, unlike the co-culture with benign pseudocyst fluid which demonstrated a decreased IFN- γ expression. However, these results were not statistically significant (**Figure 4.7G**).

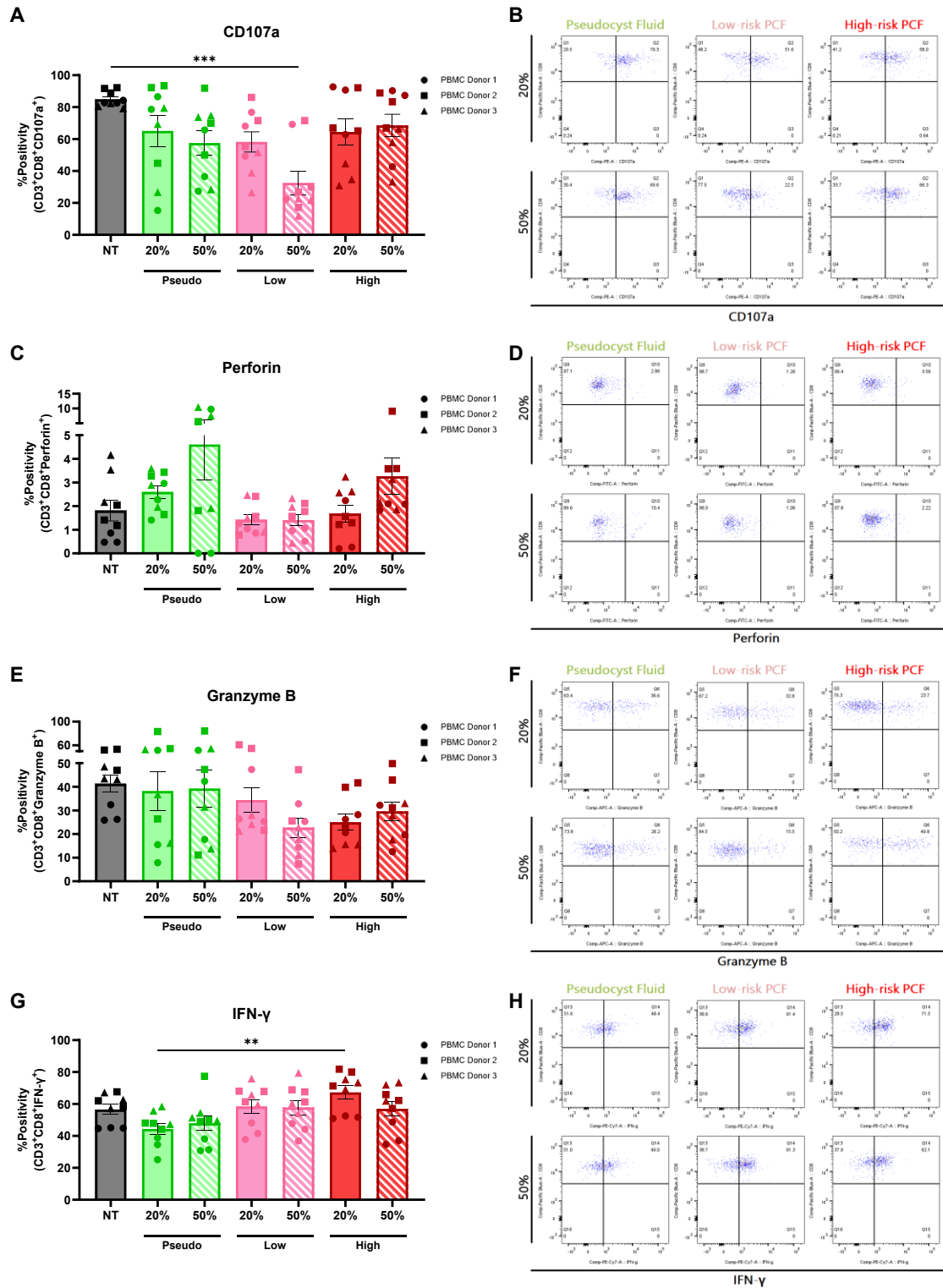


Figure 4.7: The cytotoxic capacity of cytotoxic CD8⁺ T cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) and 50% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess CD107a⁺ (A, B), perforin⁺ (C, D), granzyme B⁺ (E, F) and IFN-γ⁺ (G, H) expression in viable

CD3⁺CD8⁺ T cells. Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as ** $p \leq 0.01$, and *** $p \leq 0.001$].

4.4.8 Changes in the cytotoxic capacity of CD8⁺ T cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.

A 48-hour co-culture with risk-stratified PCF, resulted in a significantly decreased expression of CD107a in CD8⁺ T cells following a 20% (v/v) co-culture and 50% (v/v) co-culture with low-risk PCF compared to the non-treated cRPMI control (20% (v/v) Low-risk vs Control: $96.48 \pm 0.75\%$ vs. $99.48 \pm 0.06\%$, $p=0.0015$ and 50% (v/v) Low-risk vs Control: $91.59 \pm 2.99\%$ vs. $99.48 \pm 0.06\%$, $p=0.0001$) (**Figure 4.8A**). Similarly, the frequency of CD107a⁺ CD8⁺ T cells was also significantly decreased following a 50% (v/v) co-culture with high-risk PCF in comparison to the non-treated cRPMI control ($94.91 \pm 1.77\%$ vs. $99.48 \pm 0.06\%$, $p=0.0022$) (**Figure 4.8A**).

Co-culture of CD8⁺ T cells with risk-stratified PCF for 48 hours resulted in similar outcomes as seen in the 24-hour experimental setup. Although no significance was found, intracellular perforin expression was again only detected at levels below 10% in CD8⁺ T cells irrespective of co-culture with low-risk PCF, high-risk PCF and benign pseudocyst fluid (**Figure 4.8C**).

Intracellular granzyme B expression was not significantly altered in CD8⁺ T cell populations following a 48-hour co-culture with PCF (**Figure 4.8E**).

Similarly, intracellular IFN- γ expression in CD8⁺ T cells was not significantly altered after a 48-hour co-culture with risk-stratified PCF, irrespective of cancer risk stratification (**Figure 4.8G**).

Overall, this data indicates that the cytotoxic capacity of CD8⁺ T cells was markedly enhanced after a 48-hour exposure to benign pseudocyst fluid and pre-malignant PCF, regardless of cancer risk stratification. Consequently, differences in expression of cytotoxic markers on CD8⁺ T cells between co-culture conditions are more apparent in the 24-hour co-culture experiments.

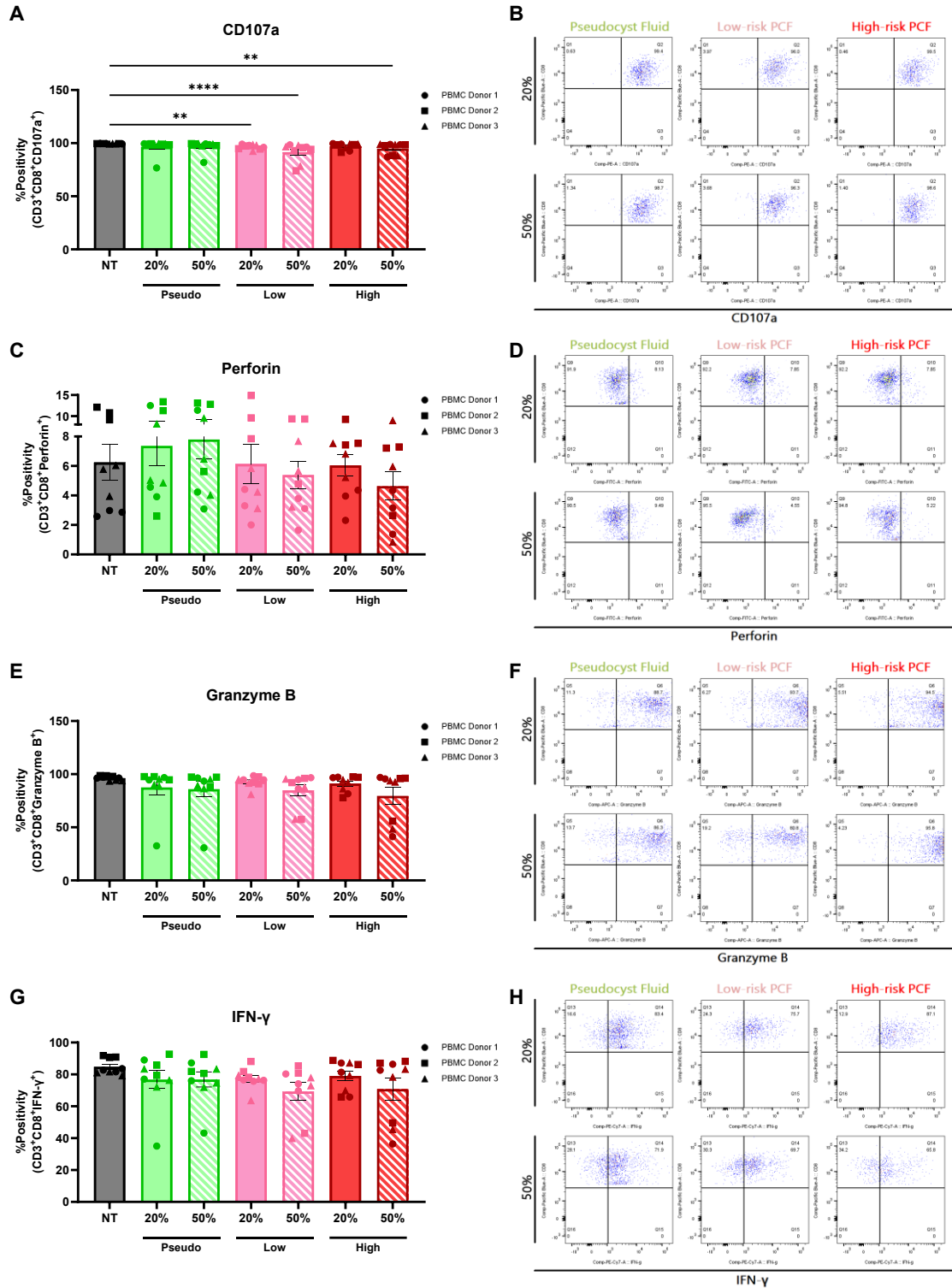


Figure 4.8: Changes in the cytotoxic capacity of CD8⁺ T cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 48 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) and 50% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 48 hours after activation. Flow cytometric staining was carried out to assess CD107a⁺ (A,

B), perforin⁺ (C, D), granzyme B⁺ (E, F) and IFN- γ ⁺ (G, H) expression in viable CD3⁺CD8⁺ T cells. Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as ** $p \leq 0.01$, and **** $p \leq 0.0001$].

4.4.9 The cytotoxic capacity of NK cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF.

After a 24-hour co-culture with risk-stratified PCF, CD107a expression was significantly decreased in NK cell populations after 20% (v/v) and 50% (v/v) co-culture with low-risk PCF in comparison to the non-treated cRPMI control (20% (v/v) Low-risk vs Control: $25.84 \pm 4.52\%$ vs. $54.67 \pm 2.11\%$, $p=0.0046$ and 50% (v/v) Low-risk vs Control: $19.33 \pm 3.05\%$ vs. $54.67 \pm 2.11\%$, $p=0.0002$) (**Figure 4.9A**). Furthermore, the frequency of CD107a⁺ NK cells was found to be significantly increased following 50% (v/v) co-culture with high-risk PCF in comparison to low-risk PCF ($49.73 \pm 5.01\%$ vs. $19.33 \pm 3.05\%$, $p=0.0174$) (**Figure 4.9A**).

However, after a 24-hour co-culture with risk-stratified PCF, NK cells showed significantly decreased perforin expression after 50% (v/v) co-culture with benign pseudocyst fluid compared to the non-treated cRPMI control ($42.80 \pm 7.67\%$ vs. $70.07 \pm 1.93\%$, $p=0.0148$) (**Figure 4.9C**). This study identified a trend towards a significant decrease in intracellular perforin expression in NK cells co-cultured with 50% (v/v) low-risk PCF compared to the non-treated cRPMI control ($56.93 \pm 2.38\%$ vs. $70.07 \pm 1.93\%$, $p=0.0527$) (**Figure 4.9C**). Although not significant, a similar trend was observed in the frequency of perforin⁺ NK cells when treated with high-risk PCF (**Figure 4.9C**).

After a 24-hour co-culture with risk-stratified PCF, NK cells showed significantly decreased granzyme B expression, after 50% (v/v) co-culture with benign pseudocyst fluid compared to the non-treated cRPMI control ($65.44 \pm 8.49\%$ vs. $93.91 \pm 0.32\%$, $p=0.0008$) (**Figure 4.9E**). Data from this study also demonstrated a trend towards a significant decrease of intracellular granzyme B expression in NK cells co-cultured with 20% (v/v) benign pseudocyst fluid compared to the non-treated cRPMI control ($84.90 \pm 2.34\%$ vs. $93.91 \pm 0.32\%$, $p=0.0624$) (**Figure 4.9E**). Similarly, the frequency of granzyme B⁺ NK cells was significantly decreased following a 24-hour co-culture with 20% (v/v) and 50% (v/v) low-risk PCF compared to the non-treated cRPMI control (20% (v/v) Low-risk vs Control: $86.48 \pm 0.70\%$ vs. $93.91 \pm 0.32\%$, $p=0.0464$ and 50% (v/v) Low-risk vs Control: $79.30 \pm 2.71\%$ vs. $93.91 \pm 0.32\%$, $p=0.0001$) (**Figure 4.9E**). Furthermore, there

was a trend towards a significant decrease in the frequency of intracellular granzyme B in NK cells co-cultured with 50% (v/v) pre-malignant high-risk PCF compared to the non-treated cRPMI control ($85.24 \pm 2.20\%$ vs. $93.91 \pm 0.32\%$, $p=0.0527$) (**Figure 4.9E**). Intracellular IFN- γ expression was not significantly altered in NK cell populations following a 24-hour co-culture with PCF, irrespective of cancer risk stratification (**Figure 4.9G**).

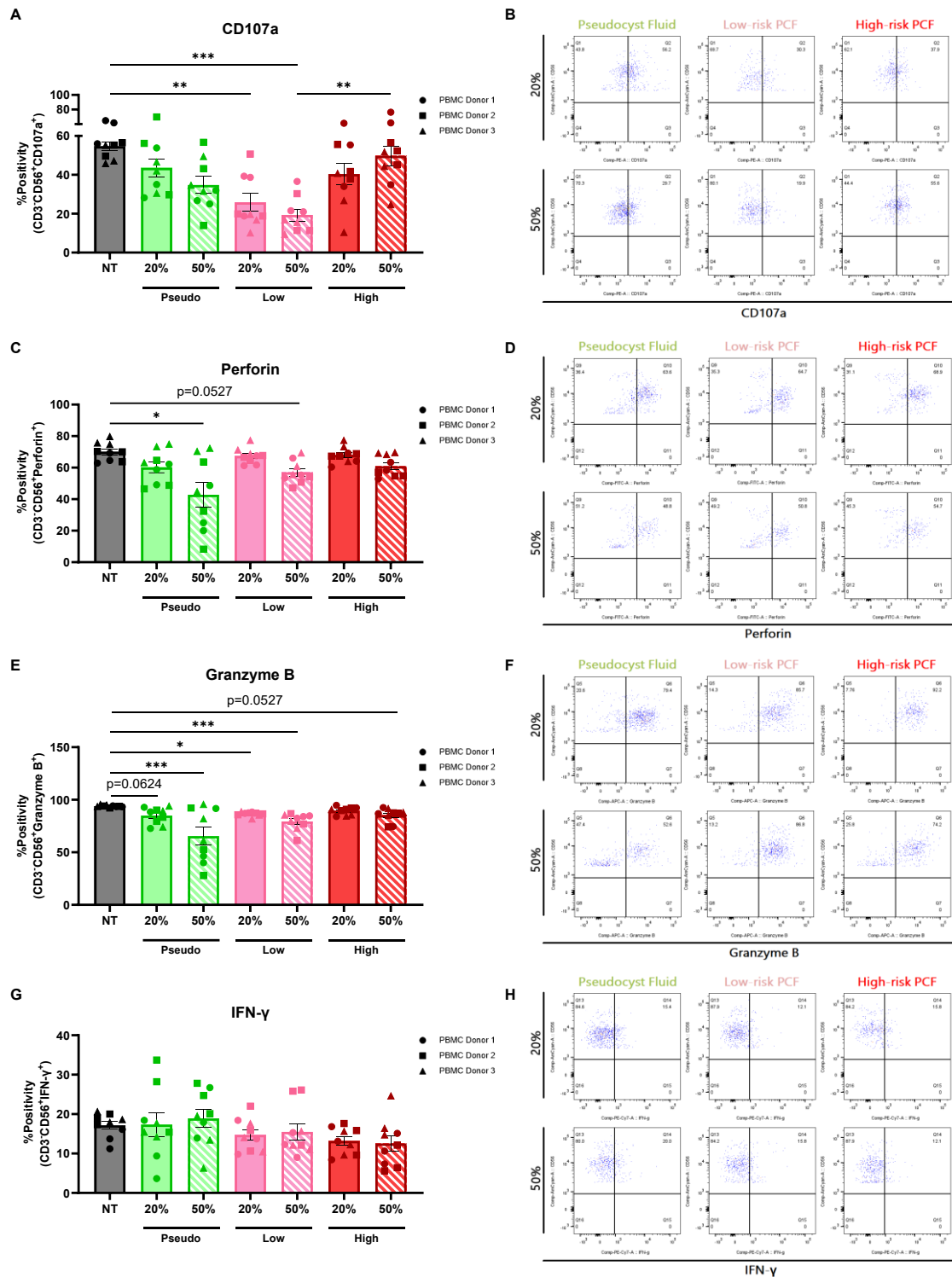


Figure 4.9: The cytotoxic capacity of NK cells is altered by a 24-hour co-culture with benign pseudocyst fluid and pre-malignant PCF. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) and 50% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess CD107a⁺ (A, B), perforin⁺ (C, D), granzyme B⁺ (E, F) and IFN-γ⁺ (G, H) expression in viable CD3⁺CD56⁺ NK cells. Graphical data are representative of mean percentage positivity ± SEM. Statistical

significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$].

4.4.10 Changes in the cytotoxic capacity of NK cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions.

Similar to what was seen in the 24-hour experimental setup, CD107a expression, was significantly decreased in NK cell populations after 20% (v/v) and 50% (v/v) co-culture with low-risk PCF in comparison to the non-treated cRPMI control (20% (v/v) Low-risk vs Control: $78.12 \pm 4.93\%$ vs. $96.58 \pm 0.59\%$, $p=0.0209$ and 50% (v/v) Low-risk vs Control: $71.31 \pm 6.58\%$ vs. $96.58 \pm 0.59\%$, $p=0.0028$) (**Figure 4.10A**).

Although not significant, perforin expression was detected at lower levels in NK cells that were co-cultured with PCF for 48 hours, regardless of cancer risk stratification, in comparison to the non-treated cRPMI control (**Figure 4.10C**).

NK cells showed significantly decreased granzyme B expression, following a 48-hour co-culture with 20% (v/v) and 50% (v/v) low-risk PCF compared to the non-treated cRPMI control (20% (v/v) Low-risk vs Control: $73.79 \pm 1.56\%$ vs. $84.01 \pm 1.49\%$, $p=0.0426$ and 50% (v/v) Low-risk vs Control: $72.47 \pm 2.43\%$ vs. $84.01 \pm 1.49\%$, $p=0.0287$) (**Figure 4.10E**).

Similar to data recorded for perforin expression, the data indicates an overall decreased IFN- γ expression, in NK cells after a 48-hour co-culture with risk-stratified PCF, regardless of cancer risk stratification (**Figure 4.10G**).

Similar to what was observed in CD8⁺ T cells, the cytotoxic capacity of NK cells was highly upregulated following a 48-hour co-culture with pre-malignant PCF, independent of cancer risk stratification. As a result, differences in expression of cytotoxic markers on NK cells between co-culture conditions are more apparent in 24-hour co-culture experiments.

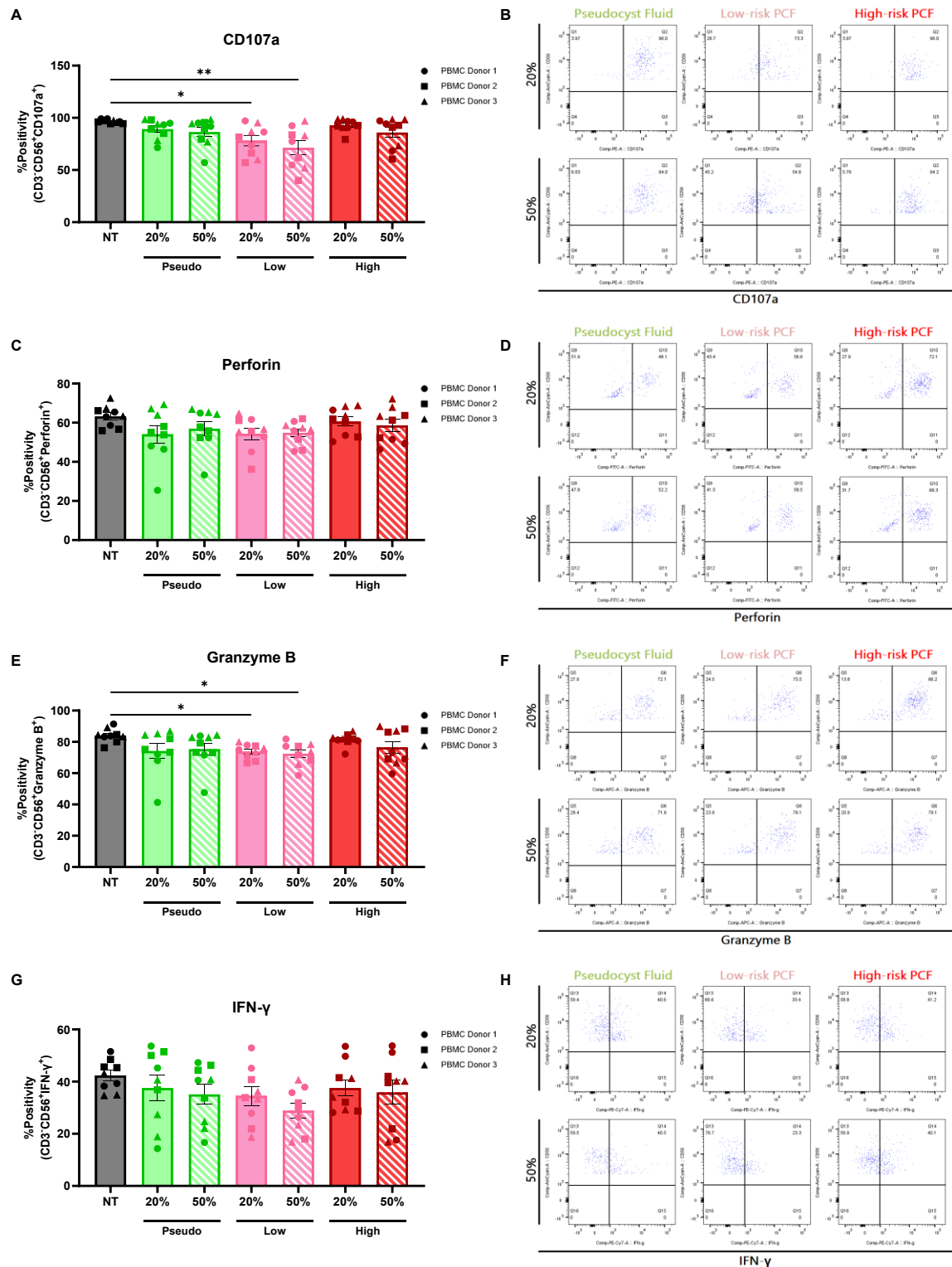


Figure 4.10: Changes in the cytotoxic capacity of NK cells were masked after a prolonged exposure to risk-stratified PCF due to upregulation of degranulation and cytotoxic markers, regardless of co-culture conditions. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 48 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) and 50% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 48 hours after activation. Flow cytometric staining was carried out to assess CD107a⁺ (A,

B), perforin⁺ (C, D), granzyme B⁺ (E, F) and IFN- γ ⁺ (G, H) expression in viable CD3⁺CD56⁺ NK cells. Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, and ** $p \leq 0.01$].

4.4.11 Effector cell-mediated cytotoxicity is promoted upon exposure to benign pseudocyst fluid and low-risk PCF but remains unaffected by high-risk PCF.

Lymphocyte killing assays were performed to investigate the effect of risk-stratified PCF on functional effector cell-mediated cytotoxicity on PC cells.

Lymphocyte-mediated cytotoxicity, as shown by decreased cell survival, in BxPC-3 PC cells was promoted upon exposure to benign pseudocyst fluid and pre-malignant low-risk PCF compared to high-risk PCF (Mean % survival $\pm$ SEM; Pseudocyst fluid vs. High-risk PCF: $60.18 \pm 2.21\%$ vs. $86.78 \pm 2.57\%$, $p=0.0049$ and Low-risk PCF vs. High-risk PCF: $50.60 \pm 6.08\%$ vs. $86.78 \pm 7.71\%$, $p=0.0006$) (**Figure 4.11A**). This effect was also recorded in Panc-1 cells co-cultured with benign pseudocyst fluid and pre-malignant low-risk PCF compared to high-risk PCF (Pseudocyst fluid vs. High-risk PCF: $73.28 \pm 1.76\%$ vs. $91.76 \pm 1.64\%$, $p=0.0030$ and Low-risk PCF vs. High-risk PCF: $67.67 \pm 4.23\%$ vs. $91.76 \pm 1.64\%$, $p=0.0006$) (**Figure 4.11B**). Importantly, this lymphocyte-mediated cytotoxicity was not promoted by co-culture with high-risk PCF.

