

2019-11-14

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Dhanda, Ashwin

<http://hdl.handle.net/10026.1/14602>

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10.5009/gnl19035

Gut and Liver

The Editorial Office of Gut and Liver

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# *Ex vivo* T cell cytokine expression predicts survival in patients with severe alcoholic hepatitis

Ashwin D. Dhanda<sup>1,2</sup>, Euan Yates<sup>2</sup>, Lauren P. Schewitz-Bowers<sup>3</sup>, Philippa J. Lait<sup>3</sup>, Richard W.J. Lee<sup>3</sup>, Matthew E. Cramp<sup>12</sup>

<sup>1</sup>South West Liver Unit, University Hospitals Plymouth NHS Trust, Plymouth, UK

<sup>2</sup>Hepatology Research Group, University of Plymouth, Plymouth, UK

<sup>3</sup>Translational Health Sciences, University of Bristol, Bristol, UK

## *Corresponding author:*

Dr Ashwin Dhanda  
South West Liver Unit, Level 7, Derriford Hospital  
University Hospitals Plymouth NHS Trust  
Plymouth PL6 8DH  
United Kingdom  
Email: ashwin.dhanda@plymouth.ac.uk  
Tel: +44 1752 432723  
Fax: +44 1752 517576

Running title: T cell cytokines in alcoholic hepatitis

## *Author contributions:*

Study concept: AD, RL; Performed experimental work: EY, LSB, PL; Statistical analysis: AD, PL; Study supervision: RL, MC; Drafting manuscript: AD, LSB; Finalising manuscript: AD, RL, MC

## Abstract

### Aim

Alcoholic hepatitis (AH) is an acute inflammatory liver condition with high early mortality. Steroids have proven short-term survival benefit but non-responders have the worst outcomes. There is a clinical need to identify these high-risk individuals at presentation. T cells have been implicated in alcoholic hepatitis and steroid responsiveness. We aimed to measure *ex vivo* T cell cytokine expression as a candidate biomarker of outcome in patients with AH.

### Methods

Consecutive patients with AH (bilirubin >80µmol/L and AST:ALT>1.5 in heavy alcohol consumers with discriminant function [DF]≥32), were recruited from University Hospitals Plymouth NHS Trust. T cells were obtained and stimulated *ex vivo* before cytokine expression was determined by flow cytometry and protein multiplex analysis.

### Results

Twenty-three patients were recruited (10 male; median age 51; baseline DF 67; 30% 90-day mortality). Compared to non-survivors at day 90, T cells from survivors had higher baseline intracellular IL-10:IL-17A ratio (0.43 v 1.20; p=0.02). Multiplex protein analysis identified IFNγ and TNFα as independent predictors of 90-day mortality (p=0.04 and p=0.01 respectively). The ratio of IFNγ/TNFα was predictive of 90-day mortality (1.4 v 0.2; p=0.03).

### Conclusions

These data demonstrate the potential utility of T cell cytokine release assays performed on pre-treatment blood samples as biomarkers of survival in severe AH. Our key findings were that both the ratio of intracellular IL-10 to IL-17A and the ratio of IFNγ to TNFα in culture supernatants were predictors of 90-day mortality. This offers the promise of developing T cell based diagnostic tools for risk stratification.

Keywords: Alcoholic hepatitis, T cells, cytokines, biomarker

## Introduction

Alcoholic hepatitis (AH) is an acute inflammatory condition, which carries a high mortality of 30% within 90 days<sup>1</sup>. Steroid treatment is the only therapy with proven short-term survival benefit<sup>2</sup>. However, the worst outcomes are in the 30-40% of patients with a poor response to steroid treatment<sup>3</sup>, and there is a pressing need to identify these high-risk individuals at presentation.

