

Chapter 4. The immune microenvironment of appendiceal tumour peritoneal disease

Chapter 4 presents a transcriptomic analysis focusing on the immune compartment of the ATPD TME. The preliminary analysis of immune cell aggregates was presented as a poster at ASCO Gastrointestinal Cancers Symposium 2023(Abstract 217):

Strach, M. C., Yeung, N., Lin, H.-M., Ansari, N., Koh, C., Shin, J.-S., Kench, J., Horvath, L. and Mahon, K. L. Characteristics of immune-infiltrating cells in the tumor microenvironment of appendiceal cancer with peritoneal disease. *J Clin Oncol.* 2023;41(4_suppl):217–217. Doi: [10.1200/jco.2023.41.4_suppl.217](https://doi.org/10.1200/jco.2023.41.4_suppl.217)

4.1 Introduction

The normal human peritoneal microenvironment contains a high proportion of T cells and antigen-presenting macrophages which suggests it is poised for effective immune responses.²⁸⁴ The peritoneal tumour microenvironment has not been well characterised for ATPD, but data suggests an active and immunologically complex environment.^{238, 513-515} The immune microenvironment can be illustrated conceptually by the immunoediting paradigm. This refers to the dynamic process whereby the immune system influences the tumour in a way that promotes tumour progression. The three phases comprise: (1) elimination, where there is immunosurveillance leading to an effective immune response against the tumour, (2) equilibrium, where immunotolerance develops via mechanisms of evolving tumour immune-resistance, and (3) escape, where host immune defences are overwhelmed.^{516, 517} Tumour-associated features such as tertiary lymphoid structures (TLS) comprised of B cell-rich aggregates surrounded by T cells, similar to lymphoid follicles, impact on anti-tumoural immunity in other cancers.³¹² There is currently no role for immunotherapies for the treatment of ATPD, outside of clinical trials. Yet, immunotherapies have significantly prolonged patient survival in other cancers so identifying effective immune-modulating strategies for patients with ATPD is a high priority.

The primary objective of this study was to characterise the immune microenvironment of ATPD. This was done to: (1) identify the immune subsets present, (2) to elucidate the balance of anti-tumoural versus pro-tumoural immunity in the context of the immunoediting phases comparing LG and HG ATPD, and (3) to understand the composition of observed lymphoid aggregates. This builds on the previously presented single cell atlas of ATPD to describe the immune components as well as evaluate the influence of the mucinous microenvironment on the transcriptomic profile.

4.2 Methods

4.2.1 Patient cohort

Tissue and clinical data were collected from 33 patients with ATPD as previously described in Chapter 3. All samples were formalin-fixed and paraffin-embedded (FFPE) prior to H&E staining. Immune cell immunohistochemical (IHC) analysis was performed on 29 patients with ATPD (15 LG and 14 HG). Informed consent was obtained as previously stated under HREC approved protocols (Chapter 3 and [Appendix 2](#)).

4.2.2 Transcriptomic analysis

Transcriptomic profiling of the immune compartment of ATPD was performed using scRNAseq and spatial transcriptomic profiling methods (Chapter 3, Figure 3.1).

4.2.3 Immune cell assessment

FFPE tissues of patients with ATPD were reviewed for the presence of immune cell infiltrates (Figure 3.1). Tissue sections, stained with H&E, were reviewed using digitised slides on Qupath (v0.4.3) and characterised according to size, cell number, location and structure. Structural descriptors included infiltrating immune cells (no defined structure), or cell aggregates (Chapter 3, Figure 3.2D). Immune cell aggregates were initially defined based on H&E appearance where developing aggregates were partly rounded and developed aggregates were rounded. The descriptors of immature and mature aggregates (TLS) were also used to describe the presence of a potential germinal centre, identified on H&E or by confirmation using IHC markers (Figure 3.1).

Characterising the immune microenvironment in ATPD

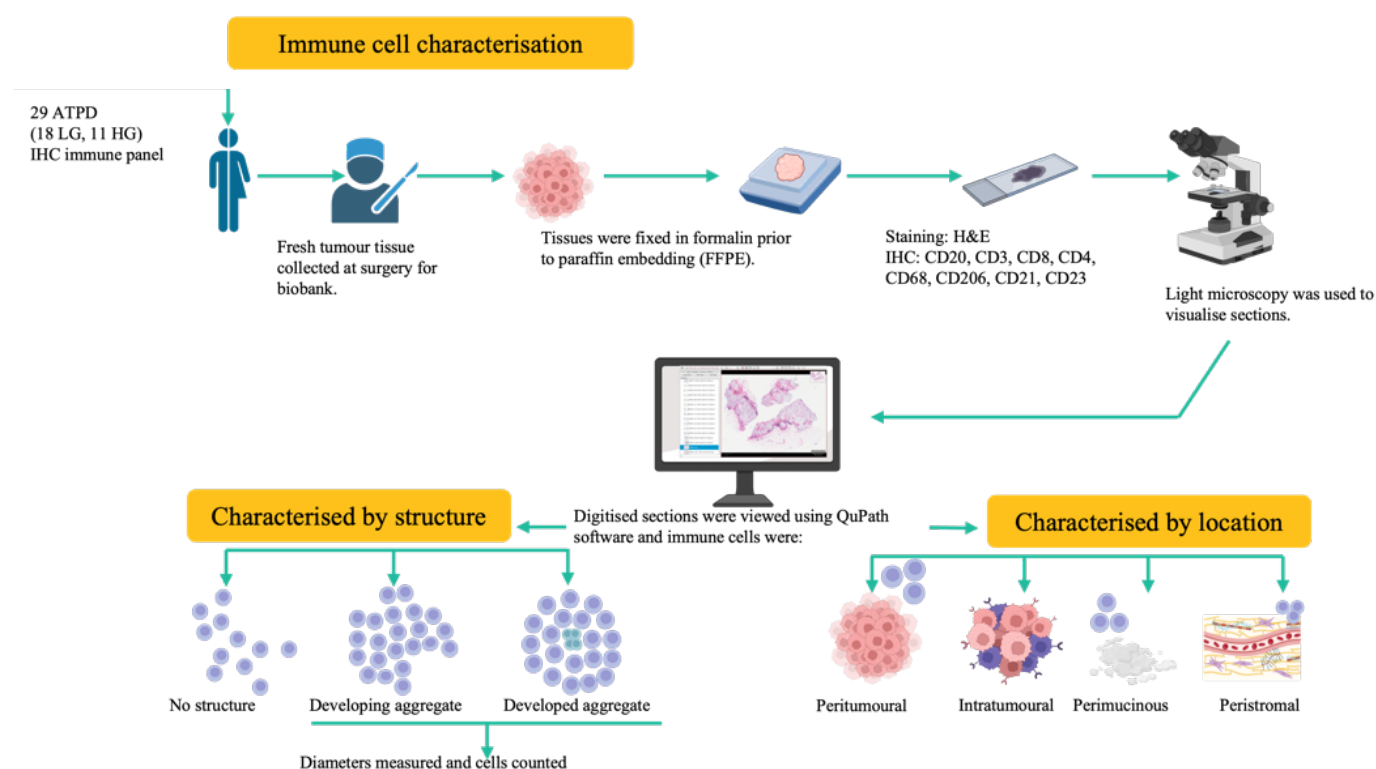


Figure 4.1 | Outline of characterisation of the immune cell microenvironment of ATPD

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Tumour tissue sections from ATPD were viewed and immune infiltrating cells characterised by location, structure and IHC panel of antibodies against immune markers.

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease.

Image created by BioRender.com.

4.2.4 Immunohistochemical immune cell panel

Samples with immune cells were further stained with a panel of antibodies against immune markers: anti-CD3 (polyclonal, Agilent Dako #A045229-2), anti-CD4 (clone EP204, Cell Signalling Technology #48274), anti-CD8 (clone C8/144B, Cell Signalling Technology #70306), anti-CD20 (clone L26, Agilent Dako #M0755), anti-CD21 (clone 2G9, ThermoFisher Scientific #MA5-11417), anti-CD23 (clone SP23, ThermoFisher Scientific #MA5-14572), anti-CD206 (clone E2L9N, Cell Signalling Technology #91992) and anti-CD68 (clone KP1, Abcam #Ab16702) (Table 4.1). Only selected samples with immune aggregates present on H&E were stained with anti-CD21 and anti-CD23, as these markers defined the presence of a germinal centre representing a mature aggregate (TLS). IHC staining was performed using a Leica Bond RX automated stainer (Leica Biosystems) with brown staining 3,3'-diaminobenzidine (DAB) chromogen for primary mouse antibodies and pink staining (Fast red chromogen) for primary rabbit antibodies. These were optimised as single stains (Bond polymer refine detection kit, Leica Biosystems), or as dual stains for CD20/CD3 (ChromoPlex 1 dual detection kit, Leica Biosystems). The final dilution of antibodies are indicated in Table 4.1. Descriptive statistics were used to evaluate immune cell aggregates, with unpaired t-tests to assess significance between the difference of means between immune cell aggregate diameters and cell counts.

Table 4.1 | Summary of antibodies used for immune cell immunohistochemistry[Back to paragraph](#)

Immune cell	Antigen	Antibody (anti-human)	Dilution	HIER (mins)	Epitope retrieval solution	Antibody incubation (mins)
T cell	CD3	Rabbit	1:400	20	ER2	60
Helper T cell	CD4	Rabbit	1:100	20	ER2	60
Cytotoxic T cell	CD8	Mouse	1:1000	20	ER2	60
B Cell	CD20	Mouse	1:3000	20	ER2	60
Germinal centre B cell	CD23	Rabbit	1:200	20	ER2	60
Follicular dendritic cell	CD21	Mouse	1:100	20	ER2	60
Macrophage	CD68	Mouse	1:5000	20	ER1	60
M2 macrophage	CD206	Rabbit	1:500	20	ER2	60

Abbrev. HIER, heat-induced epitope retrieval.

4.2.5 Deconvolution of immune cell types of the TME

An estimation of the cell type abundance of the spatial profiling of the TME was performed using the SpatialDecon package (v1.12.3).⁵¹⁸ This used a log-normal regression algorithm to compare gene expression in the experimental dataset with a reference matrix ('safeTME') of expected expression profiles designed to estimate the presence and abundance of immune and stromal cells in the tumour microenvironment. The safeTME matrix is estimated to have less than 20% transcripts attributed to cancer cells, to avoid misclassification.⁵¹⁹ The algorithms used have been optimised for the lower signal and higher background of spatial gene expression data. The normalised data is integrated with a matrix of expected background signal for each data point estimated from the negative control probes.