This data suggests that patients with high-risk PCLs have less effective cytotoxic lymphocytes as a result of the soluble microenvironment of high-risk PCF, which likely impacts their ability to prevent aggressive PC development and progression.

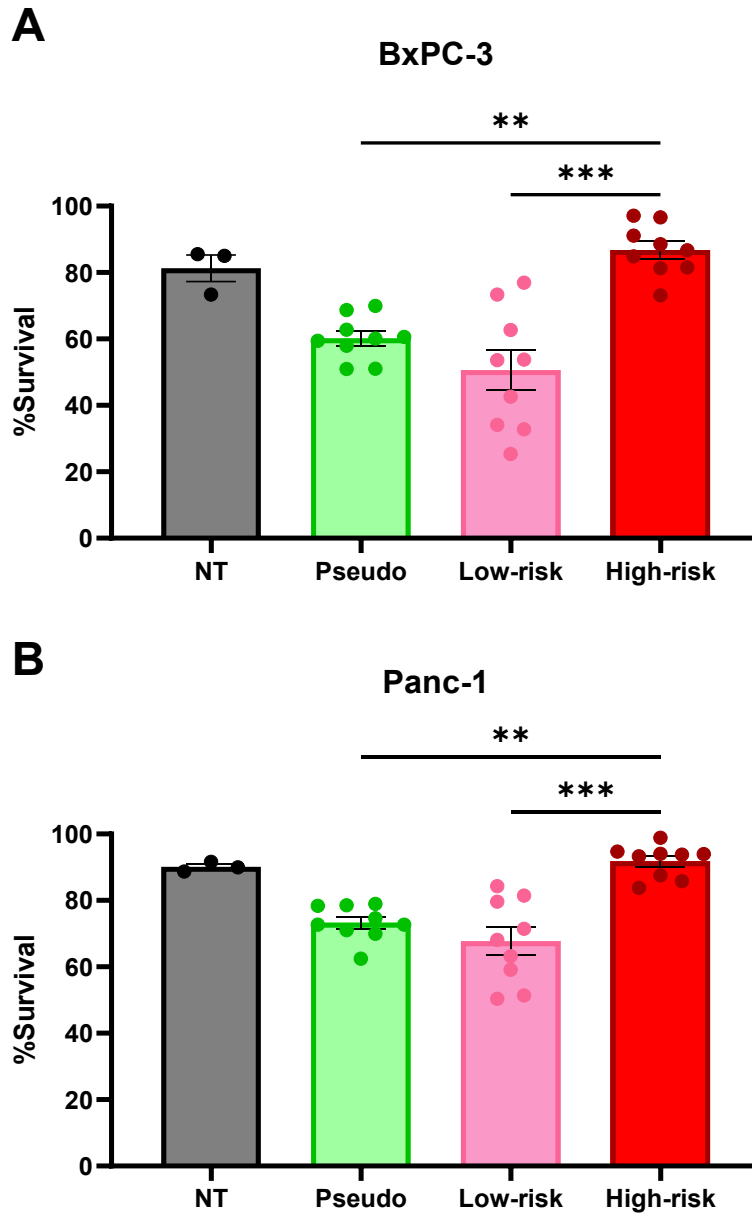


Figure 4.11: Effector cell-mediated cytotoxicity is promoted upon exposure to benign pseudocyst fluid and low-risk PCF but remains unaffected by high-risk PCF. Bar charts indicate percentage survival of BxPC-3 pancreatic cancer cells (n=3) (A) and Panc-1 pancreatic cancer cells (n=3) (B) co-cultured with cRPMI as a non-treated control (n=3), benign pseudocyst fluid (n=3), pre-malignant low-risk PCF (n=3), and pre-malignant high-risk PCF (n=3) in an effector:target (E:T) ratio of 5:1 (25,000:5000 cells) for 48 hours at 37°C. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as **p≤0.01, and ***p≤0.001].

4.5 Discussion

T cells and NK cells are largely implicated in anti-tumour immunity, and their effector function is crucial to eliminate tumour cells. It is well established that the anti-tumour immune response in PC is ineffective as effector immune cells are excluded from the TME through desmoplasia or dysregulated chemokine release^[290, 291]. This thesis focuses on investigating the impact of soluble immune factors within risk-stratified PCF on the function and phenotype of T cells and NK cells. Importantly, by assessing the effects of exposure to risk-stratified PCF on effector immune cells, this study provides evidence that immune dysregulation may begin even before the early stages of malignancy.

The viability of effector immune cells plays a key role in not only influencing the immune landscape but also allowing these cells to elicit effective anti-tumour immune responses. This study is the first of its kind to identify a significant decrease in the frequency of viable NK cells exposed to benign pseudocyst fluid compared to pre-malignant PCF. Similarly, benign pseudocyst fluid also impacted the viability of CD4⁺ T cells and CD8⁺ T cells. However, exposure to pre-malignant PCF did not adversely impact the viability of these effector immune populations, highlighting a clear distinction between the biological effects of benign inflammatory and pre-malignant cyst microenvironments. These findings suggest that benign pseudocysts, which arise in the context of inflammation and pancreatic tissue damage, contain soluble factors that may directly impair immune cell viability. Chapter 3 of this thesis revealed that benign pseudocyst fluid contained increased concentrations of inflammatory markers, including IL-1 β , TNF- α , IL-6 and IL-8, compared to pre-malignant PCF. Pancreatic pseudocyst fluid is also known to be enriched in digestive enzymes, including amylase, lipases and proteases, due to leakage from damaged pancreatic ducts^[292-294]. Such enzymes possess the capacity to disrupt cellular membranes and degrade extracellular and cell-surface proteins, thereby compromising immune cell integrity and survival^[295]. In addition to enzymatic activity, the physicochemical properties of benign pseudocyst fluid may further contribute to immune cell impairment. Pancreatic secretions are characteristically alkaline in nature in order to neutralise acidic gastric chyme under physiological conditions^[293]. Alterations in pH within benign pseudocyst fluid may therefore perpetuate cellular stress and reduce cell viability. In the literature at present, there are no studies that report the effects of benign pseudocyst fluid and pre-malignant PCF on T cell and NK cell viability in pre-malignant lesions. However, results presented in this thesis suggest that abundant

digestive enzymes and the elevated pH within benign pseudocyst fluid may perpetuate cellular stress and reduce cell viability. Conversely, exposure to pre-malignant PCF did not adversely affect T cell or NK cell viability, permitting immune cell survival which may allow for sustained immune surveillance and signalling.

The activation and differentiation states of T cells and NK cells are also key determinants of effective anti-tumour immunity. Data from this study shows that there was a decreased frequency of CD27, CD45RA and CD62L on CD4⁺ T cells and CD8⁺ T cells and decreased CD45RA and CD62L expressed on NK cells when co-cultured with benign pseudocyst fluid compared to the non-treated cRPMI control and pre-malignant PCF. There was little to no differential expression of these markers on T cells or NK cells when co-cultured with low-risk PCF or high-risk PCF compared to the non-treated cRPMI control. This study shows, for the first time, that the soluble microenvironment of benign pseudocyst fluid may transiently support immune activation, in contrast to pre-malignant PCF whereby effector immune cells are maintained in a naïve state. This finding was also confirmed when flow cytometric analysis of CD45RA and CD62L expression was carried out on effector immune cells isolated from healthy PBMC donors and co-cultured with pre-malignant PCF. CD45RA and CD62L are key surface markers used to delineate lymphocyte maturation into naïve (CD45RA⁺CD62L⁺), central memory (CD45RA⁻CD62L⁺), effector memory (CD45RA⁻CD62L⁻) and terminally differentiated effector memory (CD45RA⁺CD62L⁻) subsets^[296]. Within this thesis, it was found that low-risk and high-risk pre-malignant PCF maintains T cells in a more naïve state, whereas benign pseudocyst fluid permits activation and differentiation of T cells to become central memory and effector memory phenotypes, both of which are important for eliciting an immune response. Existing literature has provided evidence that immune cells, particularly T cells, are frequently maintained in a naïve state in cancer where activation is hindered by mediators of the TME as a key mechanism of cancer immune evasion^[264]. Maintenance of a naïve-like phenotype under pre-malignant conditions, as in the case of PCLs, may also represent a mechanism of immune evasion, limiting the formation of central and effector memory T cell subsets that are essential for eliciting robust anti-tumour responses.

CD103, also known as integrin α E β 7, is a transmembrane heterodimer complex involved in cell-cell or cell-matrix interactions and acts as a tissue resident memory marker^[297, 298].

CD103 mediates cell adhesion, migration and lymphocyte homing of cells through interaction with E-cadherin, which is broadly expressed on epithelial cells^[299]. Findings from this study show that CD103 expression is suppressed on CD4⁺ T cells, CD8⁺ T cells and NK cells upon exposure to benign pseudocyst fluid, and high-risk PCF to a lesser extent, compared to the non-treated cRPMI control. This suppression suggests that soluble immune factors within benign pseudocyst fluid and high-risk PCF may impair lymphocyte adhesion and migration within epithelial tissues and potentially dampens tissue-resident effector populations. These results contrast with findings reported in tumour settings. Webb *et al.* used flow cytometry to assess the frequency of T cells expressing CD103 in primary ascites fluid from patients with high-grade serous ovarian cancer and found that patients with advanced serous ovarian cancer had profoundly elevated frequencies of CD103-expressing CD8⁺ cells in ascites fluid^[300]. Similarly, Gorchs *et al.* demonstrated that CD8⁺ T cells expressing integrin CD103 accumulate in the central parts of the PC tumour, present an exhausted phenotype with high expression of immune checkpoint markers PD-1 and TIM-3, and have higher expression of chemokine receptors chemokine (C-C motif) receptor 5 (CCR5) and chemokine (C-X-C motif) receptor 6 (CXCR6)^[301]. Collectively, these studies indicate that CD103⁺ T cells can persist within established TMEs but may adopt a dysfunctional, exhausted phenotype under chronic antigen exposure and inflammatory stimulation. In contrast, the suppression of CD103 observed in this study following exposure to pre-malignant PCF may reflect an early regulatory immune mechanism that prevents the establishment of tissue-resident cytotoxic T or NK cell populations within cystic lesions.

The upregulation of inhibitory receptors is a defining feature of T cell exhaustion in the context of chronic infections and cancer^[228]. Within the TME, the presence of immunosuppressive cytokines, regulatory factors and persistent tumour antigen stimulation drive sustained and elevated expression of cell surface inhibitory immune checkpoint receptors such as CTLA-4, PD-1, TIM-3 and LAG-3 on tumour-infiltrating lymphocytes^[302]. Engagement of these inhibitory receptors with their respective ligands on cancer cells progressively diminishes effector functions and proliferative capacity^[228, 303, 304]. Little is understood about the effect of pre-malignant PCF on T cell activation and exhaustion. However, data from this study demonstrates that T cells and NK cells exposed to pre-malignant PCF acquire an exhausted phenotype, as is also seen in the non-treated cRPMI control, indicated by the upregulation and co-expression of inhibitory immune

checkpoint receptors PD-1, LAG-3 and TIM-3. In comparison, CD4⁺ T cells, CD8⁺ T cells and NK cells exposed to benign pseudocyst fluid exhibit a less exhausted phenotype compared to cells exposed to pre-malignant PCF or the non-treated cRPMI control. This suggests that soluble mediators in benign pseudocyst fluid may be preventing immune cell exhaustion. Conversely, soluble immune factors within pre-malignant PCF may instead prime effector cells towards immune dysfunction even before malignant transformation to PC. A number of studies investigating the immune profile of resected tissue from IPMN lesions by immunohistochemical analysis, have shown that immune landscapes evolve during precursor lesion progression, with increasing expression of immunoregulatory pathways^[111, 305]. Indeed, early IPMN lesions often show richer T cell infiltration that is lost in higher grade lesions, and other studies report immune activity in low-grade lesions that transitions to immunosuppression^[250, 305]. These observations reinforce the idea that immune suppression is not only a feature of invasive PC but may be initiated earlier. Elucidating the immunomodulatory factors within the soluble microenvironment of pre-malignant PCLs that specifically drive lymphocyte exhaustion could guide the development of targeted immunotherapeutic strategies. These strategies could potentially block immunosuppressive mediators such as IL-2, IL-10 and TGF- β , all of which were identified in pre-malignant high-risk PCF in Chapter 3. An alternative therapeutic approach to overcoming immune cell exhaustion in pre-malignant PCLs could involve supplementing pro-inflammatory cytokines such as IL-6 or TNF- α , also identified in Chapter 3, within the lesion to prevent immune exhaustion and sustain early anti-tumour immune surveillance. In a study carried out by Kane *et al.*, the proteome and transcriptome of PCF and patient-matched serum was extensively profiled, and a differential expression analysis revealed eight proteins that were significantly upregulated in high-risk PCF compared to low-risk PCF^[275]. One such protein identified in this multi-omic analysis was LGAL3, otherwise recognised as galectin-3^[275]. Emerging evidence has shown that galectin-3 binds to the inhibitory receptor LAG-3 on terminally differentiated T cells^[306]. Additionally, galectin-3 expression was also shown to be associated with increased activation of pro-inflammatory signalling pathways in CD8⁺ T cells^[306-309]. These findings together suggest that galectin-3 may also be a promising target to overcome immune cell exhaustion in high-risk pre-malignant PCLs.

Mature T cells can be classified as cytotoxic CD8⁺ T cells and CD4⁺ helper T cells (Th), which further differentiate into Th1, Th2, Th17 and Tregs^[272]. CD4⁺ Th1 cells secrete the

pro-inflammatory cytokine IFN- γ , which activates and supports CD8⁺ T cell cytotoxicity, while CD4⁺ Th2 cells exhibit pro-tumour functions by producing a range of cytokines, sustaining fibrosis through ECM and collagen deposition, and contributing to the differentiation of macrophages into a M2-immunosuppressive phenotype^[310]. The impact of soluble immune factors within benign pseudocyst fluid, low-risk PCF and high-risk PCF on T helper cell phenotypes remains widely understudied. However, the data from this study shows that activated T cells employed a predominant Th1 phenotype following co-culture with PCF, regardless of cancer risk stratification status indicating that PCF is perhaps not a key player in the initiation of the phenotypic switch from anti-tumour Th1 phenotype to a pro-tumour Th2 phenotype. Unlike the data generated from this study, previous investigations have shown that in PC, Th1-polarised CD4⁺ T cells are less frequent at the tumour site compared to Th2-polarised CD4⁺ T cells and their ratio correlates with patient survival^[246, 311]. In an independent study carried out by Roth *et al.* whereby immunohistochemical analysis of surgically resected tissue from IPMN lesions was performed, data showed that the relative abundance of Th1, Th2, Th9 and Th17 cell infiltrates in low-grade lesions decreases during IPMN progression to high-grade lesions and invasive carcinoma, unlike Treg subsets of CD4⁺ T cells, which increase during progression^[111]. Additionally, other studies have shown that the frequency of FoxP3⁺CD4⁺ Treg cells significantly increases during the malignant transformation of IPMN lesions and furthermore constitute a major player in the immunosuppressive microenvironment of PC and are often used as a negative prognostic biomarker in PC^[39, 43, 241, 311]. However, it is important to highlight the nature of these studies focusing on the immune landscape of resected tissue from precursor lesions and how it differs greatly to this study which is focusing on the immune component of pre-malignant PCF and how the soluble immune microenvironment influences T cell function and phenotype upon exposure. Although differing from what is recorded in literature, the findings from this study suggest that pre-malignant PCF alone may not be enough to drive the Th1 to Th2 phenotypic switch. Instead, additional elements such as stromal cell signalling, robust antigen exposure, or cell-cell contact *in vivo* may be required to drive this phenotypic shift.

As CD107a is a marker of cytotoxic degranulation, it was hypothesised that PCF with increased risk of PC development would impair the cytotoxic function of effector immune cells to overcome the anti-tumour immune response. Unexpectedly, the cytotoxic

potential, in terms of CD107a expression, of effector CD8⁺ T cells and NK cells was found to be downregulated irrespective of whether they were co-cultured with pseudocyst fluid, low-risk PCF or high-risk PCF. However, degranulation was depleted the most in the healthy cells co-cultured with low-risk PCF. Interestingly, in a study by Jun *et al.*, NK cells were isolated from PC patients and co-cultured with target cells and degranulation was subsequently analysed by flow cytometry^[312]. This study revealed that there was a progressive impairment of NK cell cytotoxic CD107a degranulation, which was associated with disease progression in PC^[312]. Tran *et al.* used multi-parameter flow cytometry to assess proliferation markers, functional markers and cytokine expression in activated CD4⁺ T cells and CD8⁺ T cells following co-culture with ascites fluid in ovarian cancer^[276]. This study found that pre-malignant ovarian ascites fluid induced a dramatic inhibition of CD107a degranulation in cytotoxic CD8⁺ T cells^[276]. In the context of pre-malignant PCLs, the cytotoxic degranulation process may be used as a mechanism to minimise local tissue damage that would otherwise be mediated by the release of cytotoxic granules from the cytolytic lysosome. This suppression of cytotoxic degranulation might also paradoxically favour the survival of dysplastic or malignant transformed epithelial cells, facilitating PCL persistence or progression.

Interestingly, intracellular expression of perforin was increased in CD8⁺ T cells that were co-cultured with benign pseudocyst fluid and high-risk PCF compared to the non-treated cRPMI control. Diminished intracellular perforin expression in CD8⁺ T cells co-cultured with low-risk PCF may be indicative of an effective degranulation of cytolytic granules and pore-forming capacity. Furthermore, NK cells demonstrated an elevated perforin expression compared to what was observed in CD8⁺ T cells, which may be a direct reflection of the decreased degranulation capacity of these cells after co-culture with PCF. In the PC setting, Yu *et al.* has shown that patients diagnosed with PC have decreased circulating CD8⁺ T cells in addition to decreased perforin expression within these cells compared to healthy subjects^[313]. Peng *et al.* also used flow cytometry to investigate surface receptor expression and cytotoxic granules of NK cells in patients with PC and found that the frequency of perforin-expressing circulating NK cells was significantly reduced in PC patients compared to healthy controls^[314, 315]. The data generated within this thesis suggests that even at a pre-malignant stage, the soluble immune microenvironment of PCF, specifically benign pseudocyst fluid and high-risk PCF, is

sufficient to impair cytotoxic degranulation in CD8⁺ T cells and NK cells causing these cell populations to retain higher levels of intracellular perforin.

Granzyme B elicits a cytotoxic effect and mediates apoptosis by cleaving caspase proteins in the target cell^[316]. At present, very little is known about granzyme B expression in precursor lesions of PC. This study demonstrates that there is an overall diminished expression of granzyme B, particularly in CD8⁺ T cells co-cultured with pre-malignant PCF compared to the non-treated cRPMI control. There is little to no difference in granzyme B expression observed in NK cells co-cultured with pseudocyst fluid, low-risk PCF or high-risk PCF when compared to the non-treated cRPMI control. It is likely that other granzymes, such as granzyme A and granzyme M, are also playing a role in the granzyme-mediated cytotoxic mechanisms of NK cells^[317]. However, the effect of benign and pre-malignant PCF on these granzyme molecules has yet to be studied. Data from this study suggests that overall, the soluble immune factors within PCF are sufficient to impair the cytotoxic killing capacity of healthy effector immune cells, through granzyme B production and retention, even before progression to invasive cancer. It is also worth considering that effector immune cells such as CD8⁺ T cells may be taking advantage of the impaired cytotoxic degranulation and granzyme B release caused by pre-malignant PCF, in order to prevent cytolytic damage to malignant target cells and permit the progression of PCLs to invasive PC. PC is widely known to be an immunologically “cold” tumour, and so existing literature reports that the immune cell infiltrate is mostly immunosuppressive Treg cells and MDSCs, while only a small proportion of the immune cell infiltrate are granzyme B⁺ cytotoxic CD8⁺ T cells^[223, 283, 318].

The cytokine, IFN- γ incites a pro-inflammatory microenvironment to recruit immune cells and factors to initiate apoptosis in target cells^[248, 319]. Data within this study demonstrates little to no differential expression of IFN- γ on T cells or NK cells when co-cultured with PCF compared to the non-treated cRPMI control. However, when differences in IFN- γ expression was assessed between risk cohorts of PCF, an increasing stepwise IFN- γ production was identified with increasing risk of cancer development particularly in cytotoxic CD8⁺ T cells after a 24-hour co-culture with pseudocyst fluid, low-risk PCF and high-risk PCF. These results potentially reflect a dysregulated inflammatory microenvironment within low-risk PCF and high-risk PCF that may be stimulating the synthesis of immune checkpoint inhibitory molecules, and specifically in

high-risk PCF, potentially suppressing the cytotoxic function of T cells and NK cells and furthermore recruiting immunosuppressive Treg and MDSC populations^[320-323].

Data from this study also shows that lymphocyte-mediated cytotoxicity, as indicated by decreased percentage cell survival, in PC cells was promoted upon exposure to benign pseudocyst fluid and pre-malignant low-risk PCF compared to high-risk PCF and the non-treated cRPMI control. Importantly, this lymphocyte-mediated cytotoxicity was not promoted by co-culture with pre-malignant high-risk PCF. A number of studies have shown that early PanIN lesions and low-grade IPMN lesions contain more abundant and less exhausted CD8⁺ T cells and CD4⁺ T cells, indicating that immune surveillance is more active at pre-malignant stages, while progression to invasive PDAC is marked by effector immune cell exclusion and T cell exhaustion that limit effective anti-tumour responses^[290, 324, 325]. These observations align with the findings in this study, implying that benign pseudocyst fluid and low-risk PCF harbour inflammatory mediators or stimuli that sustains lymphocyte-mediated cytotoxicity, whereas high-risk PCF may contain soluble factors that promote immunosuppression and effector immune cell exhaustion or otherwise lack sufficient soluble immunomodulatory mediators that promote lymphocyte-mediated cytotoxicity. This project has carried out an in-depth multiplex screen of risk-stratified PCF for immunomodulatory mediators including cytokines, adhesion molecules and soluble immune checkpoint molecules as discussed in Chapter 3, in addition to soluble chemokines and growth factors investigated in Chapter 5. However, further mechanistic and functional assays are needed to identify specific targets for restoring cytotoxic immune surveillance in the local microenvironment of high-risk PCLs.

This investigation into the effect of pre-malignant PCF on the cytotoxic capacity of effector immune cells, particularly towards PC cells suggests that high-risk PCF mediates dysregulated cytotoxic degranulation. Conversely, low-risk PCF may utilise an impaired cytotoxic response to minimise local inflammation and tissue damage. These differential immune profiles may reflect divergent paths towards PC initiation, and they suggest that candidate soluble mediators or checkpoint pathways could potentially serve as early biomarkers of PC progression or therapeutic targets. Restoring effector function and preventing premature exhaustion may ultimately promote the infiltration of functional effector immune cells into pancreatic lesions.

4.6 Study limitations

This study is the first to document the effect of risk-stratified PCF on effector immune cell phenotype and function. However, no study is without its limitations.

One such limitation of the data presented in this chapter relates to the availability of appropriate control samples. In this study, pseudocyst fluid was used as a benign comparator for pre-malignant PCLs. However, pseudocysts may not be representative of the most appropriate control as they are not classified as true cysts. Unlike pre-malignant PCLs, benign pseudocysts lack an epithelial lining and typically arise from a background of inflammation, such as pancreatitis. Consequently, their influence on the immune microenvironment is likely to differ substantially from that of pre-malignant lesions, which are not primarily inflammation-driven. More appropriate benign cyst comparators would include lymphoepithelial cysts, simple retention cysts or serous cystadenomas. However, such samples were not available for inclusion in this study.

A further limitation of this study concerns the volume of PCF available for co-culture experiments. Smaller lesions yielded reduced PCF volumes, which restricted co-culture capacity and limited the number of cells included in co-culture assays. As a result, in flow cytometric analyses the percentage frequency may be more susceptible to variability for less abundant markers.

