**The Pathological Society & Jean Shanks Foundation Pathological Research Training
Fellowship – final report**

Title:
The effects of hypoxia on the anti-tumour immune response

Name and address:
Dr Philip Macklin, Ratcliffe/Pugh laboratory, Nuffield Department of Medicine Research Building, University of Oxford Old Road Campus, Roosevelt Drive, Headington, Oxford, OX3 7FZ

Background and aims:
It is my hypothesis that tumour hypoxia contributes to an impaired anti-tumour immune response and thus to poor clinical outcomes. In this project, I utilised image analysis of human tumour histology samples and murine models to evaluate the effects of hypoxia and activation of the hypoxia-inducible factor (HIF) system on anti-tumour immunity.

Results:
During this fellowship, I completed three major strands of work.
1. In order to identify robust immunohistochemical markers of hypoxia, I completed a systematic literature review of studies comparing the expression of endogenous and exogenous hypoxia markers in human tumours. In general, concordance was not strong, highlighting the complexities of quantifying tumour hypoxia and underlining the value of samples labelled with pimonidazole, an exogenous hypoxia marker.
2. I validated and utilised a novel image analysis pipeline developed by Dr Joshua Bull and Professor Helen Byrne (Wolfson Centre for Mathematical Biology, University of Oxford) to apply spatial statistics to histology images in order to describe better spatial patterns of tumour-infiltrating leukocytes [1]. I demonstrated comparable performance in cell counting to both human experts and currently available image analysis software. I then used this pipeline to analyse the distribution, relative to hypoxia, of tumour infiltrating leukocytes in pimonidazole-treated head and neck squamous cell carcinoma (HNSCC) samples (**Figure 1**) before comparing the observed distributions to those in clear cell renal cell carcinoma (CCRCC), a tumour characterised by perturbed oxygen sensing. Leukocyte-specific distribution patterns were shared by both tumour types, with lymphoid cells largely restricted to well oxygenated perivascular regions and myeloid cells being more diffusely distributed including within profoundly hypoxic necrotic regions. I then demonstrated that tumour blood vessels, semaphorin 3A (SEMA3A; an endothelial and neuronal guidance protein) and CD8$^+$ cytotoxic T lymphocytes co-localise within CCRCC samples, in keeping with *in vitro* and *in vivo* mechanistic evidence from collaborators demonstrating that SEMA3A binding to neuropilin-1 induces cytoskeletal paralysis in tumour-specific CD8$^+$ cytotoxic T lymphocytes and thereby prevents their migration into tumour cell nests [2]. Next, I utilised this pipeline to evaluate immunohistochemically labelled hypoxia, vascular and immunological markers in lung cancer samples from participants in the Atovaquone as Tumour HypOxia Modifier (ATOM) clinical trial evaluating whether atovaquone, an oxidative phosphorylation inhibitor, reduces tumour hypoxia. Although all markers were equivalent between groups (**Figure 2**), given that atovaquone-treated tumours were more hypoxic at baseline imaging, this may actually represent a meaningful finding (Skwarski et al., 2020 – manuscript under submission).
3. I undertook histological phenotyping of transgenic mice in which HIF was up-regulated through reversible prolyl hydroxylase domain-containing protein 2 (*Phd2*) gene silencing. Surprisingly, after five weeks of *Phd2* knockdown, an autoimmune phenotype developed with evidence of lymphadenopathy, splenomegaly and multi-organ leukocyte infiltration. These findings share several abnormalities with the Scurfy mouse, a mouse model of the human IPEX (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome in which autoimmune phenomena occur as a result of *Foxp3* mutation and associated regulatory T cell dysfunction. Subsequent analyses have also demonstrated defective regulatory T cell function in our transgenic mice [3]. Based upon these observations, I then tested whether *Phd2* knockdown would promote an anti-tumour immune response. To investigate this, I utilised two syngeneic murine tumour models that grow in our C57BL/6J transgenic mouse lines. Disappointingly, *Phd2* knockdown beginning on day 3 after tumour cell inoculation did not lead to a change in tumour growth rate for either E0771 (mammary gland carcinoma) or LL/2 (Lewis lung carcinoma) tumours (**Figure 3**).