4.3 Results

4.3.1 Patients and tissue samples

Of 33 patients with peritoneal tissue evaluated, four patients had no immune cells evident on H&E-stained FFPE sections and were excluded from further immune cell evaluation, leaving 29 patients for further analysis (18 LG, 11 HG). Patients were classified according to the presence of immune cell aggregates (n=23) and these cases were evaluated further. The spatial transcriptomic analysis was most representative for LG ATPD as it included 24 immune cell segments from 11 patients with LG ATPD compared to 11 immune cell segments from four patients with HG ATPD. One patient (#5) did not have any tumour present and was classified as a 'normal' control. Due to overlapping analysis of specimens, the clinicopathological characteristics are detailed in the previous chapter (Chapter 3, Table 3.4).

4.3.2 Transcriptomic profiles of immune cells

The majority cell type in the single cell atlas belonged to the immune compartment (Figure 4.2A-C). All major immune cell lineages were present within the TME of ATPD, but there was a notable preponderance of peritoneal T cells and myeloid cells (Figure 4.2D-F). This was consistent with the evaluation of the immune segments from the spatial profiling analysis following deconvolution using the safeTME reference (Figure 4.3). Numerous immune cell subsets were present in each immune segment consistent with an immunologically rich peritoneal milieu which has been previously documented in other disease states. Annotation of the immune cell populations by marker genes is displayed in [Appendix 3](#) Supplementary Figures SF3.2-3.6. Additionally, macrophage and dendritic cells dominate myeloid subsets.^{284, 520, 521} The presence of fibroblasts (CAFs) in some designated immune segments highlights the challenges of spatial annotation of intermingled cell types, but also raises the possibility of the presence of leucocytes with fibroblastic properties such as monocyte-derived fibrocytes.^{522, 523} Furthermore, this is consistent with the close relationship between CAFs and immune cells, where their inter-cellular signalling shapes the TME.⁴²²

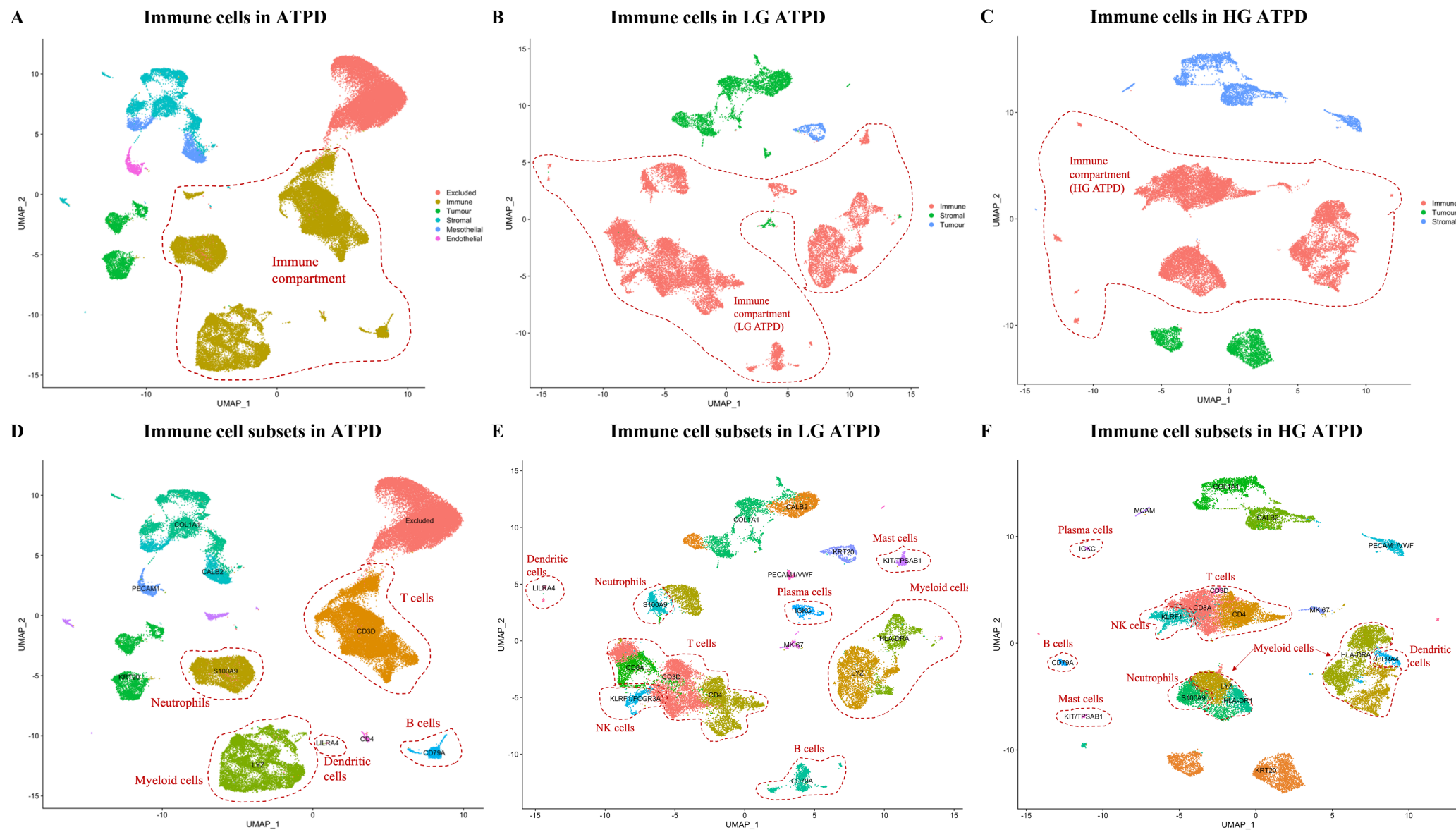


Figure 4.2 | The immune compartment and immune cell subsets of ATPD atlas

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UMAP visualisations show the immune cell compartment (A-C) and immune cell subsets (D-F) for all ATPD (A,D) and LG (B,E) and HG (C,F) ATPD.

Abbrev. UMAP, uniform manifold approximation projection; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; NK, natural killer.

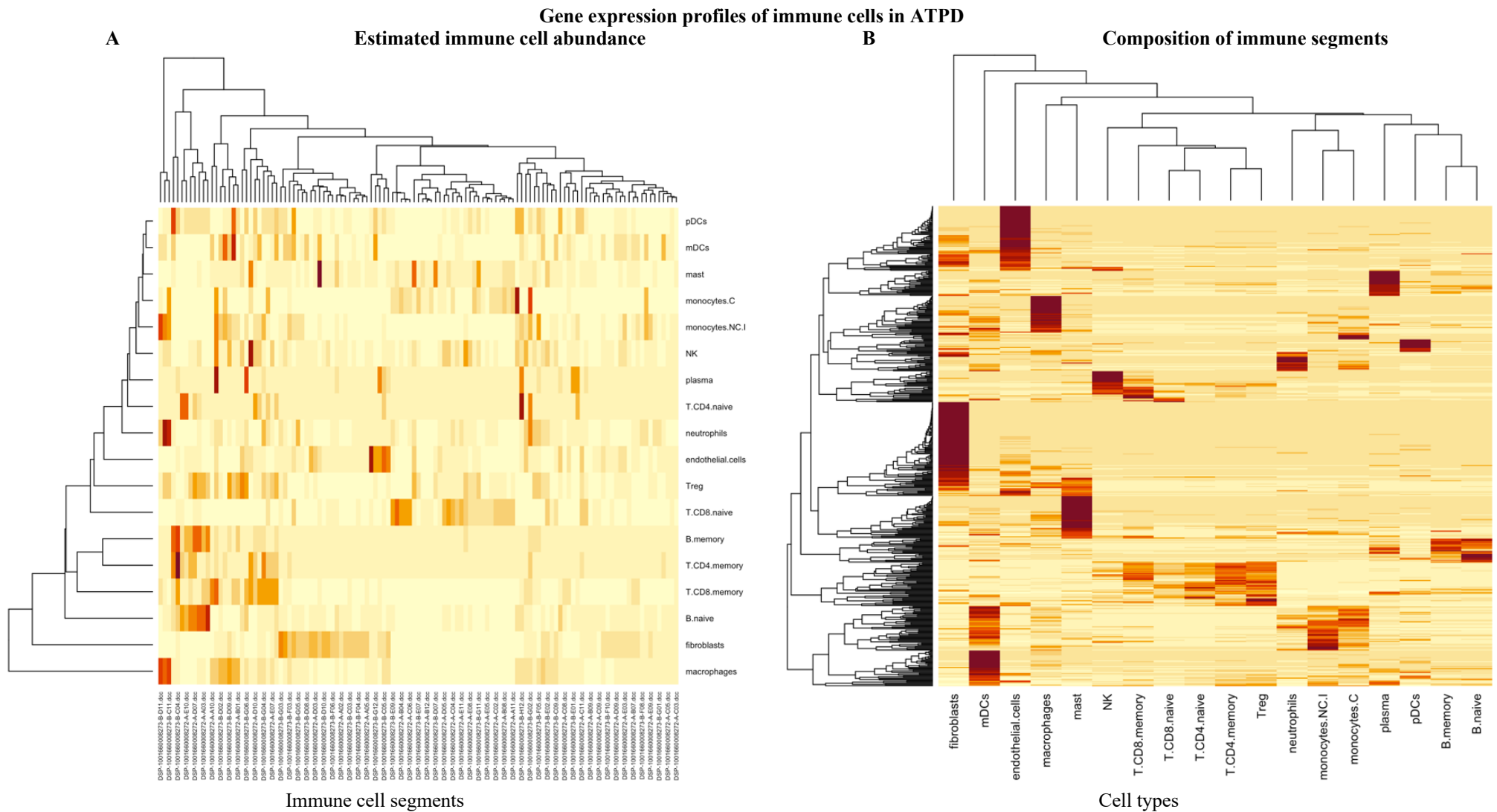


Figure 4.3 | Deconvolution of genetically profiled ATPD immune segments by spatial profiling using a reference dataset

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Heatmaps show the estimated abundance of TME cell subsets per immune segment based on gene expression (A) and TME cell-type composition of the global immune infiltrate of ATPD (B). Red/orange is high and yellow is low expression.

Abbrev. ATPD, appendiceal tumour peritoneal disease; TME, tumour microenvironment; pDCs, plasmacytoid dendritic cells; cDCs, conventional dendritic cells; monocytes.C, classical monocytes; monocytes.NC, non-classical monocytes; NK, natural killer; Treg, regulator T cell.

