



Stimpson, M. L., Lait, P. J. P., Schewitz-Bowers, L. P., Williams, E. L., Thirlwall, K., Lee, R. W. J., & Bradbury, C. A. (2020). IL-10 and IL-17 Expression by CD4+ T Cells is Altered in Corticosteroid Refractory Immune Thrombocytopenia (ITP). *Journal of Thrombosis and Haemostasis*, 18(10), 2712-2720. <https://doi.org/10.1111/jth.14970>

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**Title: IL-10 and IL-17 Expression by CD4<sup>+</sup> T Cells is Altered in Corticosteroid Refractory Immune Thrombocytopenia (ITP)**

Running title: CD4<sup>+</sup> T cells in Corticosteroid Refractory ITP

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## **Essentials**

- Corticosteroids are widely used for autoimmune/inflammatory disease, but responses are variable
- *In vitro*, CD4<sup>+</sup> T cells from steroid refractory ITP patients express a lower IL-10:IL-17 ratio
- We confirm this finding in an independent cohort of patients with autoimmune disease
- Cytokine expression of CD4<sup>+</sup> T cells is likely important for clinical corticosteroid response

## **Abstract**

### **Background**

Corticosteroids remain the first line treatment for patients with Immune Thrombocytopenia (ITP). However, 20-30% of patients do not respond to treatment at tolerable doses. This variation in corticosteroid efficacy is replicated in other autoimmune diseases and may have an adaptive immune basis.

### **Objective**

To test the hypothesis that CD4<sup>+</sup> T cell responses to corticosteroids are different in patients with clinically defined corticosteroid refractory ITP.

### **Methods**

In this prospective cohort study, CD4<sup>+</sup> T cells from patients with ITP were cultured in the presence or absence of dexamethasone (Dex). Intracellular cytokine expression was then quantified by flow cytometry and compared with patients' clinical response to corticosteroid treatment. A control cohort of patients with autoimmune uveitis was also studied to evaluate whether our findings were limited to ITP or are potentially generalizable across autoimmune diseases.

### **Results**

The ratio of interleukin (IL)-10 to IL-17 expression following CD4<sup>+</sup> T cell culture with Dex was able to discriminate between ITP patients with a clinically defined complete (n=33), partial (n=12) or non-response (n=11) to corticosteroid treatment (p=0.002). These findings were replicated in patients with autoimmune uveitis (complete response n=14, non-response n=22; p=0.01)

### **Conclusions**

There is a relative abrogation of IL-10 and persistence of IL-17 expression in the CD4<sup>+</sup> T cells of patients who clinically fail corticosteroid therapy. This observation has potential to inform both our mechanistic understanding of the action of corticosteroids in the treatment of ITP, and as a biomarker for steroid refractory disease, with potential application across a range of haematological and non-haematological conditions.

**Key words**

Autoimmunity, Cytokines, Glucocorticoids, Immune Thrombocytopenia, Lymphocytes

## Introduction

Corticosteroids are one of the most widely prescribed drugs in medicine. However, the doses needed to achieve disease remission varies enormously across inflammatory and autoimmune conditions and between individuals with a significant proportion of patients failing to respond at tolerable doses. Corticosteroids are also associated with a plethora of metabolic, endocrine, gastrointestinal, psychological and musculoskeletal side-effects [1]. In haematology, corticosteroids are standard treatment for many conditions including immune thrombocytopenia (ITP), autoimmune haemolytic anaemia, acquired haemophilia, thrombotic thrombocytopenic purpura, myeloma and lymphoid cancers. ITP is an autoimmune illness with a risk of bleeding due to thrombocytopenia that results from increased consumption and decreased production of platelets [2]. Recent guidelines continue to recommend high dose corticosteroids as first line treatment for ITP but responses are heterogeneous with 20-30% of patients refractory to treatment and the burden of corticosteroid side-effects remains a major clinical challenge [3].

