A Novel Flow Cytometry-Based Method to Study Lymphocytes present in Low Cell Numbers infiltrating Non-Functional and Growth-Hormone Secreting Pituitary Adenomas

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INTRODUCTION

Non-functional pituitary adenomas (NFPA) are non-hormone secreting pituitary tumours while growth hormone-secreting pituitary adenomas (GHPA) are active pituitary tumours which can cause acromegaly. Although these tumours are usually benign, they can cause pressure on optic nerces and/or dysregulation of hormone secretion. Understanding the diversity of immune cells infiltrating the microenvironment of tumours is important given that cancer immunotherapy is becoming a preferred therapeutic strategy. However knowledge of the immune landscape in pituitary adenomas is still lacking. We therefore developed an acoustic-assisted flow cytometry based method to identify immune cell populations in fresh NFPA and GHPA.

METHODS

Fresh tumour tissue was used immediately following transsphenoidal surgery and enzymatically digested to cell suspensions suitable for flow cytometry as shown in Figure 1. The cells were stained with antibodies against cell surface markers and then fixed and permeabilised followed by staining with antibodies against intracellular cell markers. The multicolour panel of antibodies used ito identify immune cells of lymphocytic and myeloid lineage is given in Table 1. The cell suspensions were then loaded onto an Attune NxT Cytometer (Thermofisher) and data analysis performed on FlowJo Version 10.8.1. The gating strategy followed to identify lymphocytes and macrophages is depicted in Figure 3. Finally, we looked into the histology data to check for any correlations with the flow cytometry data.

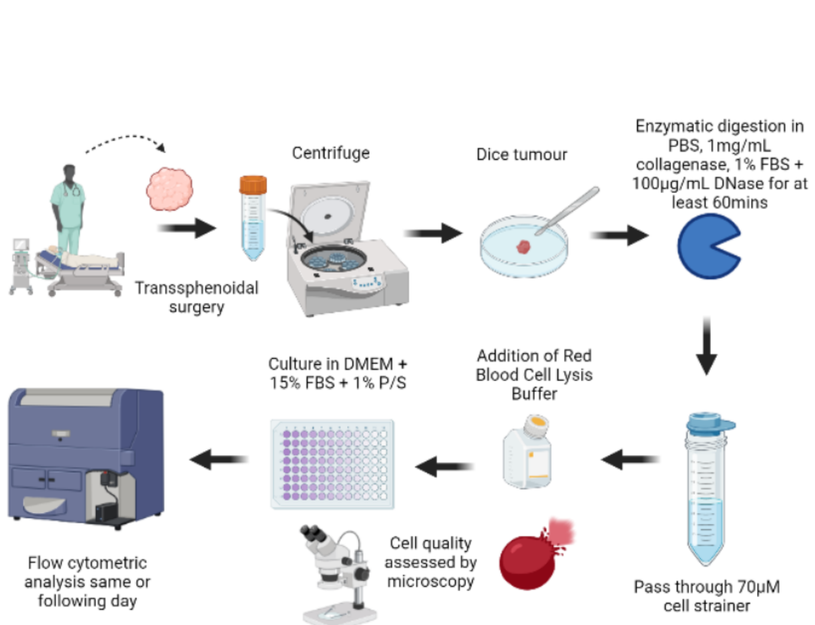

Figure 1: Method used to obtain single cell suspension from fresh NFPA and GHPA

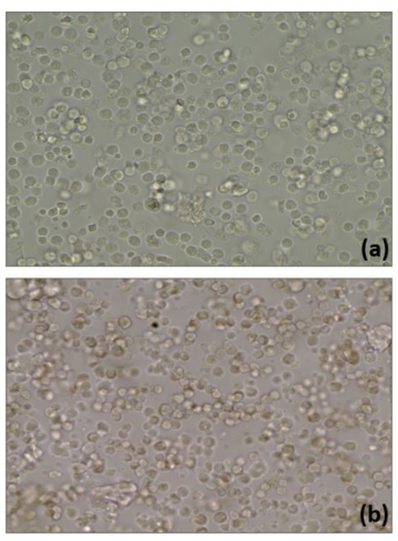

Figure 2: Cell suspensions obtained from (a) NFPA and from (b) GHPA after enzymatic digestion of the tumour tissue. *Magnification: x400*

	Antibody	Expression
Lymphoid lineage	CD45	All Leucocytes
	CD3	T cells
	CD8a	Cytotoxic T cells
	CD4	Helper T cells
	T-bet	Th1 transcription factor
	GATA3	Th2 transcription factor
	FOXP3	Treg transcription factor
	CD19	B cells
	CD56	Natural Killer cells
	CD44	Activated T cells
	TIM-3	Exhausted T cells
	PD-1	Activated T cells
Myeloid lineage	CD11b	Myeloid cells
	CD68	Macrophages
	CD64	M1 macrophages
	CD163	M2 macrophages
Pituitary cell markers	PTTG	Pituitary tumour cells
	Pit-1	Lactotrophs, somatotrophs & thyrotrophs
	S100	Folliculostellate cells