4.7 Graphical summary

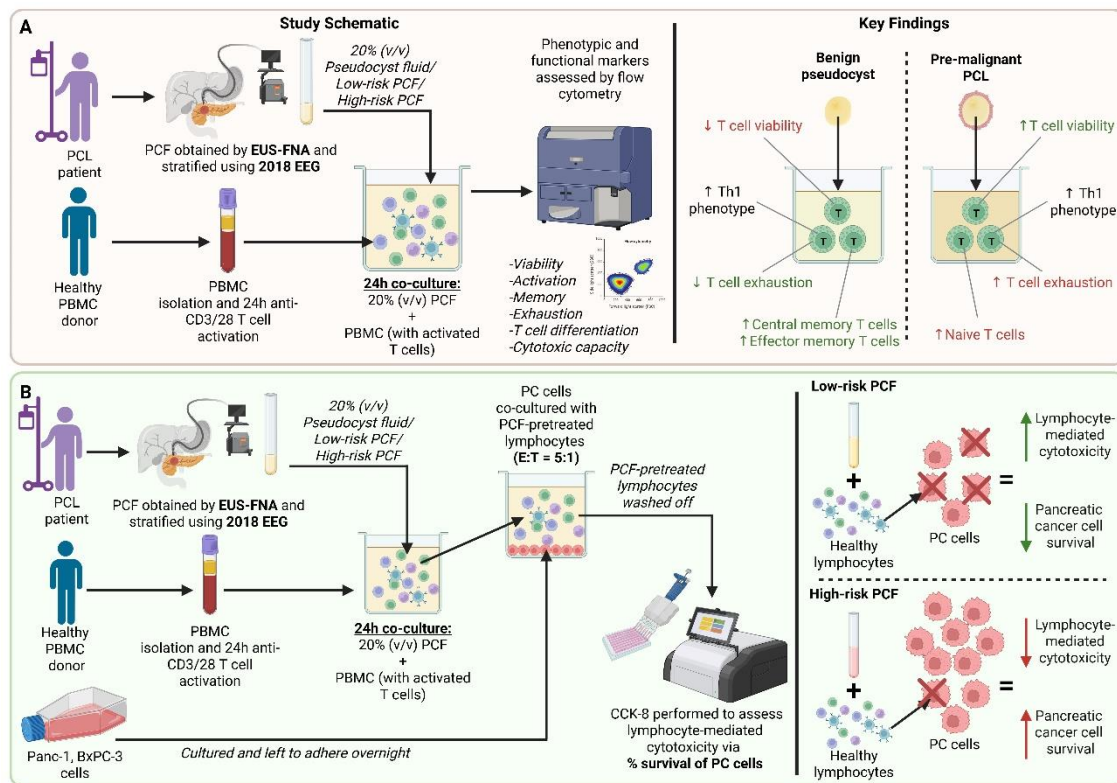


Figure 4.12: Graphical summary of study findings from results chapter 4. (A) PCF was isolated from risk-stratified PCL patients by EUS-FNA and co-cultured with healthy PBMCs for 24 hours. Phenotypic analysis was carried out by flow cytometry to assess the impact of PCF on T cell phenotype and function. Benign pseudocyst fluid decreased (↓) T cell viability and permitted T cell differentiation into central memory and effector memory subsets. Pre-malignant PCF maintained T cells in a naïve state. Pre-malignant PCF also promoted an exhausted phenotype in T cell subsets. (B) T cells from healthy PBMCs were activated and pre-treated for 24 hours with risk-stratified PCF. These PCF-pre-treated PBMCs were co-cultured with PC cells lines for 48 hours. A CCK-8 assay was then used to assess lymphocyte-mediated cytotoxicity on PC cells. Low-risk PCF promoted (↑) lymphocyte-mediated cytotoxicity, resulting in decreased (↓) PC cell survival. High-risk PCF decreased (↓) lymphocyte-mediated cytotoxicity, resulting in higher (↑) PC cell survival.

Chapter 5:
**Chemotactic effects of risk-stratified pancreatic
cyst fluid on effector immune cells**

5.1 Introduction

Current cancer risk stratification guidelines exhibit a vast lack of consensus on the appropriate and ideal use of surveillance and surgical intervention between institutions and geographical locations. This lack of consensus emphasises the need for more robust and standardised set of PCL characterisation guidelines to minimise unnecessary surgical intervention while ensuring appropriate surveillance and/or surgical intervention for patients at a high-risk of PC progression^[95, 166-170]. PCLs are precursor lesions of PC and offer a unique clinical opportunity for early detection and intervention before malignant transformation. However, despite this potential, PCLs remain poorly defined biologically and their risk of progression to PC is often difficult to predict due to limited understanding of the molecular and immunological mechanisms underlying the pathogenesis of pre-malignant PCLs^[72]. Identifying immune biomarkers within pre-malignant PCLs will aid to further define stratification guidelines and provide high-risk patients with the appropriate surveillance and timely intervention.

Chemotaxis is a process by which the movement of cells is directed in response to an extracellular chemical gradient^[326]. This process underlies the ability of immune cells to migrate to sites of infection, inflammation and tissue damage^[326, 327]. Chemokines drive the migration of leukocytes by binding to cognate G protein-coupled chemokine receptors expressed on the surface of cells^[328]. Chemotaxis is tightly regulated in healthy cells and is a process mediated specifically by chemokines, growth factors and their cognate receptors^[326]. However, during malignant progression, dysregulated chemokine signalling contributes to tumour progression, dissemination and poor immunotherapy responses in immune-excluded pancreatic tumours^[329-333]. Currently, the influence of PCL-secreted chemokines and growth factors on PC initiation, promotion and progression, in addition to immune cell migration, has yet to be elucidated.

The infiltration of immune cells into tissues, including in the TME, is a highly dynamic, multistep process^[334, 335]. This process involves immune cell activation in circulation, chemotactic guidance towards target tissues, adhesion to vascular endothelium, transmigration through endothelial barriers, and eventual infiltration into tissue^[334, 335]. Adhesion factors and integrins are critical regulators of chemotaxis and immune cell recruitment as a result of their capacity to act as the molecular “bridges” to permit circulating leukocytes to detect chemotactic cues, arrest at the endothelium, and migrate

into tissues^[258, 336, 337]. Altered expression of adhesion molecules or chemokine receptors can drastically influence immune cell trafficking, often resulting in an immune landscape that favours tumour escape. In the context of PCLs, dysregulation of adhesion and chemotactic signalling may represent one of the earliest immunomodulatory events that sets the stage for immune exclusion and immune escape in pancreatic carcinogenesis.

In a recent multi-omic study by Kane *et al.* investigating biomarkers predictive of patients at high risk of PC development, LGALS3, otherwise known as galectin-3, was found to be significantly upregulated in high-risk PCF compared to low-risk PCF^[275]. Galectin-3, a β -galactoside-binding lectin and ICAM-1 are two key immunomodulatory molecules involved in cell adhesion, proliferation and cell survival processes^[338, 339]. In the TME, galectin-3 interacts with integrins and various glycoproteins to modulate cell-cell and cell-matrix interactions^[340, 341]. It is reported to be frequently overexpressed in malignant tissues and has been linked to tumour growth, angiogenesis, metastasis, and immune suppression^[309, 342-344].

Similarly, ICAM-1 which is expressed on endothelial and epithelial cells has been shown to facilitate leukocyte trafficking and immune synapse formation^[345, 346]. Under physiological conditions, ICAM-1 facilitates leukocyte rolling, adhesion, and transmigration through the endothelium, serving as a crucial mediator of immune cell infiltration during inflammation^[345, 347]. However, in the TME, ICAM-1 expression can become dysregulated, paradoxically contributing to tumour invasion and metastasis by promoting adhesive interactions that favour tumour cell survival and dissemination^[259, 348]. Altered secretion or surface expression of galectin-3 and ICAM-1 within the epithelium lining of pre-malignant lesions may contribute to differential immune cell recruitment and immune evasion. As differences in galectin-3 and ICAM-1 expression have not previously been examined in risk-stratified pre-malignant PCLs, investigating these factors may yield novel insights into how chemotactic mechanisms are modulated during early tumourigenesis.

This study aims to address these important gaps in research by investigating the chemotactic migration of effector immune cells towards risk-stratified PCF. By characterising the soluble immunomodulatory profiles across pseudocyst fluid, low-risk PCF and high-risk PCF, this study also aims to delineate specific chemotactic and

adhesion-related cues that drive immune cell migration and infiltration towards PCF in the pre-malignant setting. Incorporating differential immunobiological features of pre-malignant PCLs into current risk stratification frameworks will enable appropriate longitudinal surveillance, to more accurately identify patients at a high-risk of PC development as candidates for earlier surveillance, and avoid unnecessary surgical interventions in low-risk cases. Additionally, the identification of immune targets within risk-stratified PCF may present potential avenues for novel immunotherapies to be used at pre-malignant stages to boost anti-tumour immunity in order to prevent PC development and late-stage diagnoses.

5.2 Hypothesis

Soluble immunomodulatory mediators within risk-stratified PCF differentially impacts effector immune cell migration.

5.3 Specific Aims

1. Study effector immune cell migration towards benign pseudocyst fluid, pre-malignant low-risk PCF and pre-malignant high-risk PCF.
2. Examine whether exposure to benign pseudocyst fluid or pre-malignant PCF alters expression of chemokine receptors on T cells derived from healthy PBMC donors.
3. Profile the soluble microenvironment of risk-stratified PCF to explore the immunomodulatory factors involved in mediating chemotactic effects.
4. Investigate the expression of galectin-3 in normal pancreatic cells and PC cell lines to identify and understand the role it may play in PC progression.

5.4 Results

5.4.1 Low-risk PCF exhibits higher effector immune cell migration compared to high-risk PCF and benign pseudocyst fluid.

The desmoplastic microenvironment in PC alters the structure and composition of the dense ECM, in addition to disrupting the adhesive capacity causing exclusion of anti-tumour effector immune cells. Furthermore, dysregulated chemokine release contributes to poor immunotherapy responses in immune-excluded pancreatic tumours. Chemokines are known to guide the migration of immune cells into tissues. However, the influence of PCL-secreted chemokines, cytokines and growth factors on PC initiation, promotion and progression is yet to be elucidated. In this study, functional Boyden chamber chemotaxis assays were performed to elucidate key chemokine pathways that drive immune cell migration within the soluble microenvironments of PCLs and surrounding tissues.

This study found that there was a significantly higher frequency of CD4⁺ T cell migration towards low-risk PCF compared to the serum-free RPMI control, a finding that is not seen in the case of high-risk PCF or benign pseudocyst fluid (Mean no. of migrated cells $\pm$ SEM; 1825.00 $\pm$ 556.50 migrated cells vs. 15.04 $\pm$ 4.97 migrated cells, $p=0.0047$) (**Figure 5.1A**). There was a significant increase in CD4⁺ T cell migration towards the 20% FBS RPMI positive chemotaxis control compared to the serum-free RPMI negative control, benign pseudocyst fluid and high-risk PCF (20% FBS RPMI vs. SF RPMI: 3111.00 $\pm$ 488.80 migrated cells vs. 15.04 $\pm$ 4.97 migrated cells, $p=0.0001$; 20% FBS RPMI vs. Pseudocyst fluid: 3111.00 $\pm$ 488.80 migrated cells vs. 194.20 $\pm$ 84.07 migrated cells, $p=0.0169$; 20% FBS RPMI vs. High-risk PCF: 3111.00 $\pm$ 488.80 migrated cells vs. 254.30 $\pm$ 108.50 migrated cells, $p=0.0149$) (**Figure 5.1A**). Although not significant, CD4⁺ T cells exhibited a higher frequency of cell migration towards low-risk PCF compared to high-risk PCF (1825.00 $\pm$ 556.50 migrated cells vs. 254.30 $\pm$ 108.50 migrated cells, $p=0.6453$) and benign pseudocyst fluid (1825.00 $\pm$ 556.50 migrated cells vs. 194.20 $\pm$ 84.10 migrated cells, $p=0.6989$) (**Figure 5.1A**).

Similarly, there was a significantly higher frequency of CD8⁺ T cell migration towards low-risk PCF compared to the serum-free RPMI control, which was also not observed in the case of high-risk PCF or benign pseudocyst fluid (582.10 $\pm$ 161.80 migrated cells vs. 5.01 $\pm$ 2.13 migrated cells, $p=0.0136$) (**Figure 5.1C**). There was a significant increase in CD8⁺ T cell migration towards the 20% FBS RPMI positive chemotaxis control compared to the serum-free RPMI negative control and benign pseudocyst fluid (20% FBS RPMI

vs. SF RPMI: 921.50 ± 126.80 migrated cells vs. 5.01 ± 2.13 migrated cells, $p=0.0001$; 20% FBS RPMI vs. Pseudocyst fluid: 921.50 ± 126.80 migrated cells vs. 49.55 ± 23.32 migrated cells, $p=0.0072$) (**Figure 5.1C**). $CD8^+$ T cells showed a non-significant increased frequency of cell migration towards low-risk PCF compared to high-risk PCF (582.10 ± 161.80 migrated cells vs. 126.70 ± 54.30 migrated cells, $p=0.9999$) and benign pseudocyst fluid (582.10 ± 161.80 migrated cells vs. 49.55 ± 23.32 migrated cells, $p=0.6380$) (**Figure 5.1C**).

There was a significant increase in NK cell migration towards RPMI supplemented with 20% FBS compared to the serum-free RPMI negative control and benign pseudocyst fluid (20% FBS RPMI vs. SF RPMI: 4622.00 ± 525.90 migrated cells vs. 55.74 ± 14.53 migrated cells, $p=0.0001$; 20% FBS RPMI vs. Pseudocyst fluid: 4622.00 ± 525.90 migrated cells vs. 32.56 ± 7.14 migrated cells, $p=0.0001$) (**Figure 5.1E**). NK cell migration was significantly higher towards low-risk PCF compared to benign pseudocyst fluid (582.80 ± 133.70 migrated cells vs. 32.56 ± 7.32 migrated cells, $p=0.0409$) and non-significantly higher when compared to high-risk PCF (582.80 ± 133.70 migrated cells vs. 369.60 ± 104.90 migrated cells, $p=0.9999$) (**Figure 5.1E**).

These preliminary results indicate that the migration of effector immune cell populations towards high-risk PCF is dysregulated compared to low-risk PCF.

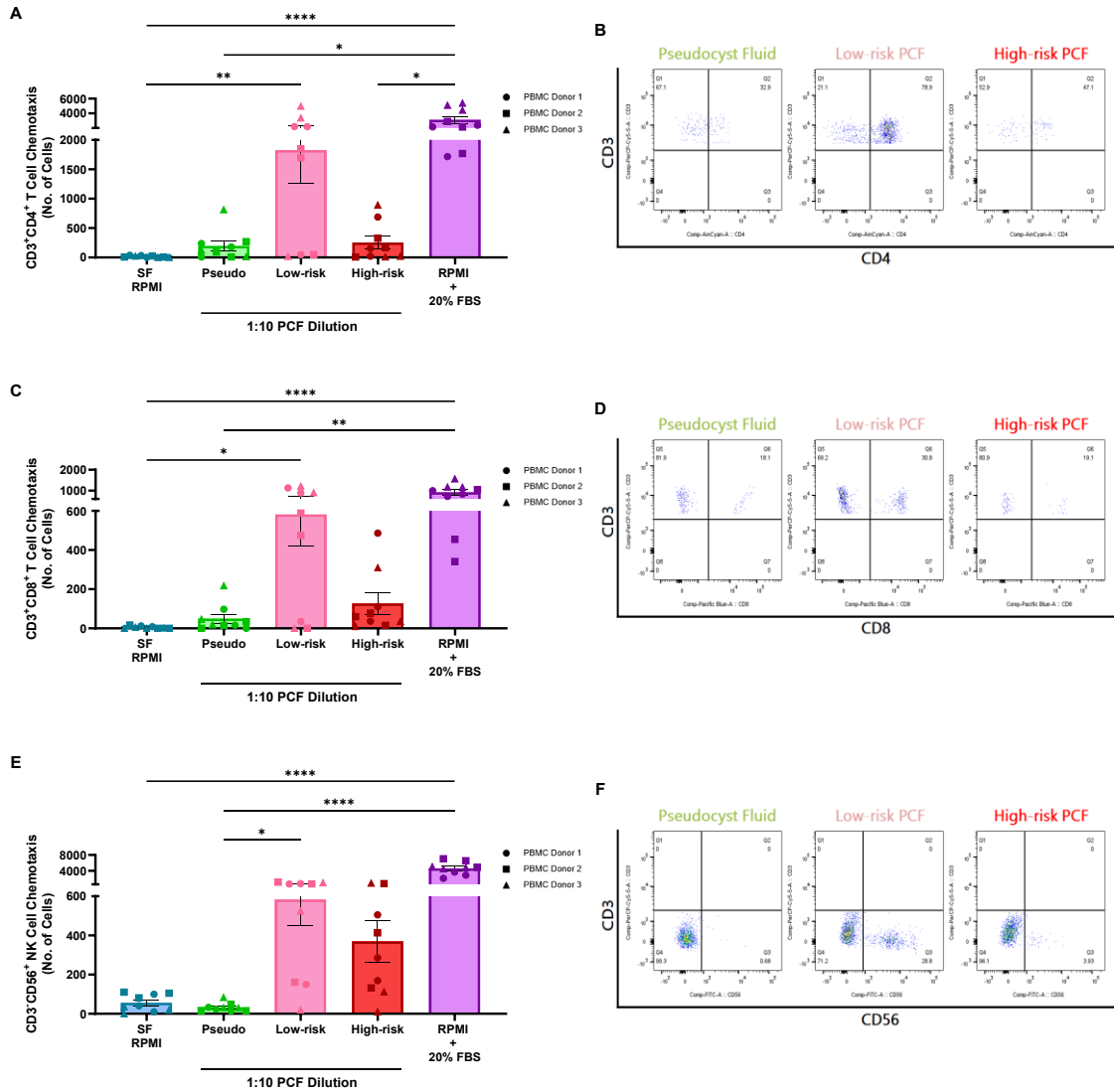


Figure 5.1: Low-risk PCF exhibits higher effector immune cell migration compared to high-risk PCF and benign pseudocyst fluid. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. The Boyden chamber chemotaxis assay was used to assess immune cell migration towards benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) diluted 1:10 with serum-free RPMI media. Flow cytometric staining was carried out to assess migration of viable CD3⁺CD4⁺ T cells (A), viable CD3⁺CD8⁺ T cells (C) and viable CD3⁺CD56⁺ NK cells (E). Representative dot plots show migrated CD3⁺CD4⁺ T cells (B), CD3⁺CD8⁺ T cells (D) and CD3⁺CD56⁺ NK cells (F) towards risk-stratified PCF. Graphical data are representative of mean no. of migrated cells $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.0001$].

5.4.2 Lower concentrations of high-risk PCF do not permit effector immune cell migration.

To further investigate if it is the soluble immune components within low-risk PCF that is promoting the migration of effector immune cells, as opposed to an inhibitory factor within high-risk PCF that may be hindering effector immune cell migration, a titration of high-risk PCF was carried out. Functional Boyden chamber chemotaxis assays were performed using titrated concentrations (1:10, 1:100 and 1:1000 dilutions) of high-risk PCF.

CD4⁺ T cells exhibited a non-significant trend of decreasing frequency of cells migrating towards decreasing concentrations of high-risk PCF. Although not significant, there is an evident decrease in the frequency of migrated CD4⁺ T cells exposed to high-risk PCF diluted 1:10 with serum-free RPMI compared to cells exposed to high-risk PCF diluted 1:100 with serum-free RPMI (756.90 ± 304.40 migrated cells vs. 243.30 ± 90.04 migrated cells, $p=0.9999$) (**Figure 5.2A**). Similarly, a non-significant decrease was identified in the frequency of migrated CD4⁺ T cells when cells were exposed to high-risk PCF diluted 1:100 compared to cells exposed to high-risk PCF diluted 1:1000 (243.20 ± 90.00 migrated cells vs. 73.22 ± 14.93 migrated cells, $p=0.9999$) (**Figure 5.2A**). There was a significant increase in CD4⁺ T cell migration towards the 20% FBS RPMI positive chemotaxis control compared to CD4⁺ T cells exposed to the negative serum-free RPMI control, high-risk PCF diluted 1:100 and high-risk PCF diluted 1:1000 (20% FBS RPMI vs. SF RPMI: 7590.00 ± 908.30 migrated cells vs. 62.33 ± 9.20 migrated cells, $p=0.0001$; 20% FBS RPMI vs. 1:100 high-risk PCF: 7590.00 ± 908.30 migrated cells vs. 243.20 ± 90.04 migrated cells, $p=0.0059$; 20% FBS RPMI vs. 1:1000 high-risk PCF: 7590.00 ± 908.30 migrated cells vs. 73.22 ± 14.93 migrated cells, $p=0.0002$) (**Figure 5.2A**).

CD8⁺ T cells also showed a non-significant trend of decreasing frequency of cell migration towards decreasing concentrations of high-risk PCF. Although not significant, there is an evident decrease in the frequency of migrated CD8⁺ T cells exposed to high-risk PCF diluted 1:10 compared to cells exposed to high-risk PCF diluted 1:100 (217.80 ± 83.80 migrated cells vs. 127.20 ± 44.81 migrated cells, $p=0.9999$) (**Figure 5.2C**). This non-significant decrease was also identified in CD8⁺ T cell migration when cells were exposed to high-risk PCF diluted 1:100 compared to cells exposed to high-risk PCF diluted 1:1000 (127.20 ± 44.81 migrated cells vs. 36.22 ± 9.27 migrated cells, $p=0.9999$) (**Figure 5.2C**). Similar to what was observed in CD4⁺ T cells, CD8⁺ T cell migration was

significantly increased towards the 20% FBS RPMI positive chemotaxis control compared to CD8⁺ T cells exposed to the serum-free RPMI negative chemotaxis control, high-risk PCF diluted 1:100 and high-risk PCF diluted 1:1000 (20% FBS RPMI vs. SF RPMI: 1218.00 ± 83.43 migrated cells vs. 32.78 ± 3.94 migrated cells, $p=0.0002$; 20% FBS RPMI vs. 1:100 High-risk PCF: 1218.00 ± 83.43 migrated cells vs. 127.20 ± 44.81 migrated cells, $p=0.0140$; 20% FBS RPMI vs. 1:1000 High-risk PCF: 1218.00 ± 83.43 migrated cells vs. 36.22 ± 9.27 migrated cells, $p=0.0001$) (**Figure 5.2C**).

NK cells exhibited a non-significant trend of decreasing frequency of cell migration towards decreasing concentrations of high-risk PCF. Although not significant, there is an evident decrease in the frequency of migrated NK cells exposed to high-risk PCF diluted 1:10 compared to cells exposed to high-risk PCF diluted 1:100 (328.40 ± 157.10 migrated cells vs. 166.00 ± 84.16 migrated cells, $p=0.9999$) (**Figure 5.2E**). Similarly, a non-significant decrease was identified in the frequency of migrated NK cells when cells were exposed to high-risk PCF diluted 1:100 compared to cells exposed to high-risk PCF diluted 1:1000 (166.00 ± 84.16 migrated cells vs. 98.11 ± 28.05 migrated cells, $p=0.9999$) (**Figure 5.2E**). This study found that there was also a significant increase in NK cell migration towards the positive RPMI control supplemented with 20% FBS compared to the serum-free RPMI chemotaxis negative control, high-risk PCF diluted 1:10, high-risk PCF diluted 1:100 and high-risk PCF diluted 1:1000 (20% FBS RPMI vs. SF RPMI: 12939.00 ± 1992.00 migrated cells vs. 176.80 ± 76.87 migrated cells, $p=0.0116$; 20% FBS RPMI vs. 1:10 High-risk PCF: 12939.00 ± 1992.00 migrated cells vs. 328.40 ± 157.10 migrated cells, $p=0.0065$; 20% FBS RPMI vs. 1:100 High-risk PCF: 12939.00 ± 1992.00 migrated cells vs. 166.00 ± 84.16 migrated cells, $p=0.0003$; 20% FBS RPMI vs. 1:1000 High-risk PCF: 12939.00 ± 1992.00 migrated cells vs. 98.11 ± 28.05 migrated cells, $p=0.0020$) (**Figure 5.2E**).

This data suggests that within PCLs, the presence of chemoattractant factors within high-risk PCF alone may not be enough to induce immune cell migration but in fact, the concentration of these factors is indeed important.

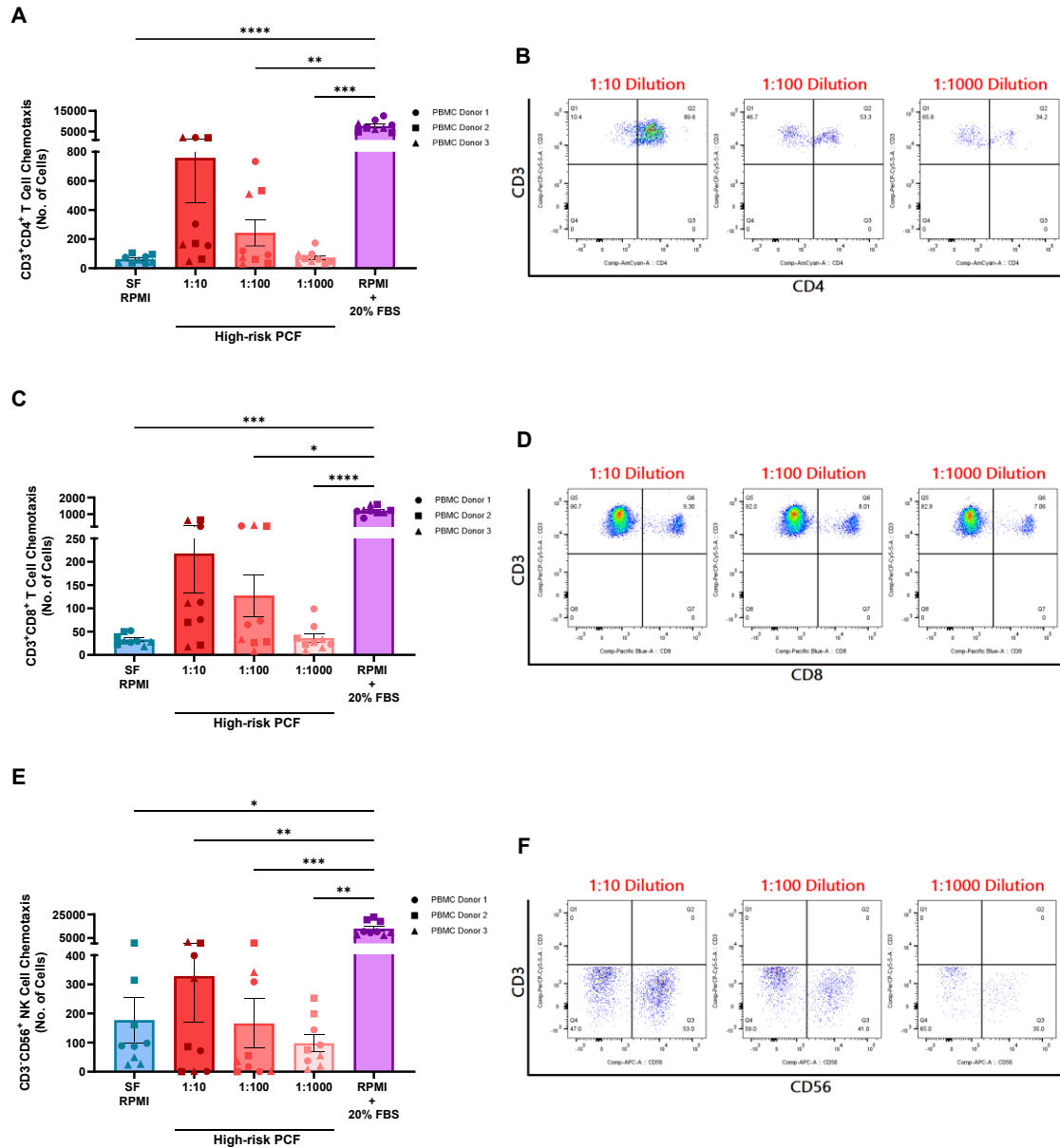


Figure 5.2: Lower concentrations of high-risk PCF do not permit effector immune cell migration. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. The Boyden chamber chemotaxis assay was used to assess immune cell migration towards high-risk PCF (n=3) diluted 1:10, 1:100 or 1:1000 with serum-free media. Flow cytometric staining was carried out to assess migration of viable CD3⁺CD4⁺ T cells (A), viable CD3⁺CD8⁺ T cells (C) and viable CD3⁺CD56⁺ NK cells (E). Representative dot plots show migrated CD3⁺CD4⁺ T cells (B), CD3⁺CD8⁺ T cells (D) and CD3⁺CD56⁺ NK cells (F) towards titrated high-risk PCF. Graphical data are representative of mean no. of migrated cells $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$].

