A defect in the Nrf2/HO-1 pathway and Nox2 activation results in regulatory T cell mitochondrial dysfunction in alcohol related cirrhosis

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1 INTRODUCTION

There has been a global rise in mortality due to alcohol related liver disease in the western world, with Alcohol Related Cirrhosis (ARC) being a major indication for transplantation[1].

The molecular and cellular events underlying the progression of liver disease remain poorly understood, with immune-mediated injury and oxidative stress highlighted as factors driving disease progression[2,3].

The effects of oxidative stress are mediated at least in part by nuclear factor E2- related factor 2 (Nrf2), a transcription factor that regulates an array of antioxidant genes in the liver. In response to inflammation and reactive oxygen species (ROS), Nrf2 upregulates the cytoprotective enzyme, heme oxygenase-1 (HO-1) [Scheme 1][4].

In this study we focus on regulatory T cells (CD4+CD25+FOXP3+ T cells, Tregs), known for their function in modulating immune responses during tissue inflammation by suppressing T cell responses and limiting myeloid cell activation, and for having a key role in preventing autoimmunity and tissue injury[5].

2 AIM

Here we report a previously unrecognized functional deficit in circulating regulatory T cells (Tregs) isolated from patients with alcohol-related cirrhosis (ARC), correlating with disease severity. We propose a mechanism to explain this defect, showing that a dysregulated Nrf2 pathway and subsequent NADPH oxidase 2 (Nox2) activation leads to Treg mitochondrial dysfunction and loss of suppressor function with the aim of influencing the design of novel targeted therapies.

3 METHOD

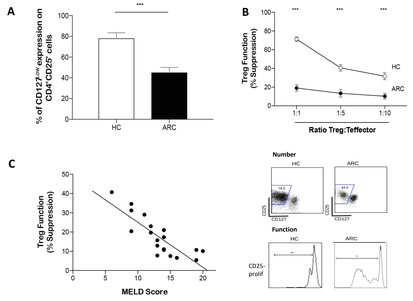
- Tregs were isolated from the peripheral blood of a total of 40 patients with ARC on the transplant waiting list at King's College Hospital and compared with age and sex matched Healthy Controls.
- Treg suppressor function was assessed using a CFSE dilution assay when co-cultured with Teffectors (Teffs).The assay was conducted at different Treg:Teff ratios
- Western blots were used to quantify Nrf2 and HO-1 expression.
- Imagestream® was used to assess Nrf2 translocation.
- Zinc Protoporphyrin (ZnPP) was used as a specific competitive inhibitor of HO-1

Tregs isolated from spleens of Nrf2 KO/WT mice were assessed by flow cytometry.

- The Seahorse Platform was used to measure glycolysis (extracellular acidification rate; ECAR) and oxidative phosphorylation (oxygen consumption rate; OCR) post 24hrs stimulation with anti-CD3/CD28.
- Nox2 activation was studied by confocal microscopy, assessing the co-localization of p47phox and gp91phox.

4 RESULTS

Figure 1. Tregs Isolated from Patients with Alcohol Related Cirrhosis (ARC) are Deficient in Number and Lack Suppressor Function



(A) Graph shows the relative frequency of CD127Low on freshly purified regulatory T cells from peripheral blood- 20 HCs and 20 ARC patients. (B) graph displaying the suppressor function of freshly isolated Tregs from 20 HCs and 20 ARC patients. (C) Graph denoting correlation between Treg Suppressor function and disease severity, as calculated by the model for end stage (MELD) liver disease score.

Scheme 1: Regulation of Heme Oxygenase-1 (HO-1) Signaling

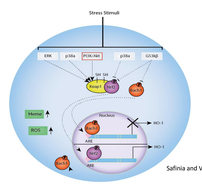
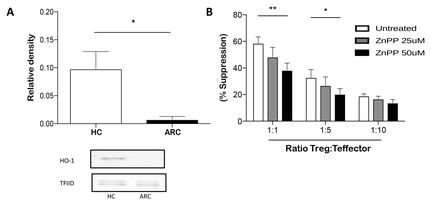


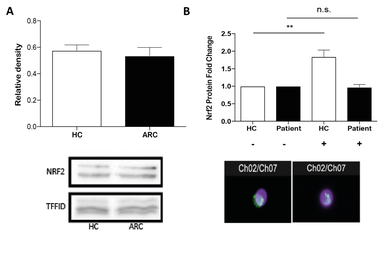
Diagram depicts the major signaling pathways involved in the regulation of HO-1 gene expression through the transcription factors (TF) Nrf2 and Bach1. At baseline the transcription repressor, Bach1, constitutively binds to the ARE preventing HO-1 gene expression. However, upon activation of certain signaling cascades and high levels of intracellular heme and stress stimuli, the TF Nrf2 dissociates from its complex with Keap1and translocates to the nucleus, displacing Bach1 at the antioxidant response element. As a result HO-1 expression is promoted.

