CD4$^+$ T Cells from Patients with Glucocorticoid-Refractory Immune Thrombocytopenia Have Altered Cytokine Expression

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Short Title: CD4$^+$ T Cell cytokine expression and steroid response in ITP

Consensus clinical guidelines recommend high dose glucocorticoids as first line therapy for adult patients with Immune Thrombocytopenia (ITP).[1] However, frequent side effects and heterogeneous responses are clinical challenges with approximately 20-30% failing to respond at tolerable doses.[2] Although addition of mycophenolate or rituximab to glucocorticoid may increase response rates, quality of life was worse with mycophenolate and more adverse events were associated with rituximab.[3,4] Therefore, it would be clinically valuable to predict those patients expected to fail glucocorticoid monotherapy who may benefit from early additional treatment.

Studies have suggested that CD4$^+$ T helper cell subsets from ITP patients respond differently to glucocorticoid treatment *in vitro*. Cells expressing the pro-inflammatory cytokines interleukin (IL)-17 and interferon (IFN)-γ in the absence of the anti-inflammatory cytokine IL-10 are resistant to inhibition.[5-7]

Furthermore, we recently reported that activated CD4$^+$ T-cells from glucocorticoid refractory ITP patients showed a relative abrogation of IL-10 with persistent IL-17 in response to *in vitro* glucocorticoid compared to responsive patients.[8] However, this study was limited by the heterogeneity of the ITP patient cohort in terms of disease stage and treatment.

Here, we aimed to prospectively validate our findings in a cohort of newly-diagnosed ITP patients recruited to the laboratory sub-study of the FLIGHT trial (ClinicalTrials.gov number:NCT03156452).

Following written informed consent, peripheral blood (20mls) was collected in EDTA containing tubes at baseline (when randomised; n=87) and at 2 months follow up (n=80). Full methods of the FLIGHT trial were previously published[9] – in brief, patients were randomised to receive glucocorticoid (dexamethasone or prednisolone) alone or in combination with mycophenolate. A glucocorticoid response (R; n=34) was defined as platelet count of >30x10^9/L and at least a 2-fold increase from baseline with glucocorticoid monotherapy. Non-responsive (NR) patients were defined by platelet count of <30x10^9/L, or less than a 2-fold increase from baseline with glucocorticoid therapy either alone (n=11) or in combination with MMF (n=2).

$CD4^+$ T cells were isolated from peripheral blood using a RosetteSep™ Human $CD4^+$ T-cell enrichment cocktail (StemCell Technologies, Canada). Cytokine expression was examined immediately *ex vivo* by 4-hour incubation with PMA (20ng/ml), ionomycin (1µM) and GolgiStop™ (2µM). Alongside this, $CD4^+$ T-cells were activated with Human T Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, UK) and cultured *in vitro* in presence of recombinant IL-2 (Roche, UK) and dexamethasone, a relatively pure glucocorticoid (1µM; Sigma-Aldrich,UK), for 96 hours under tissue culture conditions with PMA, ionomycin and GolgiStop™ added for the last 4 hours of culture. Intracellular cytokine staining was measured by flow cytometry using a BD Fortessa X20 (BD Biosciences). Masked analysis was carried out using FlowJo software (version 10; Tree Star Software, USA). Statistical analysis was carried out using GraphPad Prism software (version 9; GraphPad Software, USA).

Following *in vitro* culture with dexamethasone treatment, $CD4^+$ T-cells from baseline samples of R patients compared to NR patients showed higher proportions of IL-10^+ cells, no significant difference in IL-17^+ cells and a higher IL-10:IL-17 ratio (Table 1). An area under the receiver operating characteristic (ROC) curve of 0.7115 (95% CI 0.5301 to 0.8930, p=0.0358) demonstrates significant discrimination of the IL10:IL17 ratio between R and NR patients. This is consistent with our previous findings.[8] There were no significant differences in the expression of IL-4, IL-22, IFN-γ, GM-CSF or TNF between R and NR patients.

$CD4^+$ T-cells examined immediately *ex vivo* from baseline samples of R patients compared to NR patients, also demonstrated a higher IL-10:IL-17 ratio although with no significant difference in the individual proportions of IL-10^+ and IL-17^+cells (Figure 1A-D). The *ex vivo* IL-10:IL-17 ratio shows discrimination between patient cohorts, with an AUROC of 0.7889 (95% CI 0.6432 to 0.9346, p=0.0038; Figure 1E). NR patients had higher proportions of IL-4^+cells than R patients with no significant differences in any of the other cytokines.