The main actions of corticosteroids on the immune system are mediated by activation of the glucocorticoid receptor (GR). As such, *in vitro* studies are typically conducted with a pure glucocorticoid such as dexamethasone. The GR has near ubiquitous expression in the body, and binding of glucocorticoids to the GR results in a variety of cellular responses, including the transactivation of anti-inflammatory genes and transrepression of pro-inflammatory genes [4]. However, the biological basis underlying the variable response to corticosteroids is not well understood. Several molecular mechanisms have been proposed, including defective GR binding and nuclear translocation, increased expression of the inhibitory isoform GR $\beta$ , and polymorphisms in accessory proteins [5]. However, no strong associations with known GR polymorphisms have been found in ITP [6].

More broadly, there is increasing evidence to suggest that the intrinsic sensitivity of an individual's lymphocytes may be a major factor in determining their response to corticosteroids [7]. Early studies of adaptive immunity in corticosteroid responsiveness focused on CD4<sup>+</sup> T helper (Th) cells, and more recently the importance of cytokine expression within these populations has been revealed. In particular, Hawrylowicz *et al* have demonstrated a failure to upregulate interleukin (IL)-10 in corticosteroid resistant asthma patients [8, 9] while others have shown a bias towards pro-inflammatory IL-17 expression over interferon (IFN)- $\gamma$  [10, 11]. This has been corroborated in a range of autoimmune conditions [12-14]. Furthermore, a number of cytokine gene polymorphisms have been associated with the effectiveness of corticosteroid therapy in ITP, including specific IL-10 and IFN- $\gamma$  genotypes [15, 16]. These data infer an adaptive immune basis to diversity of clinical corticosteroid responsiveness.

We therefore hypothesized that the same phenomenon would be observed in ITP and sought to develop a functional T cell assay to quantify upregulation of IL-10 after exposure to dexamethasone in parallel with IL-17 expression. In this preliminary study, we tested this assay in a mixed cohort of ITP patients, and, to evaluate the potential generalizability of this work to other autoimmune diseases, in parallel, we evaluated a control cohort of patients with autoimmune eye inflammation (uveitis). Our goal was to interrogate the continuum of corticosteroid response in patients from an adaptive immune T cell perspective, both to shed light on underlying mechanisms of disease and to assess the potential for this to be the basis of a T cell biomarker to predict corticosteroid responsiveness.

## Methods

### *Patient cohorts*

Patients with a diagnosis of ITP aged 16 years and over were recruited following informed written consent in accordance with the Declaration of Helsinki (study registration details: <https://www.isrctn.com/ISRCTN95606674>, NHS Research Ethics Committee ref: 15/LO/2088). Patients with a complete response (CR) were defined as achieving a platelet count of  $>100 \times 10^9/L$  with single agent, first line oral prednisolone treatment (1mg/kg daily) within 2 weeks of treatment initiation, whereas non-responsive (NR) patients were defined by a platelet count of  $<30 \times 10^9/L$ , or less than a 2-fold increase from baseline after 2 weeks of treatment. If patients achieved a platelet count of  $30-100 \times 10^9/L$  and 2-fold increase from baseline they were classified as a partial responder (PR). At time of recruitment, patients could be receiving any ITP-directed therapy (Table 1).

Control cohorts of autoimmune uveitis patients and healthy donors were also recruited following informed consent (UK NHS Research Ethic Committee reference: 04/Q2002/84). The definition of corticosteroid resistance in autoimmune uveitis was failure to taper to a dose of 10mg/day prednisolone as previously described [12].

### *Cell isolation and culture*

Peripheral blood was acquired by venepuncture, and CD4<sup>+</sup> T cells isolated through negative selection using a RosetteSep™ Human CD4<sup>+</sup> T cell enrichment cocktail (StemCell Technologies, Canada) as per the manufacturer's protocol. Purified CD4<sup>+</sup> T cells were resuspended to a concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 growth medium containing 10% FCS, L-Glutamine and Penicillin/Streptomycin. Cells were activated with Human T Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, UK) and 50IU/ml recombinant human IL-2 (Roche, UK), and cultured with or without dexamethasone (Dex;  $1 \times 10^{-6}M$ ) for 96 hours (unless otherwise stated) under tissue culture conditions. Unstimulated cells at the point of isolation (day 0) were also examined as a pre-culture sample.