5.4.3 Effector immune cell migration is increased towards high-risk PCF following a spike-in of recombinant fractalkine/RANTES/I-TAC chemokine cocktail.

To further investigate if immune cell migration can be reinstated towards high-risk PCF, a select number of biologically relevant recombinant chemokines were spiked into high-risk PCF and compared to a non-spiked high-risk PCF control. Functional Boyden chamber chemotaxis assays were performed to assess and quantify immune cell migration towards non-spiked high-risk PCF and high-risk PCF spiked with a chemokine cocktail of 100 ng/mL fractalkine, RANTES and I-TAC.

Although not significant, the addition of recombinant chemokines fractalkine, RANTES and I-TAC did increase CD4⁺ T cell migration towards high-risk PCF compared to non-spiked (non-treated) high-risk PCF (863.10 ± 358.80 migrated cells vs. 738.80 ± 389.00 migrated cells, p=0.9999) (**Figure 5.3A**). This study found that there was also a significant increase in CD4⁺ T cell migration towards the 20% FBS RPMI positive chemotaxis control compared to the serum-free RPMI negative control and towards non-spiked high-risk PCF, a finding not observed when high-risk PCF was spiked with fractalkine, RANTES and I-TAC chemokines (20% FBS RPMI vs. SF RPMI: 10284.00 ± 1164.00 migrated cells vs. 10.91 ± 5.23 migrated cells, p=0.0001; 20% FBS RPMI vs. NT High-risk PCF: 10284.00 ± 1164.00 migrated cells vs. 738.80 ± 389.00 migrated cells, p=0.0051) (**Figure 5.3A**).

A similar effect was seen when CD8⁺ T cell migration towards high-risk PCF was non-significantly increased when spiked with a recombinant chemokine cocktail of fractalkine, RANTES and I-TAC, compared to non-spiked high-risk PCF (779.20 ± 482.40 migrated cells vs. 224.50 ± 99.84 migrated cells, p=0.9999) (**Figure 5.3C**). This study also found that there was a significant increase in CD8⁺ T cell migration towards the 20% FBS RPMI positive chemotaxis control compared to serum-free RPMI and towards non-spiked high-risk PCF, which was not seen in the case of chemokine-spiked high-risk PCF (20% FBS RPMI vs. SF RPMI: 10506.00 ± 1352.00 migrated cells vs. 69.96 ± 45.51 migrated cells, p=0.0001; 20% FBS RPMI vs. NT High-risk PCF: 10506.00 ± 1352.00 migrated cells vs. 224.50 ± 99.84 migrated cells, p=0.0013) (**Figure 5.3C**).

NK cell migration was non-significantly increased towards high-risk PCF spiked with the recombinant chemokine cocktail, compared to non-spiked high-risk PCF (510.10 ± 260.30 migrated cells vs. 277.20 ± 121.70 migrated cells, p=0.9999) (**Figure 5.3E**). Similar to what was observed in T cells, NK cell migration was significantly increased

towards the positive RPMI control supplemented with 20% FBS compared to the serum-free RPMI chemotaxis negative control and towards non-spiked high-risk PCF and chemokine-spiked high-risk PCF (20% FBS RPMI vs. SF RPMI: 11282.00 ± 2117.00 migrated cells vs. 115.10 ± 29.33 migrated cells, $p=0.0004$; 20% FBS RPMI vs. NT High-risk PCF: 11282.00 ± 2117.00 migrated cells vs. 277.30 ± 121.70 migrated cells, $p=0.0033$; 20% FBS RPMI vs. chemokine-spiked High-risk PCF: 11282.00 ± 2117.00 migrated cells vs. 510.10 ± 260.30 migrated cells, $p=0.0033$) (**Figure 5.3E**).

These findings suggest that chemotactic dysregulation in high-risk PCLs is not driven by the presence of inhibitory factors, which would have otherwise increased lymphocyte migration upon titration, but rather by a reduced proportion of key chemokines within the PCF which may limit recruitment of effector immune cells. Consequently, restoring chemokine stores, like in the case of fractalkine, RANTES and I-TAC, may represent a potential therapeutic strategy to enhance immune cell infiltration in pre-malignant lesions with a high risk of PC progression.

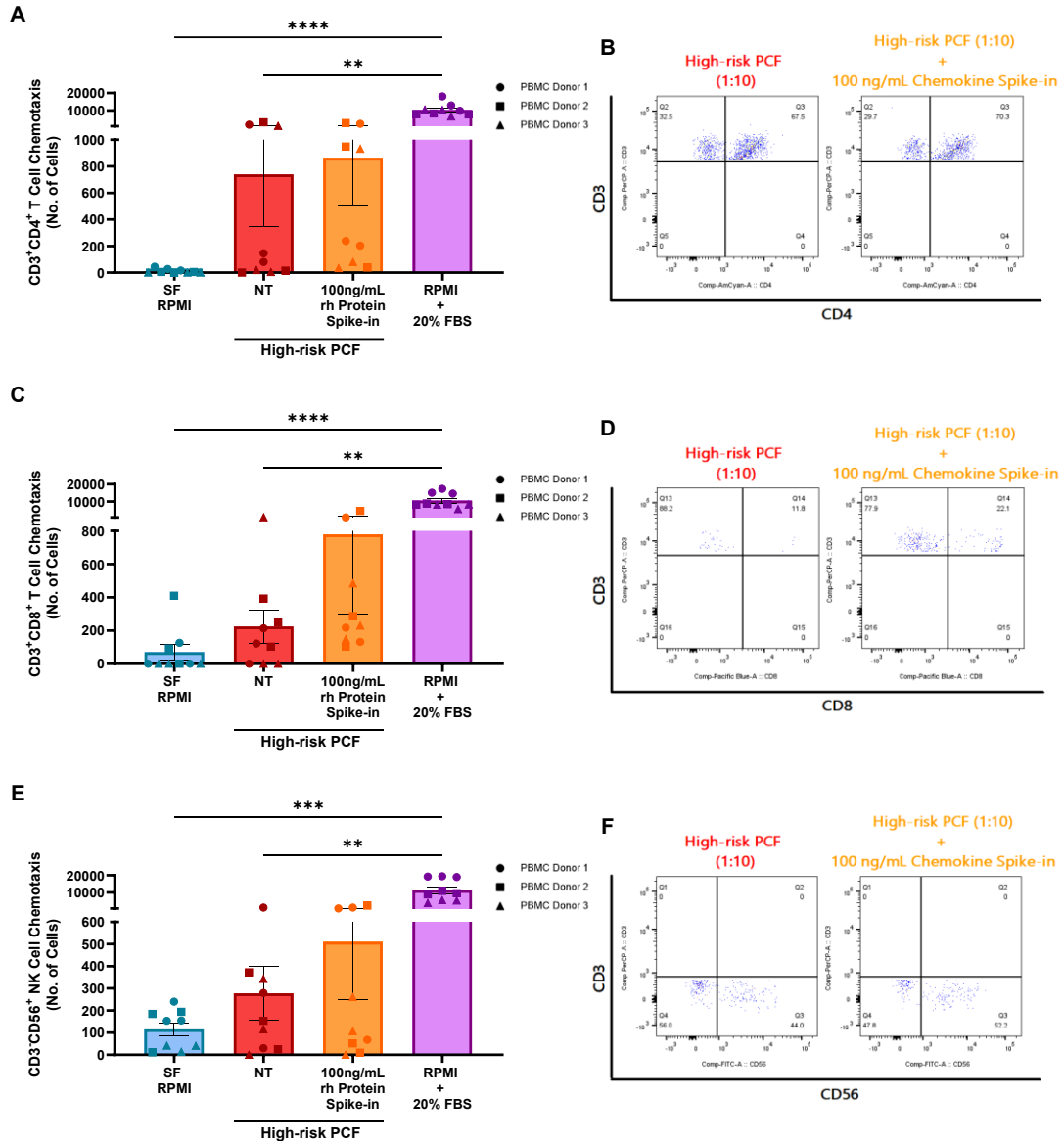


Figure 5.3: Effector immune cell migration is increased towards high-risk PCF following a spike-in of recombinant fractalkine/RANTES/I-TAC chemokine cocktail. PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. The Boyden chamber chemotaxis assay was used to assess immune cell migration towards high-risk PCF (n=3) and high-risk PCF spiked with 100ng/mL recombinant chemokines (fractalkine, RANTES, I-TAC) (n=3). Flow cytometric staining was carried out to assess migration of viable CD3⁺CD4⁺ T cells (A), viable CD3⁺CD8⁺ T cells (C) and viable CD3⁺CD56⁺ NK cells (E). Representative dot plots show migrated CD3⁺CD4⁺ T cells (B), CD3⁺CD8⁺ T cells (D) and CD3⁺CD56⁺ NK cells (F) towards non-treated high-risk PCF and high-risk PCF spiked with recombinant chemokines. Graphical data are representative of mean no. of migrated cells $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as **p $\leq$ 0.01, ***p $\leq$ 0.001, and ****p $\leq$ 0.0001].

5.4.4 Soluble innate immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.

To further elucidate how the constituents of risk-stratified PCF affect effector immune cell migration, this study investigated the soluble immune profiles of PCF. A custom flow cytometry-based multiplex ELISA was employed to screen PCF and patient-matched sera for a range of soluble chemokines, cytokines and growth factors associated with the promotion and progression of cancer. Further screening was carried out on additional PCF samples using a 54-plex MSD multiplex ELISA.

This initial screen of soluble innate immune cell-associated chemokines in risk-stratified PCF and patient-matched serum samples, focused on the expression and concentration of eotaxin, eotaxin-3, MCP-1, MCP-4, MDC, MIF, MIP-1 α , MIP-1 β , MIP-3 α and IL-8. These chemokines play a key role in recruiting innate immune cells such as eosinophils, monocytes, macrophages, neutrophils and basophils. Data from this study showed that there was a significantly higher concentration of MCP-4 in high-risk serum compared to matched high-risk PCF (Mean concentration $\pm$ SEM; 125.20 ± 49.39 pg/mL vs. 35.12 ± 2.17 pg/mL, $p=0.0312$) (**Figure 5.4D**). A similar result was observed for MDC concentration, which was significantly increased in high-risk serum compared to matched high-risk PCF (5589.00 ± 778.60 pg/mL vs. 16.31 ± 10.55 pg/mL, $p=0.0156$) (**Figure 5.4E**). Conversely, the concentration of IL-8 was significantly decreased in low-risk serum compared to matched low-risk PCF (2.46 ± 0.58 pg/mL vs. 246.30 ± 139.40 pg/mL, $p=0.0312$) (**Figure 5.4I**). Furthermore, this study identified a trend towards a significant increase in the concentration of MIF in serum from pseudocyst patients compared to patient-matched pseudocyst fluid samples (22.08 ± 1.09 pg/mL vs. 16.64 ± 0.60 pg/mL, $p=0.0625$) (**Figure 5.4F**). When the concentration of soluble innate cell-associated chemokines was investigated between risk-stratified serum samples, MIF concentration was shown to be significantly increased in serum isolated from high-risk PCL patients compared to serum from low-risk PCL patients (26.72 ± 4.20 pg/mL vs. 19.14 ± 0.43 pg/mL, $p=0.0317$) (**Figure 5.4F**). Although not significant, this effect was also observed across risk-stratified serum samples for the expression of eotaxin, eotaxin-3, MCP-4, MIP-1 α and MIP-1 β (**Figure 5.4A, B, D, G, H**). This study showed that there were no significant differences in the concentration of soluble innate cell-associated chemokines between risk-stratified PCF. However, in cases such as eotaxin-3, MCP-1, MCP-4, MIF and MIP-1 α , the concentration of these analytes was non-significantly

increased in low-risk PCF compared to benign pseudocyst fluid and high-risk PCF (**Figure 5.4B, C, D, F, G**).

This effect was mirrored in an independent screening study using the MSD multiplex ELISA, specifically in the case of chemokines: eotaxin, eotaxin-3, MCP-1, MCP-4, MIP-1 α and MIP-1 β (**Figure 5.4J-M, O, P**). Indeed, data from this study also revealed a trend towards a significant increase in the concentration of MDC in pre-malignant low-risk PCF compared to pre-malignant high-risk PCF (1155.00 ± 187.60 pg/mL vs. 2.11 ± 1.84 pg/mL, $p=0.0512$) (**Figure 5.4N**). Eotaxin exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (91.76 ± 22.44 pg/mL vs. 0.47 ± 0.08 pg/mL, $p=0.0219$) (**Figure 5.4J**). Additionally, there was a significantly higher concentration of MIP-1 β in low-risk PCF compared to high-risk PCF (70.97 ± 17.20 pg/mL vs. 0.49 ± 0.23 pg/mL, $p=0.0338$) (**Figure 5.4P**). **Table 5.1** also details the non-significant data for the concentration of soluble innate immune cell-associated chemokines in risk-stratified PCF and patient-matched sera.

Increased concentrations of soluble innate immune cell-associated chemokines in PCLs with a low-risk of PC progression may suggest that a more active innate immune environment may play a protective role in preventing progression of PCLs to PC.

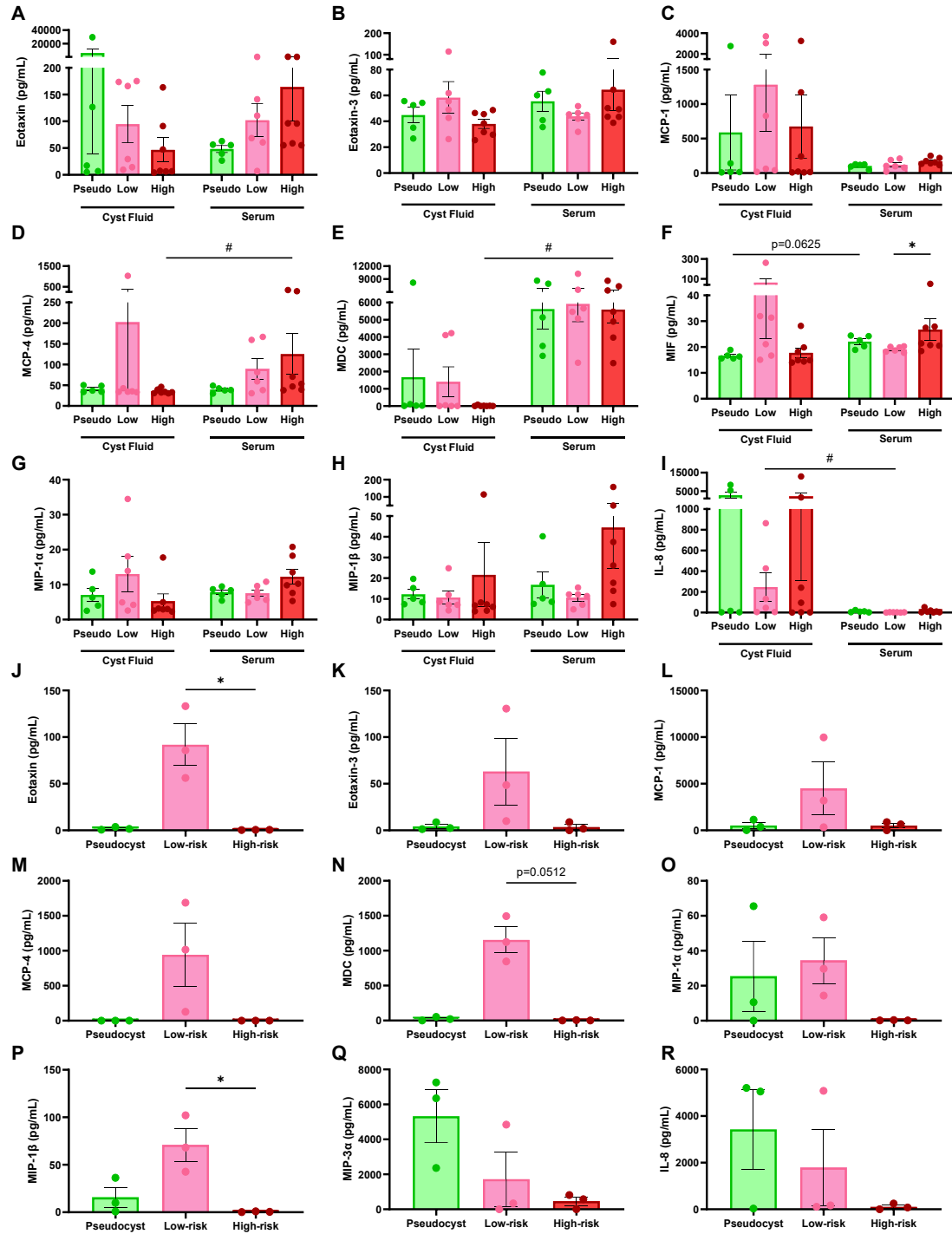


Figure 5.4: Soluble innate immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration. Pseudocyst fluid (n=5), low-risk PCF (n=6) and high-risk PCF (n=7) and patient matched sera were screened for the expression of soluble innate immune cell-associated chemokines involved in the promotion and progression of cancer using a custom flow cytometry-based multiplex ELISA; Eotaxin (A), Eotaxin-3 (B), MCP-1 (C), MCP-4 (D), MDC (E), MIF (F), MIP-1α (G), MIP-1β (H) and IL-8 (I). Further screening was carried out in a cohort of pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) using an MSD multiplex ELISA; Eotaxin (J), Eotaxin-3 (K), MCP-1 (L), MCP-4 (M), MDC (N), MIP-1α (O), MIP-1β (P), MIP-3α (Q) and IL-8 (R).

3 α (Q) and IL-8 (R). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as * $p \leq 0.05$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum) [significance defined as # $p \leq 0.05$].

Table 5.1: Mean concentration of soluble innate immune cell-associated chemokines in PCF and patient-matched serum.

		PCF				Serum				PCF vs. Serum
ELISA Platform	Analyte	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	<i>p-value Non-parametric paired t-test</i>
<i>Innate immune cell-associated chemokines</i>										
Assay Genie	Eotaxin (pg/mL)	5944.00	94.63	46.65	<i>NS</i>	48.28	102.20	163.90	<i>NS</i>	<i>NS</i>
	Eotaxin-3 (pg/mL)	44.83	58.34	37.97	<i>NS</i>	55.49	43.74	64.34	<i>NS</i>	<i>NS</i>
	MCP-1 (pg/mL)	588.00	1282.00	674.20	<i>NS</i>	102.70	117.20	170.10	<i>NS</i>	<i>NS</i>
	MCP-4 (pg/mL)	40.73	201.70	35.12	<i>NS</i>	39.37	89.22	125.20	<i>NS</i>	<i>0.0312 (Hv.H)</i>
	MDC (pg/mL)	1670.00	1399.00	16.31	<i>NS</i>	5620.00	5913.00	5589.00	<i>NS</i>	<i>0.0156 (Hv.H)</i>
	MIF (pg/mL)	16.64	62.93	17.76	<i>NS</i>	22.08	19.14	26.72	<i>0.0317 (Lv.H)</i>	<i>0.0625 (Pv.P)</i>
	MIP-1 α (pg/mL)	7.05	13.03	5.23	<i>NS</i>	7.81	7.53	12.22	<i>NS</i>	<i>NS</i>
	MIP-1 β (pg/mL)	12.17	10.67	21.62	<i>NS</i>	16.77	10.54	44.43	<i>NS</i>	<i>NS</i>
	IL-8 (pg/mL)	2767.00	246.30	2122.00		9.74	2.46	13.88	<i>NS</i>	<i>0.0312 (Lv.L)</i>
MSD	Eotaxin (pg/mL)	1.95	91.76	0.47	<i>0.0219 (Lv.H)</i>					
	Eotaxin-3 (pg/mL)	4.14	63.02	3.54	<i>NS</i>					
	MCP-1 (pg/mL)	514.50	4499.00	510.00	<i>NS</i>					
	MCP-4 (pg/mL)	0.52	943.10	0.08	<i>NS</i>					
	MDC (pg/mL)	24.58	1155.00	2.11	<i>0.0512 (Lv.H)</i>					
	MIP-1 α (pg/mL)	25.35	34.42	0.22	<i>NS</i>					
	MIP-1 β (pg/mL)	15.69	70.97	0.49	<i>0.0338 (Lv.H)</i>					
	MIP-3 α (pg/mL)	5324.00	1727.00	463.40	<i>NS</i>					
	IL-8 (pg/mL)	3430.00	1787.00	108.00	<i>NS</i>					

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. MCP=monocyte chemoattractant protein. MDC=macrophage-derived chemokine. MIF=macrophage inhibitory factor. MIP=macrophage inflammatory protein. IL=interleukin. NS=non-significant. Lv.H=low-risk vs. high-risk. Pv.P=pseudo vs. pseudo. Hv.H=high-risk vs. high-risk. Lv.L=low-risk vs. low-risk.

5.4.5 Soluble adaptive immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration.

The concentration of soluble adaptive immune cell-associated chemokines was also assessed in the screening of risk-stratified PCF and patient-matched sera. Adaptive immune cell-associated chemokines are essential for the recruitment of T cells, B cells and NK cells. This initial screening of soluble adaptive immune cell-associated chemokines in risk-stratified PCF and patient-matched sera examined the expression and concentration of fractalkine, RANTES, IP-10, I-TAC, SDF-1, TARC, TSLP, and IL-16. These chemokines are key mediators in the recruitment of adaptive immune cells, including CD4⁺ T helper cells, CD8⁺ cytotoxic T cells, B cells, and NK cells.

The concentration of fractalkine, a T-cell-associated chemokine involved in the homing of effector immune cells, was shown to be significantly increased in serum isolated from patients with high-risk PCLs compared to serum from patients with benign pseudocysts (550.00 ± 245.40 pg/mL vs. 96.76 ± 19.06 pg/mL, $p=0.0418$) (**Figure 5.5A**). Fractalkine was also significantly increased in high-risk serum compared to patient-matched high-risk PCF (550.00 ± 245.40 pg/mL vs. 46.07 ± 21.67 pg/mL, $p=0.0312$) (**Figure 5.5A**). RANTES, a T cell-associated chemokine, had a significantly higher concentration in high-risk serum compared to patient-matched high-risk PCF (13331.00 ± 933.60 pg/mL vs. 263.40 ± 254.90 pg/mL, $p=0.0156$) (**Figure 5.5B**). There was also a significantly higher concentration of RANTES in low-risk serum compared to low-risk PCF (14032.00 ± 629.50 pg/mL vs. 2563.00 ± 2546.00 pg/mL, $p=0.0312$) (**Figure 5.5B**). Data from this study also identified a trend towards a significant decrease in the concentration of IP-10 in serum from pseudocyst patients compared to patient-matched benign pseudocyst fluid (11.07 ± 0.73 pg/mL vs. 14.87 ± 2.68 pg/mL, $p=0.0625$) (**Figure 5.5C**). I-TAC was shown to have a significantly increased concentration in serum isolated from patients with high-risk PCLs compared to matched high-risk PCF (1421.00 ± 912.50 pg/mL vs. 12.66 ± 3.29 pg/mL, $p=0.0312$) (**Figure 5.5D**). Additionally, there was a trend towards a significant decrease in the concentration of SDF-1 in low-risk serum compared to patient-matched low-risk PCF (25.92 ± 0.29 pg/mL vs. 41.22 ± 5.64 pg/mL, $p=0.0625$) (**Figure 5.5E**). The concentration of thymus and activation regulated chemokine (TARC) was significantly increased in high-risk serum compared to high-risk PCF (67.04 ± 19.65 pg/mL vs. 7.88 ± 1.00 pg/mL, $p=0.0156$) (**Figure 5.5F**). Thymic stromal

lymphopoietin (TSLP), a Th2-associated chemokine, was also shown to have a significantly increased concentration in serum from patients with high-risk PCLs compared to matched high-risk PCF (12.90 ± 2.70 pg/mL vs. 8.93 ± 0.31 pg/mL, $p=0.0156$) (**Figure 5.5G**). There were no significant differences in the concentration of IP-10 and SDF-1 between risk-stratified PCF samples, risk-stratified serum samples or between matched-patient PCF and serum samples (**Figure 5.5C, E**).