Conclusions:

Human tumour studies
Leukocyte-specific distribution patterns were shared by both tumour types, with lymphoid cells largely restricted to well oxygenated perivascular regions and myeloid cells being more diffusely distributed. This raises the possibility that hypoxic tumour regions represent immunoprivileged niches which foster immune evasion and resistance to immunotherapy. Furthermore, the existence of broadly comparable distributions between a tumour type in which HIF stabilisation is focal and consequent upon microenvironmental oxygenation (HNSCC) and another with diffuse, oncogenic HIF activation (CCRCC) suggests that these distributions result primarily from leukocyte intrinsic processes, as opposed to as a consequence of HIF activation within tumour cells themselves. This is in keeping with the role of endothelial SEMA3A in restricting the migration of CD8$^+$ cytotoxic T lymphocytes into less vascularised tumour regions.

Murine tumour model studies
Whilst long-term *Phd2* knockdown leads to autoimmune disease in the mouse, mediated through defective regulatory T cell function, this intervention does not alter the rate of growth of subcutaneous syngeneic tumours.

How closely have the original aims been met?:

Year one milestones:
1. Complete human head and neck tumour study – completed, further samples accessed from collaborators to increase patient numbers in work to be completed after PhD;
2. Refine mouse cell lines – wild-type mouse cancer cell lines used in experiments and protocol to induce tumours in our transgenic mice with methylcholanthrene also developed;
3. Commence mouse experiments – syngeneic murine breast and lung cancer models optimised.

Year two milestones:
1. Continue mouse experiments – *Phd2* knockdown experiments completed and analysed by flow cytometry and immunohistochemistry;
2. Gain further experience in data analysis including flow cytometry, functional assays, gene expression profiling and digital image analysis – main emphasis was placed on the development/optimisation of digital image analysis algorithms to assess immune cell spatial distribution (work ongoing on further manuscripts relating to this collaboration);
3. Perform transcriptomics analysis – single-cell RNA sequencing experiment of *Phd2* knockdown regulatory T cells performed by colleagues to gain mechanistic insights into their dysfunction.

Year three milestones:
1. Test hypotheses on human tumour samples and/or in mice – human immunohistochemistry studies expanded to include CCRCC samples and ATOM study samples;
2. Submit thesis and complete viva examination – thesis submitted in November 2020 and viva examination scheduled for January 2021.

Outputs:

Publications
1. 'Hypoxia and HIF pathway in cancer and the placenta' **Macklin P**, McAuliffe J, Pugh C, Yamamoto A (2017), *Placenta*, 56:8-13
2. 'Systemic silencing of PHD2 causes reversible immune regulatory dysfunction' Yamamoto A, Hester J, **Macklin P**, Kawai K, Uchiyama M, Biggs D, Bishop T, Bull K, Cheng X, Cawthorne E, Coleman M, Crockford T, Davies B, Dow L, Goldin R, Kranc K, Kudo H, Lawson H, McAuliffe J, Milward K, Scudamore C, Soilleux E, Issa F, Ratcliffe P, Pugh C (2019), *Journal of Clinical Investigation*, 129(9):3640-3656
3. 'Recent advances in the biology of tumour hypoxia with relevance to diagnostic practice and tissue-based research' **Macklin P**, Yamamoto A, Browning L, Hofer M, Adam, J, Pugh C (2020), *Journal of Pathology*, 250(5):593-611
4. 'Combining multiple spatial statistics enhances the description of immune cell localisation within tumours' Bull J, **Macklin P**, Quaiser T, Braun F, Waters S, Pugh C, Byrne H (2020), *Scientific Reports*, 10(1):18624

Awards
- Senior Scholarship (2018-19), Lincoln College, University of Oxford

Figures

Figure 1 – Analysis of immune cell spatial distribution with respect to regions of tumour hypoxia in human head and neck squamous cell carcinoma