$CD4^+$ T-cells examined following *in vitro* culture with dexamethasone from two-month follow-up samples of R patients compared to NR patients again demonstrated higher IL-

10:IL-17 ratio (AUROC 0.7642, 95% CI 0.5830 to 0.9454, *p*=0.0096) with higher proportions of IL-10$^+$ cells but no significant difference in IL-17$^+$ cells.

CD4$^+$ T-cells examined immediately *ex vivo* from samples at 2 months follow-up of R patients compared to NR patients, also demonstrated a significant difference in the IL-10:IL-17 ratio, with lower proportions of IL-17$^+$ cells and no significant difference in IL-10$^+$cells. There were no significant differences in the other cytokines studied.

These results provide evidence of an adaptive immune basis in the clinical diversity of glucocorticoid responsiveness, particularly related to IL-10 and IL-17 cytokine expression. While limited by a relatively small number of patients, the prospective cohort of newly diagnosed patients with blood sampling at two time points and robust clinical response data are strengths. We confirm our previous findings where CD4$^+$ T-cells from NR patients have a reduced IL-10:IL-17 ratio following *in vitro* treatment with glucocorticoids. Furthermore, we have expanded on these observations and shown that immediately *ex vivo* CD4+ T-cells also demonstrate a reduced IL-10:IL-17 ratio in NR patients. Results from two-month follow-up samples are consistent with this finding. This suggests that CD4$^+$ T-cells from NR patients are predisposed to produce sustained low levels of IL-10 and higher levels of IL-17.

A key mechanism underlying glucocorticoid efficacy is likely to be the induction of IL-10 in a range of immune cell types, including CD4$^+$ and CD8$^+$ T-cells[10,11], and B-cells.[12] Indeed, failure to upregulate IL-10 in response to glucocorticoids has been identified in glucocorticoid-resistant asthma.[7] CD4$^+$ T-cells upregulating IL-10 in response to glucocorticoids often co-produce Th17-associated cytokines (IL-17, IL-22 and IFN-γ).[13] These “non-pathogenic Th17 cells” are important in limiting inflammation and autoimmunity.[14] Contrastingly, Th17 cells that do not upregulate IL-10 in response to glucocorticoid have been shown to be both highly pathogenic and glucocorticoid resistant.[5,6]

It may be possible to develop the observed *ex vivo* T-cell phenotype into a clinically applicable biomarker to help predict NR patients, which could then inform the clinical decision to initiate alternative therapy early. Our data may also be applicable to other autoimmune illnesses treated with glucocorticoids[8] and highlight the potential importance of CD4$^+$ T cells in both ITP pathogenesis and therapy.