### *Intracellular staining*

PMA (20ng/ml), ionomycin (1 $\mu$ M) and GolgiStop (2 $\mu$ M; BD Biosciences, USA) were added to the cell culture for the last 4 hours. Cells were then stained with a Live/Dead viability dye (ThermoFisher Scientific) for 30 minutes before fixation and permeabilization using BD Cytfix/Cytoperm Kit (BD Biosciences) for 15 minutes. Intracellular cytokine expression was measured using the following antibodies: IL-10-eFluor660 (JES3-9D7; ThermoFisher Scientific), IL-17-BrilliantViolet™ 605 (BL168; BioLegend, USA), and IFN $\gamma$ -PerCP-Cy5.5 (4S.B3; ThermoFisher Scientific). Fluorescence was then measured by flow cytometry using a BD Fortessa X20 or BD LSR II (BD Biosciences).

### *Data analysis*

Analysis was performed using FlowJo software (TreeStar Software, USA) and carried out in a masked fashion so that the assessor was blind to the clinical response of the patient. Live singlets were gated, and the percentage of cytokine positive cells determined by using fluorescence minus one (FMO) controls. The proportion of IL-10<sup>+</sup> and IL-17<sup>+</sup> cells was used to calculate the IL-10:IL-17 ratio. Statistical analysis was carried out using GraphPad Prism

software (San Diego, CA). Mann-Whitney U tests were performed between groups, and a P value of <0.05 was considered significant.

## Results and Discussion

We initially aimed to characterize the kinetics of CD4<sup>+</sup> T cell cytokine expression in the presence of dexamethasone in cells isolated from healthy controls. An increase in the proportion of IL-10-producing CD4<sup>+</sup> T cells compared to T cell activation alone was demonstrated, with a significant difference seen from 4 days of culture onwards (Figure 1A). This IL-10 expression occurred in a dose-dependent manner (Figure 1B). The day 4 timepoint also showed the least differential of IL-17 (Figure 1C), and the greatest suppression of IFN- $\gamma$  (Figure 1D), which is consistent with other reports [9]. Consequently, the day 4 timepoint was chosen for this assay.

To determine whether clinical non-responsiveness to corticosteroid treatment in ITP patients correlated with the *in vitro* cytokine response of CD4<sup>+</sup> T cells, 56 adults with ITP were recruited from University Hospitals Bristol NHS Foundation Trust. In total, 33 CR, 11 NR, and 12 PR patients were recruited, with a median age of 53 years (range 16-85), and 26 females (Table 1). Dexamethasone treatment *in vitro* resulted in a significant increase in the proportion of IL-10<sup>+</sup> CD4<sup>+</sup> T cells compared to T cell activation across all patient groups (Figure 2A, B). In contrast, the proportion of IL-17<sup>+</sup> CD4<sup>+</sup> T cells following dexamethasone treatment was only increased in CR patients (Figure 2C). Dexamethasone did not suppress IFN- $\gamma$  expression after 4 days culture regardless of clinical response to corticosteroid treatment (Figure 2D). In contrast this was suppressed in healthy donors (Figure 1D) and the maintenance of IFN- $\gamma$  in ITP therefore potentially reflects underlying immune activation in the context of disease. Nonetheless, when comparing the change in intracellular expression of individual cytokines after culture with dexamethasone in CD4<sup>+</sup> T cells from NR, PR and CR patients, neither IL-10, IL-17 or IFN- $\gamma$  expression in isolation was discriminatory. However, when the ratio of IL-10 to IL-17 in CD4<sup>+</sup> T cells was examined, this was significantly elevated in dexamethasone treated CD4<sup>+</sup> T cells from CR compared with NR patients (6.02 +/- 2.98 CR vs 3.22 +/- 1.75 NR, p=0.002; Figure 2E, F). This IL-10:IL-17 ratio was also able to distinguish between CR and NR patients with an area under the receiver operating characteristic (ROC) curve of 0.81, indicating significant predictive power (95% confidence intervals 0.668, 0.952, p=0.002) (Figure 2G). We have interpreted this *in vitro* observation as a glucocorticoid-dependent phenomenon, rather than a reflection of disease severity, as there was no significant difference in the expression of IL-10, IL-17 or IFN- $\gamma$  between CR, PR and NR groups directly *ex vivo*, pre-culture (Figure 3A-D).