4.3.2.1 Identification of immune cell subsets in the TME

The dominant T cell and myeloid populations identified in the single cell atlas described in Chapter 3 were studied, in order to elucidate the balance of pro-tumoural versus anti-tumoural immunity comparing LG to HG ATPD. On evaluation of the ATPD single cell UMAP combining LG and HG tissue samples (Chapter 3, Figure 3.5B), the overlapping immune cell populations between LG and HG were mostly myeloid populations. The main difference was the predominant T cell population in LG ATPD (the small contribution from subjects #28 and #29 with HG ATPD is more easily identified in Figure 3.6B). LG ATPD also had a more prominent B cell population than HG ATPD (Chapter 3, Figure 3.5B). Thus, in this study myeloid cell populations from LG and HG ATPD shared similar transcriptomic profiles. The direct comparison of relative cell abundance between LG and HG ATPD per subject is displayed in Figure 4.4. It is also important to note that this study was performed on a small number of patients and tissue sampling may not reflect the full microenvironmental heterogeneity. The transcriptomic analysis provides observational insights to develop hypotheses for future research.

Assessment of abundance of immune cell types in ATPD

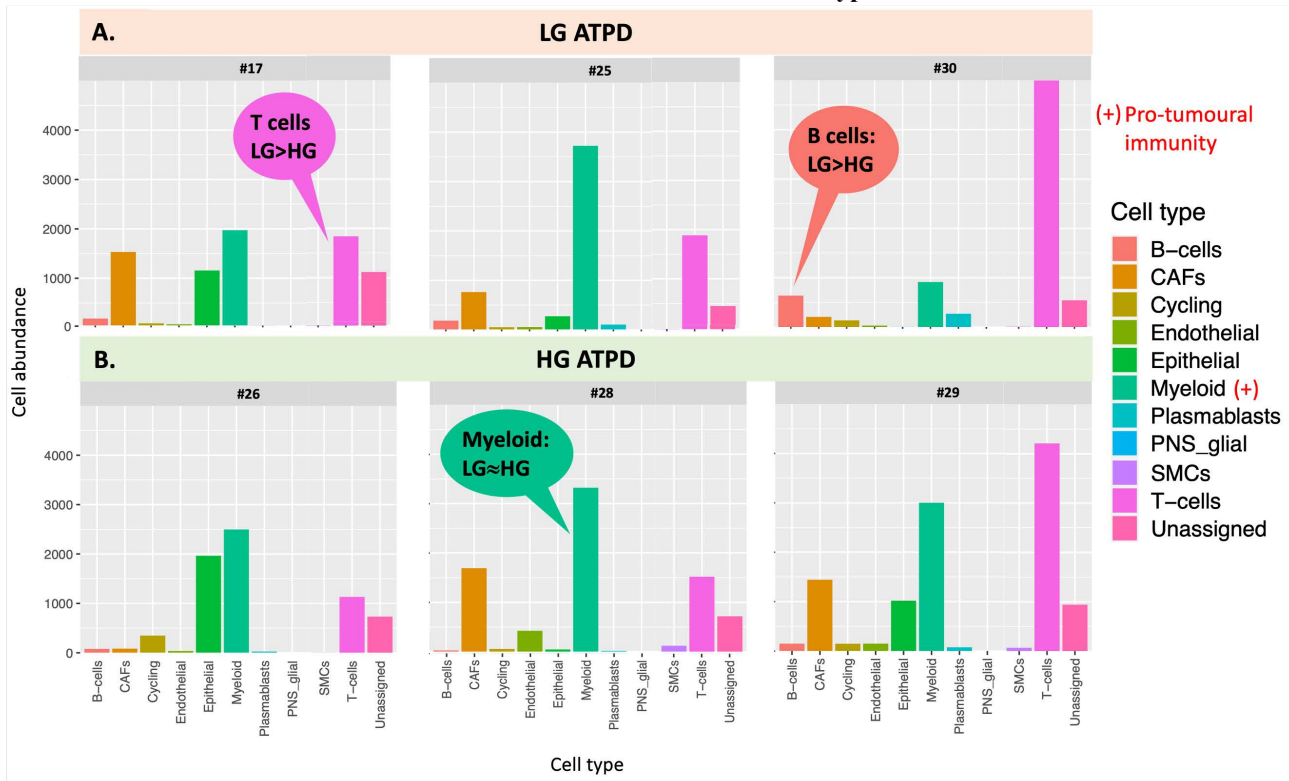


Figure 4.4 | Assessment of cell type abundance in ATPD

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Bar plots represent the raw cell number for major cell lineages focusing on the relative abundance of T cells, myeloid cells and B cells in LG (A) and HG ATPD (B).

Abbrev.ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; CAFs, cancer-associated fibroblasts; PNS, peripheral nervous system, SMCs, smooth muscle cells.

4.3.2.2 Pro-tumoural T and myeloid cells in ATPD

a) Regulatory T cells and CD4 Th2 differentiation

Within the CD4 T cell subset (Chapter 3, Figure 3.9), there were cells expressing genes indicating T helper type 2 differentiation (Th2), with a similar distribution in LG and HG ATPD (*IL4R*; Figure 4.5A,B). Their presence suggests pro-tumoural immunity as they are known to secrete anti-inflammatory mediators including IL-4, IL-5, IL-9, IL-10 and IL-13.^{524, 525} There was also a population of Tregs in both LG and HG ATPD (*FOXP3*; Figure 4.5B,C), which are known to promote an immunosuppressive TME.^{288, 526} Gene set enrichment analysis indicated upregulated genes associated in LG ATPD with ‘IL2 STAT5 signaling’ and in HG ATPD with ‘cytokine signaling in immune system’ ($P_{adj} < 0.05$). This transcriptomic profile is consistent with activation of Th2 and regulatory differentiation programs in ATPD.

Immunosuppressive T cell subsets in ATPD

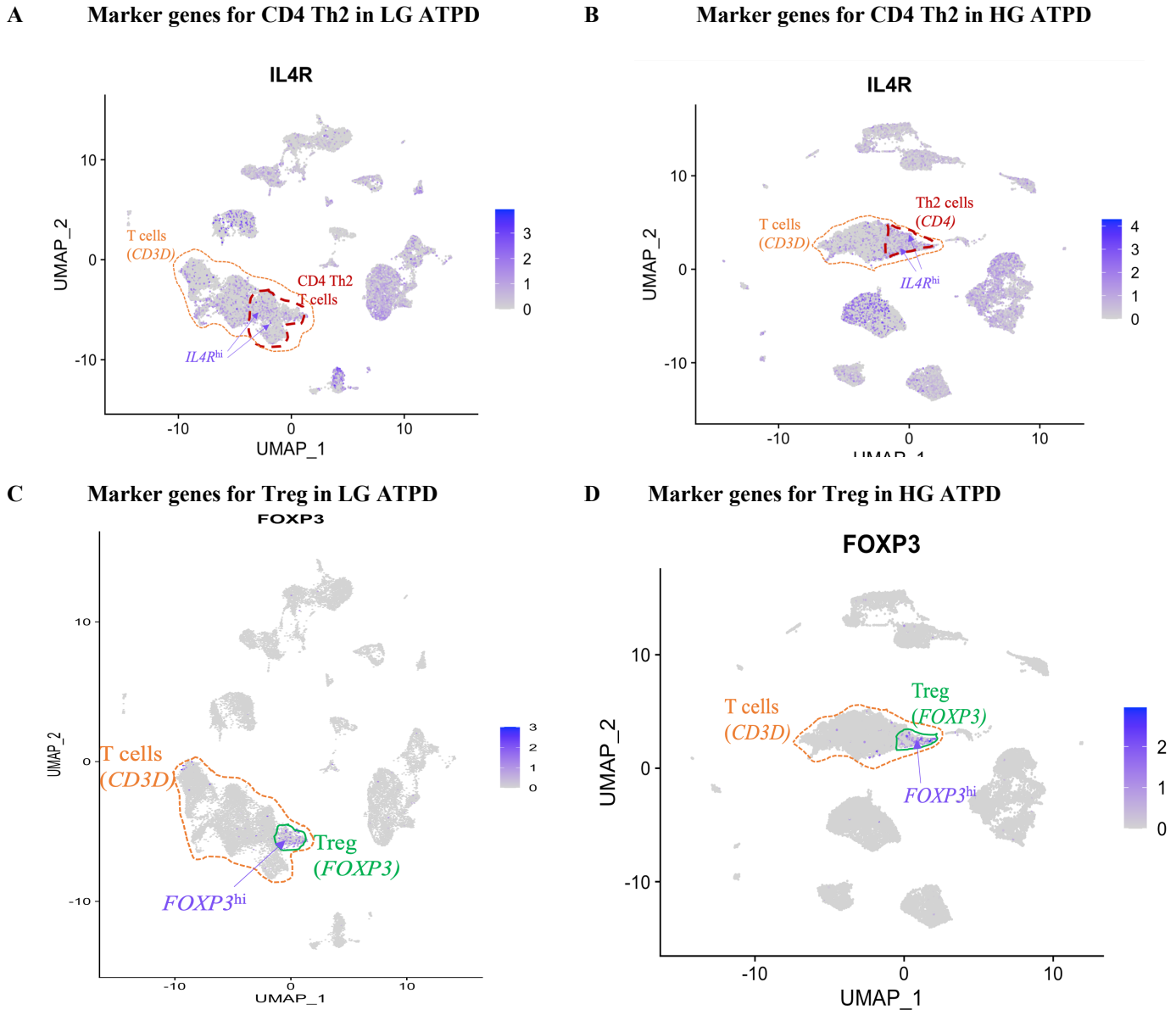


Figure 4.5 | Immunosuppressive T cell subsets in ATPD and expression of pro-tumoural genes

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Feature plots (UMAPs identifying cells expressing single genes of interest in blue) show T cell subsets of CD4 Th2 cells (*IL4R*; **A,B**) and Tregs (*FOXP3*; **C,D**) in LG (**A,C**) and HG ATPD (**B,D**).

Abbrev. UMAP, uniform manifold approximation projection, ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; UMAP, uniform manifold approximation projection, Th2, T helper type 2; Treg, regulatory T cells.

b) M2 macrophage polarisation

M2 (*MRC1*) macrophages are depicted in the UMAPs in Figure 4.6A,B. M2 macrophages are classified by an *in vitro* activation state that is highly immunosuppressive and tumour-promoting.^{298, 527} Gene set enrichment analysis suggested antigen presentation activity in the M2 macrophages ($P_{\text{adj}} < 0.05$; Figure 4.6C,D).^{298, 528} HG ATPD was upregulated genes were also enriched for genes associated with ‘complement component C1q complex’ ($P_{\text{adj}} < 0.05$; Figure 4.6C). *CIQC* expressing tumour-associated macrophages have been previously reported to interact with T cell subsets, suggesting a role for modulation of anti-tumoural T cell responses.³⁰¹ To add complexity, tumour-associated macrophages can co-express gene profiles of M1 and M2 states.³⁰¹ Therefore, to confirm that M2 gene expression (*MRC1*) resulted in expression of its CD206 protein marker, immunostaining with anti-CD206 was performed. This confirmed the presence of M2 macrophages in ATPD tissues (Figure 4.6C,D). Therefore, the ATPD microenvironment may be influenced by the immunosuppressive M2 macrophage subset.