In the independent screening study using the MSD multiplex ELISA, TARC, a chemokine that binds to CCR4 to function as a potent chemoattractant for Th2 cells, exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (153.40 ± 70.87 pg/mL vs. 2.15 ± 0.90 pg/mL, $p=0.0338$) (**Figure 5.5I**). Furthermore, there was a trend towards a significant increase in the concentration of IP-10 in pre-malignant low-risk PCF compared to pre-malignant high-risk PCF (1242.00 ± 311.60 pg/mL vs. 0.58 ± 0.32 pg/mL, $p=0.0512$) (**Figure 5.5H**). Additionally, a trend towards a significant increase in the concentration of IL-16 was also observed in low-risk PCF compared to PCF from patients with high-risk PCLs (1048.00 ± 448.2 pg/mL vs. 1.52 ± 1.25 pg/mL, $p=0.0688$) (**Figure 5.5K**). Although not significant, this effect was also recorded for the concentration of TSLP (**Figure 5.5J**). Non-significant data for the concentration of soluble adaptive immune cell-associated chemokines in risk-stratified PCF and patient-matched sera are detailed in **Table 5.2**.

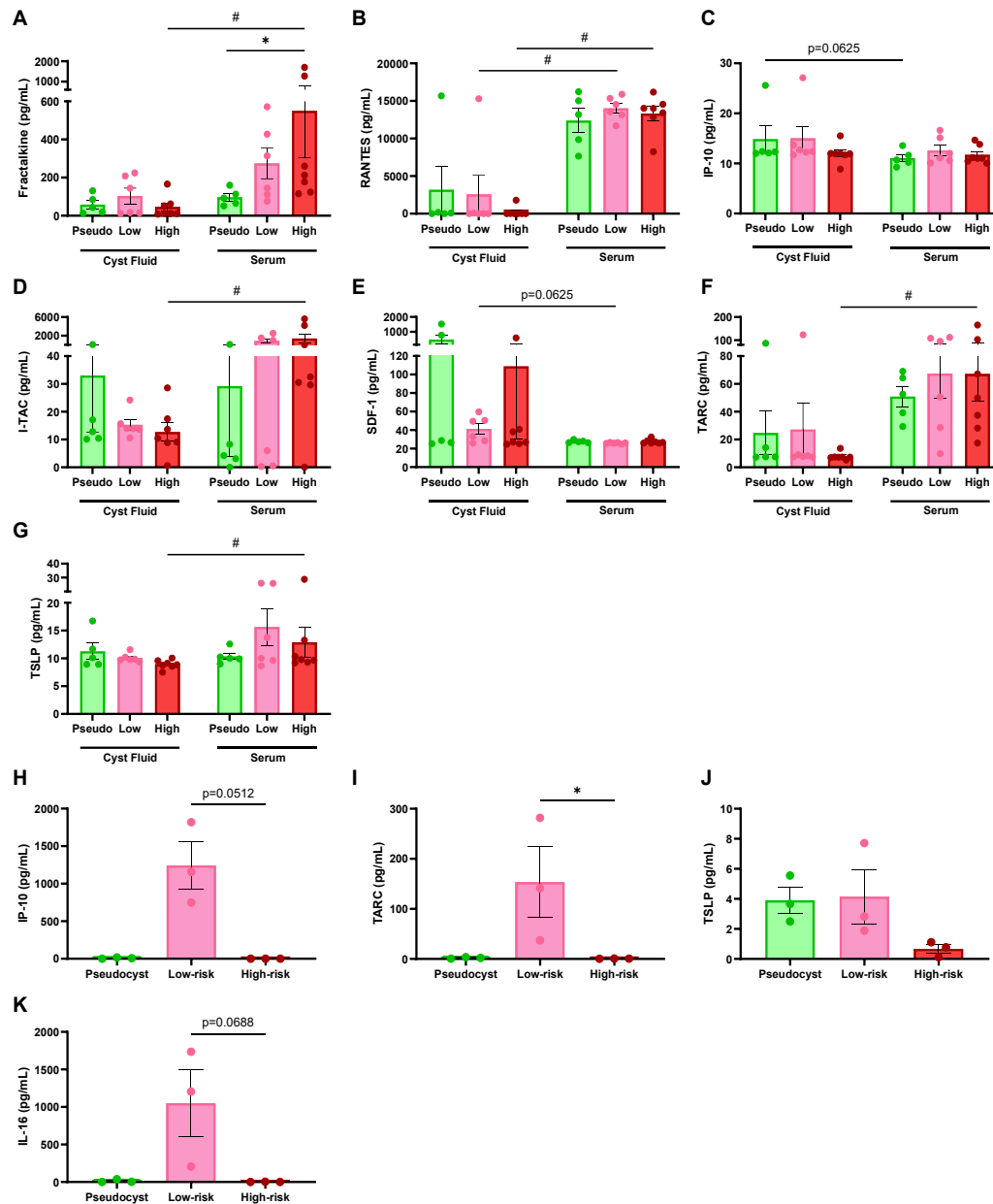


Figure 5.5: Soluble adaptive immune cell-associated chemokines are differentially expressed across risk-stratified PCF potentially influencing effector immune cell migration. Pseudocyst fluid (n=5), low-risk PCF (n=6) and high-risk PCF (n=7) and patient matched sera were screened for the expression of soluble adaptive immune cell-associated chemokines involved in the promotion and progression of cancer using a custom flow cytometry-based multiplex ELISA; Fractalkine (A), RANTES (B), IP-10 (C), I-TAC (D), SDF-1 (E), TARC (F), and TSLP (G). Further screening was carried out in an additional cohort of pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) using an MSD multiplex ELISA; IP-10 (H), TARC (I), TSLP (J) and IL-16 (K). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as * $p \leq 0.05$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum) [significance defined as # $p \leq 0.05$].

Table 5.2: Mean concentration of soluble adaptive immune cell-associated chemokines in PCF and patient-matched serum.

		PCF				Serum				PCF vs. Serum
ELISA Platform	Analyte	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	<i>p-value Non-parametric paired t-test</i>
<i>Adaptive immune cell-associated chemokines</i>										
Assay Genie	Fractalkine (pg/mL)	58.47	103.70	46.07	NS	96.76	274.20	550.00	0.0418 (Pv.H)	0.0312 (Hv.H)
	RANTES (pg/mL)	3184.00	2563.00	263.40	NS	12393	14032	13331	NS	0.0312 (Lv.L) 0.0156 (Hv.H)
	IP-10 (pg/mL)	14.87	14.96	11.99	NS	11.07	12.58	11.74	NS	0.0625 (Pv.P)
	I-TAC (pg/mL)	33.06	15.35	12.66	NS	29.21	845.80	1421.00	NS	0.0312 (Hv.H)
	SDF-1 (pg/mL)	474.30	41.22	108.90	NS	27.68	25.92	27.56	NS	0.0625 (Lv.L)
	TARC (pg/mL)	24.69	27.27	7.88	NS	50.90	67.42	67.04	NS	0.0156 (Hv.H)
	TSLP (pg/mL)	11.27	10.09	8.93	NS	10.36	15.65	12.90	NS	0.0156 (Hv.H)
MSD	IP-10 (pg/mL)	8.22	1242.00	0.58	0.0512 (Lv.H)					
	TARC (pg/mL)	2.15	153.40	0.62	0.0338 (Lv.H)					
	TSLP (pg/mL)	3.91	4.14	0.67	NS					
	IL-16 (pg/mL)	13.41	448.20	1.52	0.0688 (Lv.H)					

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. RANTES=regulated on activation, normal T cell expressed and secreted. IP-10=interferon- γ -induced protein 10. I-TAC=interferon-inducible T-cell alpha chemoattractant. SDF-1=stromal cell-derived factor 1. TARC=thymus and activation-regulated chemokine. TSLP=thymic stromal lymphopoietin. IL=interleukin. NS=non-significant. Lv.H=low-risk vs. high-risk. Pv.H=pseudo vs. high-risk. Pv.P=pseudo vs. pseudo. Hv.H=high-risk vs. high-risk. Lv.L=low-risk vs. low-risk.

5.4.6 Soluble angiogenic and growth factors are differentially expressed across risk-stratified PCF.

To better understand how risk-stratified PCF influences PCL progression to PC, the custom flow cytometry-based multiplex ELISA screen was also used to measure the concentration of angiogenic and growth factors, typically associated with cancer growth and progression, in both PCF and patient-matched serum samples. To broaden the scope of analysis, additional PCF samples were subsequently screened using a 54-plex MSD multiplex ELISA, allowing for a more extensive evaluation of the angiogenic and growth factor landscape of risk-stratified PCLs.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to have a significantly increased concentration in serum isolated from patients with high-risk PCLs compared to matched high-risk PCF (47.26 ± 23.71 pg/mL vs. 13.97 ± 1.61 pg/mL, $p=0.0312$) (**Figure 5.6B**). Additionally, there was a trend towards a significant increase in the concentration of GM-CSF in serum from patients with benign pseudocysts

compared to patient-matched pseudocyst fluid (23.35 ± 1.32 pg/mL vs. 14.83 ± 1.88 pg/mL, $p=0.0625$) (**Figure 5.6B**). The concentration of TGF- β 1 was significantly increased in high-risk serum compared to high-risk PCF (35373.00 ± 3845.00 pg/mL vs. 3333.00 ± 3159.00 pg/mL, $p=0.0156$) (**Figure 5.6C**). Moreover, this study revealed a trend towards a significant increase of TGF- β 1 concentration in low-risk serum compared to patient-matched low-risk PCF (38051.00 ± 6902.00 pg/mL vs. 4516.00 ± 2515.00 pg/mL, $p=0.0625$) (**Figure 5.6C**). Platelet-derived growth factor-AB heterodimer (PDGF-AB), had a significantly higher concentration in high-risk serum compared to patient-matched high-risk PCF (24777.00 ± 962.30 pg/mL vs. 91.40 ± 32.32 pg/mL, $p=0.0156$) (**Figure 5.6D**). There was also a significantly higher concentration of PDGF-AB in low-risk serum compared to low-risk PCF (22413.00 ± 4013.00 pg/mL vs. 1325.00 ± 1297.00 pg/mL, $p=0.0312$) (**Figure 5.6D**). There were no significant differences in the concentration of vascular endothelial growth factor A (VEGF-A) and hepatocyte growth factor (HGF) between risk-stratified PCF samples, risk-stratified serum samples or between matched-patient PCF and serum samples (**Figure 5.6A, E**).

In the additional screen using the 54-plex MSD multiplex ELISA, VEGF exhibited significantly higher concentrations within low-risk PCF compared to high-risk PCF (8528.00 ± 3532.00 pg/mL vs. 173.50 ± 166.60 pg/mL, $p=0.0219$) (**Figure 5.6F**). VEGF-A was also shown to have a significantly increased concentration in low-risk PCF compared to high-risk PCF (660482.00 ± 328016.00 pg/mL vs. 5620.00 ± 5090.00 pg/mL, $p=0.0219$) (**Figure 5.6G**). This study also identified a trend towards a significant increase in the concentration of VEGF-C in low-risk PCF compared to high-risk PCF (20290.00 ± 9497.00 pg/mL vs. 693.20 ± 639.20 pg/mL, $p=0.0688$) (**Figure 5.6H**). Similarly, the concentration of placental growth factor (PlGF), a member of the VEGF family that plays a crucial role in angiogenesis, was significantly increased in low-risk PCF compared to high-risk PCF (1522.00 ± 428.80 pg/mL vs. 0.00 ± 0.00 pg/mL, $p=0.0458$) (**Figure 5.6L**). Although not significant, this effect was also noted for the concentration of VEGF-D, GM-CSF, basic FGF, Tie-2 and Flt-1 (**Figure 5.6I-K, M, N**). **Table 5.3** details the non-significant data for the concentration of soluble angiogenic and growth factors in risk-stratified PCF and patient-matched sera.

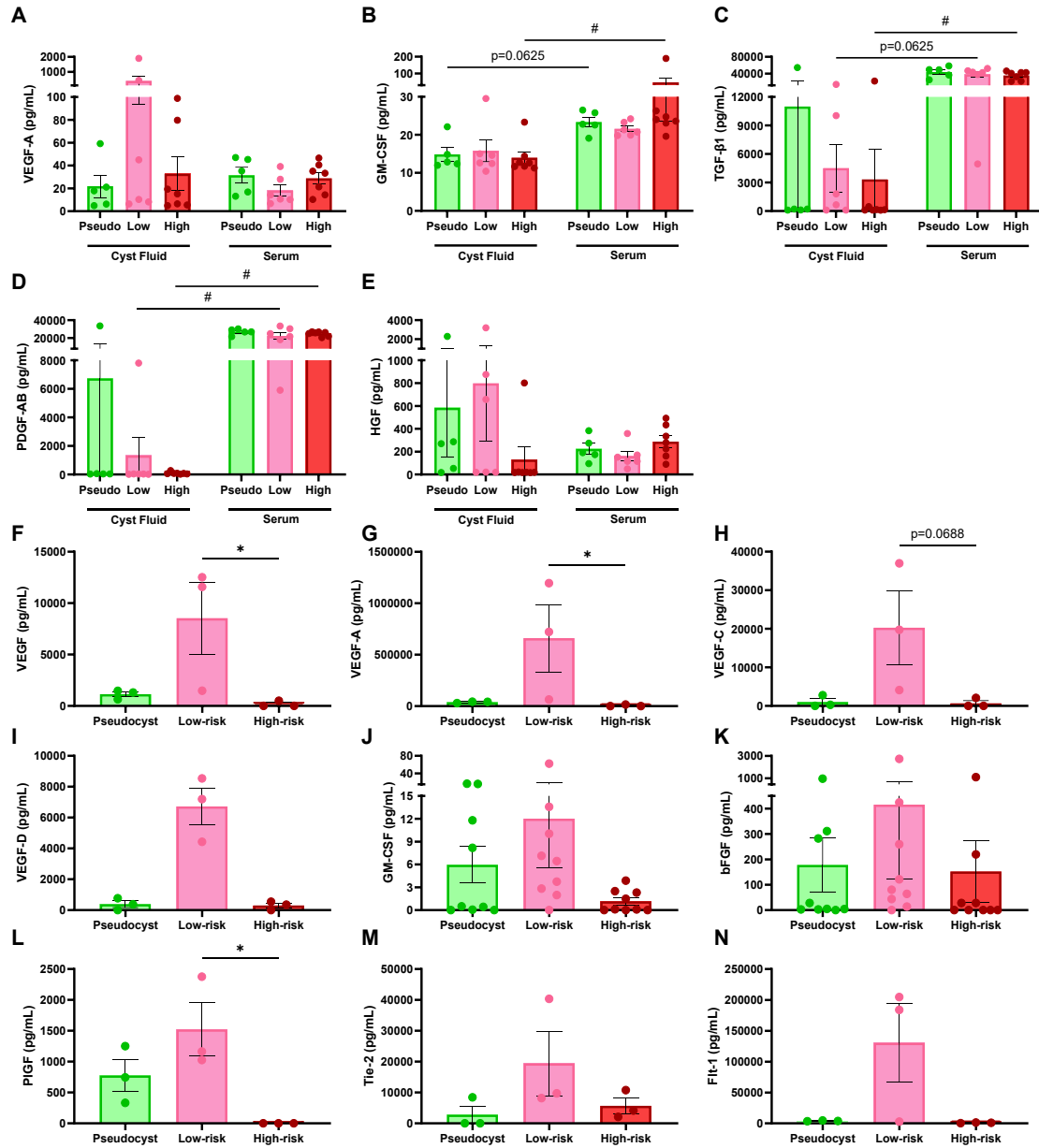


Figure 5.6: Soluble angiogenic and growth factors are differentially expressed across risk-stratified PCF. Pseudocyst fluid (n=5), low-risk PCF (n=6) and high-risk PCF (n=7) and patient matched sera were screened for the expression of soluble angiogenic and growth factors using a custom flow cytometry-based multiplex ELISA; VEGF-A (A), GM-CSF (B), TGF- β 1 (C), PDGF-AB (D), and HGF (E). Further screening was carried out in a cohort of pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) using an MSD multiplex ELISA (where additional samples were screened via MSD multiplex ELISA; Sample numbers: pseudocyst fluid (n=9), low-risk PCF (n=9) and high-risk PCF (n=9)); VEGF (F), VEGF-A (G), VEGF-C (H), VEGF-D (I) GM-CSF (J), basic FGF (K), PlGF (L), Tie-2 (M) and Flt-1 (N). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as * $p \leq 0.05$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum) [significance defined as # $p \leq 0.05$].

Table 5.3: Mean concentration of soluble angiogenic and growth factors in PCF and patient-matched serum.

		PCF				Serum				PCF vs. Serum
ELISA Platform	Analyte	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	Pseudo cyst	Low-risk	High-risk	<i>p-value Non-parametric one-way ANOVA</i>	<i>p-value Non-parametric paired t-test</i>
<i>Angiogenic and growth factors</i>										
Assay Genie	VEGF-A (pg/mL)	21.86	400.90	32.83	NS	31.52	18.13	28.76	NS	NS
	GM-CSF (pg/mL)	14.83	15.78	13.97	NS	23.35	21.56	47.26	NS	0.0312 (Hv.H) 0.0625 (Pv.P)
	TGF-β1 (pg/mL)	10993	4516	3333	NS	43790	38051	35373	NS	0.0156 (Hv.H) 0.0625 (Lv.L)
	PDGF-AB (pg/mL)	6738.00	1325.00	91.40	NS	26960	22413	24777	NS	0.0312 (Lv.L) 0.0156 (Hv.H)
	HGF (pg/mL)	583.40	797.50	130.40	NS	223.30	160.10	288.00	NS	NS
MSD	VEGF (pg/mL)	1140.00	8528.00	173.50	0.0219 (Lv.H)					
	VEGF-A (pg/mL)	38114.00	660482.00	5620.00	0.0219 (Lv.H)					
	VEGF-C (pg/mL)	1017.00	20290.00	693.20	0.0688 (Lv.H)					
	VEGF-D (pg/mL)	384.00	6721.00	300.60	NS					
	GM-CSF (pg/mL)	5.95	12.01	1.15	NS					
	FGF (pg/mL)	178.10	415.80	152.50	NS					
	PlGF (pg/mL)	775.90	1522.00	0.00	0.0458 (Lv.H)					
	Tie-2 (pg/mL)	2807.00	19421.00	5679.00	NS					
	Flt-1 (pg/mL)	3944.00	130459.00	897.80	NS					

PCF=pancreatic cyst fluid. ELISA=enzyme-linked immunosorbent assay. VEGF=vascular endothelial growth factor. GM-CSF=granulocyte-macrophage colony-stimulating factor. TGF=transforming growth factor. PDGF-AB=platelet-derived growth factor AB subunits. FGF=fibroblast growth factor. PlGF=placental growth factor. NS=non-significant. Lv.H=low-risk vs. high-risk. Hv.H=high-risk vs. high-risk. Pv.P=pseudo vs. pseudo. Lv.L=low-risk vs. low-risk.

5.4.7 Chemokine receptor expression is altered following co-culture with pre-malignant PCF.

It is known that chemokines greatly influence the immune infiltrate of the TME by driving chemotaxis of immune cell populations and their respective receptors^[349]. However, the influence of PCL-secreted chemokines, cytokines and growth factors on chemokine receptor expression and the associated effect on tumourigenesis is yet to be elucidated. This study assessed the expression of specific chemokine receptors in T cell populations, by flow cytometry, following co-culture with risk-stratified PCF. This is key to establish the ability of T cells to migrate in response to the chemotactic cues released into the PCF.

The MFI of chemokine (C-C motif) receptor 1 (CCR1), a receptor for ligands such as MIP-1α, RANTES, MCP-1 and eotaxin-3, was found to be significantly decreased on

CD4⁺ T cells following a 24-hour co-culture with high-risk PCF compared to benign pseudocyst fluid (Mean MFI $\pm$ SEM; 2703.00 $\pm$ 43.30 MFI vs. 3417.00 $\pm$ 213.20 MFI, $p=0.0040$) (**Figure 5.7A**). However, there was no significant difference in CCR1 expression on CD4⁺ T cells co-cultured with low-risk PCF when compared to high-risk PCF (**Figure 5.7A**).

Similarly, when compared to CD8⁺ T cells co-cultured with pseudocyst fluid, CCR1 expression was significantly decreased on cells co-cultured with low-risk PCF (3264.00 $\pm$ 81.30 MFI vs. 2856.00 $\pm$ 87.50 MFI, $p=0.0104$) and high-risk PCF (3264.00 $\pm$ 81.30 MFI vs. 2799.00 $\pm$ 36.90 MFI, $p=0.0032$) (**Figure 5.7B**). However, there was no significant difference in CCR1 expression on CD8⁺ T cells co-cultured with low-risk PCF compared to high-risk PCF (**Figure 5.7B**).

The expression of CCR2, a chemokine receptor for MCP-1, MCP-4 and eotaxin-3, was found to be non-significantly increased on CD4⁺ T cells following a 24-hour co-culture with benign pseudocyst fluid and low-risk PCF (**Figure 5.7D**). CD4⁺ T cells co-cultured with high-risk PCF for 24 hours non-significantly decreased the MFI of CCR2 compared to the non-treated cRPMI control, benign pseudocyst fluid and low-risk PCF (**Figure 5.7D**).

Similar trends in the MFI of CCR2 on CD8⁺ T cells was observed following a 24-hour co-culture with benign pseudocyst fluid, low-risk PCF and high-risk PCF (**Figure 5.7E**). Data from this study revealed a trend towards a significant decrease in the MFI of CCR2 expressed on CD8⁺ T cells co-cultured with high-risk PCF compared to low-risk PCF (1838.00 $\pm$ 122.30 MFI vs. 2396.00 $\pm$ 96.12 MFI, $p=0.0567$) (**Figure 5.7E**).

There was no significant difference in the MFI of CCR6, a chemokine receptor for MIP-3 α , on CD4⁺ T cells and CD8⁺ T cells co-cultured with PCF for 24 hours, irrespective of cancer risk stratification (**Figure 5.7G, H**).

The expression of CXCR3, a receptor for ligands such as IP-10 and I-TAC, was found to be significantly increased on the surface of CD4⁺ T cells following a 24-hour co-culture with low-risk PCF compared to with benign pseudocyst fluid (9109.00 $\pm$ 709.00 MFI vs. 6306.00 $\pm$ 606.60 MFI, $p=0.0121$) (**Figure 5.7J**). Although not significant, high-risk PCF also induced an increase in the MFI of CXCR3 compared to the non-treated media control and benign pseudocyst fluid. CXCR3 expression on CD4⁺ T cells co-cultured with high-

risk PCF was decreased when compared to low-risk PCF. However, this differential MFI of CXCR3 was found to be non-significant (**Figure 5.7J**).

Although similar trends were seen for the MFI of CXCR3 on CD8⁺ T cells, compared to CD4⁺ T cells, these differences were not statistically significant (**Figure 5.7K**).

CXCR4, a chemokine receptor for SDF-1 and MIF, was found to be significantly increased on CD4⁺ T cells following a 24-hour co-culture with risk-stratified PCF compared to the non-treated cRPMI media control. Specifically, when compared to CD4⁺ T cells co-cultured with cRPMI media, CXCR4 expression was significantly increased on cells co-cultured with benign pseudocyst fluid (5170.00 ± 108.30 MFI vs. 6459.00 ± 347.70 MFI, $p=0.0163$), low-risk PCF (5170.00 ± 108.30 MFI vs. 6421.00 ± 358.90 MFI, $p=0.0289$) and high-risk PCF (5170.00 ± 108.30 MFI vs. 6499.00 ± 360.20 MFI, $p=0.0163$) (**Figure 5.7M**). However, there were no significant differences between the MFI of CXCR4 on CD4⁺ T cells co-cultured with benign pseudocyst fluid, pre-malignant low-risk PCF or pre-malignant high-risk PCF (**Figure 5.7M**).

Similarly, CXCR4 expression was found to be significantly increased on CD8⁺ T cells following a 24-hour co-culture with risk stratified PCF compared to the cRPMI media control. Compared to CD8⁺ T cells co-cultured with cRPMI media, CXCR4 expression was significantly increased on cells co-cultured with benign pseudocyst fluid (6723.00 ± 74.86 MFI vs. 8408.00 ± 369.20 MFI, $p=0.0112$) and low-risk PCF (6723.00 ± 74.86 MFI vs. 8623.00 ± 519.30 MFI, $p=0.0089$). Furthermore, this study also revealed a trend towards a significant increase in the MFI of CXCR4 on CD8⁺ T cells co-cultured with high-risk PCF compared to the non-treated cRPMI control (8166.00 ± 453.30 MFI vs. 6723.00 ± 74.86 MFI, $p=0.0688$) (**Figure 5.7N**). No significant differences were identified in the MFI of CXCR4 between CD8⁺ T cells co-cultured with benign pseudocyst fluid, pre-malignant low-risk PCF or pre-malignant high-risk PCF (**Figure 5.7N**).

CX₃CR1, a chemokine receptor for fractalkine, was found to be non-significantly decreased on the surface of CD4⁺ T cells following a 24-hour co-culture with benign pseudocyst fluid compared to the cRPMI media control, low-risk PCF and high-risk PCF (**Figure 5.7P**). Additionally, there was no significant difference in the MFI of CX₃CR1 on CD4⁺ T cells co-cultured with low-risk PCF compared to high-risk PCF (**Figure 5.7P**).

Conversely, the MFI of CX₃CR1 was non-significantly increased on CD8⁺ T cells following a 24-hour co-culture with benign pseudocyst fluid compared to the non-treated cRPMI media control, low-risk PCF and high-risk PCF (**Figure 5.7Q**). However, there was no significant difference in the MFI of CX₃CR1 on CD8⁺ T cells co-cultured with low-risk PCF compared to high-risk PCF (**Figure 5.7Q**).