Furthermore, to confirm the generalizability of these findings, we recruited a second cohort of 36 corticosteroid-treated patients with autoimmune uveitis. Fourteen of these patients had a complete response, and 22 were non-responsive in accordance with previously published criteria [12]. The *in vitro* response to dexamethasone of CD4<sup>+</sup> T cells from uveitis patients replicated our findings in ITP, with CR patients showing increased IL-10<sup>+</sup> (Figure 4A, B) and decreased IL-17<sup>+</sup> (Figure 4A, C) CD4<sup>+</sup> T cells compared to NR patients, resulting in a significant difference in the ratio of IL-10<sup>+</sup> to IL-17<sup>+</sup> cells in CR patients compared to NR

patients (3.19 +/- 2.25 CR vs 1.74 +/- 2.14 NR, p=0.01; Figure 4E, F), and an area under the ROC curve of 0.76 (95% confidence intervals 0.599, 0.914, p=0.01; Figure 4G). In addition, consistent with ITP, there was no difference in the proportion of IFN- $\gamma$ <sup>+</sup> T cells between CR and NR uveitis patients (Figure 4D).

In common with other immune-driven diseases, these data strongly support the concept of an adaptive immune basis for clinical heterogeneity in patients' response to corticosteroid treatment in ITP. We have refined this to CD4<sup>+</sup> T helper cell subsets, demonstrating a reduced IL-10:IL-17 ratio in NR patients. This reflects a relative abrogation of IL-10 and persistent IL-17 expression in patients who fail to respond.

There is increasing evidence that the dysregulation of CD4<sup>+</sup> T cells plays a central role in the pathogenesis of ITP. Several studies have identified an imbalanced Th1/Th2 ratio, with increased Th1 cells and an associated rise in the production of pro-inflammatory cytokines such as IFN- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  [17-19]. Additionally, increased numbers of pro-inflammatory Th17 cells, and reduced numbers of regulatory T cells (Tregs) have been described in patients with ITP [20-22]. The clinical efficacy of glucocorticoids in ITP may therefore be in part due to their capacity to rebalance these T helper populations through the induction of the anti-inflammatory cytokine IL-10. Indeed, high dose dexamethasone treatment has been reported to restore a normal Th1/Th2 ratio, reduce Th17 cells, and increase the number of Tregs [23]. A subset of CD45RA<sup>+</sup>FoxP3<sup>hi</sup> Tregs with high IL-10 expression has also been correlated with greater sensitivity to corticosteroid treatment [24]. Corticosteroids can also upregulate IL-10 expression in other cell types, including CD8<sup>+</sup> T cells [25, 26] and B cells [27]. Indeed, the frequency of IL-10<sup>+</sup> B cells positively correlates to a high Treg/Th17 ratio and a good treatment outcome in ITP patients [28].

CD4<sup>+</sup> T cells that upregulate IL-10 in response to dexamethasone often co-produce Th17-associated cytokines, including IL-17, IL-22, and IFN- $\gamma$  [9]. These IL-10-producing Th17 cells have been termed "non-pathogenic" Th17 cells, and are important in limiting inflammation and autoimmunity [29]. In contrast, a subset of CD4<sup>+</sup> T cells that co-express IL-17 and IFN- $\gamma$ , so-called "pathogenic" Th17 cells, have been shown to be unresponsive to corticosteroids. These cells do not express IL-10 [11] and their proliferation is not suppressed by dexamethasone [10]. Thus, in patients who fail to respond to corticosteroids by upregulating IL-10, we hypothesize that there may be a preferential outgrowth of IL-17<sup>+</sup>IFN- $\gamma$ <sup>+</sup> pathogenic Th17 cells, contributing to persistent, corticosteroid refractory disease.