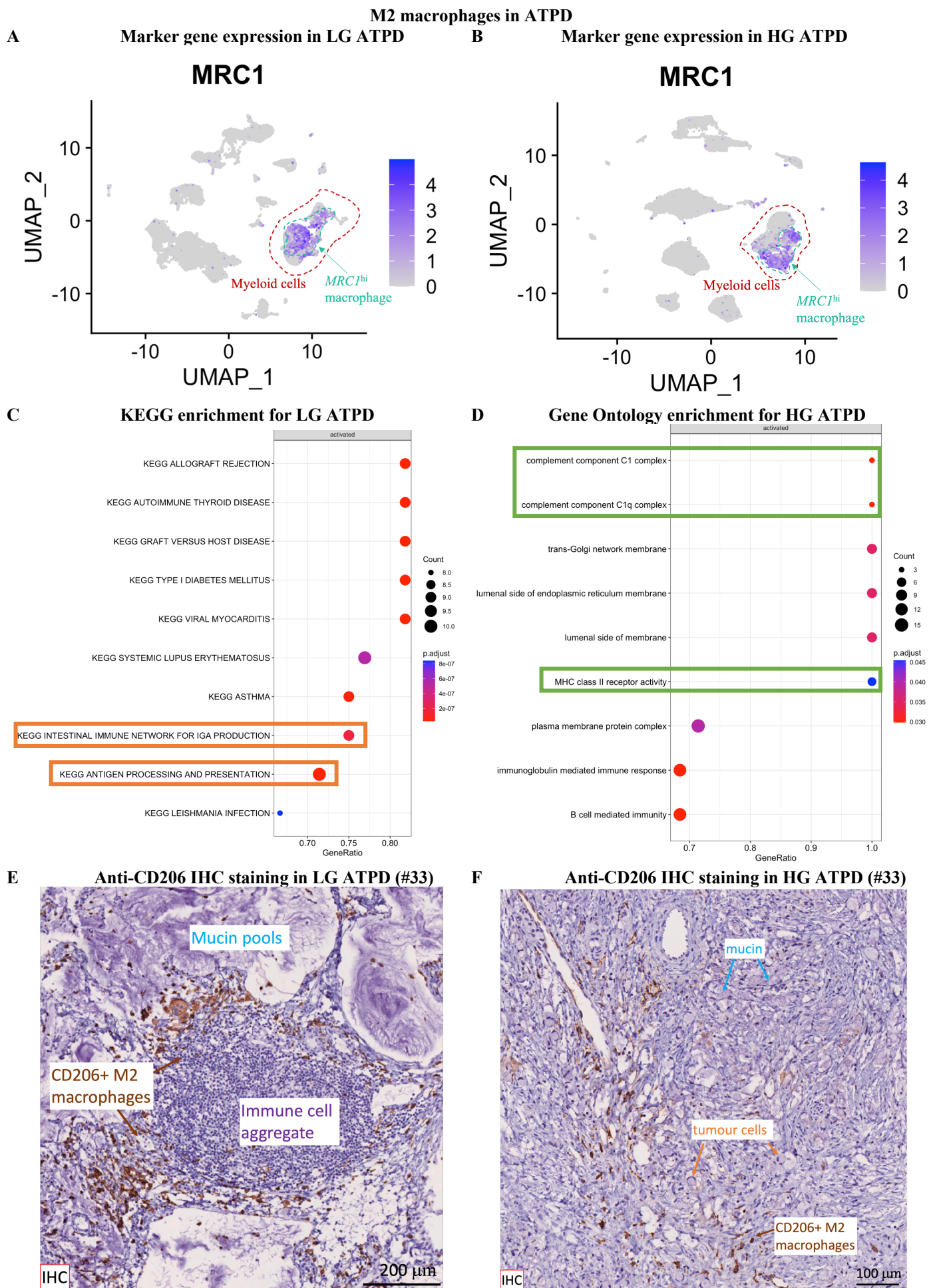


Figure 4.6 | Assessment of M2 macrophages and functional pathway enrichment

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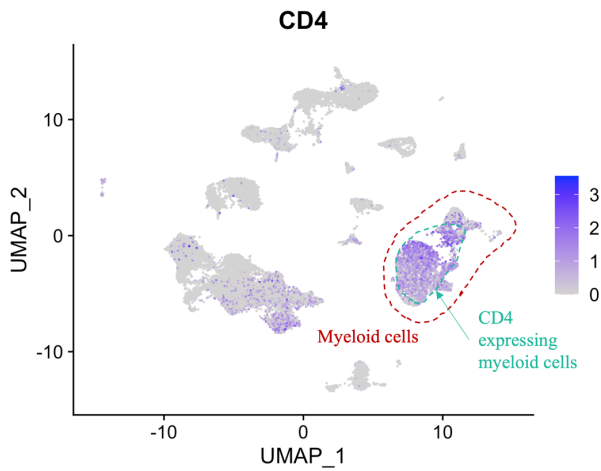
Feature plots show the expression of marker genes for M2 macrophages (*MRC1* [CD206]) in LG (A) and HG ATPD (B). Colour scale represents the log-normalised gene expression. Dotplots shows the Gene Ontology enrichment for M2 macrophage cluster from LG (C) and HG ATPD (D). The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted $p < 0.05$). Histological sections shows anti-CD206 immunostaining (brown) of M2 macrophages in LG (E) and HG ATPD (F). Abbrev. UMAP, uniform manifold approximation projection, ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of genes and genomes.

c) Peritoneal resident macrophages

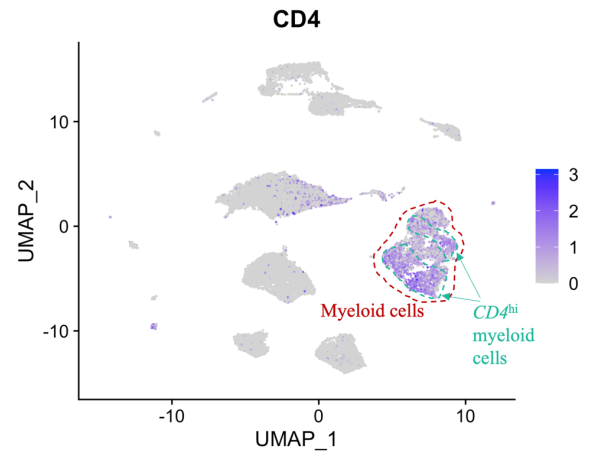
Peritoneal resident tissue macrophages (*CD4*, *LYVE1*) are depicted in the UMAPs in Figure 4.7. Peritoneal macrophages co-expressing *CD4* and *TIMD4* (T-cell immunoglobulin and mucin domain containing 4, encodes Tim-4) have been documented previously.^{300, 529} *TIMD4* expression was confirmed in LG ATPD (Figure 4.7C), but was less evident in HG ATPD (Figure 4.7D). *LYVE1* is a canonical marker gene for tissue resident macrophages, and LG had more cells expressing this than HG ATPD (Figure 4.7E,F).⁵³⁰ The gene set enrichment analysis in addition to antigen presenting activity also suggested upregulated genes associated with the ‘intestinal immune network for IgA production’ ($P_{adj} < 0.05$; Figure 4.6C). This suggests peritoneal resident tissue macrophages may play a role in repair responses.^{531, 532} An excessive immune response directed towards repair may explain overproduction of mucin as an aberrant repair response.

Peritoneal resident tissue macrophages (RTMs) in ATPD

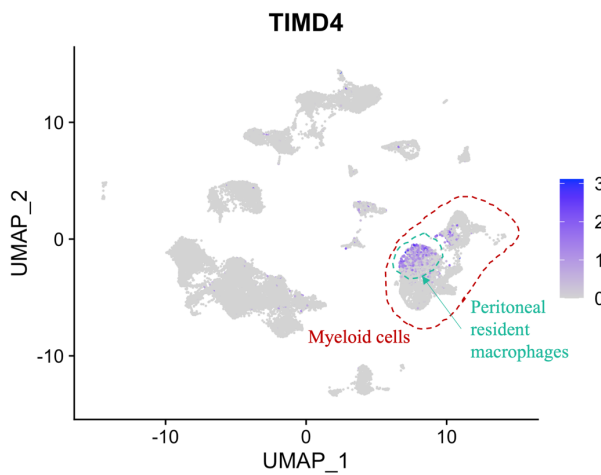
A Marker gene expression for RTMs in LG ATPD



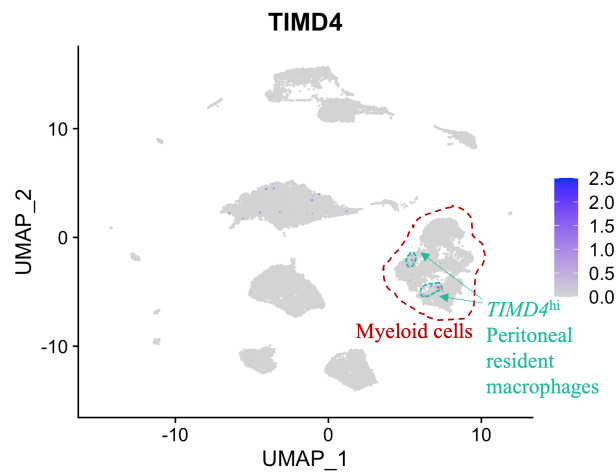
B Marker gene expression for RTMs in HG ATPD



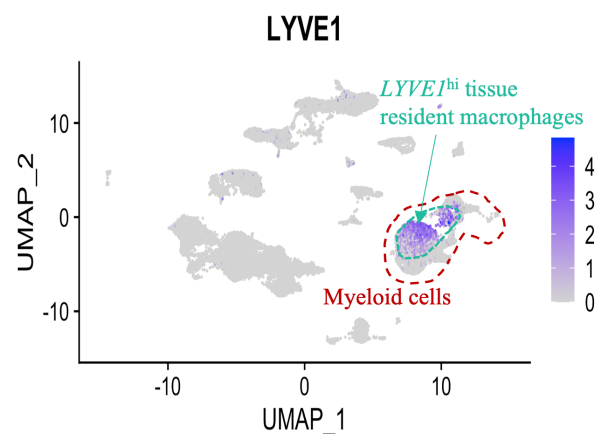
C



D



E



F

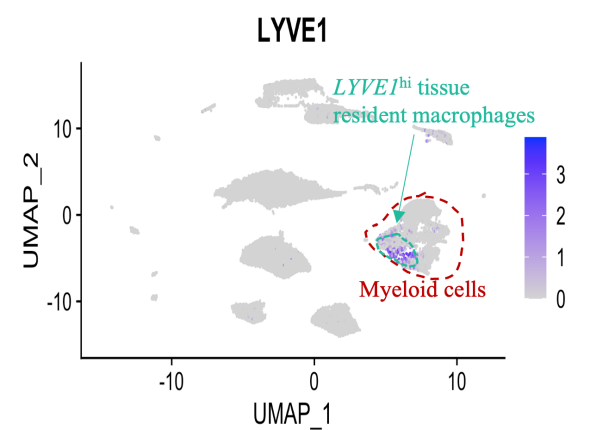


Figure 4.7 | Assessment of peritoneal tissue resident macrophages in ATPD

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Feature plots show the expression of marker genes *CD4* (A,B), *TIMD4* (C,D) and *LYVE1* (E,F) for peritoneal tissue resident macrophages for LG (A,C,E) and HG ATPD (B,D,F). Colour scale represents the log-normalised gene expression.