(A) Comparison of densities of CD8+, FOXP3+ and CD68+ cells in different tumour compartments. (B) Illustration of trends for each tumour corresponding to the densities presented in (A). Interestingly, whilst the decrease in immune cell density in hypoxic tumour regions was consistent across each tumour for CD8+ and FOXP3+ cells, an increase in CD68+ cells in pimonidazole labelled areas was observed in 3/16 tumours, suggesting that under certain circumstances, macrophages are more able to localise within hypoxic tumour regions than the two T lymphocyte subsets. (C) Representative histological images demonstrating the presence of CD68+, but not CD8+ or FOXP3+, cells within a tumour region labelled by pimonidazole (necrosis outlined by dashed lines).
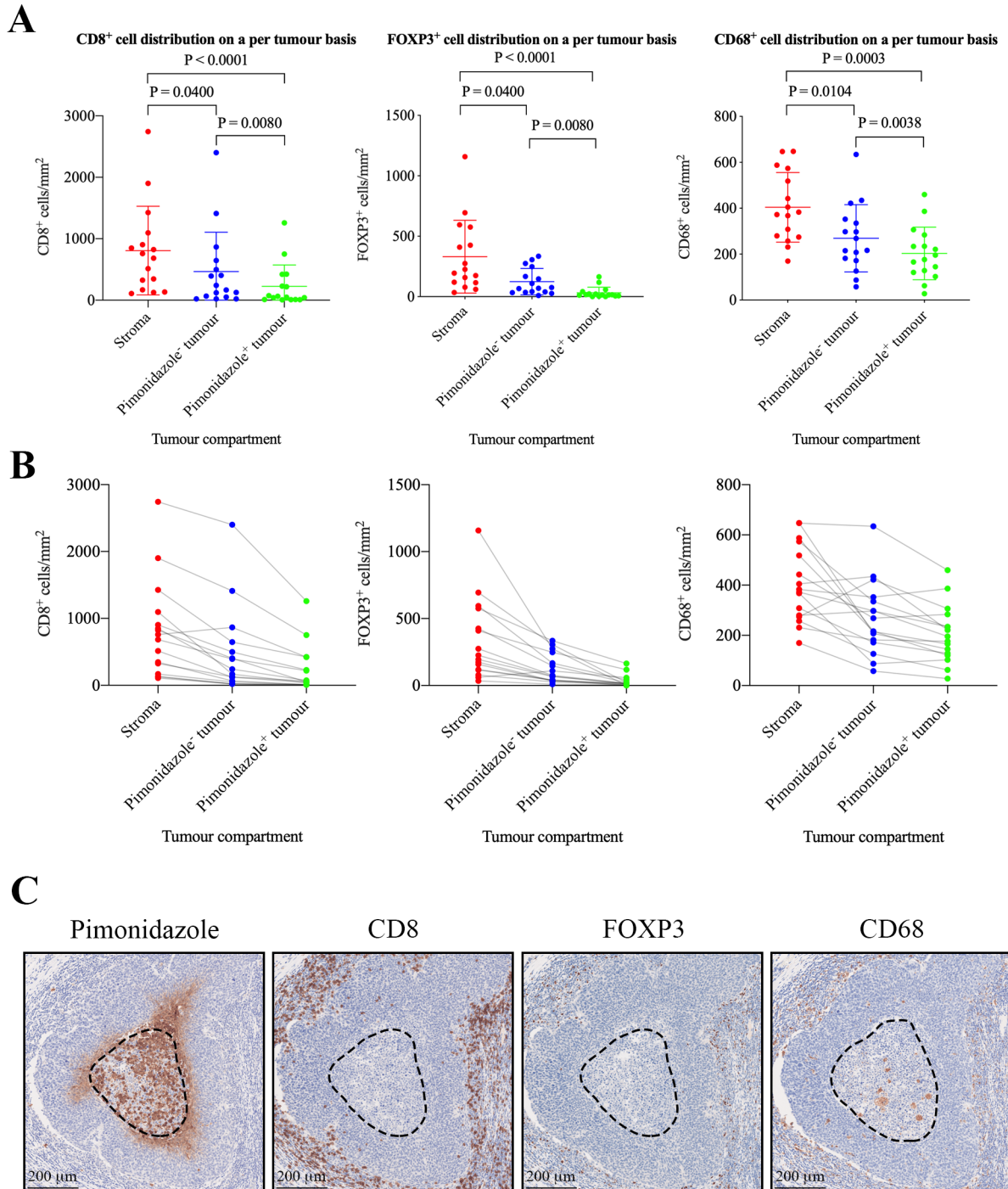
Figure 2 – Effects of atovaquone administration on hypoxia, vascular and immunological markers in the ATOM study