A limitation of this study is the mixed cohort of ITP patients at various stages of disease duration and receiving a range of treatments, as well as the low number of NR patients recruited. Whilst there are no clear indications that age, gender, or chronicity of disease have significantly influenced our findings (Table 1), it would be necessary to prospectively validate this finding in a larger cohort of newly diagnosed ITP patients. For a small number of patients (n=9, 4 CR, 3 PR and 2 NR), a repeat sample was taken 1 to 13 months after the initial sample. The IL-10:IL-17 ratio was not significantly changed between the two time points (paired t-test; mean of differences -1.138, S.D. of differences 3.028, p=0.2922), despite the fact that all but one of the patients had changed treatment regime at the second time point. However, a larger prospective cohort is required to definitively evaluate whether the results of our 4-day in vitro culture are independent of patients' clinical treatment.



There is also some evidence that ITP patients who have anti-GPIIb complex antibodies are more resistant to corticosteroid therapy [30]; it would therefore be interesting to examine the relationship between CD4<sup>+</sup> T cell cytokine expression and the presence of anti-GPIIb antibodies in patients in future studies.

Overall, these results contribute to our mechanistic understanding of the action of, and resistance to corticosteroids, with potential broad applicability across a wide range of autoimmune and inflammatory diseases. These findings may also inform the development of a predictive biomarker of clinical corticosteroid refractory disease. In ITP, a CD4<sup>+</sup> T cell-based biomarker would benefit patients by enabling earlier initiation of alternative interventions in those unlikely to respond to corticosteroid. This would improve overall therapeutic success by minimizing the time during which patients have dangerously low platelet counts, reducing bleeding risk, and avoiding toxicity of ineffective corticosteroid treatment in refractory patients.

### **Contributions**

M.L. Stimpson, P.P.J. Lait, L.P. Schewitz-Bowers, E.L. Williams, and K.F. Thirlwall performed experiments; M.L. Stimpson analysed results and made the figures; M.L. Stimpson., P.P.J. Lait, E.L. Williams, R.W.J. Lee and C.A. Bradbury designed the research; and M.L. Stimpson, R.W.J. Lee and C.A. Bradbury wrote the paper; all authors reviewed and approved the final manuscript.

### **Acknowledgements**

We thank Andrew Herman and Lorena Sueiro Ballesteros for their flow cytometry support. This research was funded by Above and Beyond, Elizabeth Blackwell Institute for Health Research grant RJ6317 and ITP Support Association grant 55258. PJPL, LPS-B, ELW and RWJL were also funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health

### **Conflict of Interest Disclosures:**

RWJL and LPS-B are named inventors on a US patent application (US15/106,411) entitled 'Conjugates for treating inflammatory disease and identification of patients likely to benefit from such treatment'. No other authors have conflicts of interest that are relevant to this work.

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<b>Characteristic</b>	<b>All patients (n=56)</b>	<b>Complete Response (n=33)</b>	<b>Partial Response (n=12)</b>	<b>No Response (n=11)</b>
Age -- median (range)	53 (16-85)	50 (17-84)	38 (26-85)	61 (16-84)
Female -- no. (%)	26 (46)	16 (48)	7 (58)	3 (27)
ITP duration -- no. (%)				
Newly diagnosed	21 (38)	16 (48)	3 (25)	2 (18)
Chronic	35 (63)	17 (52)	9 (75)	9 (82)
Current treatment -- no. (%)				
None	34 (61)	22 (67)	9 (75)	3 (27)
Corticosteroid	7 (13)	5 (15)	1 (8)	1 (9)
TPO RA	7 (13)	3 (9)	2 (17)	2 (18)
MMF	8 (14)	3 (9)	0 (0)	5 (45)

**Table 1. Characteristics of ITP patients**

**Figure 1. Kinetics of IL-10, IL-17, and IFN- $\gamma$  expression by CD4<sup>+</sup> T cells in the presence of glucocorticoids.** IL-10 (A, B) IL-17 (C) and IFN- $\gamma$  (D) intracellular cytokine expression of CD4<sup>+</sup> T cells from healthy controls were assessed by flow cytometry using a BD LSR II, and percentage of cells positive for each cytokine shown. Cells were activated in the presence (black circles) or absence (clear circles) of  $1 \times 10^{-6}$ M dexamethasone (Dex) for the indicated number of days. The percentage of IL-10<sup>+</sup> CD4<sup>+</sup> T cells in response to a dose titration of Dex was compared at day 4 (B). Cumulative data shown, n=4. \*p<0.05, \*\* p<0.01.