Abbrev. RTMs, resident tissue macrophages; ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade.

d) *Myeloid-derived suppressor cells*

Tumour-associated neutrophils were canonically identified by *S100* genes (Figure 4.8A,B). In parallel to tumour-associated macrophages, these cells are classified as N1 (anti-tumoural) and N2 (pro-tumoural).⁵³³ Overlapping with the neutrophil population were genetically similar cells with features of myeloid-derived suppressor cells (MDSCs) identified by *ITGAM* (CD11b; Figure 4.8A,B). These cells are highly immunosuppressive.⁵³⁴ Gene set enrichment of the MDSC population in LG ATPD indicated that upregulated genes were associated with ‘fatty acid binding’, ‘icosanoid binding’ and ‘arachidonic acid binding’ ($P_{\text{adj}} < 0.05$; Figure 4.8C). This was consistent with a role for fatty acid metabolism in immune cells which influences and can reprogramme the TME to drive anti-tumour inflammation.^{535, 536} In HG ATPD, these cells were enriched for genes associated with ‘RAGE receptor binding’, ($P_{\text{adj}} < 0.05$; Figure 4.8D). Receptor for advanced glycosylation endpoint (RAGE) ligands are thought to influence oncogenic pathways including MAPK, NF- κ B and TNF α (as described in Chapter 3).⁵³⁷ Overall, both tumour-associated neutrophils and MDSCs contribute to the immunosuppressive myeloid cell population present in ATPD.

Neutrophil and MDSC subsets in ATPD

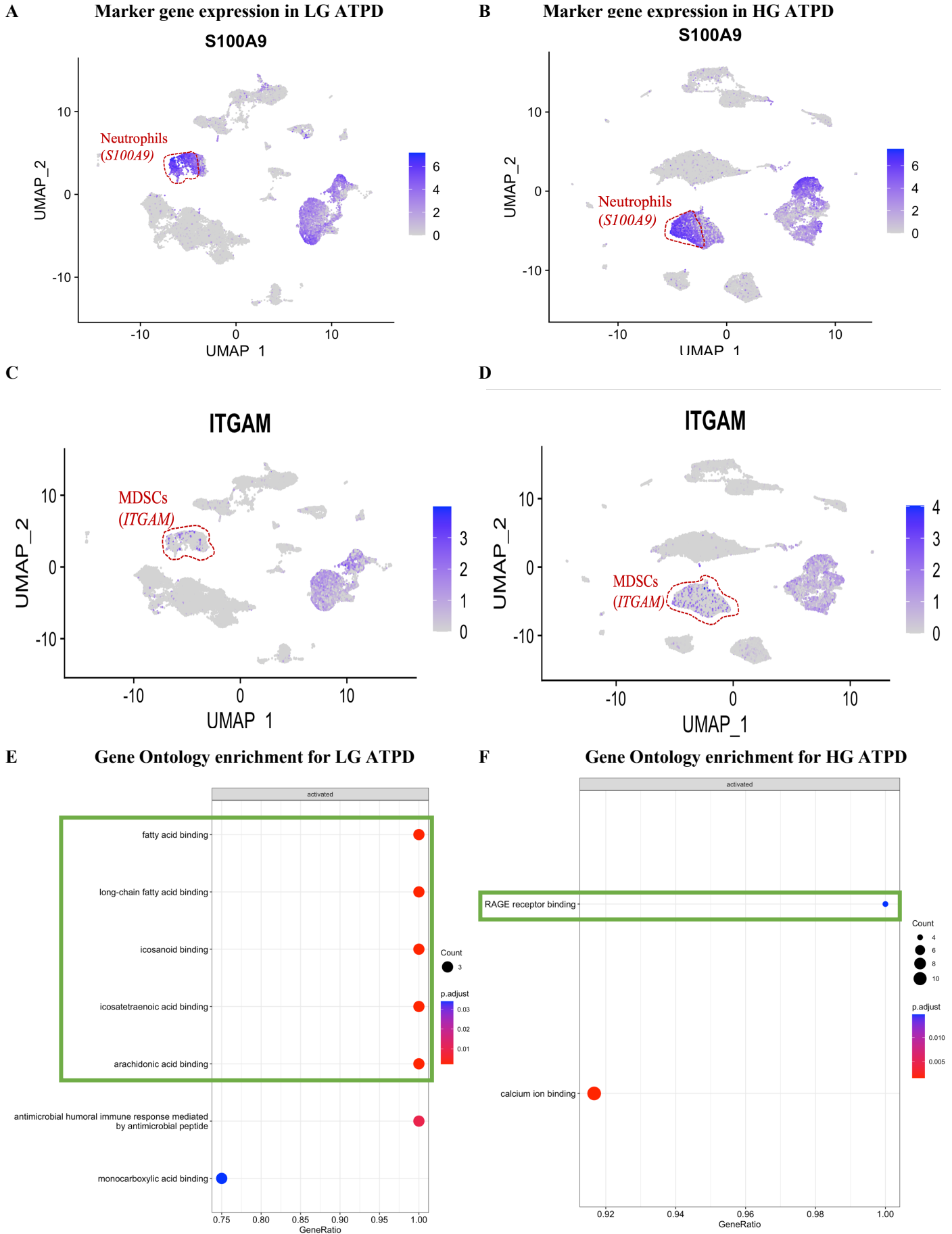


Figure 4.8 | Assessment of tumour associated neutrophil and myeloid derived suppressor cells

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Feature plots show the expression of marker genes for neutrophil (*S100A9*) and MDSCs (*ITGAM*) for LG (A,C) and HG ATPD (B,D). Colour scale represents the log-normalised gene expression. The dotplots shows Gene Ontology theme enrichment for the neutrophil/MDSCs in LG (E) and HG (F). The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted $p < 0.05$).

Abbrev. UMAP, uniform manifold approximation projection, ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade, MDSCs, myeloid-derived suppressor cells.

4.3.3 Influence of the mucinous microenvironment in LG ATPD

A hallmark of the ATPD microenvironment is the presence of mucin. Mucin is a normal bodily substance when secreted on mucosal membranes with a role to protect and heal.⁴²⁶ However, its presence in the peritoneal space is pathological. Thus, the abnormal mucin could influence the ATPD immune microenvironment. Firstly, it could trigger an immune response aimed at clearing both mucin and secreting tumour cells. Secondly, mucin may elicit an immunotolerant response that would allow for ongoing disease activity and may provide physical protection to secreting tumour cells. These processes may interact and lead to a net immune response favouring the tumour. The disease process could also be exacerbated if a pro-tumoural immune response activated ‘protective’ mechanisms leading to a further accumulation of mucin (i.e. an aberrant repair attempt). Transcriptomic differences between immune cells under different microenvironmental conditions were investigated to elucidate how mucin may impact the balance of tumoural immunity.

There were no statistically significant differentially expressed genes between immune segments comparing peri-mucinous (immune segments near mucin) to peri-tumoural (near tumour) and intra-tumoural (immune cells infiltrating into tumour cells) microenvironments ($FDR > 0.05$). Peri-mucinous compared to both peri-tumoural and compared to intra-tumoural immune segments were enriched for genes associated with ‘TNFa signaling via NFkB’ and ‘FCGR3A mediated IL10 synthesis’ ($P_{adj} < 0.05$; Figure 4.9B,C). These findings suggest that the differing microenvironments may have an impact on shaping the tumoral immune response. Additionally, the presence of mucin promotes a pro-tumoural immune response.