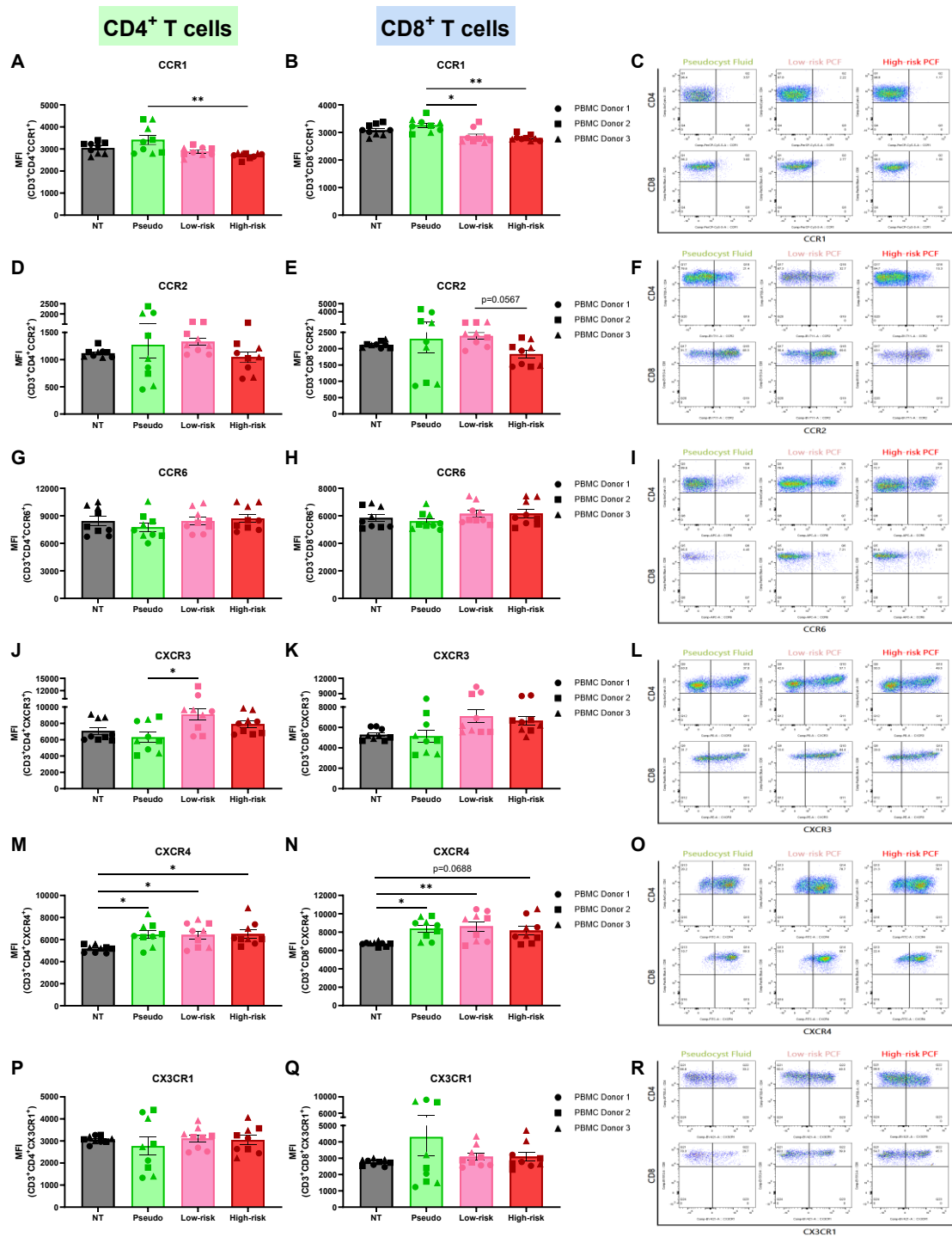


Figure 5.7: Chemokine receptor expression is altered following co-culture with pre-malignant PCF.

PBMCs were isolated from peripheral whole blood of healthy donors (n=3) and activated for 24 hours in the presence of plate-bound anti-CD3/anti-CD28 antibodies. PBMCs were co-cultured at 20% (v/v) with benign pseudocyst fluid (n=3), low-risk PCF (n=3) and high-risk PCF (n=3) for 24 hours after activation. Flow cytometric staining was carried out to assess CCR1⁺ (A, B, C), CCR2⁺ (D, E, F), CCR6⁺ (G, H, I), CXCR3⁺ (J, K, L), CXCR4⁺ (M, N, O) and CX₃CR1⁺ (P, Q, R) expression on the surface of viable CD3⁺CD4⁺ T cells (A, D, G, J, M, P) and viable CD3⁺CD8⁺ T cells (B, E, H, K, N, Q). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using a non-parametric one-way ANOVA (Kruskal-Wallis) with a Dunn's post-hoc test [significance defined as *p $\leq$ 0.05, and **p $\leq$ 0.01].

5.4.8 Under stress conditions, CD54 expression is increased in intermediary pancreatic cells but remains unaffected in normal pancreatic cells and PC cells.

To further understand how PCF impacts chemotactic immune responses, it's important to also consider how normal pancreatic epithelial and PC cells may themselves interact with immune cells. This study assessed the extracellular and intracellular expression of CD54 basally and under stress conditions by flow cytometry on normal H6c7-HPDE pancreatic epithelial cells, intermediary hTERT-HPNE pancreatic cells, BxPC-3 PC cells and Panc-1 PC cells. CD54, otherwise recognised as ICAM-1, is a cell surface protein that plays a crucial role in the immune system, particularly in leukocyte adhesion and extravasation^[347]. Understanding how the expression of adhesion molecules changes along the normal-neoplastic-cancer axis and also under stress conditions may provide insight into how immune cell migration may be facilitated by an appropriate chemotactic signal and adhesion molecules to permit lymphocyte-epithelial adhesion and extravasation.

There were no significant changes in cell surface CD54 frequency in normal pancreatic H6c7-HPDE cells cultured under normal conditions compared to cells cultured under hypoxia-induced stress (**Figure 5.8A**). Similarly, the frequency of intracellular CD54 remained unchanged in H6c7-HPDE cells when cultured under hypoxia-induced stress (**Figure 5.8B**). The frequency of CD54 in H6c7-HPDE cells cultured under normal conditions was significantly decreased intracellularly compared to cell surface expression (Mean frequency (%) $\pm$ SEM; 80.93 $\pm$ 2.65% vs. 97.10 $\pm$ 0.26%, p=0.0016) (**Figure 5.8C**). Similarly, CD54 expression in H6c7-HPDE cells cultured under stress conditions

was significantly decreased intracellularly compared to cell surface expression ($81.73 \pm 2.69\%$ vs. $96.47 \pm 0.83\%$, $p=0.0030$) (**Figure 5.8C**).

The frequency of cell surface CD54 on intermediary hTERT-HPNE cells was significantly increased in cells cultured under stress conditions compared to the basal frequency ($37.53 \pm 9.36\%$ vs. $30.33 \pm 8.15\%$, $p=0.0358$) (**Figure 5.8E**). The frequency of intracellular CD54 in these intermediary cells was also non-significantly increased in cells cultured under stress conditions compared to normal culture conditions (**Figure 5.8F**). Although not significant, the frequency of CD54 in hTERT-HPNE cells cultured under both normal and stress conditions was decreased intracellularly compared to the cell surface expression (**Figure 5.8G**).

There were no significant changes in the cell surface expression or intracellular expression of CD54 in BxPC-3 cancer cells cultured under normal or stress conditions (**Figure 5.8I-L**).

Similarly, there were no significant changes in the surface expression or intracellular expression of CD54 in Panc-1 cancer cells cultured under hypoxia-induced stress conditions compared to normal culture conditions (**Figure 5.8M, N**). However, this analysis showed that when Panc-1 cells were cultured under hypoxia-induced stress, the frequency of CD54 was significantly decreased intracellularly compared to cell surface expression ($72.72 \pm 3.45\%$ vs. $90.37 \pm 3.62\%$, $p=0.0402$) (**Figure 5.8O**).

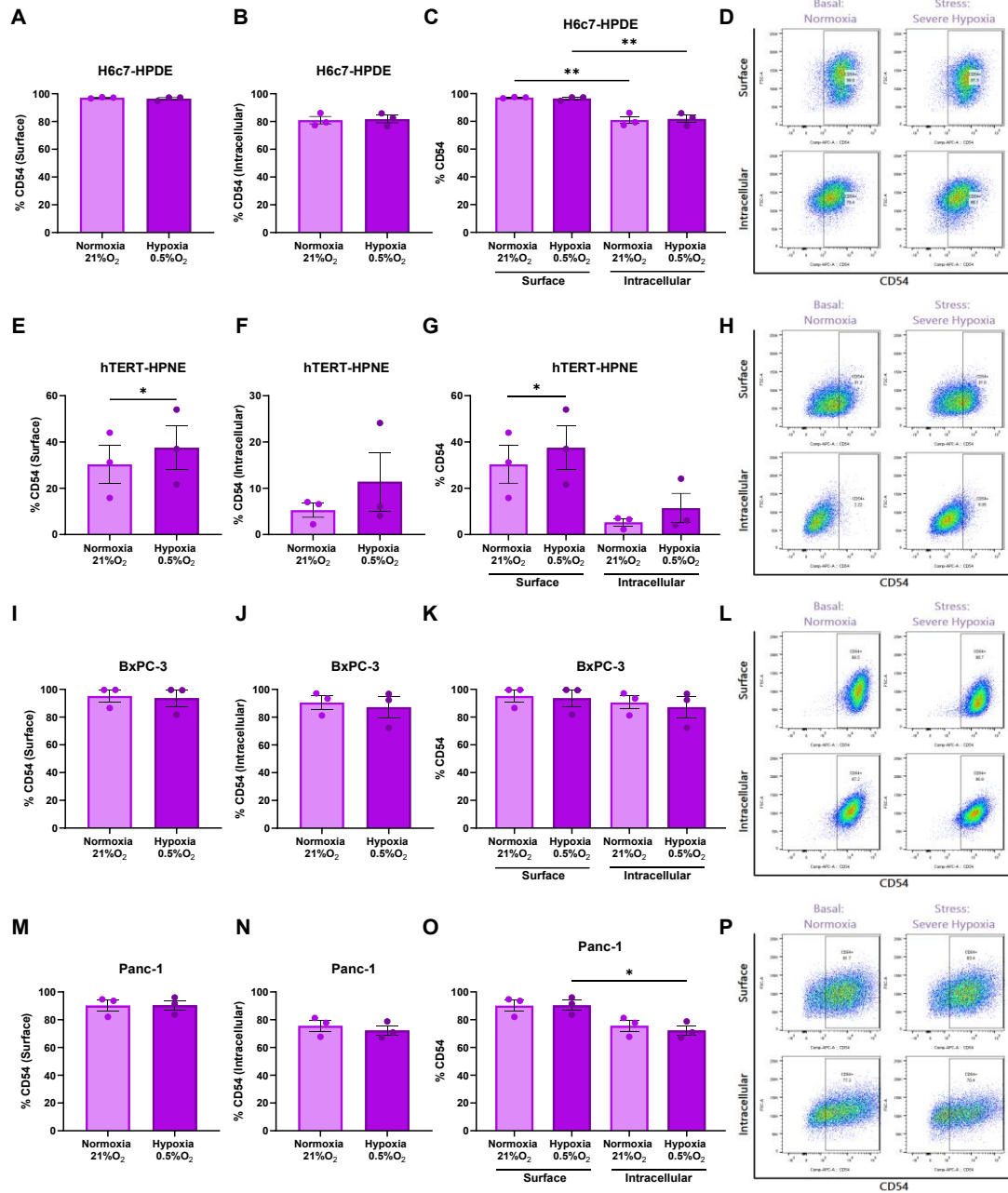


Figure 5.8: Under stress conditions, cell surface and intracellular CD54 expression is increased in intermediary pancreatic cells but remains unaffected in normal pancreatic cells and PC cells. Pancreatic cell lines (H6c7-HPDE, hTERT-HPNE, BxPC-3 and Panc-1) were cultured under basal conditions (normoxia: 21% O₂, 37°C) or stress conditions (severe hypoxia: 0.5% O₂, 37°C) for 24 hours. Flow cytometric staining was carried out to assess CD54⁺ expression on viable normal H6c7-HPDE cells (A, B, C, D), viable intermediary hTERT-HPNE cells (E, F, G, H), viable pancreatic cancer BxPC-3 cells (I, J, K, L), and viable pancreatic cancer Panc-1 cells (M, N, O, P). Graphical data are representative of mean percentage positivity $\pm$ SEM. Statistical significance was assessed using paired, parametric t-test [Surface expression; normoxia vs. hypoxia and Intracellular expression; normoxia vs. hypoxia. Significance defined as *p $\leq$ 0.05] and parametric ordinary one-way ANOVA [surface expression vs intracellular expression. Significance defined as *p $\leq$ 0.05, and **p $\leq$ 0.01].

5.4.9 Under stress conditions, cell surface galectin-3 expression is decreased in intermediary pancreatic cells and PC cells but upregulated in normal pancreatic cells.

A study carried out by Kane *et al.* to investigate multi-omic biomarkers predicting patients at a high risk of PC development found that LGALS3, the gene that encodes for the galectin-3 protein, was significantly upregulated in high-risk PCF compared to low-risk PCF^[275]. Galectin-3 is a β -galactoside-binding lectin that plays a multifaceted role in cell adhesion, proliferation and tumour cell survival^[350, 351]. Its biological functions are determined not only by its oligomeric state in the extracellular milieu but also by its subcellular localisation, as monomeric galectin-3 can reside in either the cytoplasm or the nucleus^[352, 353]. Broadly expressed across numerous cell types and tissues, galectin-3 can localise intracellularly within the nucleus and cytoplasm or be secreted via a non-classical pathway to the cell surface and extracellular milieu^[354, 355]. Through specific interactions with a range of intracellular and extracellular binding partners, galectin-3 regulates diverse biological processes and has been implicated in multiple physiological and pathological conditions, including immune responses, neoplastic transformation and metastasis^[354-356]. Within this study, the extracellular and intracellular expression of galectin-3 was assessed basally and under stress conditions (severe hypoxia) by flow cytometry on normal H6c7-HPDE pancreatic epithelial cells, intermediary hTERT-HPNE pancreatic cells, BxPC-3 PC cells and Panc-1 PC cells.

Although not significant, the frequency of cell surface galectin-3 in normal H6c7-HPDE cells was increased when the cells were cultured under stress conditions compared to the basal frequency (**Figure 5.9A**). There was no significant change in the intracellular expression of galectin-3 in H6c7-HPDE cells (**Figure 5.9B**). However, the basal frequency of galectin-3 in H6c7-HPDE cells cultured under normal conditions was significantly increased intracellularly compared to cell surface expression ($97.03 \pm 0.15\%$ vs. $0.04 \pm 0.001\%$, $p=0.0001$) (**Figure 5.9C**). Similarly, the frequency of galectin-3 in H6c7-HPDE cells cultured under hypoxic stress was significantly increased intracellularly compared to the surface expression ($94.07 \pm 0.87\%$ vs. $0.07 \pm 0.02\%$, $p=0.0001$) (**Figure 5.9C**).

Contrary to what was found in normal pancreatic cells, the frequency of cell surface galectin-3 on intermediary hTERT-HPNE pancreatic cells was significantly decreased in

cells cultured under hypoxic stress conditions compared to the basal frequency ($1.11 \pm 0.32\%$ vs. $1.91 \pm 0.29\%$, $p=0.0015$) (**Figure 5.9E**). There was no significant change in the intracellular expression of galectin-3 in hTERT-HPNE cells (**Figure 5.9F**). The basal frequency of galectin-3 in hTERT-HPNE cells cultured under normal conditions was significantly increased intracellularly compared to cell surface expression ($73.27 \pm 9.13\%$ vs. $1.91 \pm 0.29\%$, $p=0.0001$) (**Figure 5.9G**). Similarly, the frequency of galectin-3 in these intermediary cells cultured under hypoxic stress conditions was significantly increased intracellularly compared to the surface expression ($69.73 \pm 5.29\%$ vs. $1.11 \pm 0.32\%$, $p=0.0001$) (**Figure 5.9G**).

The frequency of cell surface galectin-3 on pancreatic cancer BxPC-3 cells was significantly decreased in cells cultured under hypoxic stress conditions compared to the basal frequency ($6.38 \pm 0.57\%$ vs. $8.92 \pm 0.85\%$, $p=0.0187$) (**Figure 5.9I**). There was also no significant change in the intracellular expression of galectin-3 in BxPC-3 cells (**Figure 5.9J**). The basal frequency of galectin-3 in BxPC-3 cells cultured under normal conditions was significantly increased intracellularly compared to cell surface expression ($93.70 \pm 3.92\%$ vs. $8.92 \pm 0.85\%$, $p=0.0001$) (**Figure 5.9K**). Similarly, the frequency of galectin-3 in these PC cells cultured under hypoxic stress conditions was significantly increased intracellularly compared to the surface expression ($91.13 \pm 6.72\%$ vs. $6.38 \pm 0.57\%$, $p=0.0001$) (**Figure 5.9K**).

Although not-significant, frequency of cell surface galectin-3 on pancreatic cancer Panc-1 cells, mirrors what is seen in BxPC-3 cells and was decreased in cells cultured under hypoxic stress conditions compared to the basal frequency (**Figure 5.9I**). Additionally, there was no significant change in the intracellular expression of galectin-3 in Panc-1 cells (**Figure 5.9J**). The basal frequency of galectin-3 in Panc-1 cells cultured under normal conditions was significantly increased intracellularly compared to cell surface expression ($94.07 \pm 1.34\%$ vs. $0.80 \pm 0.07\%$, $p=0.0001$) (**Figure 5.9K**). Similarly, the frequency of galectin-3 in Panc-1 cells cultured under hypoxic stress conditions was significantly increased intracellularly compared to the surface expression ($90.47 \pm 2.23\%$ vs. $0.57 \pm 0.07\%$, $p=0.0001$) (**Figure 5.9K**).

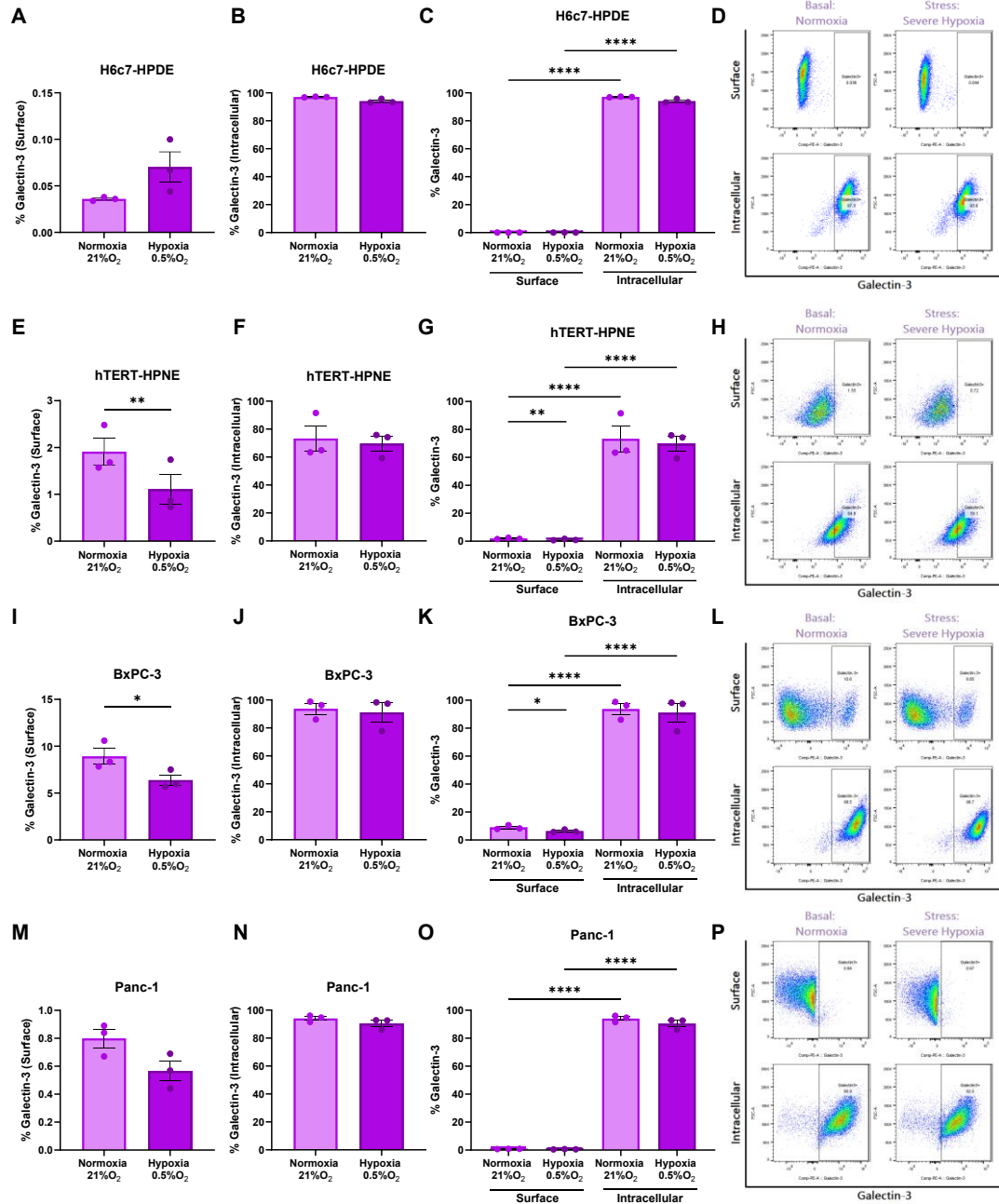


Figure 5.9: Under stress conditions, cell surface galectin-3 expression is decreased in intermediary pancreatic cells and PC cells but upregulated in normal pancreatic cells. Pancreatic cell lines (H6c7-HPDE, hTERT-HPNE, BxPC-3 and Panc-1) were cultured under basal conditions (normoxia: 21% O₂, 37°C) or stress conditions (severe hypoxia: 0.5% O₂, 37°C) for 24 hours. Flow cytometric staining was carried out to assess Galectin-3⁺ expression on viable normal H6c7-HPDE cells (A, B, C, D), viable intermediary hTERT-HPNE cells (E, F, G, H), viable pancreatic cancer BxPC-3 cells (I, J, K, L), and viable pancreatic cancer Panc-1 cells (M, N, O, P). Graphical data are representative of mean percentage positivity ± SEM. Statistical significance was assessed using paired, parametric t-test [Surface expression; normoxia vs. hypoxia and Intracellular expression; normoxia vs. hypoxia. Significance defined as *p ≤ 0.05, and **p ≤ 0.01] and parametric ordinary one-way ANOVA [surface expression vs intracellular expression. Significance defined as *p ≤ 0.05, **p ≤ 0.01, and ****p ≤ 0.0001].

5.4.10 Soluble galectin-3 is increased in high-risk PCF and predominantly localises in the cytoplasmic compartment of pancreatic cell lines.

Building upon previous findings reported by Kane *et al.* and also from data generated within this study, the present study aims to quantify the protein concentration of soluble galectin-3 in risk-stratified PCF and patient-matched sera, and to determine its subcellular localisation within pancreatic cell lines. Defining the specific intracellular distribution of galectin-3 may provide further insight into its functional roles within pancreatic epithelial cells and its potential contribution to the progression of PCLs towards established PC.

Data from this study found that the concentration of soluble galectin-3 was significantly increased in low-risk PCF compared to benign pseudocyst fluid (Mean concentration $\pm$ SEM; 499.70 ± 133.40 ng/mL vs. 79.97 ± 64.20 ng/mL, $p=0.0223$) (**Figure 5.10A**). Within PCF samples used in this analysis, there is a distinct stepwise increase in soluble galectin-3 concentration with increased risk of PC progression (**Figure 5.10A**).

Although not significant, serum samples isolated from patients with low-risk PCLs also showed a higher concentration of soluble galectin-3 compared to serum samples isolated from patients with benign pseudocysts. However, unlike what is seen in PCF, the concentration of soluble galectin-3 was decreased in serum from high-risk PCL patients compared to serum isolated from patients with benign pseudocysts or low-risk PCLs (**Figure 5.10A**).

To verify the efficiency of cytoplasmic and nuclear fractionation in pancreatic cell lines, cytoplasmic and nuclear extracts derived from normal pancreatic H6c7-HPDE cells and pancreatic cancer Panc-1 cells were subjected to Western blot analysis. GAPDH, a cytosolic marker, was used to confirm cytoplasmic extraction while histone H3, a nuclear marker confirmed nuclear extraction. Data from this analysis confirmed that the cytoplasmic and nuclear fractions were extracted adequately, as shown by positive expression of GAPDH in cytoplasmic extracts and whole cell lysates while positive histone H3 expression was exclusively confirmed in nuclear extracts and whole cell lysates (**Figure 5.10B**). Furthermore, minimal cross-contamination between the cytoplasmic and nuclear fractions was observed, indicating that the extraction procedure effectively isolated the two cellular compartments (**Figure 5.10B**).

When the concentration of soluble galectin-3 was assessed in different cellular components of normal pancreatic H6c7-HPDE cells, it was found that there was a significantly higher concentration of soluble galectin-3 in the cytoplasmic extract compared to in the nuclear compartment (287.40 ± 32.16 ng/mL vs. 51.24 ± 7.43 ng/mL, $p=0.0001$) (**Figure 5.10C**). Similarly, a significantly higher concentration of soluble galectin-3 was seen in the cytoplasmic fraction of H6c7-HPDE cells compared to what was found in whole cell lysates of these cells (287.40 ± 32.16 ng/mL vs. 61.65 ± 13.40 ng/mL, $p=0.0001$) (**Figure 5.10C**). Furthermore, soluble galectin-3 was significantly upregulated in the cytoplasmic fraction of normal H6c7-HPDE cells compared to cell culture supernatant (287.40 ± 32.16 ng/mL vs. 0.78 ± 0.20 ng/mL, $p=0.0001$) (**Figure 5.10C**).