Equivalence of hypoxia (pimonidazole & CA IX), vascular (CD31 & CD146) and immunological (CD8, FOXP3, CD68 & PD-L1) markers between treatment groups.
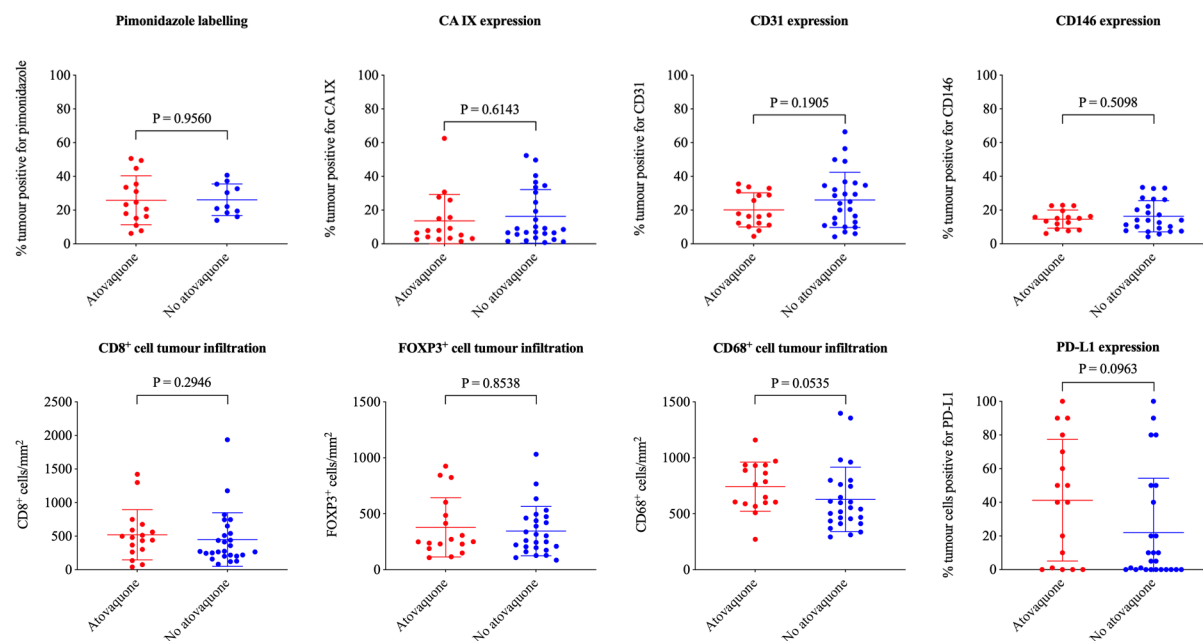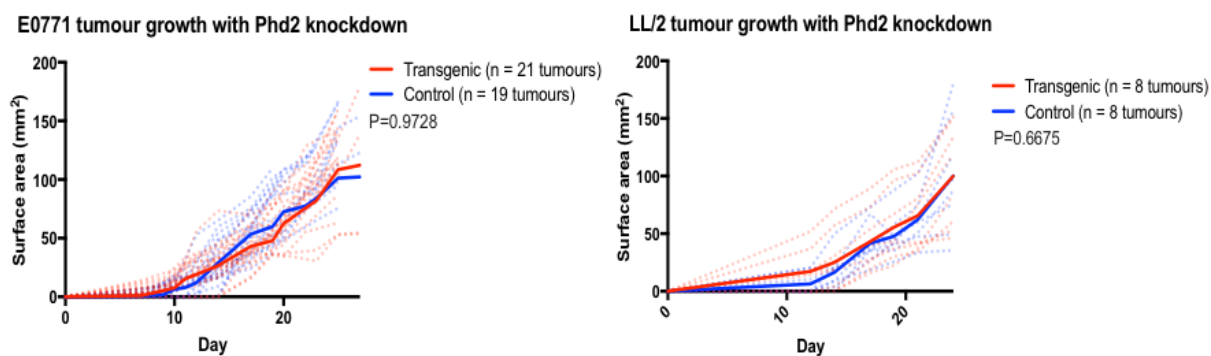

Figure 3 – Effect of *Phd2* knockdown on growth of subcutaneous syngeneic tumours
No difference in tumour growth is apparent between transgenic (Phd2 knockdown) and control mice in either the E0771 or LL/2 tumour model. Solid lines represent group mean whereas dashed lines represent individual tumours.

References
1.   Bull JA, Macklin PS, Quaiser T, et al. Combining multiple spatial statistics enhances the description of immune cell localisation within tumours. Sci Rep. 2020;10(1):18624.
2.   Barnkob MB, Michaels YS, André V, et al. Semaphorin 3A induces cytoskeletal paralysis in tumor-specific CD8+ T cells. bioRxiv. 2019:849083.
3.   Yamamoto A, Hester J, Macklin PS, et al. Systemic silencing of PHD2 causes reversible immune regulatory dysfunction. J Clin Invest. 2019;129(9):3640-56.