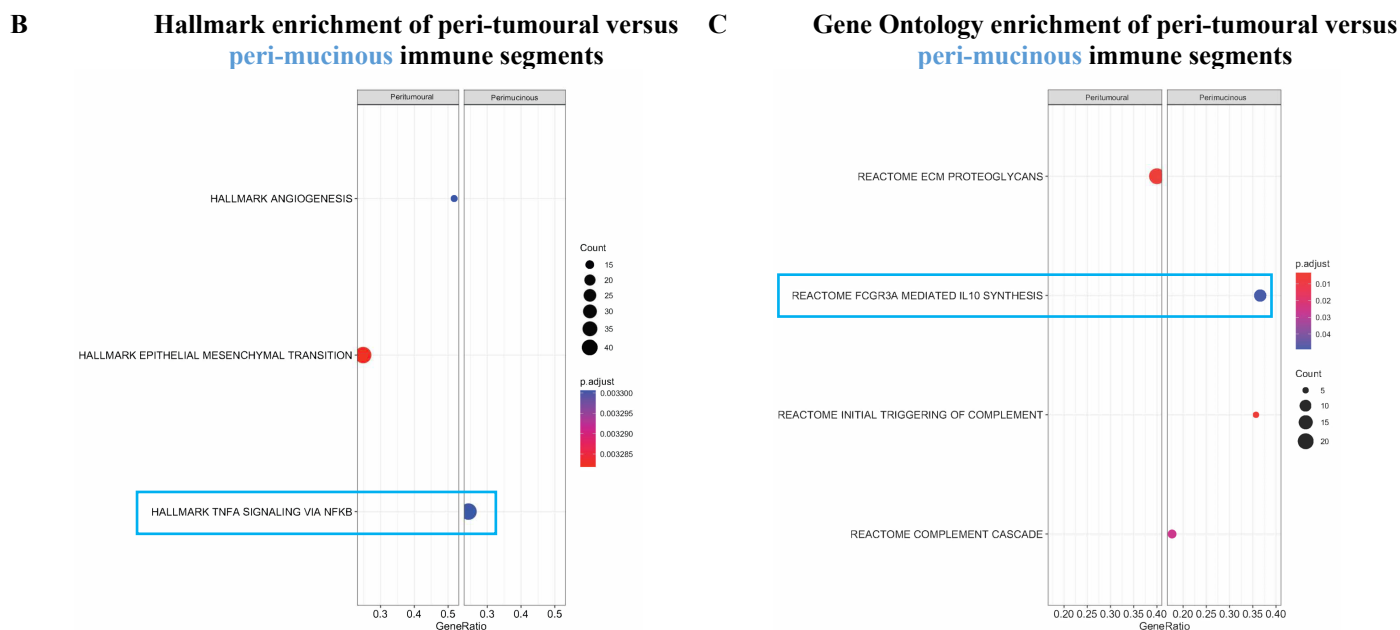
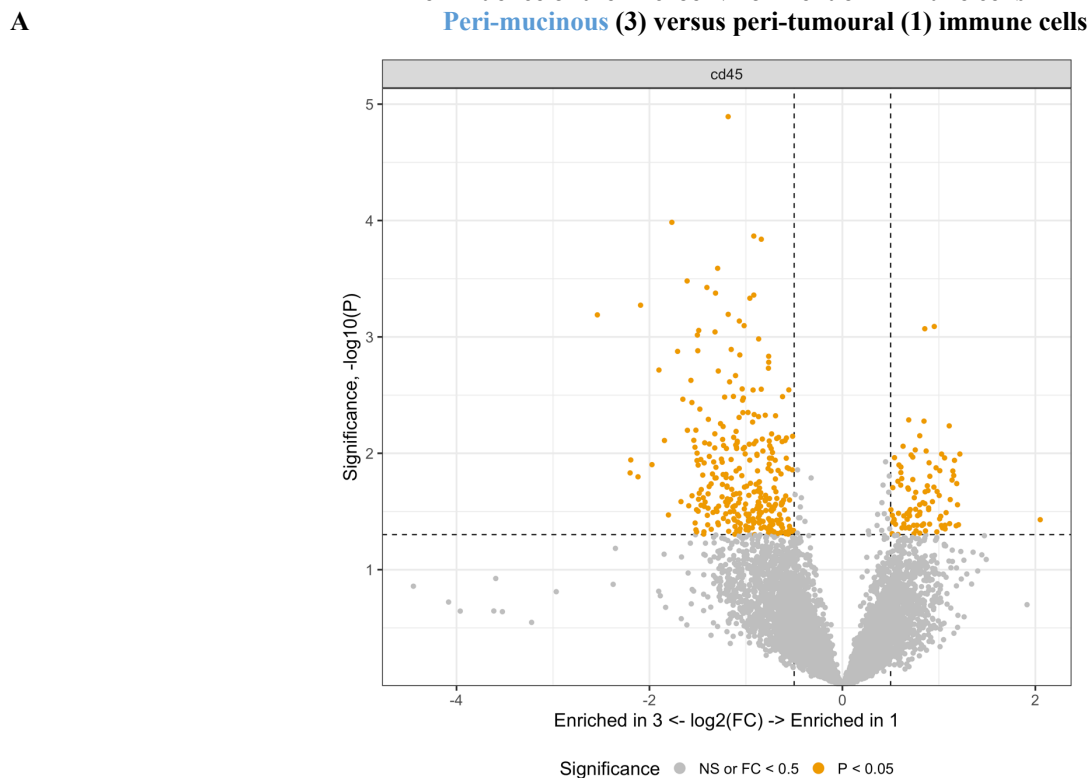


Figure 4.9 | Differential gene expression and functional pathway enrichment of peri-tumoural versus peri-mucinous immune segments

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The volcano plot shows the differential gene expression (A) between peri-mucinous and peri-tumoural immune cell microenvironments (by $\log_2(FC)$). Significantly differentially expressed genes are defined as $\log_2(FC) > 0.5$ and FDR p-value < 0.05 . Dotplots show the Hallmark (B) and Gene Ontology (C) themes comparing peri-tumoural to peri-mucinous microenvironments. The top 10 significantly enriched gene signatures are shown (Benjamini-Hochberg adjusted $p < 0.05$).

Abbrev FC, fold change; FDR, false discovery rate.

4.3.4 Tertiary lymphoid structures (TLS) in ATPD

4.3.4.1 Characterisation of structural morphology of immune cells in ATPD

The tumour immune microenvironment can be thought of spatially as being composed of different cellular neighbourhoods.^{397, 538} This is a helpful concept as it explains how the TME can be physically divided into pro- and anti-tumoural areas which are not only determined by the cell type and their interactions between cells and extracellular structures, but also by their structural arrangement. The physical appearance of the immune microenvironment was evaluated to assess the impact of neighbourhood as defined by structure and microenvironmental location.

4.3.4.2 Baseline characteristics of immune cells in ATPD

The characteristics of immune cell morphological structures in ATPD are displayed in Table 4.2. Examples of sections showing the location and structure of immune cells are demonstrated in Figure 4.10. Overall immune cell aggregates had a mean diameter of 249 μm (range 75 to 680 μm) and a mean cell count of 1442 cells (range 46 to 7830) (Table 4.3). Immune aggregates from LG ATPD (mean cell count 1341) were significantly less cellular compared to those from ‘normal’ peritoneal tissue (mean cell count 2912; $p=0.0091$), although there were only four immune aggregates from one normal sample for the comparison.

Table 4.2 | Classification of infiltrating immune cells in the ATPD microenvironment

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Immune aggregates	Number of areas with infiltrating immune cells on H&E stains (% of total)			
	Normal	LG ATPD	HG ATPD	All ATPD
Patient cases (n)	1	11	6	18
Total	5 (100)	56 (100)	27 (100)	88 (100)
Developing	1 (20)	8 (14)	14 (52)	23 (26)
Developed	3 (60)	44 (79)	9 (33)	61 (69)
None (infiltrating)	1 (20)	4 (7)	4 (15)	9 (10)

Abbrev. ATPD, appendiceal tumour peritoneal disease; LG, low grade; HG, high grade; H&E, haematoxylin and eosin

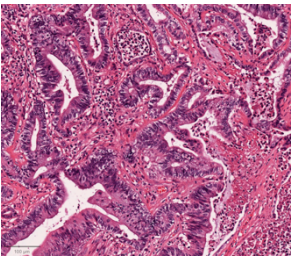
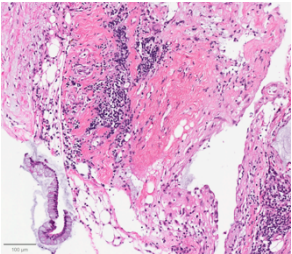
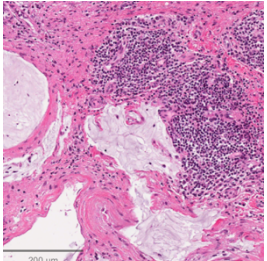
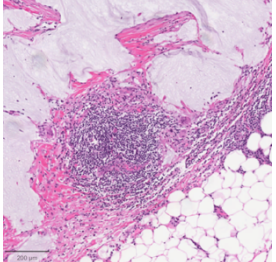
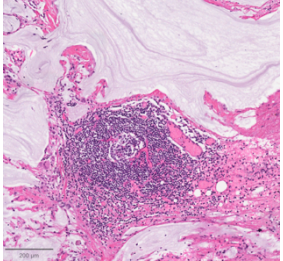
	Location	Histological appearance	Structure
A	Intra-tumoural		Infiltrating immune cells
B	Peri-tumoural		Developing immune cell aggregate
C	Peri-mucinous		Developing immune cell aggregate
D	Peri-mucinous		Developed aggregate (no germinal centre)
E	Peri-mucinous		Developed aggregate (potential germinal centre)

Figure 4.10 | ATPD tissues showing different microenvironments and structures of immune infiltrating cells

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H&E stained tissue sections from ATPD showing intra-tumoural (**A**), peri-tumoural (**B**) and peri-mucinous (**C-E**) locations and infiltrating (**A**), developing aggregate (**B, C**) and developed aggregate (**D,E**) structures of immune cells.

Abbrev. ATPD, appendiceal tumour peritoneal disease; H&E, haematoxylin and eosin.

Table 4.3 | Summary of immune cell aggregate characteristics with comparison of ATPD to normal peritoneal tissue
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	n	Mean diameter of immune aggregate (µm) (range)	p-value (ATPD vs normal)	Mean number of immune cells (range)	p-value (ATPD vs normal)
All	85	249 (75-680)	-	1442 (46-7830)	-
LG ATPD	79	250 (80-520)	0.097	1341 (46-6490)	0.0091*
HG ATPD	42	226 (75-680)	0.15	1422 (83-7830)	0.17
Normal	4	346 (2929-471)	ref	2912 (1697-4275)	ref

*Denotes significant p<0.05.