Similar to what was identified in normal pancreatic H6c7-HPDE cells, the concentration of soluble galectin-3 was significantly higher in the cytoplasmic fractions extracted from intermediary pancreatic hTERT-HPNE cells compared to the extracted nuclear fraction (245.40 ± 44.63 ng/mL vs. 33.17 ± 3.79 ng/mL, $p=0.0008$) (**Figure 5.10D**). There was also a significantly higher concentration of soluble galectin-3 in the cytoplasmic fraction of hTERT-HPNE cells compared to whole cell lysates (245.40 ± 44.63 ng/mL vs. 99.83 ± 2.63 ng/mL, $p=0.0071$) (**Figure 5.10D**). Furthermore, soluble galectin-3 was significantly upregulated in the cytoplasmic fraction of normal hTERT-HPNE cells compared to the cell culture supernatant (245.40 ± 44.63 ng/mL vs. 0.20 ± 0.01 ng/mL, $p=0.0003$) (**Figure 5.10D**). Whole cell lysates of these intermediary pancreatic hTERT-HPNE cells were also shown to have a significantly higher concentration of soluble galectin-3 compared to that of the cell culture supernatant (99.83 ± 2.63 ng/mL vs. 0.20 ± 0.01 ng/mL, $p=0.0408$) (**Figure 5.10D**).

This study found that when the concentration of soluble galectin-3 was assessed in isolated fractions from pancreatic cancer BxPC-3 cells, there was a significantly higher concentration of soluble galectin-3 in the cytoplasmic fraction compared to in the nuclear fraction (283.70 ± 39.42 ng/mL vs. 23.74 ± 3.20 ng/mL, $p=0.0040$) (**Figure 5.10E**). Similarly, a significantly higher concentration of soluble galectin-3 was recorded in the cytoplasmic fraction of BxPC-3 cells compared to isolated cell culture supernatant (283.70 ± 39.42 ng/mL vs. 0.69 ± 0.08 ng/mL, $p=0.0036$) (**Figure 5.10E**). Whole cell lysates from BxPC-3 cells exhibited a significantly higher concentration of soluble

galectin-3 compared to that found within the nuclear fraction (292.50 ± 64.16 ng/mL vs. 23.74 ± 3.20 ng/mL, $p=0.0040$) (**Figure 5.10E**). Furthermore, the concentration of soluble galectin-3 was significantly higher in whole cell lysates from BxPC-3 cells compared to cell culture supernatant (292.50 ± 64.16 ng/mL vs. 0.69 ± 0.08 ng/mL, $p=0.0035$) (**Figure 5.10E**).

Consistent with the findings in pancreatic cancer BxPC-3 cells, there was a significantly higher concentration of soluble galectin-3 in the cytoplasmic fraction isolated from Panc-1 cells compared to that found in the nuclear fraction of these cells (111.00 ± 13.44 ng/mL vs. 7.47 ± 0.73 ng/mL, $p=0.0040$) (**Figure 5.10F**). Moreover, a significant upregulation of soluble galectin-3 was observed in the cytoplasmic fraction of Panc-1 cells compared to isolated cell culture supernatant (111.00 ± 13.44 ng/mL vs. 0.09 ± 0.01 ng/mL, $p=0.0036$) (**Figure 5.10F**). There was also a significantly higher concentration of soluble galectin-3 in whole cell lysates isolated from Panc-1 cells compared to that found within the nuclear fraction of these cells (97.62 ± 16.53 ng/mL vs. 7.47 ± 0.73 ng/mL, $p=0.0040$) (**Figure 5.10F**). In addition, whole cell lysates from Panc-1 cells exhibited a significantly higher concentration of soluble galectin-3 compared to that found within cell culture supernatant (97.62 ± 16.53 ng/mL vs. 0.09 ± 0.01 ng/mL, $p=0.0035$) (**Figure 5.10F**).

Overall, these findings suggest that an upregulated concentration of soluble galectin-3 in PCF is linked with an increased risk of PC development, and that galectin-3 is predominantly localised within the cytoplasmic compartment of pancreatic cell lines, irrespective of their classification as normal, intermediary or cancerous.

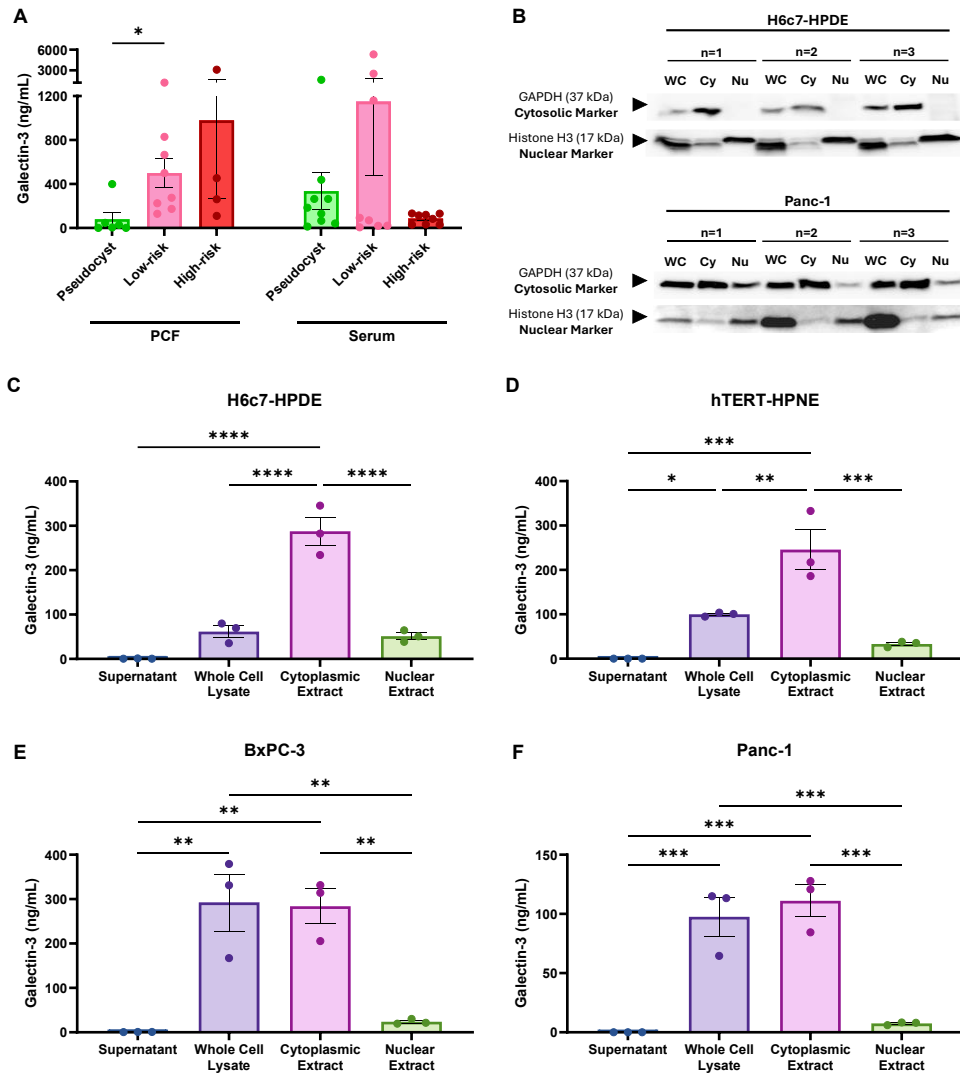


Figure 5.10: Soluble galectin-3 is increased in high-risk PCF and predominantly localises in the cytoplasmic compartment of pancreatic cell lines. Pseudocyst fluid (n=12), low-risk PCF (n=11), high-risk PCF (n=9) and patient matched sera were screened for the expression of soluble galectin-3 by standard ELISA (A). Cytoplasmic and nuclear extracts from pancreatic cells lines were isolated to further investigate the localisation of galectin-3 within normal pancreatic H6c7-HPDE cells, intermediary pancreatic hTERT-HPNE cells and pancreatic cancer cell lines BxPC-3 and Panc-1. Western blot analysis confirms fractionation efficiency in H6c7-HPDE samples and Panc-1 samples (B). Further screening of soluble galectin-3 expression was carried out in cell line supernatants (n=3), whole cell lysates (n=3), cytoplasmic extracts (n=3) and nuclear extracts (n=3) from H6c7-HPDE cells (C), hTERT-HPNE cells (D), BxPC-3 cells (E) and Panc-1 cells (F). Graphical data are representative of mean $\pm$ SEM. Statistical significance was assessed using non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-hoc test (PCF vs. PCF/ Serum vs. Serum) [significance defined as $*p \leq 0.05$] and non-parametric paired t-test (Wilcoxon matched-pairs signed rank test) (PCF vs. Serum). For data involving cell lines, statistical significance was assessed using an ordinary one-way ANOVA [significance defined as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$].

5.5 Discussion

It is now well established that PC is an “immunologically cold” and/or immunosuppressed cancer type. Infiltration of immune cells into the pancreatic tumour site is largely hindered by the desmoplastic microenvironment, which ultimately prevents an effective anti-tumour immune response^[29]. A number of PCs arise from pre-malignant fluid-filled cysts, but it has not yet been established how these cysts have the ability to progress to cancer development, while others do not. This study aims to understand the differences in chemotactic signalling, expression levels of soluble immune factors within benign pseudocyst fluid, low-risk PCF and high-risk PCF, and to ascertain how these differences may contribute to the development of PC in patients with high-risk PCLs.

Migration of immune cells is particularly important for the resolution of infection, inflammation and dysplasia^[357]. In this study, a significant increase in the migration of CD4⁺ T cells, CD8⁺ T cells and NK cells was identified towards PCF from PCL patients with a low risk of PC development. However, this effect was not observed for pre-malignant high-risk PCF or benign pseudocyst fluid suggesting that low-risk PCLs contain soluble immune factors that actively recruit effector immune cells, whereas high-risk lesions and pseudocysts lack those cues or contain inhibitory factors. This functional data is consistent with recent immune-profiling studies that describe a dynamic immune landscape during progression from precursor lesion to invasive PC^[46, 111, 197, 305]. In one such study, Roth *et al.* carried out spatial distribution analyses of surgically resected tissue from low-grade and high-grade IPMN lesions and IPMN-associated PC using immunohistochemistry and found that low-grade lesions exhibited elevated lymphocyte infiltration with an environment permissive to immune surveillance, while progression to high-grade lesions and invasive PC was associated with immune suppression, myeloid expansion and T cell exclusion^[111]. Similarly, Enzler *et al.* carried out multiplex immunohistochemical mapping of precursor lesions and found abundant CD8⁺ T cell infiltration in IPMNs but increased frequencies of immunosuppressive Treg cells in PanIN lesions and invasive PC^[46]. Importantly, this thesis differs from most tissue-based studies as it focuses on the immune compartment of pre-malignant PCF which directly interacts with the epithelial lining where malignant transformation occurs. Understanding differences in the immune profiles of PCF may provide a more dynamic representation of the local immune microenvironments of low-risk and high-risk PCLs and the

interactions between PCF-associated immune cell populations and neoplastic cells in the epithelium at the earliest stages of malignant progression.

Findings from this study indicate that the migration of CD4⁺ T cells, CD8⁺ T cells and NK cells towards high-risk PCF was not influenced by potential inhibitory proteins within the PCF, which would have otherwise permitted immune cell migration towards high-risk PCF at lower concentrations. These results suggest that it is the concentration of soluble immune factors within low-risk PCF that is influencing immune cell migration and not that of high-risk PCF. However, this immune cell migration can also be upregulated towards high-risk PCF upon the addition of chemokines such as fractalkine, RANTES and I-TAC. Further work is needed to fully elucidate what other chemokines and growth factors would be required to reinstate effector immune cell migration towards high-risk PCF to the extent observed towards low-risk PCF. Indeed, the soluble immune factors secreted into or within low-risk PCF may potentially be advantageously used to recruit effector immune cells to the lesion site to circumvent PC development and progression. Conversely, chronic inflammation is considered to be a contributing factor to the initiation and development of multiple cancers^[358, 359]. As such, in the case of pre-malignant lesions, perhaps this immune-active microenvironment, rich in chemokines and growth factors that act as signals for recruiting effector immune cells, may actually be perpetuating the progression of these lesions to high-risk PCLs and furthermore to invasive PC. Additionally, these immune markers may be incorporated as potential immunobiological parameters in the PC risk stratification guidelines of PCLs.

Identifying key immunological factors involved in facilitating the migration of effector immune cell populations towards low-risk PCF may not only elucidate the pathways used to circumvent PC development but also identify immunobiological biomarkers to more accurately distinguish between low-risk PCLs and high-risk PCLs. At present, the only biomarker approved by the FDA for the detection of PC, is serum CA19-9^[186]. However, CA19-9 is known to perform poorly as a biomarker in patients with inflammation-driven co-morbidities already associated with PC, such as pancreatitis and diabetes^[186]. A number of studies have emerged to overcome the issue of limited sensitivity and specificity of CA19-9 by investigating the applicability of multi-biomarker panels^[188, 189]. However, there has been limited work on profiling the soluble immune component of risk-stratified PCF to identify potential immunobiological biomarkers. Identifying

immune-based biomarkers may further define current PC risk stratification guidelines with quantifiable, biological parameters and provide high-risk PCL patients with the appropriate surveillance.

An exploratory study carried out by Lee *et al.* utilised microarray assays to investigate the expression of inflammatory mediator proteins (IMPs) in PCF obtained from patients with pre-malignant BD-IPMNs or benign pseudocysts, which arose from a background of inflammation, as is typically the case^[194]. Although this study was limited by sample size, the results showed differential IMP profiles for pre-malignant and benign PCLs^[194]. Eotaxin, hepatocyte growth factor (HGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were expressed at lower concentrations in high-risk pre-malignant PCLs compared to in benign pseudocysts^[194]. Data from this thesis aligns with this study whereby HGF was found to exhibit lower expression levels in PCF from patients with high-risk PCLs compared to benign pseudocyst fluid. However, the study carried out by Lee *et al.* only investigated the expression of IMPs across BD-IPMNs and pseudocysts, whereas this present study aimed to investigate benign pseudocysts, low-risk PCLs and high-risk PCLs. Similarly, a study conducted by Maker *et al.* used multiplex sandwich ELISAs to investigate the expression of cytokines in human IPMN cyst fluid with low-, moderate- or high-grade dysplasia to determine anti-tumour immune responses^[104]. The results demonstrated significantly higher concentrations of pro-inflammatory cytokines IL-1 β , IL-5 and IL-8 in the presence of high-grade dysplasia and invasive carcinoma compared to low-risk PCLs with low-grade or moderate dysplasia^[104]. The results from this present study align with these findings recorded in literature, whereby expression of IL-8 is increased in high-risk PCF compared to PCF from patients with a low-risk of PC development. Interestingly, the analytes with a higher expression in low-risk PCF (fractalkine, TARC, IL-6, TSLP, eotaxin-3, MCP-1, MCP-4, MIF, MIP-1 α , MDC, HGF, VEGF-A, GM-CSF and IL-8) also act as chemotactic cues for cell types other than T cells and NK cells including, monocytes, dendritic cells, eosinophils and macrophages^[360, 361]. Population markers for these specific cell types were not used in the flow cytometric analysis of the migration assay. However, further studies investigating the migration of these cell populations would validate these findings from the multiplex ELISA screen and add to the existing knowledge of migration of different immune cell populations towards PCF.

Chemokines are a family of low molecular weight proteins that function as chemotactic agents to regulate the migration, infiltration, and accumulation of immune cells^[328]. As a result of poor immune infiltration and a non-inflamed phenotype, PC is widely considered as an “immunologically cold” tumour that is refractory to immunotherapy treatment^[221, 362]. However, in an early study by Thomas *et al.*, immunohistochemical analysis was performed on surgically resected PanIN lesions which determined that there was an increased expression of CXCR4 with progression from normal pancreatic ductal tissue to PanIN lesions with high-grade dysplasia^[363]. To date, not many investigations have been carried out to investigate chemokine receptor expression in cystic lesions such as IPMNs and MCNs. Furthermore, the landscape of chemokine signalling influenced by pre-malignant PCLs has yet to be elucidated, particularly in relation to how these signalling pathways mitigate or promote PC progression. This present study is one of the first to look at the differential effect of the soluble immune microenvironment of risk-stratified PCF on chemokine receptor expression on the surface of healthy T cells. Data from this investigation reveals that chemokine receptors CCR2, CXCR3, CXCR4 and CX₃CR1 were expressed at higher levels on T cells co-cultured with low-risk PCF compared to high-risk PCF.

The chemokines associated with CCR2, CXCR3, CXCR4 and CX₃CR1 chemokine receptors, namely fractalkine, RANTES, IL-10, I-TAC, MIP-1 α , MCP-1, MCP-2, eotaxin-3, IP-10, I-TAC and MIF were also found to be expressed at higher levels in low-risk PCF compared to high-risk PCF. These results suggest that these chemokine signalling pathways are at play in low-risk PCLs to recruit immune cells in order to potentially induce an anti-tumour immune response whereas in high-risk PCLs with more dysregulated chemokine signalling, the tumourigenic pathway is undisturbed allowing PC to progress. It is also plausible that low-risk PCLs are more immunologically active, thereby inducing damage in healthy cells through inflammation and a skewing of recruited immune cells towards a pro-tumour phenotype, and as a result, can evolve to high-risk lesions with time.

Another critical implication of the findings from this thesis is the potential for therapeutic intervention. Data from this study has shown that in high-risk PCF, migration of effector immune cell subsets can be reinstated upon the addition of recombinant chemokines fractalkine, RANTES and I-TAC. In the case of PCLs that harbour a chemokine-depleted microenvironment that fail to recruit immune effector cells, strategies to enhance

chemotactic function or sustain effector T cell infiltration could be applied in this pre-malignant setting. For example, intralesional immunotherapy or chemokine modulation, similar to approaches trialled in melanoma, may prove effective in pre-emptively targeting high-risk PCLs prior to malignant transformation to induce an immune response and recruit effector immune cells^[364].

Within this study, CD54 or otherwise known as ICAM-1, was found to be primarily expressed on the surface of pancreatic cells, although a high expression was also observed intracellularly in normal pancreatic cells and PC cells. Although intermediary pancreatic cell lines had lower expression of ICAM-1 compared to normal and PC cells, the cell surface and intracellular ICAM-1 expression was upregulated in hTERT-HPNE cells when co-cultured under hypoxic stress conditions, a response not observed in other cell lines. This data suggests that hypoxia may play a role in priming pre-malignant pancreatic cells for interaction with immune or stromal cells via ICAM-1 expression. Hypoxia is a robust environmental stress and as a result, upregulating ICAM-1 could facilitate immune cell adhesion, cytokine signalling, or survival signalling in the pre-malignant setting.

This current study also demonstrated a stepwise increase in the concentration of soluble galectin-3 in PCF samples with increased risk of PC progression. This was in line with data reported by Kane *et al.* in which the proteome and transcriptome of PCF was extensively profiled, and a differential expression analysis revealed that as part of an eight protein biomarker panel, LGALS3 was one such protein significantly upregulated in high-risk PCF compared to low-risk PCF^[275]. Additionally, increased galectin-3 levels have been observed across a number of malignant tumours and has shown potential as a potential diagnostic and prognostic marker for some cancers^[352, 365]. Galectin-3 promotes tumour growth and metastasis through multiple mechanisms which include cellular transformation and invasion, angiogenesis, and resistance to cancer therapeutics^[339, 352, 366-368]. Furthermore, galectin-3 has been shown to be implicated in tumour immune tolerance by impairing the function of tumour-infiltrating lymphocytes and inducing T cell apoptosis^[369, 370]. Galectin-3 also plays a role in suppressing anti-tumour immunity by recruiting monocytes and macrophages, impairing NK cell function, and inhibiting T cell activation through apoptosis, TCR downregulation and crosslinking^[371-373]. Galectin-3 has been shown to mediate CD8⁺ T cell suppression through the inhibitory protein LAG-3, and its deletion enhances effector T cell responses after GM-CSF vaccination^[306]. The experimental findings within this thesis show that galectin-3 expression is primarily

localised intracellularly across normal H6c7-HPDE pancreatic cells, intermediary hTERT-HPNE pancreatic cells, BxPC-3 and Panc-1 pancreatic cancer cells. Intracellular galectin-3 is important for cell survival due to its ability to block the intrinsic apoptotic pathway^[374, 375]. In this regard, it has been demonstrated that cytoplasmic galectin-3 in cancer cells is usually associated with an aggressive phenotype, in opposition to nuclear galectin-3^[376]. Galectin-3 is a therapeutic target in the development of anti-cancer drugs due to its role in promoting cancer^[377, 378]. A number of galectin-3 inhibitors have been developed, some of which have demonstrated promising anti-cancer characteristics^[377, 378]. Early-phase clinical trials are evaluating galectin-3 inhibitors in combination with peptide vaccines or immune checkpoint blockade with pembrolizumab or ipilimumab in metastatic melanoma, underscoring its designation as a next-generation targeted therapy^[379]. The accumulation of these findings in addition to data reported in literature highlights the potential therapeutic relevance of targeting galectin-3, specifically using inhibitors to block high expression in high-risk PCLs which may prevent PC cell survival and progression.

Pancreatic tumourigenesis has been described as a progressive loss of immune surveillance culminating in the immune-excluded phenotype established in advanced PC^[111]. Data from this study suggests that lesions with a low risk of PC progression can also be characterised by a complex immune landscape, with increased concentrations of chemokines, cytokines, growth factors and effector immune cell migration, which is furthermore not observed with high-risk PCF. This aligns with multi-step carcinogenesis models proposed in colorectal and cervical cancers, where pre-malignant lesions initially elicit robust immune responses that are subsequently attenuated^[380, 381]. It is plausible that PCLs follow a similar trajectory whereby low-risk lesions may initially attract immune cells and illicit heightened immunological activity. However, over time, chronic inflammation and microenvironmental changes may potentially drive immune exhaustion, tolerance, and ultimately progression to high-risk PCLs. Thus, the local immune microenvironment within low-risk PCLs may represent an early, immunologically active phase of PC development. In contrast, high-risk lesions may reflect a later stage of disease progression in which key immunological changes, like depletion of soluble immune factors and loss of chemotactic mechanisms, have already occurred.

In conclusion, this thesis identifies differential expression levels of immune targets between benign pseudocyst fluid, pre-malignant low-risk PCF and pre-malignant high-risk PCF. The in-depth multiplex ELISA screen established that there is an increased expression of soluble immune factors in low-risk PCF which may influence the recruitment of effector immune cells to the PCL site and act as a contributing factor to immune evasion and progression to PC. T cells are vitally important in the anti-tumour immune response. However, the infiltration of immune cells into the local microenvironment of high-risk PCLs is hindered by a dysregulated chemokine release, similar to what is exhibited in established PC with dense desmoplastic stroma. The differential soluble immune profiles identified within this study should be used to further elucidate potential immune pathways that could be therapeutically targeted to enhance immune cell infiltration into pancreatic tumour tissue, in addition to more accurately identifying patients at a higher risk of PC development.

5.6 Study limitations

This study provides novel insight into immune cell migration towards benign and pre-malignant PCLs and highlights a potential role for PCF in influencing chemotactic signalling within the PCL microenvironment. Some limitations should be considered when interpreting these findings.

A limitation of this study is how chemotaxis assays provide key insights into migratory potential but do not fully recapitulate *in situ* dynamics such as immune cell adhesion, activation status, or the influence of the ECM. Similar to the limitations also mentioned in Chapter 4, the limited volume of PCF restricted the volume available for functional assays. The Boyden chamber transwell chemotaxis assay requires a relatively large volume within the lower chamber. Therefore, dilution of PCF samples was necessary to meet assay specifications. This dilution likely reduced the concentration of soluble chemotactic mediators within the PCF samples and may have attenuated immune cell migration, potentially underestimating the true chemotactic capacity of the PCL microenvironment. Additionally, due to the limited volume of PCF, individual effects of chemokines supplemented into high-risk PCF could not be investigated and instead a combination of recombinant chemokines was chosen based on earlier findings.

In addition, migration analyses were limited to phenotypic identification of CD4⁺ T cells, CD8⁺ T cells, and NK cells. While these populations represent key effector immune cell populations, other immune subsets are also likely to respond to soluble chemokine milieu associated with risk-stratified PCLs. Myeloid cell populations, Treg cells, and antigen-presenting cells such as dendritic cells, macrophages and B cells were not investigated and should be incorporated in future studies to provide a more comprehensive assessment of immune cell recruitment to PCLs.

Furthermore, this study examined the effect of risk-stratified PCF on chemokine receptor expression using immune cells isolated from healthy donors. While this allowed controlled assessment of PCF-mediated chemokine signalling, it may not fully reflect the phenotype of immune cells conditioned by the PCL microenvironment *in vivo*. Future work should therefore characterise chemokine receptor expression directly on the immune infiltrates of PCF, in addition to the epithelial lining of the cyst, to more accurately define the mechanisms mediating immune cell recruitment in PCLs. Furthermore, longitudinal studies on recurring PCLs or PCLs that have progressed could also be used to validate the prognostic value of specific immune profiles in predicting malignant transformation.