			Clinical Response		
Time point	**Condition**	**Cytokine**	**R** **Mean % (S.D)**	**NR** **Mean % (S.D)**	**R vs NR** **Significance**
Baseline	*In vitro* culture with Dex	IL-4	3.93 (2.33)	2.94 (0.95)	0.1606
		IL-10	8.93 (3.94)	6.01 (4.24)	**0.0094** **
		IL-17	1.58 (1.2)	1.53 (0.73)	0.7202
		IL-22	1.01 (0.61)	1.53 (1.85)	0.8832
		IFNg	5.15 (2.34)	6.11 (2.84)	0.3618
		GM-CSF	5.59 (3.76)	5.81 (3.5)	0.8235
		TNF	19.04 (10.01)	15.59 (8.01)	0.27
		IL-10:IL-17	8.36 (7.06)	4.73 (3.8)	**0.0341** *
	Immediate *ex vivo* phenotype	**IL-4**	3.12 (2.03)	4.92 (2.73)	**0.0361** *
		IL-10	0.78 (0.48)	0.55 (0.3)	0.1323
		IL-17	0.72 (0.48)	0.92 (0.56)	0.1726
		IL-22	0.47 (0.4)	0.63 (0.35)	0.0666
		IFNg	7.78 (3.83)	11.15 (6.91)	0.078
		GM-CSF	7.98 (5.59)	8.56 (7.34)	0.7521
		TNF	60.59 (14.42)	54.76 (18.55)	0.3887
		IL-10:IL-17	1.31 (0.8)	0.64 (0.29)	**0.003** **
Two-month follow-up	*In vitro* culture with Dex	IL-4	3.31 (1.36)	3.17 (1.03)	0.6681
		IL-10	9.75 (3.75)	7.04 (2.63)	**0.0168** *
		IL-17	1.58 (0.74)	1.9 (0.93)	0.279
		IL-22	1.18 (0.85)	1.45 (0.78)	0.2668
		IFNg	6.48 (2.44)	7.96 (2.28)	0.0643
		GM-CSF	6.13 (3.36)	7.52 (2.94)	0.0914
		TNF	22.05 (7.76)	23.52 (8.28)	0.9072
		IL-10:IL-17	7.34 (4.07)	4.51 (2.75)	**0.0086** **
	Immediate *ex vivo* phenotype	IL-4	3.95 (1.74)	4.25 (2.27)	0.9068
		IL-10	0.93 (0.52)	0.88 (0.49)	0.8055
		IL-17	0.71 (0.44)	1.26 (0.57)	**0.0108** *
		IL-22	0.73 (1.19)	1.3 (1.37)	0.1068
		IFNg	8.82 (4.87)	12.93 (6.97)	0.1546
		GM-CSF	8.45 (4.66)	11.43 (6.13)	0.2218
		TNF	58.49 (12.55)	54.7 (13.32)	0.6189
		IL-10:IL-17	1.58 (1.06)	0.73 (0.28)	**0.0154** *

Table 1: **Cytokine expression of CD4 T-cells from ITP patients.**

Intracellular cytokine expression of $CD4^+$ T-cells from ITP patients activated with anti-CD3/CD28 beads for 96 hours in the presence of $1x10^{-6}$ M dexamethasone (*in vitro* culture

with Dex), or directly *ex vivo* (immediate *ex vivo* phenotype) was assessed by flow cytometry on a BD Fortessa X20. The mean proportion of positive cells and the S.D. is shown. Patients are divided by their clinical response into those recruited to the corticosteroid alone arm who responded (R), or patients recruited to both the glucocorticoid only or glucocorticoid + mycophenolate arm and had no response (NR) to treatment. Samples taken at baseline (R n=34, NR n=13) and two months follow-up (R n=29 , NR n=10) are shown. Groups were compared by Mann Whitney U and significance indicated; a *p* value of less than 0.05 was taken as significant.

Figure 1 $CD4^+$ T-cells from newly diagnosed ITP patients who responded to glucocorticoid treatment exhibit a significantly different IL-10:IL-17 ratio compared to patients who did not respond. $CD4^+$ T-cells were isolated at the baseline timepoint from newly diagnosed ITP patients recruited to the FLIGHT clinical trial. Intracellular cytokine expression was assessed directly *ex vivo* by flow cytometry. Representative flow cytometry plots for IL-10 and IL-17 expression (A) and cumulative data for the percentage of $IL\text{-}10^+$ (B) and $IL\text{-}17^+$ $CD4^+$ T-cells (C) and the IL-10:IL-17 ratio (D) are shown. Patients were categorised as having a response (R, n=34) or no response (NR, n=13) to corticosteroids. Area under receiver operating characteristic (AUROC) curve for IL-10:IL-17 ratio in predicting the clinical response of ITP patients to corticosteroid treatment (E). AUROC is 0.7889 (95% confidence interval 0.6432 to 0.9346, p=0.0038). Mean +/- S.D. is shown. **p<0.01.

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Conflict-of-interest disclosure

RWJL and LPS-B are inventors of an IL-17 based method to identify patients likely to be resistant to glucocorticoid treatment (US Patent App. 15/106,411). The other authors have no conflicts to declare.

Ethics statement

Ethical approval from NRES Committee South West (IRAS number 225959). EudraCT Number: 2017-001171-23.

Author contribution statement

CB and RL jointly led this research. CB led the FLIGHT Clinical trial, JP and IT were responsible for the clinical trial delivery (Cardiff CTU), RG is the trial statistician and has provided expert input into statistical analysis of the data. MS led the laboratory processing of samples and wrote the first draft of the manuscript. JW wrote the final version of the manuscript. PL, EW and LSB contributed to sample analysis. All authors have made valuable contributions to the research design, delivery and provided feedback on the manuscript.

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