T cells have long been implicated in the pathogenesis of AH and we have previously reported that suppression of lymphocyte proliferation correlates with clinical outcome following steroid treatment<sup>4,5</sup>. Given the barriers to the development of this radiation-based assay for clinical application and the precedent of functional cytokine release assays for other clinical applications<sup>6</sup>, we sought to evaluate T cell cytokine expression as a candidate biomarker of AH mortality.

## Case report

### *Study design*

The study protocol was approved by the UK's Health Research Authority (reference: 15/LO/1501) and all participants provided written informed consent. Consecutive patients with severe AH, defined as recent onset jaundice with bilirubin > 80 µmol/L and AST:ALT > 1.5 in heavy alcohol consumers (> 60 g or 80 g ethanol / day in females and males respectively) with a discriminant function (DF) score ≥ 32, were recruited from University Hospitals Plymouth NHS Trust. Patients received standard care including steroid treatment in the absence of active infection, hepatorenal syndrome or gastrointestinal haemorrhage.

### *T cell isolation, stimulation and statistical analysis*

Whole blood samples were taken before steroid treatment was started with CD4<sup>+</sup> T cells isolated by negative selection (Stemcell Technologies, Cambridge, UK) and cultured for 4 days in supplemented

media with interleukin (IL)-2 (Sigma-Aldrich, Poole, UK) and T cell receptor stimulation (anti-CD3/CD28 microbeads; Thermo Fisher Scientific, Loughborough, UK). Cells cultured without T cell receptor stimulation were included as controls. T cell receptor stimulation with anti-CD3/CD28 microbeads was selected as a standardized method of T cell activation, which has been optimized for use in other conditions<sup>7</sup>. For the final 4 hours, cultures were stimulated with T cell mitogen phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma) with golgi export inhibitor (BD Biosciences, Oxford, UK). Cells were fixed, permeabilised and stained with fluorescently labelled antibodies to IL-10, IL-17A and interferon gamma (IFN $\gamma$ ) (Thermo Fisher Scientific), quantified on a BD Accuri flow cytometer (BD Biosciences) and analysed in FlowJo (FlowJo LLC, Ashland, OR, USA). Protein concentration in cell culture supernatants prior to the final 4 hour stimulation was analysed in duplicate for CCL20, GM-CSF, IFN $\gamma$ , IL-10, IL-12p70, IL-17A, IL-21, IL-23, IL-4, IL-6 and TNF $\alpha$  using a magnetic bead array (R&D Systems, Minneapolis, MN) on a Luminex 200 analyser (Luminex Corp, Austin, TX). Statistical analysis was performed using IBM SPSS version 24 (IBM, Armonk, NY). The primary clinical outcome was death within 90 days of presentation. The data presented are median values and comparisons were made with non-parametric tests.

### *Findings*

23 consecutive patients were recruited between April 2016 and November 2017 (10 male; median age 51, baseline DF 67, model for end-stage liver disease [MELD] score 19). 90-day mortality was 30%. Four patients did not receive steroids because of active infection. Samples were obtained prior to steroid treatment in all cases at a median of 5 days from hospital admission. Median time from admission to steroid treatment was 6 days.

Compared to non-survivors at day 90, CD4<sup>+</sup> T cells from non-survivors had a higher baseline intracellular IL-10:IL-17A ratio (percent of T cells expressing IL-10: 2.4% [non-survivors] v 1.6% [survivors], p=0.07; IL-17A: 2.1% [non-survivors] v 3.5% [survivors], p=0.08; median IL-10:IL-17A ratio

1.20 [non-survivors] v 0.43 [survivors];  $p=0.02$ ; **figure 1A**) with an area under the curve of the receiver operating characteristic (AUROC) score of 0.82 (95% confidence interval 0.64-1.00). The proportion of T cells expressing IFN $\gamma$  was similar between groups (14.2% v 11.9%;  $p=0.55$ ). In comparison, expression of cytokines in control cultures was lower than stimulated conditions: IL-10: 0.2% v 1.8%; IL-17: 2.5% v 3.3%; IFN $\gamma$ : 6.2% v 13.3% (all  $p=0.07$ ; Wilcoxon signed ranks test).