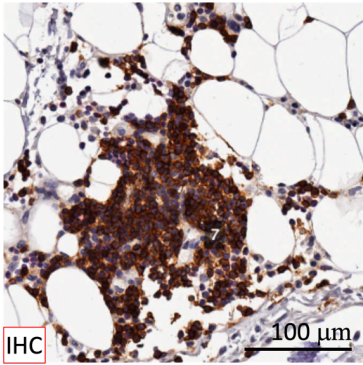
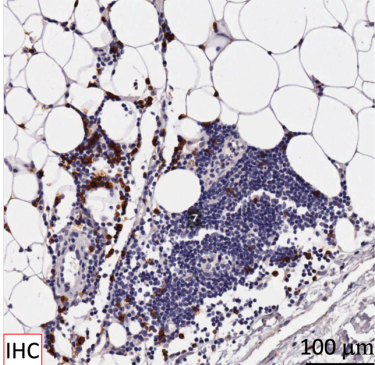
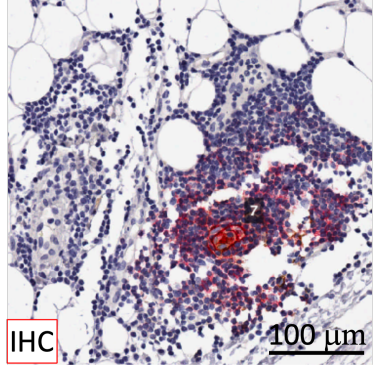
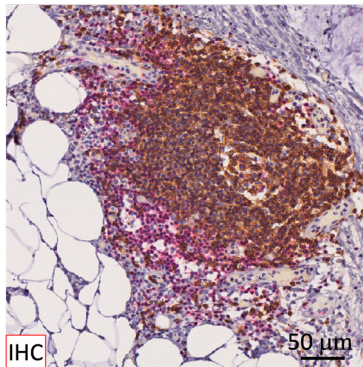
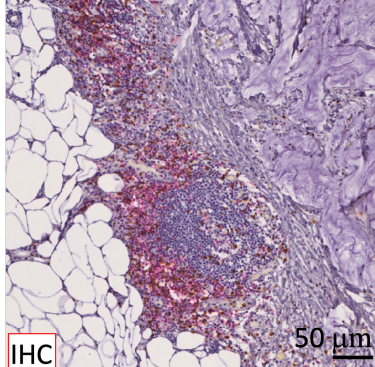
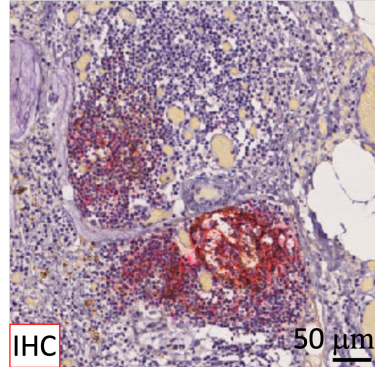
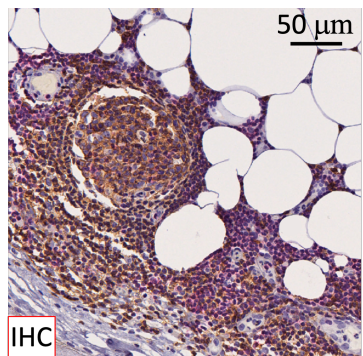
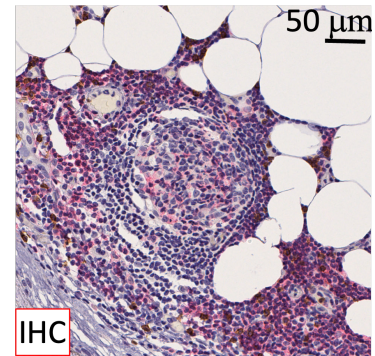
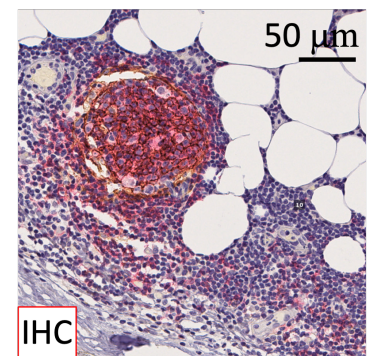
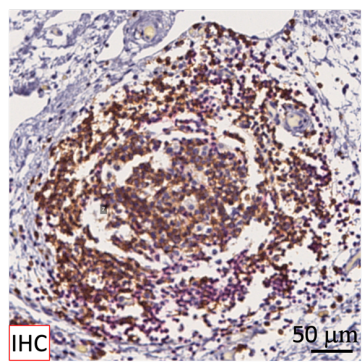
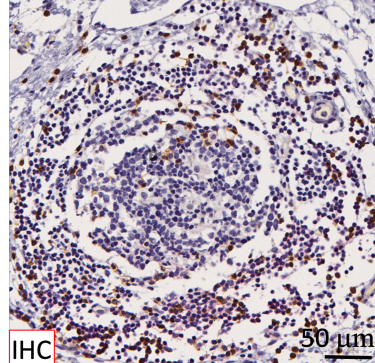
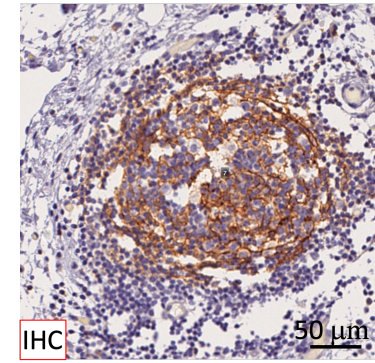
Abbrev. ATPD, appendiceal peritoneal disease; LG, low grade; HG, high grade.

4.3.4.3 IHC evaluation of immune cell subsets and tertiary lymphoid structures

Developing and developed immune cell aggregates were present in the ATPD microenvironment, with evidence of the generation of tertiary lymphoid structures (TLS) on IHC. Twelve cases (40%) exhibited developed aggregates (10 LG and two HG) on H&E-stained tissues. Germinal centre formation (CD21⁺CD23⁺ lymphocytes) in developed (mature) aggregates from eight cases (seven LG and one HG) is consistent with the capacity for *in situ* antibody production and anti-tumoural immune response (Figure 4.11). This is associated with improved clinical outcomes in other tumour types.^{310-312, 314, 539-541}

These findings highlight that the immune cells, more so in the LG than the HG ATPD microenvironment, are competing to establish pro- or anti-tumoural immunity. This is consistent with the overall findings supporting immunotolerance and the state of ‘equilibrium’ in LG ATPD and in contrast, an immunosuppressive microenvironment consistent with immune ‘evasion’ in HG ATPD.

Immune cell aggregates immunohistochemistry

ID #	LG ATPD		
	CD20/CD3 A	CD8/CD4 B	CD21/CD23 C
1 #3			
2 #8			
3 #11			
4 #13			

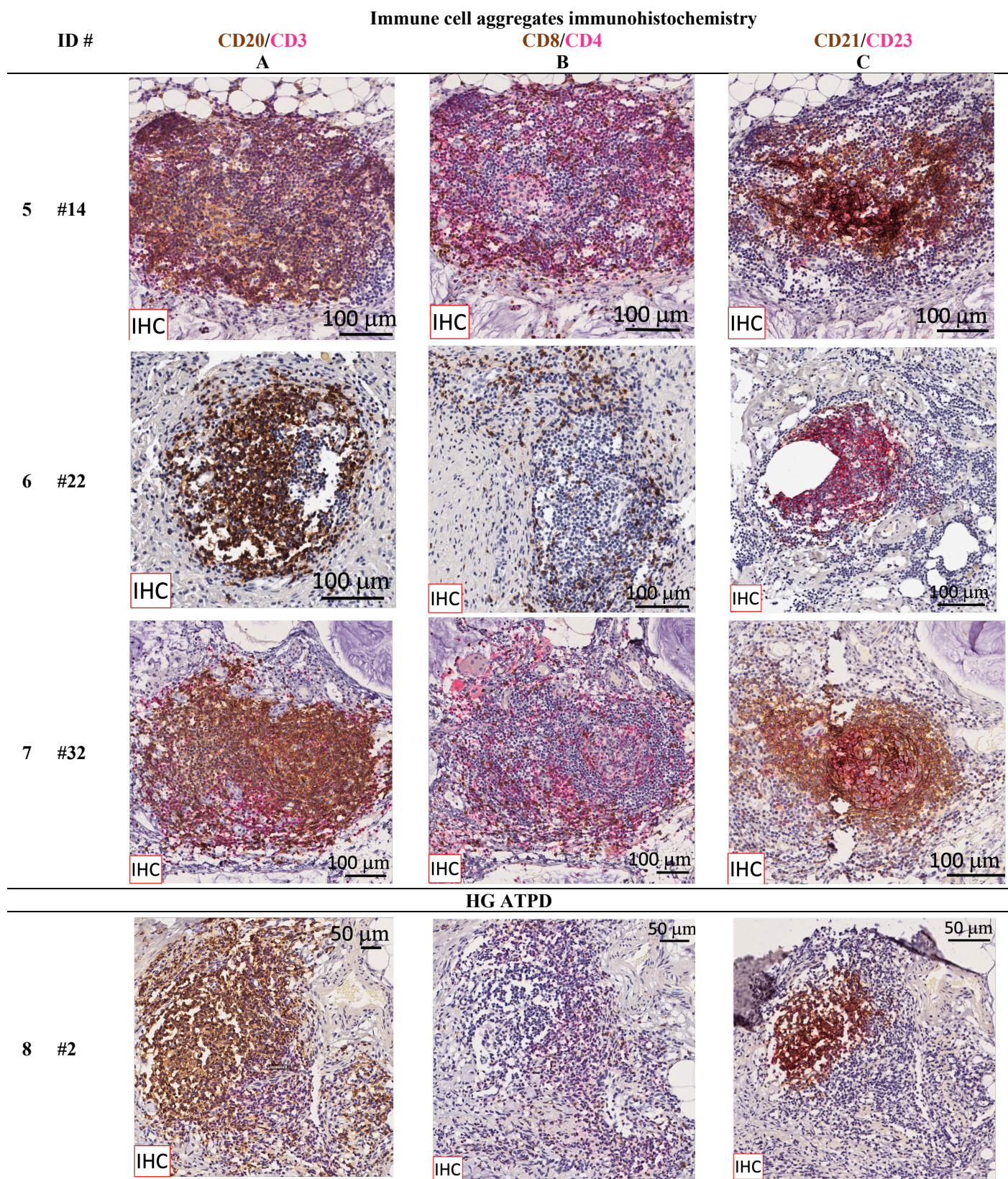


Figure 4.11 | Tissue sections from ATPD stained with anti-immune antibodies

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Columns show immune cell staining for B cells (CD20+; brown) and T cells (CD3+; pink) (A), CD8 T cells (brown) CD4 T cells (pink) (B), follicular dendritic cell (CD21; brown) and germinal centre B cell (CD23; pink) (C). Rows correspond to patient case ID for LG ATPD (1-7) and HG ATPD (8).

Abbrev. LG, low grade; HG, high grade; ATPD, appendiceal tumour peritoneal disease.

4.4 Discussion

4.4.1 Summary of findings

This study illustrates the complexity of the immune microenvironment of ATPD. Pro-tumoural immunity is favoured and shared by LG and HG ATPD, with evidence of the presence and activity of potent immunosuppressive T and myeloid subsets. The mucinous microenvironment appears to foster this. A novel finding from this study was the observation of TLS with germinal centre formation, confirmed on IHC staining. Thus, ATPD appears to have elements of underlying anti-tumoural immunity, hindered by the presence of pro-tumoural contributors. This paves the way for therapeutics that bolster the existing anti-tumour responses while suppressing the pro-tumoural signalling.