**Figure 2. CD4<sup>+</sup> T cells from CR, PR and NR ITP patients exhibit distinct IL-10 and IL-17 profiles in response to *in vitro* glucocorticoids.** Intracellular cytokine expression of CD4<sup>+</sup> T cells from ITP patients activated for 4 days in the presence or absence of  $1 \times 10^{-6}$ M dexamethasone (Dex) were assessed by flow cytometry on a BD Fortessa X20. Representative flow cytometry plots for IL-10 and IL-17 (A) and cumulative data for the percentage of IL-10<sup>+</sup> (B), IL-17<sup>+</sup> (C) and IFN- $\gamma$ <sup>+</sup> (D) CD4<sup>+</sup> T cells and the IL-10:IL-17 ratio (E) from ITP patients who clinically showed complete response (CR; n=33), partial response (PR; n=12), or no response (NR; n=11) to corticosteroid treatment. Box plot of the IL-10:IL-17 ratio of CD4<sup>+</sup> T cells treated *in vitro* with dexamethasone from CR, PR and NR ITP patients (F). Area under receiver operating characteristic (AUROC) curve for IL-10:IL-17 ratio in predicting the clinical response of ITP patients to corticosteroid treatment (G). AUROC is 0.81 (95% confidence interval 0.668 to 0.952). (A-E) Mean +/- S.D. is shown. (F) Median, interquartile range, minimum and maximum values. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Figure 3. CD4<sup>+</sup> T cells from CR, PR and NR patients do not have distinct differences in cytokine levels directly *ex vivo*.** IL-10 (A) IL-17 (B) and IFN- $\gamma$  (C) intracellular cytokine expression, plus the calculated ratio of IL-10 to IL-17 expression (D) in CD4<sup>+</sup> T cells isolated directly *ex vivo* from patient blood stimulated with PMA, ionomycin and GolgiStop for 4 hours were assessed on a BD Fortessa X20. The percentage of all cells positive for each cytokine is shown. Patients were categorised by clinical response to corticosteroid treatment; complete response (CR; n=25), partial response (PR; n=10), or no response (NR; n=10). Mean +/- S.D. is shown.

**Figure 4. CD4<sup>+</sup> T cells from corticosteroid non-responsive autoimmune uveitis patients demonstrate reduced IL-10:IL-17 ratio.** Intracellular cytokine expression of CD4<sup>+</sup> T cells from autoimmune uveitis patients activated for 4 days in the presence or absence of  $1 \times 10^{-6}$ M dexamethasone (Dex) were assessed by flow cytometry on a BD LSR II. Representative flow cytometry plots for IL-10 and IL-17 (A) and cumulative data for the percentage of IL-10<sup>+</sup> (B), IL-17<sup>+</sup> (C) and IFN $\gamma$ <sup>+</sup> (D) CD4<sup>+</sup> T cells and the IL-10:IL-17 ratio (E) from ITP patients who clinically showed complete response (CR; n=14) or no response (NR; n=22) to corticosteroid treatment. Box plot of the IL-10:IL-17 ratio of CD4<sup>+</sup> T cells treated *in vitro* with dexamethasone from CR and NR uveitis patients (F). Area under receiver operating characteristic (AUROC) curve for IL-10:IL-17 ratio in predicting the clinical response of ITP patients to corticosteroid treatment (G). AUROC is 0.76 (95% confidence interval 0.599 to 0.914). (A-F) Mean +/- S.D. is shown. (F) Median, interquartile range, minimum and maximum values. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.