Table 1: Antibody panel used to identify immune cells

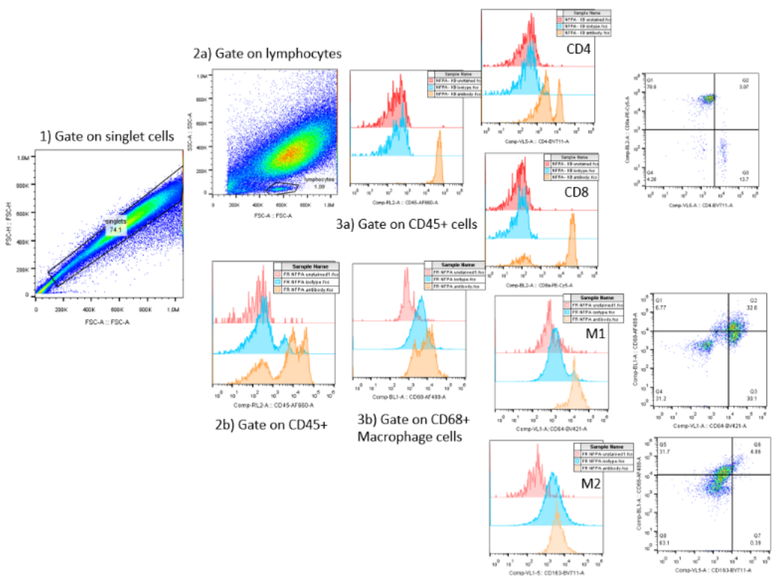

Figure 3: Gating strategy used

RESULTS

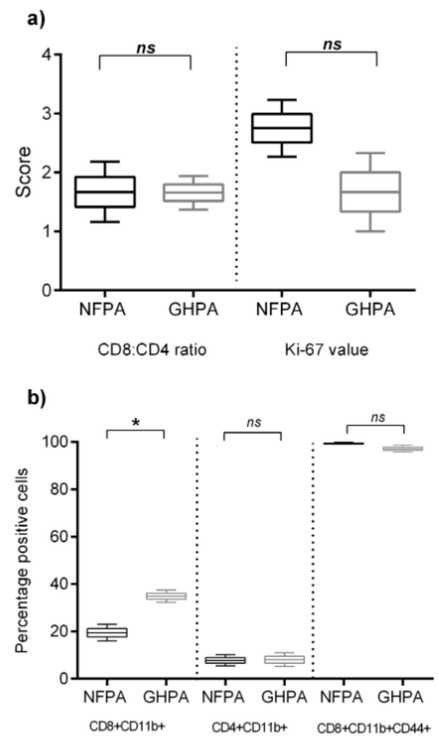

Figure 4: Box and whisker plots showing *(a)* CD8:CD4 ratio and Ki-67 values obtained from NFPA (*n* = 5) and GHPA (*n* = 4). No statistical significant correlation was found between CD8:CD4 ratio and Ki-67 values when analysed using Pearson's correlation coefficient. Plot (*b*) shows the percentage of CD45+ lymphocytes that were CD8+CD11b+ and CD4+CD11b+. Plot also shows the percentage of CD8+CD11b+ that were also CD44+. Data is presented as mean ± SEM and differences between the two tumour types tested using student t-test.

		SAMPLES	MEAN %
Total leucocytes (CD45+ cells)	NFPA	5	7.62
	GHPA	4	9.84
Cytotoxic T cells CD8+ cells	NFPA	5	34.24
	GHPA	4	48.13
Helper T cells (CD4+ cells)	NFPA	5	24.86
	GHPA	4	30.05
B cells (CD19+ cells)	NFPA	3	2.51
	GHPA	3	0.89
Nautral killer cells (CD3-CD56+ cells)	NFPA	3	16.42
	GHPA	3	8.84
Treg (CD4+FOXP3+)	NFPA	2	0
	GHPA	1	0
Th1:Th2 ratio CD4+T-bet+ : CD4+GATA-3+ cells)	NFPA	2	1
	GHPA	1	0.89
M1 macrophages (CD68+CD64+ cells)	NFPA	2	64.15
	GHPA	1	16.6
M2 macrophages (CD68+CD163+ cells)	NFPA	2	0
	GHPA	1	0
Folliculostellate cells (S100+ cells)	NFPA	2	82.65
	GHPA	1	20.4
PD-1+ cells	NFPA	2	0
	GHPA	1	0
TIM-3+ cells	NFPA	2	0
	GHPA	1	13.7

Table 2: Flow cytometry statistics showing mean percentage count of different types of immune cells infiltrating NFPA and GHPA. The percentage of leucocytes is being recorded as the total CD45+ cell from the singlet cell suspensions. The statistics for Natural Killer cells, B cells, Treg, Th1 and Th2 are given as percentage of the total lymphocytes gated on singlet, lymphocytic, CD45+ population. The statistics for M1 and M2 macrophages, and folliculostellate cells are given as percentage of the total CD45+CD68+ cells.

CONCLUSION

- The study has show that flow cytometry is a reliable technology to identify and quantify immune cells of lymphocytic and myeloid origin present in NFPA and GHPA.
- Leucocytic infiltrates, especially lymphocytes, were detected in all specimen.
- The CD8:CD4 ratio was equal to or higher than 1 in 3 out of 5 NFPA and in all GHPA with no statistical significant difference in the ratio between the two tumour types.
- No statistical significant correlation was found between CD8:CD4 ratios and Ki-67 values.
- Some CD8+ cells also expressed the myeloid marker CD11b with GHPA recording a statistically significant higher percentage than NFPA possibly suggesting a novel population of cytotoxic T cells.
- Expression of CD11b seemed to be restricted only to activated CD8+ cells as these also expressed the activation marker CD44 in both tumour types (> 93%).
- Other useful information on immune cells infiltrating both types of pituitary adenomas was revealed however sample size was too small to make any inferences. Nonetheless, these preliminary results provided directions for further research.
- Future studies shall focus on increasing sample number and validating the findings using other technologies.

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