5.7 Graphical summary

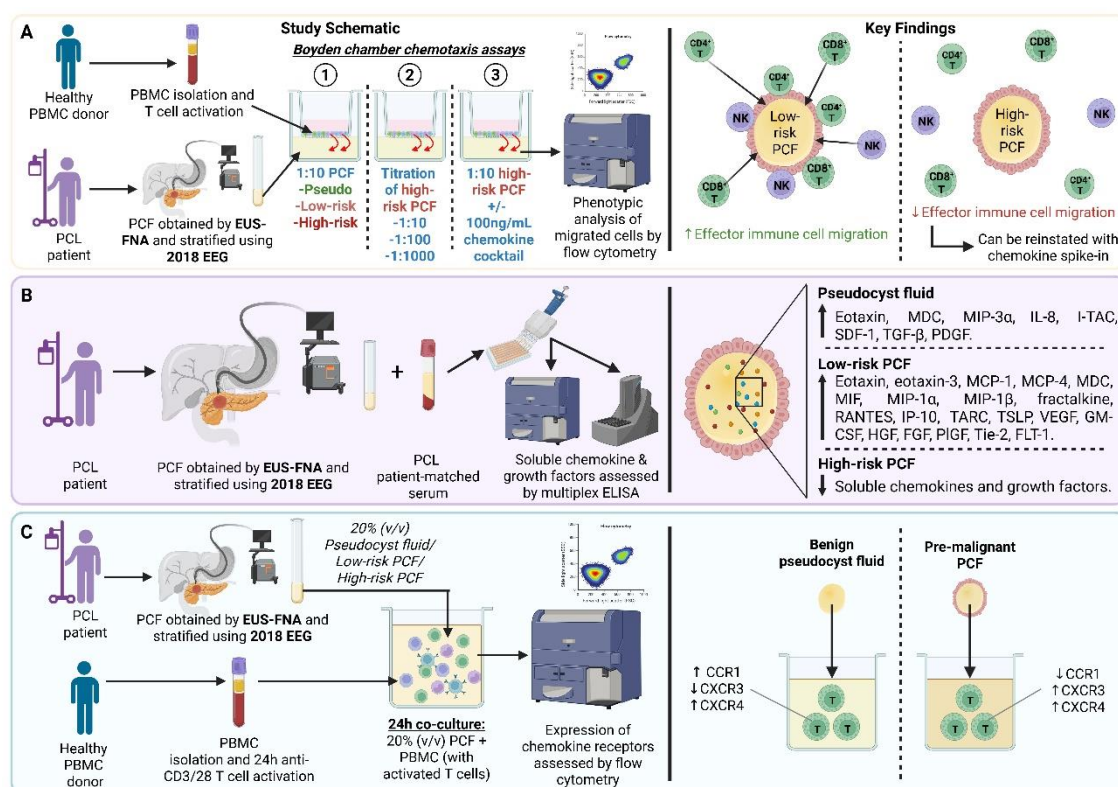


Figure 5.11: Graphical summary of study findings from results chapter 5. (A) Boyden chamber chemotaxis assays were performed to assess immune cell migration towards risk-stratified PCF. Increased (↑) effector immune cell migration was observed towards low-risk PCF, but not towards high-risk PCF. A titration of high-risk PCF under the same experimental set-up showed that inhibitory factors were not at play and migration must be dependent on the concentration of specific chemokines. Immune cell migration could be reinstated towards high-risk PCF upon the addition of a chemokine cocktail of fractalkine, RANTES and I-TAC. (B) The concentration of soluble chemokines and growth factors in PCF and patient-matched serum was assessed by multiplex ELISA. Low-risk PCF was characterised by an immune-active microenvironment with increased (↑) levels of chemokines and growth factors. High-risk PCF had decreased (↓) levels of soluble chemokines and growth factors. (C) PCF was isolated from risk-stratified PCL patients by EUS-FNA and co-cultured with healthy PBMCs for 24 hours. Flow cytometry was used to assess the impact of PCF on chemokine receptor expression. CCR1 and CXCR3 were differentially expressed between effector immune cells co-cultured with benign pseudocyst fluid and pre-malignant PCF.

Chapter 6:

Concluding discussion and future directions

6.1 Discussion

PC remains one of the most lethal malignant diseases worldwide, with a dismal 5-year survival rate of approximately 13%^[1]. This poor prognosis is largely due to challenges in early detection and treatment, as PC typically presents at advanced stages when clinical symptoms are vague and non-specific^[5-7]. The aggressive biology of the disease and its limited response to standard-of-care therapies in late stages further contribute to its high mortality^[15, 16, 22]. Early identification of individuals at risk of PC development is therefore critical to improving patient outcomes.

One promising avenue for early detection lies in the study of PCLs, which are fluid-filled structures that develop within or on the surface of the pancreas. While many PCLs are benign, others such as IPMNs and MCNs possess the ability to undergo malignant transformation and can be regarded as precursor lesions of PC^[51, 52, 55, 382, 383]. The prevalence of PCLs has vastly increased over recent years as a result of improved quality and frequency of imaging technology used in routine examinations^[384]. However, although PC is traditionally characterised as an “immunologically cold” cancer, relatively little is known about the immune microenvironment within these pre-malignant lesions^[43, 46, 111]. Gaining insight into immune differences among these lesions may elucidate the role of local immune microenvironment of PCLs in the transition from pre-malignant lesions to invasive PC.

A number of studies have investigated the immune cell landscape of precursor lesions of PC^[43, 46, 111, 197]. However, these studies focus on the spatial distribution of immune cell populations in surgically resected tissue from PanIN lesions, IPMN lesions or IPMN-associated PC. This thesis is one of the first to explore the immune cell infiltrate of PCF isolated from PCLs by EUS-FNA and stratified by the 2018 European evidence-based guidelines. This study characterised the immune cell infiltrate and immunomodulatory secretome of risk-stratified PCL cyst fluid. Additionally, little is known about the effect of the soluble immunomodulatory secretome of risk-stratified PCF on anti-tumour effector immune cell function. As such, this thesis explored T cell and NK cell activation, exhaustion and cytotoxicity in response to exposure to benign pseudocyst fluid, low-risk PCF and high-risk PCF. Furthermore, this study aimed to elucidate key chemokine profiles in risk-stratified PCF to identify potential chemokine pathways shaping the immune cell infiltrate in pre-malignant PCLs and chemokine-based therapeutic targets

that may enhance specific effector immune cell infiltration into the local immune microenvironment of PC precursor lesions.

Data from this study firstly showed that the immune cell infiltrate of pre-malignant PCF was predominantly composed of adaptive immune cell populations, including CD4⁺ T cells, CD8⁺ T cells and NK cells. This immune profile suggests that adaptive immunity may play a key role in shaping the local microenvironment of pre-malignant PCLs. These findings are consistent with several studies that have explored the immune landscape of precursor lesions of PC. For example, Roth *et al.* also performed immunohistochemical analysis on surgically resected IPMN tissue samples to examine the spatial distribution of immune cell infiltrates throughout IPMN progression^[111]. Their investigation revealed that the immune microenvironment evolves from a diverse T cell landscape, predominantly composed of CD8⁺ T cells, Th1, Th2, Th17, Th22, and Treg cells in low-grade IPMNs to a primarily Treg-enriched immunosuppressive state during the progression to invasive PC^[111]. The same study also identified the formation of tertiary lymphoid structures in the surrounding stroma of IPMNs, which accumulate immunosuppressive cell types and subsequently evolve during malignant progression^[111]. These observations are further supported by recent single-cell RNA sequencing studies carried out by Bernard *et al.* which reveal that low-grade IPMNs exhibited a higher infiltration of cytotoxic CD8⁺ T cells, activated CD4⁺ T helper cells and dendritic cells, whereas higher-grade dysplastic lesions were more so dominated by immune subsets with immunosuppressive phenotypes^[197]. Similarly, Eckhoff *et al.* used multidimensional immunophenotyping to identify that the infiltrate of IPMN lesions was primarily composed of T cell and macrophage populations^[238]. In agreement, previous investigations in PC have demonstrated a spatial reorganisation of immune infiltrates, where CD8⁺ T cells accumulate in peritumoral regions, while Treg cells preferentially infiltrate intraepithelial areas of neoplastic tissue as lesions progress from pre-malignant to malignant disease^[43, 239-241]. Complementary findings were reported in a study by Enzler *et al.*, whereby multiplex immunohistochemistry was used to assess the composition of immune cell infiltrates in surgically resected tissue from PCLs and PC, which revealed pronounced CD8⁺ T cell infiltration within pre-malignant lesions^[46]. Nevertheless, further research is required to define the role of immunosuppressive cell populations such as MDSCs and Treg cells in pre-malignant PCF, particularly within high-risk lesions, as this cohort needs to be powered for further analysis. Understanding

how these populations influence immune regulation, cytokine secretion, and chemotactic signalling may provide further mechanistic insight into how immune evasion is established prior to malignant transformation.

While the data presented in this thesis aligns with these previous observations in that adaptive immune cells predominantly constitute the immune cell infiltrate of pre-malignant PCLs, this project provides a novel perspective by focusing specifically on the immune cell compartment within PCF rather than resected tissue. This distinction is crucial, as PCF is in direct contact with the epithelial lining where malignant transformation originates. As such, the immune landscape of PCF may offer a more dynamic and functionally relevant representation of immune-epithelial cell interactions occurring at the earliest stages of pancreatic tumourigenesis. Importantly, analysing PCF allows for the identification of circulating or secreted immune cell subsets that could potentially act as biomarkers for early detection or cancer risk stratification.

This study also demonstrated that CD4⁺ T cells and CD8⁺ T cells within the PCL microenvironment exhibit features consistent with an exhausted phenotype. The upregulation of exhaustion-associated markers, such as inhibitory immune checkpoint receptors PD-1, LAG-3 and TIM-3, implies that there may be chronic antigen exposure and sustained immune activation within these pre-malignant lesions, leading to diminished effector functionality^[228, 303, 304]. This finding mirrors immune exhaustion observed in advanced solid tumours and chronic infections, where continuous antigenic stimulation drives functional impairment^[228, 302]. In addition, when the impact of PCF on effector immune cell function and phenotype was investigated, it was found that the soluble immune microenvironment of pre-malignant PCLs caused healthy T cells and NK cells to acquire an exhausted phenotype, a finding not reported in benign pseudocysts.

Furthermore, soluble checkpoint molecules can be proteolytically released from immune cells or epithelial cells, and they may indicate an early engagement of inhibitory pathways that dampen cytotoxic activity^[257, 263, 264]. This study demonstrated that the local immune microenvironment of low-risk PCLs also had increased concentrations of soluble immune checkpoint molecules (PD-1, PD-L1, PD-L2 and CD40L). Importantly, the identification of such an exhausted effector immune cell phenotype within pre-malignant PCLs provides a rationale for exploring immunotherapeutic interventions aimed at reinvigorating T cell activity. Targeting immune checkpoint pathways or exhaustion-

associated mediators, such as PD-1, could therefore represent a strategy to restore anti-tumour immunity at an early stage of pancreatic tumourigenesis.

Additionally, this study carried out in-depth profiling of the soluble immune microenvironment of PCLs using multiplex ELISAs and found that, compared with benign pseudocyst fluid and high-risk PCF, low-risk PCF showed elevated levels of adhesion molecules (ICAM-1, VCAM-1, CD147), growth factors (VEGF, GM-CSF, HGF, PIGF), acute-phase and inflammatory mediators (SAA, IL-6), effector immune cell-associated cytokines (IFN- γ , IL-2, IL-12p40, IL-12/IL23p40, IL-15, TNF- β), and chemokines (eotaxin, eotaxin-3, MCP-1, MCP-4, MDC, MIF, MIP-1a, MIP-1B, fractalkine, IP-10, TARC, TSLP). This immunologically active profile suggests that there may be ongoing immune cell recruitment and activation in low-risk PCLs, which is not found in high-risk lesions. These findings were found to be consistent with prior studies of IPMNs that demonstrate an increased immune infiltration and Th1-mediated cytotoxic signalling in early-stage lesions^[111, 197, 250]. Chronic inflammation is widely recognised as a key factor involved in the initiation and progression of various malignancies^[358, 359]. In the context of pre-malignant PCLs, an active immune microenvironment rich in cytokines, chemokines, growth factors and adhesion molecules as signals for recruiting effector immune cells may paradoxically promote the progression of low-risk PCLs to high-risk PCLs and furthermore to invasive PC.

Although the precise mechanisms underlying the progression of pre-malignant PCLs from low-risk lesions to high-risk or malignant states remain unclear, current evidence suggests that this transformation may be a time-dependent process. Prolonged cyst persistence may potentially facilitate the gradual accumulation of genetic and microenvironmental alterations, in addition to an expended soluble immune microenvironment and loss of chemotactic cues that collectively promote malignant potential. However, longitudinal evidence directly supporting the evolution of low-risk PCLs into high-risk PCLs is currently limited. Further research is therefore required to elucidate changes over time in molecular and immunological factors that drive or prevent the progression of PCLs.

To investigate the effect of risk-stratified PCF on the cytotoxic function of effector immune cells, this study used CCK-8 assays to evaluate the survival of PC cells exposed to PCF-pretreated lymphocytes. A key functional observation of this study was that

lymphocyte-mediated cytotoxicity against PC cells was enhanced upon exposure to benign pseudocyst fluid and pre-malignant low-risk PCF, a response that was notably absent following exposure to high-risk PCF. This differential immunomodulatory effect suggests that the soluble immune factors within low-risk PCLs retains the capacity to stimulate cytotoxic immune responses, whereas high-risk PCL immune microenvironments are immunosuppressive and dampen lymphocyte-mediated cytotoxic effects. These findings underscore a potential immunological shift accompanying cyst progression, from an immune-active to an immune-silent state, consistent with the concept of “immune editing” in cancer evolution.

It is known that migration of immune cells is particularly important for the resolution of infection, inflammation and dysplasia^[357]. An important finding of this project was the enhanced migration of CD4⁺ T cells, CD8⁺ T cells, and NK cells towards low-risk PCF, but not towards high-risk PCF or benign pseudocyst fluid, suggesting that low-risk lesions secrete soluble factors attracting effector cells, whereas high-risk lesions lack such cues or contain inhibitory factors. These findings align with immune-profiling studies showing immune infiltration and surveillance in low-grade PCLs shifting to suppression and T cell exclusion with progression to invasive PC^[46, 111, 197, 305]. Unlike tissue-based analyses, this study focused on the PCF immune compartment that directly interacts with the epithelial lining, providing a dynamic view of early immune-epithelial crosstalk. This study showed that the absence of migration towards high-risk PCF appears to be due to a lack of chemotactic factors, rather than inhibitory factors. Notably, chemokines such as fractalkine, RANTES and I-TAC restored migration, suggesting that modulating the chemokine milieu could enhance immune recruitment. While soluble immune factors in low-risk PCF may promote anti-tumour immune surveillance, chronic immune activation could also drive lesion progression^[358, 359]. Therefore, PCF-derived immune profiles hold potential as biomarkers for PC risk stratification and as targets to manipulate immune dynamics in pre-malignant PCLs.

The observation that low-risk PCLs are highly immune active, in contrast to the immune quiescence of high-risk PCLs, supports the notion that immune surveillance may initially constrain neoplastic progression. Over time, however, PCLs may evolve mechanisms to evade immune detection and destruction, resulting in loss of immune activity characterised by decreased expression of soluble cytokines, growth factors and immune

checkpoint molecules, as well as reduced chemotactic signalling. This immune attenuation may facilitate uncontrolled cellular proliferation, tissue remodelling and ultimately malignant transformation. Understanding the molecular and cellular basis of this immunological transition could provide critical insights into the timing and targets of preventive immunotherapeutic interventions for pre-malignant PCLs.

Among the immunomodulatory mediators examined, galectin-3 emerged as a potential therapeutic target in pre-malignant pancreatic lesions. Data from this study revealed a higher concentration of soluble galectin-3 within high-risk PCF compared to low-risk PCF and benign pseudocyst fluid. This finding is consistent with the work of Kane *et al.*, who performed extensive proteomic and transcriptomic profiling of PCF and identified LGALS3, otherwise recognised as galectin-3, as one of eight proteins significantly upregulated in high-risk PCF compared to low-risk PCF^[275].

Galectin-3 has been implicated in tumour growth and metastasis through multiple mechanisms, including the promotion of cellular transformation, invasion, angiogenesis, and resistance to therapy^[339, 352, 366-368]. Galectin-3 has also been implicated in tumour-associated immune tolerance by impairing the function of tumour-infiltrating lymphocyte function and inducing T cell apoptosis^[369, 370]. In addition, galectin-3 contributes to the suppression of anti-tumour immune responses by recruiting monocytes and macrophages, impairing NK cell function, inhibiting T cell activation, and T cell receptor downregulation^[371-373]. Notably, galectin-3 can also mediate CD8⁺ T cell suppression via interaction with inhibitory immune checkpoint receptor LAG-3, and genetic deletion of galectin-3 enhances effector T cell responses following GM-CSF-based vaccination^[306]. Experimental findings from this thesis reveal that galectin-3 expression is predominantly localised intracellularly across normal H6c7-HPDE pancreatic cells, intermediary hTERT-HPNE pancreatic cells, BxPC-3 and Panc-1 pancreatic cancer cells. Intracellular galectin-3 plays a crucial role in promoting cell survival through inhibition of the intrinsic apoptotic pathway^[374, 375]. In this context, cytoplasmic localisation of galectin-3 in cancer cells has been associated with a more aggressive and invasive phenotype, in contrast to its nuclear localisation^[376]. Collectively, these findings suggest that galectin-3 supports both tumour cell survival and immune suppression during the early stages of pancreatic tumourigenesis. Consequently, targeting galectin-3 in low-risk PCLs may represent a promising therapeutic strategy to preserve immune surveillance and prevent the

immunosuppressive remodelling that accompanies progression to high-risk and malignant states.

Furthermore, as discussed, PC risk stratification guidelines for PCLs are highly contested both geographically and institutionally^[95, 100, 167-170]. PCF samples used within this study were stratified using the 2018 European evidence-based guidelines^[167]. However, as there is a lack of consensus among gastroenterologists as to the most appropriate stratification cut-offs and parameters, these classifications may not be accurate. As such, longitudinal patient data with information regarding malignant transformation of PCLs is needed. Moreover, in respect to low-risk patients, no clear cut-off exists for the interpretation of whether this was the correct classification. This further emphasises the need for the addition of measurable biomarkers to current stratification guidelines. A particularly novel implication of our study pertains to the translational potential of PCF as a liquid biopsy medium for immune surveillance and cancer risk stratification. Given the accessibility of PCF via EUS-FNA, profiling immune cell infiltrates, in addition to the chemokine and cytokine profiles of the soluble immune microenvironment, could be utilised as a biomarker platform to improve stratification of PCLs not only by risk of PC development but also by their immune landscape.

Another limitation of this study is that PCF can be limited particularly when cyst size is small and therefore the volume of PCF to work with is limited. Within this thesis, where co-cultures were carried out, PCF was diluted. However, *in vivo* the epithelial cells that constitute the cyst lining and the cellular component within the PCF would be in contact with more concentrated PCF over more sustained periods of time, which may have even more pronounced impacts.

An important limitation of this study is the lack of ideal benign PCL control samples. In this study, pseudocysts were used as a benign comparator for pre-malignant PCLs. However, despite their benign status, pseudocysts may not be representative of the most appropriate control. Unlike pre-malignant PCLs, benign pseudocysts lack an epithelial lining and typically arise from a background of pancreatitis-associated inflammation. Therefore, their immune microenvironment is likely distinct from that of pre-malignant lesions, which are not primarily driven by inflammation. Ideally, benign PCL

comparators would have included lymphoepithelial cysts, simple retention cysts, or serous cystadenomas, but such samples were not available for inclusion in this study.

In conclusion, this study reveals distinct immune landscapes across benign pseudocysts, pre-malignant low-risk PCLs and pre-malignant high-risk PCLs. Low-risk PCLs are characterised by an immune-active but regulated microenvironment that can permit lymphocyte-mediated cytotoxicity, while high-risk PCLs exhibit immune exhaustion, a depleted soluble immune microenvironment, loss of chemokine-mediated immune cell recruitment and accumulation of immunomodulatory mediators such as galectin-3. Together, these findings advance our understanding of immune dysregulation during early pancreatic tumourigenesis and suggest novel avenues for biomarker development and immunomodulatory intervention prior to malignant transformation. Furthermore, these results suggest that restoring immune surveillance and preventing immune silencing at the pre-malignant stage may represent a new paradigm in PC prevention.

6.2 Future directions

This project has identified several promising avenues for future research aimed at further elucidating the role of the local immune microenvironment of PCF in the malignant transformation of PCLs. The findings presented within this thesis highlight that the cellular and soluble immune microenvironment within PCF may be the key to understanding the progression from low-risk PCLs to high-risk PCLs and ultimately to invasive PC. A deeper understanding of how immune factors within the cystic microenvironment contribute to this mechanism of transformation could ultimately inform strategies to predict, monitor, and potentially prevent malignant development in patients harbouring PCLs. Future work should therefore aim to refine the immunological characterisation of PCF, integrate these findings with molecular and clinical data, and experimentally dissect the mechanisms through which immune modulation shapes PCL biology and progression.

Further comprehensive characterisation of the immune cell infiltrate in PCF from a broader cohort of patients with benign pseudocysts and high-risk PCLs is needed to explicitly identify differences in immune composition and activity between risk-stratified PCL cohorts. This study profiled only a limited number of benign and high-risk samples

due to difficulties in obtaining sufficient samples, which restricts the strength of comparative analyses. Future work incorporating larger patient cohorts to capture more high-risk PCL samples and benign pseudocyst samples would increase statistical power but also is crucial to be able to identify explicit differences in cellular immune composition and activity between pre-malignant low-risk and high-risk PCL cohorts. Continuing the comprehensive characterisation carried out in this thesis on the immune cell infiltrate of PCLs on a larger patient cohort will ultimately capture the heterogeneity of immune populations and their activation, exhaustion and cytotoxic phenotypes within PCF. Comparing specific immune signatures between low-risk PCLs and high-grade PCLs may reveal immune phenotypes predictive of progression.

Future work should also include expanded chemotaxis experiments incorporating additional immune markers to more precisely dissect the nature of recruited immune cell populations into the cystic microenvironment. In particular, Treg cells ($CD4^+FoxP3^+CD25^+$) and MDSCs ($CD11b^+CD33^+$) would be considered immune cell populations of interest in this context. Populations of these cell subsets have been implicated in fostering an immunosuppressive TME in PC and subsequently facilitating immune evasion and disease progression. Assessing the migration of Tregs and MDSCs in response to risk-stratified PCF derived from pre-malignant PCLs may provide functional evidence for how immune suppression is established and maintained within PCLs. Furthermore, correlating data from flow cytometry-based chemotaxis assays to the expression of soluble immunomodulatory mediators within PCF may also elucidate whether specific chemokines or soluble factors drive preferential recruitment of suppressive or effector cell types. Understanding these chemotactic dynamics may uncover potential therapeutic targets for immune modulation aimed at restoring effective anti-tumour immunity.

Parallel to this, further defining the chemokine and cytokine landscapes within PCF remains a crucial goal. While the current study has provided initial insights into differential chemotactic activity between risk-stratified PCF, a more comprehensive analysis of the chemotactic mechanisms at play is warranted. Future experiments should investigate specific chemokine profiles that could reinstate immune cell migration towards high-risk PCF to levels observed with low-risk PCF with the aim of normalising immune surveillance within the high-risk PCL microenvironment. Additionally,

chemokine receptor profiles of cells in the immune cell infiltrate of PCLs should be investigated to elucidate the key chemotactic mechanisms at play in these pre-malignant lesions.

Another interesting future direction concerns cell-cell interactions that may be occurring within the PCL microenvironment. The galectin-3/integrin axis represents a particularly intriguing candidate pathway. Galectin-3 is frequently overexpressed in malignant settings and is known to regulate cell adhesion, proliferation, and immune modulation^[309, 342-344]. Galectin-3 is known to interact with multiple integrins on both epithelial and immune cells, potentially influencing immune cell recruitment, retention, and function. Profiling integrin expression and activation on immune cells within PCF may shed light on how these cells communicate with or are modulated by epithelial cells in both low-risk PCL and high-risk PCL linings. Future studies could employ co-culture assays with pancreatic epithelial cells and immune cells pre-treated with PCF to model these interactions *in vitro* in order to further understand how galectin-3 modulates immune cell functions. This investigation may uncover potential utility in targeting galectin-3 in pre-malignant lesions. Targeting such pathways may represent a novel therapeutic avenue for reprogramming the cystic immune microenvironment towards a less permissive state for malignant transformation.

An additional technical consideration for future research would be the concentration of PCF used in experimental assays. In this current study, functional and co-culture experiments were conducted using low concentrations of PCF, primarily to conserve PCF for multiple experiments and to ensure replicates were used. However, *in vivo*, the epithelial lining and immune cell infiltrate of PCLs are continuously exposed to undiluted PCF. It is plausible that cellular responses differ under these physiological conditions. Future experiments should therefore evaluate dose-dependent effects of PCF, testing a range of concentrations. This approach may uncover concentration-dependent mechanisms of immune modulation, epithelial stress responses, or chemokine signalling that were not observable under diluted conditions.

Beyond mechanistic studies, the association between immunological experimental findings with clinically recorded data is essential. Many PCLs are discovered incidentally during routine imaging, and the clinical data was often incomplete at the time of the

patient's EUS-FNA procedure. Future investigations should aim to improve longitudinal and complete records of standard clinical data in parallel to PCL sample acquisition. Access to complete standard recorded clinical data for samples used within this study will enable correlation analysis of immune profiles with patient data to be carried out to potentially identify immune biomarkers predictive of cancer risk status.

Identifying immune pathways that contribute to the malignant transformation of PCLs opens opportunities for targeted intervention. Future studies are required to explore whether modulating chemokine signalling, blocking galectin-3-integrin interactions, or targeting immunosuppressive cell populations may prevent progression from low-risk PCLs to high-risk PCLs and ultimately to invasive PC. Such strategies may ultimately inform the development of immune-targeted therapies and complement existing management and cancer risk stratification guidelines for the prevention or early intervention of PC.

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