Ex Vivo T Cell Cytokine Expression Predicts Survival in Patients with Severe Alcoholic Hepatitis

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Alcoholic hepatitis (AH) is an acute inflammatory liver condition with high early mortality rate. Steroids improve short-term survival but nonresponders have the worst outcomes. There is a clinical need to identify these high-risk individuals at the time of presentation. T cells are implicated in AH and steroid responsiveness. We measured ex vivo T cell cytokine expression as a candidate biomarker of outcomes in patients with AH. Consecutive patients (bilirubin >80 µmol/L and ratio of aspartate aminotransferase to alanine aminotransferase >1.5 who were heavy alcohol consumers with discriminant function [DF] ≥32), were recruited from University Hospitals Plymouth NHS Trust. T cells were obtained and stimulated ex vivo. Cytokine expression levels were determined by flow cytometry and protein multiplex analysis. Twenty-three patients were recruited (10 male; median age 51 years; baseline DF 67; 30% 90-day mortality). Compared to T cells from nonsurvivors at day 90, T cells from survivors had higher baseline intracellular interleukin (IL)-10:IL-17A ratio (0.43 vs 1.20, p=0.02). Multiplex protein analysis identified interferon γ (IFNγ) and tumor necrosis factor-α (TNF-α) as independent predictors of 90-day mortality (p=0.04, p=0.01, respectively). The ratio of IFNγ to TNF-α was predictive of 90-day mortality (1.4 vs 0.2, p=0.03). These data demonstrate the potential utility of T cell cytokine release assays performed on pretreatment blood samples as biomarkers of survival in patients with severe AH. Our key findings were that intracellular IL-10:IL-17A and IFNγ:TNF-α in culture supernatants were predictors of 90-day mortality. This offers the promise of developing T cell-based diagnostic tools for risk stratification. (Gut Liver 2020;14:265-268)

Key Words: Hepatitis, alcoholic; T cells; Cytokines; Biomarker

INTRODUCTION

Alcoholic hepatitis (AH) is an acute inflammatory condition, which carries a high mortality of 30% within 90 days.1 Steroid treatment is the only therapy with proven short-term survival benefit.2 However, the worst outcomes are in the 30% to 40% of patients with a poor response to steroid treatment,3 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.4,5 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,6 we sought to evaluate T cell cytokine expression as a candidate biomarker of AH hepatitis.

CASE REPORT

1. 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 ratio of aspartate aminotransferase to alanine aminotransferase >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 hemorrhage.
2. **T cell isolation, stimulation and statistical analysis**

Whole blood samples were taken before steroid treatment was started with CD4⁺ 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.² For the final 4 hours, cultures were stimulated with T cell mitogen phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich) with Golgi export inhibitor (BD Biosciences, Oxford, UK). Cells were fixed, permeabilized and stained with fluorescently labelled antibodies to IL-10, IL-17A and interferon γ (IFNγ) (Thermo Fisher Scientific), quantified on a BD Accuri flow cytometer (BD Biosciences) and analyzed in FlowJo (FlowJo LLC, Ashland, OR, USA). Protein concentration in cell culture supernatants prior to the final 4-hour stimulation was analyzed in duplicate for CCL20, granulocyte-macrophage colony stimulating factor, IFNγ, IL-10, IL-12p70, IL-17A, IL-21, IL-23, IL-6 and tumor necrosis factor-α (TNF-α) using a magnetic bead array (R&D Systems, Minneapolis, MN, USA) on a Luminex 200 analyzer (Luminex Corp., Austin, TX, USA). Statistical analysis was performed using IBM SPSS version 24 (IBM Corp., Armonk, NY, USA). The primary clinical outcome was death within 90 days of presentation. The data presented are median values and comparisons were made with nonparametric tests.

3. **Findings**

Twenty-three consecutive patients were recruited between April 2016 and November 2017 (10 male; median age, 51 years; baseline DF, 67; Model for End-Stage Liver Disease [MELD] score, 19). Ninety-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 survivors at day 90, CD4⁺ T cells from nonsurvivors had a higher baseline intracellular IL-10:IL-17A ratio (percent of T cells expressing IL-10: 2.4% [nonsurvivors] vs 1.6% [survivors], p=0.07; IL-17A: 2.1% [nonsurvivors] vs 3.5% [survivors], p=0.08; median IL-10:IL-17A ratio: 1.20 [nonsurvivors] vs 0.43 [survivors]; p=0.02) (Fig. 1A) with an area under the curve of the receiver operating characteristic score of 0.82 (95% confidence interval, 0.64 to 1.00). The proportion of T cells expressing IFNγ was similar between groups (14.2% vs 11.9%, p=0.55). In comparison, expression of cytokines in control cultures was lower than stimulated conditions: IL-10: 0.2% vs 1.8%; IL-17: 2.5% vs 3.3%; IFNγ: 6.2% vs 13.3% (all p=0.07; Wilcoxon signed ranks test).