The preponderance of T-lineage and myeloid cell populations demonstrated in this analysis was consistent with the small amount of published literature. A study presented at a recent international conference performed scRNAseq on three patients with HG ATPD, and also found the majority of immune cells present to be myeloid and T cells.³⁸⁶ This mirrors similar findings in peritoneal metastases from colorectal and gastric cancers.^{224, 542}

4.4.2 ATPD represents a state of immune evasion

The presence of potent pro-tumoural immune cells in the ATPD TME suggests a state of immune evasion. This is consistent with lack of effective treatment responses, and in HG ATPD its aggressive clinical behaviour. This study described the genetic profile of peritoneal-derived macrophage populations and confirmed the presence of the M2 subtype in ATPD. The role of immunosuppressive subsets in peritoneal disease progression has been elucidated in other cancers. M2 macrophages were higher in patients with peritoneal dissemination of gastric cancer than those without while co-culture of M1 macrophages with gastric cancer cells resulted in proliferation and differentiation to the M2 phenotype.⁵⁴³ In turn, co-culture of gastric cancer cells with M2 macrophages resulted in increased cancer cell proliferation. A limitation of this study was the use of PBMC-derived macrophages rather than peritoneal-derived macrophages which may have altered specificity. Another study confirmed that manipulation of M2 macrophages led to the recovery of anti-tumoural immunity. Tranilast (N-3,4-Domethoxycinnamoyl]-anthranilic acid), an anti-allergy drug, was shown to inhibit M2 macrophage migration of mouse gastric cancer cells in co-culture with mouse intestinal myofibroblast cells.⁴⁵¹ Tranilast-treated mice had reduced peritoneal tumour weight as well as reduced number of M2 macrophages and higher number of CD8 T cells.⁴⁵¹ M2 macrophage directed therapies could be used to reverse the immunosuppressive microenvironment in ATPD.

In addition to the immunosuppressive M2 macrophage population, there was a *CD4* expressing population of peritoneal tissue resident macrophages co-expressing *LYVE1*, and in a smaller population in LG ATPD, *TIMD4*. The role of these cells have not been described in ATPD previously, although they have been implicated in promotion of peritoneal tumour metastasis in other cancers.⁵⁴⁴ CD4 and Tim-4 marker expression has been previously identified by transcriptomic profiling as a marker of mature intestinal resident macrophages.^{300, 545} Tim-4 interactions with CD8 T cells to impair anti-tumour immunity.⁵⁴⁶ Antibody-blockade of Tim-4 has been suggested by mouse models as an adjunct to immunotherapies, such as checkpoint inhibitors. This represents another avenue for immune-modulating therapies in ATPD.

Tumour-associated neutrophils have different functional states akin to their pro-inflammatory (anti-tumoural) and regenerative states (pro-tumoural).^{533, 547} The contradictory literature describing their role in directing the TME demonstrates this duality.⁵⁴⁸ This cell subset was defined by *S100* genes, which produce calcium-binding proteins of that are RAGE ligands. Their expression on various cancers has been associated with malignant growth and activation of oncogenic pathways including MAPK, ERK1/2, SAPK/JNK and NF- κ B.^{549, 550} In further support of pro-tumoural immunity, S100 proteins were associated with neutrophil extracellular traps.⁵⁵¹ These are formed when neutrophils expel intracellular proteins and DNA and have several tumour-promoting roles.⁵⁵² Their role in ATPD exacerbates the pro-tumoural state.

There is data supporting the role of chemokine modulation in the TME as a strategy for functional reprogramming of the immune response from pro-tumoural to anti-tumoural.^{553, 554} This strategy may manipulate the ATPD TME due to the enrichment of neutrophil and MDSC cell profiles for pathways involving eicosanoids (e.g. COX-2, PGE₂). Expression of COX-2 was reported in 61% of patients (n=149) patients with HG AT (AA, 40% with peritoneal metastases) and median OS was 57.6 months for those treated with celecoxib (a COX-2 inhibitor; n=9) compared to 55.7 months to those without celecoxib (n=21; p=0.84).¹¹⁹ Interpretation is limited based on the observatory nature of this study and the influence of confounding variables. However, it is likely that this strategy alone would not be immunologically potent enough to elicit meaningful benefit.

A more comprehensive approach to manipulating the peritoneal immune microenvironment was evaluated in a phase 2 clinical trial in 46 patients with peritoneal metastases (24 ATPD, 20 colorectal cancer and two mesothelioma) undergoing CRS/HIPEC. Patients received an adjuvant dendritic cell vaccine in combination with IFN- α , rintatolimod (a toll-like receptor [TLR]-3 agonist) and celecoxib.⁵⁵⁵ The trial was terminated early after interim analysis indicated futility but they reported an increase in circulating pro-inflammatory mediators including CXCL10/11, IL-6/8, SDF (stromal cell-derived factor)-1 α and TNF- α as well as an increase in the anti-inflammatory cytokine IL-10. This reinforces the complexity in manipulating the peritoneal immune microenvironment.

Overall, ATPD appears to lack an effective anti-tumoural immune response and the described immunosuppressive cell populations promote tumour outgrowth. Strategies that reverse the tumour-induced immunosuppression while boosting anti-tumoural responses are ideal. Furthermore, conventional cytotoxic agents also exert immunomodulatory effects. In other cancers, the combination of chemoimmunotherapy results in higher and more durable efficacy with evidence for supporting immunological competence.⁵⁵⁶ This approach may be most relevant for HG ATPD.

4.4.3 The mucinous microenvironment is pro-tumoural

In the previous chapter, the role of abnormal glycosylation of mucins in ATPD was described. This led to the hypothesis that abnormal mucin may induce a misguided protective response. Evidence for this was the enrichment in peri-mucinous immune cells for pro-tumoural gene signatures (NF- κ B, IL10). Given the findings, most notably in LG ATPD, for anti-tumoural B cells in addition to TLS presence, humoral adaptive immunity may be involved in the mucin-directed immune response. B cells have been shown to recognise tumour antigens such as p53 and post-

translational aberrantly glycosylated mucins such as MUC1 (CA15.3).^{557, 558} Antibodies from B cells may also induce NK cell antibody dependent cell-mediated cytotoxicity via the FcγRIII (encoded by *FCGR4*) based on analysis of The Cancer Genome Atlas Program data.^{397, 559} Conversely, *MUC16* expression was also seen in peritoneal mesothelial cells (Chapter 3) and MUC16 binding with siglec-9 on B cells can inhibit anti-tumour activity.⁵⁶⁰

Monoclonal antibody therapy directed to mucins (MUC1, MUC16, MUC5AC) have been investigated in multiple non-ATPD cancers.⁵⁶¹ MUC1 peptide vaccines alone have failed to promote significant cytotoxic T cell activation unless given in combination with immune adjuvants such as TLR agonists.⁵⁶¹ Cell based therapies using genetically engineered mucin-specific chimeric antigen receptors (CARs) have also been explored in preclinical and phase 1 trials, including intra-peritoneal CEA-directed CAR-T immunotherapy.⁵⁶² More extreme immunotherapeutic concepts that may achieve multifactorial immunomodulation in complex immune niches such as for peritoneal tumours, include trifunctional antibodies⁵⁶³ and multi-peptide vaccines.⁵⁶⁴ However, these therapies can be limited by life-threatening toxicity from cytokine-release syndromes and manufacturing difficulties.

4.4.4 Role of tertiary lymphoid structures in anti-tumour immunity

This analysis highlighted that developed immune cell aggregates in ATPD, consistent with findings in other cancer types, have TLSs in the microenvironment which exhibit germinal centres containing B cells, plasmablasts and plasma cells.^{539, 565-567} While granulopoiesis itself is not described as a function of a tumour TLS, there are a number of transcription factors active during granulopoiesis also involved in haematopoiesis.⁵⁶⁸ The presence of enriched genes associated with B cell pathways in immune cells from developed aggregates is consistent with the presence of tertiary lymphoid structures (TLS) in the peritoneal tissue, as these are the main cells contained in these structures.^{311, 312}

Intraperitoneal immune aggregates were observed in ATPD and while they were consistent with the immune components and structure of TLS, an alternative explanation could be that these aggregates represent ‘milky spots’. These are immune cell aggregates specific to the omentum, a protective layer of mostly adipose tissue covering the stomach and pancreas.⁵⁶⁹ These omental immune aggregates have been termed ‘milky spots’ and are thought to function as a filter for peritoneal fluid to prevent infection.⁵⁶⁹ Studies indicate these areas provide a unique microenvironment for the growth of peritoneal metastases.⁵⁷⁰ They are pro-tumoural due to the presence of immature macrophages and limited cytotoxicity of NK cells with sparse dendritic cells and B cells.^{571, 572} The immune aggregates identified in this study are favoured to be TLS for the following reasons: (1) the immune cell composition and structure determined by IHC staining, including germinal centre formation is consistent with TLS and different to the characteristics of ‘milky spots’ described in the literature and (2) the sampling of the tissue was preferentially non-omental peritoneum so to minimise the adipocyte content which could interfere with the analysis technique.⁵⁷³

4.4.5 Limitations

The immune TME provides challenges for interpretation in ATPD due to the collection of tissues samples from patients during CRS. This is major surgery with an expectation of a local inflammatory response which may bias findings to that of the innate acute inflammatory response. To overcome this challenge, non-tumour peritoneal tissue was used as a control for the effect of the surgical insult (Chapter 3). However, the presence of active adaptive

immune responses as well as the formation of mature TLS are unlikely to be affected by the acute effect of surgery and so are most representative of the immune microenvironment of ATPD. To achieve a purer assessment of the TME without the influence of the surgical immune response, peritoneal samples could be collected at the time of diagnostic laparoscopy performed prior to CRS. Sample collection at this time point could also provide an immune profile prior to the effect of drug treatments.

Another challenge related to the scRNAseq method was the time-dependent presence of certain cell populations. Neutrophils are short-lived and may not survive the time from sample collection, single cell dissociation through to single cell capture.⁵⁷⁴ Therefore, these results may underestimate the neutrophil proportions. However, this issue was mitigated by an efficient workflow for tissue collection, which was available for laboratory processing within one hour of harvesting. The single cell atlas presented draws attention to the expected and integral components of the ATPD immune microenvironment. This was consolidated by spatial transcriptomic profiling as well as the IHC analysis performed to further characterise the observed immune cell aggregates.

4.5 Conclusion

This is the first comprehensive analysis of the immune microenvironment of ATPD with transcriptomic characterisation of the composition and structure of immune cells. The ATPD TME comprises a potent pro-tumoural immunity based on an immunosuppressive immune cell composition and this is influenced by the presence of mucin. These findings suggest the immune microenvironment of ATPD represents a state of immune evasion. However, a key novel finding is the presence of mature TLS, particularly in LG ATPD, which supports the exploration of novel immunotherapeutic strategies for this disease.