Multiplex protein analysis performed on 22 of the 23 cases identified IFN $\gamma$  and TNF $\alpha$  as most differentially expressed between survivors and non-survivors at day 90 (7044 v 2741 pg/ml;  $p=0.19$  and 2610 v 4098 pg/ml;  $p=0.08$  respectively). Multivariate analysis confirmed that both IFN $\gamma$  and TNF $\alpha$  were independent predictors of 90-day mortality ( $p=0.04$  and  $p=0.01$  respectively). The ratio of IFN $\gamma$ /TNF $\alpha$  was predictive of 90-day mortality (1.4 v 0.2;  $p=0.03$ ; **figure 1B**) with an AUROC score of 0.79 (95% CI 0.58-0.99). The concentration of other mediators tested in the multiplex was similar between survivors and non-survivors. No cytokine concentration correlated with baseline DF or MELD scores. Steroid response, as determined by Lille score with a threshold of 0.45 at day 7 of steroid treatment, was not associated with differences in any cytokine measured by either flow cytometry or protein multiplex. Similarly, the ratios of intracellular IL-10/IL-17A and IFN $\gamma$ /TNF $\alpha$  were the same in Lille steroid responders and non-responders.

## Discussion

These data from a pilot prospective cohort of more than 20 patients demonstrate the potential utility of T cell cytokine release assays performed on pre-treatment blood samples as biomarkers of survival in severe AH. Our key findings were that both the ratio of intracellular IL-10 to IL-17A measured by flow cytometry in CD4 $^{+}$  cells and the ratio of IFN $\gamma$  to TNF $\alpha$  in culture supernatants were predictors of 90-day mortality. These data also demonstrate that flow cytometry and protein

multiplex are complementary techniques that can reveal different alterations in protein expression either in proportion of cells secreting a cytokine or in cytokine concentration, respectively.

The pathogenesis of AH is orchestrated by several immune subsets including neutrophils and monocytes, which are activated by gut derived signals such as endotoxin<sup>8,9</sup>. However, T cells also play a vital role in responding to antigen presenting cells and propagating inflammation in AH<sup>10</sup>. Transcriptome studies have confirmed upregulation of TNF and T cell pathways<sup>11,12</sup>. In particular, the Th17 (IL-17 expressing T cell) pathway is upregulated with high expression of related chemokines such as CCL20<sup>12,13</sup>. Furthermore, recent studies have demonstrated that monocytes activated by exposure to gut microbial signals interact with CD4<sup>+</sup> T cells to alter expression of exhaustion markers and cytokines including IFN $\gamma$ <sup>14</sup>. These data confirm the relevance of T cells in AH and support the measurement of T cell cytokines as a broad measure of immune response.

These findings offer the promise of developing T cell based diagnostic tools for risk stratification both in the context of routine clinical practice and also to inform the design of smart clinical trials seeking to test new interventions in patients most likely to fail conventional corticosteroid therapy.

## **Acknowledgements**

### *Grant support:*

AD received funding from Plymouth Hospitals Charity. AD and RL receive salary funding from the National Institute for Health Research. L.P.S-B, P.J.L and R.W.J.L are supported 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.

### *Disclosures:*

L.P.S.-B. and R.W.J.L are named inventors on a US patent application (no. 61/919,404), which incorporates biomarkers to identify patients who will benefit from treatments for inflammatory diseases



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**Figure 1:** (A) Ratio of the percentage of CD4<sup>+</sup> T cells expressing IL-10:IL-17A measured by flow cytometry (0.4 v 1.2; p=0.02) and (B) ratio of IFN $\gamma$ :TNF $\alpha$  concentration measured by protein multiplex in patients with AH alive and dead at day 90 (1.4 v 0.2; p=0.03). \*p<0.05