Multiplex protein analysis performed on 22 of the 23 cases identified IFNγ and TNF-α as most differentially expressed between survivors and nonsurvivors at day 90 (7,044 pg/mL vs 2,741 pg/mL, p=0.19 and 2,610 pg/mL vs 4,098 pg/mL, p=0.08, respectively). Multivariate analysis confirmed that both IFNγ and TNF-α were independent predictors of 90-day mortality (p=0.04 and p=0.01, respectively). The ratio of IFNγ to TNF-α was predictive of 90-day mortality (1.4 vs 0.2, p=0.03) (Fig. 1B) with an area under the curve of the receiver operating characteristic score of 0.79 (95% confidence interval, 0.58 to 0.99). The concentration of other mediators tested in the multiplex was similar between survivors and nonsurvivors. 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 IFNγ/TNF-α were the same in Lille steroid responders and nonresponders.

![Fig. 1](image-url) (A) The ratio of the percentage of CD4⁺ T cells expressing interleukin (IL)-10 to IL-17A measured by flow cytometry (0.4 vs 1.2, p=0.02) and (B) the ratio of the interferon γ (IFNγ) to tumor necrosis factor-α (TNF-α) concentrations measured by protein multiplex in patients with alcoholic hepatitis who were survivors and nonsurvivors at day 90 (1.4 vs 0.2, p=0.03). *p<0.05.
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 pretreatment 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 IFNγ to TNF-α 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. However, T cells also play a vital role in responding to antigen presenting cells and propagating inflammation in AH. Transcriptome studies have confirmed upregulation of TNF and T cell pathways. In particular, the Th17 (IL-17 expressing T cell) pathway is upregulated with high expression of related chemokines such as CCL20. Furthermore, recent studies have demonstrated that monocytes activated by exposure to gut microbial signals interact with CD4+ T cells to alter expression of exhaustion markers and cytokines including IFNγ. 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.

CONFLICTS OF INTEREST

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L.P.S. and R.W.J.L are named inventors on a US patent application (number: 61/919,404), which incorporates biomarkers to identify patients who will benefit from treatments for inflammatory diseases.

AUTHOR CONTRIBUTIONS

Study concept: A.D.D., R.W.J.L. Performed experimental work: E.Y., L.P.S., P.J.L. Statistical analysis: A.D.D., P.J.L. Study supervision: R.W.J.L., M.E.C. Drafting manuscript: A.D.D., L.P.S. Finalizing manuscript: A.D.D., R.W.J.L., M.E.C.

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REFERENCES

1. Thursz MR, Richardson P, Allison M, et al. Prednisolone or pentoxifylline for alcoholic hepatitis. N Engl J Med 2015;372:1619-1628.
2. Louvet A, Thursz MR, Kim DJ, et al. Corticosteroids reduce risk of death within 28 days for patients with severe alcoholic hepatitis, compared with pentoxifylline or placebo—a meta-analysis of individual data from controlled trials. Gastroenterology 2018;155:458-468.
3. Louvet A, Naveau S, Abdelnour M, et al. The Lille model: a new tool for therapeutic strategy in patients with severe alcoholic hepatitis treated with steroids. Hepatology 2007;45:1348-1354.
4. Dhanda AD, di Mambro AJ, Hunt VL, et al. Long-term outcome in patients with severe alcoholic hepatitis can be reliably determined using an in vitro measure of steroid sensitivity. Hepatology 2015;61:1099.
5. di Mambro AJ, Parker R, McCune A, Gordon F, Dayan CM, Collins P. In vitro steroid resistance correlates with outcome in severe alcoholic hepatitis. Hepatology 2011;53:1316-1322.
6. Diel R, Goletti D, Ferrara G, et al. Interferon-gamma release assays for the diagnosis of latent Mycobacterium tuberculosis infection: a systematic review and meta-analysis. Eur Respir J 2011;37:88-99.
7. Lee RW, Schewitz LP, Nicholson LB, Dayan CM, Dick AD. Steroid refractory CD4+ T cells in patients with sight-threatening uveitis. Invest Ophthalmol Vis Sci 2009;50:4273-4278.
8. Mookerjee RP, Stadlbauer V, Lidder S, et al. Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome. Hepatology 2007;46:831-840.
9. Vergis N, Khamri W, Beale K, et al. Defective monocyte oxidative burst predicts infection in alcoholic hepatitis and is associated with reduced expression of NADPH oxidase. Gut 2017;66:519-529.
10. Dhanda AD, Collins PL. Immune dysfunction in acute alcoholic hepatitis. World J Gastroenterol 2015;21:11904-11913.
11. Affò S, Dominguez M, Lozano JJ, et al. Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis. Gut 2013;62:452-460.
12. Affò S, Morales-Ibanez O, Rodrigo-Torres D, et al. CCL20 medi-
ates lipopolysaccharide induced liver injury and is a potential
driver of inflammation and fibrosis in alcoholic hepatitis. Gut
2014;63:1782-1792.
13. Lemmers A, Moreno C, Gustot T, et al. The interleukin-17 path-
way is involved in human alcoholic liver disease. Hepatology
2009;49:646-657.
14. Markwick LJ, Riva A, Ryan JM, et al. Blockade of PD1 and TIM3
restores innate and adaptive immunity in patients with acute alco-
holic hepatitis. Gastroenterology 2015;148:590-602.