Figure 2. ARC Tregs have Decreased Expression of Heme Oxygenase-1 (HO-1) and Modulation of HO-1 Affects Treg Suppressor Function



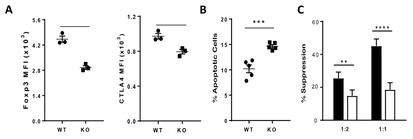
(A) Western blot and graph of HO-1 (25KDa) expression in 4 HCs and 5 ARC patients. (B) Treg suppressor function following 12 hours of culture in the presence of 25μM and 50μM and absence of the HO-1 inhibitor, ZnPP. n=9 * p<0.05 **p < 0.01

Figure 3. Tregs from Patients with Alcohol Related Cirrhosis Exhibit a Dysregulated NRF2 Transnuclear Migration



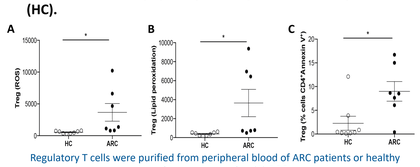
(A) Western-blot and graph of NRF2 expression in 4 HCs and 5 ARC and (B) Graph of transnuclear migration of NRF2 following 12 hours activation with prostaglandin J2 (PGJ2). Translocation was assessed by Imagestream, which relies on the spectral isolation of NRF2 images from nuclear images. The degree of nuclear translocation was assessed by quantifying the peak pixel intensity values for NRF2 staining within the digitally masked nuclear region (DAPI) in each individual sample n=5 ARC and 5 HCs **p < 0.01.

Figure 4. Tregs Isolated from Spleens of NRF2 KO Mice Display Reduced Expression of Regulatory Markers, Increased Apoptosis and Reduced Suppressive Capacities



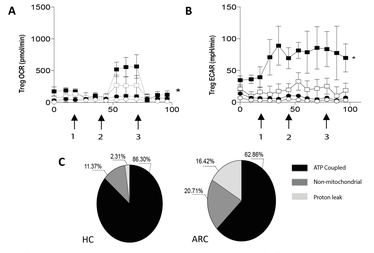
(A)Homogenized cells from spleen of WT and Nrf2-/- mice were analysed using flow cytometry. Nrf2-/- Tregs have lower expression of Foxp3 and CTLA4. Pooled data from three independent experiments are analysed (B) Tregs from spleen and pLN of WT or Nrf2-/- mice were freshly isolated and assessed for % apoptotic cells. Pooled data from three independent experiments(C) Tregs from spleen and pLN of WT or Nrf2-/- mice were freshly isolated. Polyclonal suppression assay, comparing the suppressive capacity of Nrf2-/- Tregs (Tregs:Teff = 1:2) as compared to WT Tregs. **p < 0.01 ***p < 0.001

Figure 5. Tregs from Patients with Alcohol Related Cirrhosis (ARC) have Increased Reactive Oxygen Species Production and are Prone to Apoptosis, as compared to Healthy Controls (HC).



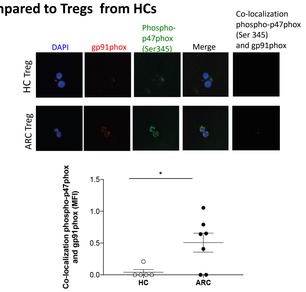
Regulatory T cells were purified from peripheral blood of ARC patients or healthy volunteers and evaluated by flow cytometry for: (A) ROS production measured by dihydroethidium, (B) Lipid peroxidation by using BodipyTM 581/591 (C) Relative number of Annexin V+ cells. * p<0.05

Figure 6. Tregs from ARC patients have increased non-mitochondrial oxygen consumption and proton leak than HC Tregs



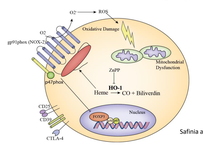
Tregs were subject to mitochondrial stress test for: (A) Oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR). Arrows indicate the injection of oligomycin A (1μM), FCCP (2.5μM), antimycin A (1μM) plus rotenone (100nM), respectively. (C) The OCR value obtained after treatment with antimycin A plus rotenone was subtracted from the OCR value under baseline (100%) to obtain the non-mitochondrial respiration. The OCR value obtained after the injection of oligomycin A was subtracted from the OCR value under baseline to obtain the ATP-coupled respiration. The proton leak was obtained by subtracting the relative values of non-mitochondrial respiration and the ATP-coupled respiration. * p<0.05

Figure 7. ARC Tregs have Increased NOX2 Activation as compared to Tregs from HCs



Tregs were purified from peripheral blood of ARC patients or healthy volunteers and evaluated for co-localization of p47phox (green) and gp91phox (red) by using confocal microscope. Representatives images on the left and graph showing the means of fluorescence intensity (MFI) for co-localized points (white) on the right. Nucleus were stained with 4,6-diamino-2-phenylindole (DAPI, blue). *P<0.05 (n=5-8).

Scheme 2: Mechanism of Treg Dysfunction in ARC



Dysregulated Nrf2/HO-1 pathway in ARC Tregs leads to increased intracellular Heme levels. As a result there is activation of NOX2 and increased ROS production by ARC Tregs. Increased ROS leads to Mitochondrial dysfunction: increased proton leak, increased non-mitochondrial OCR. AS a result Tregs express an altered metabolic profile towards glycolysis with predisposition for Tregs to become effector-like.

5 CONCLUSIONS

- Here we demonstrate for the first time that the immune dysregulation as reported in cirrhosis, extends beyond the myeloid population, to involve Tregs
- We have further shown a correlation with liver disease severity and the decreased immunoregulatory function of Tregs
- Analysis of Tregs isolated from patients with cirrhosis revealed that they expressed lower HO-1 levels than Tregs from HCs. This was associated with a defective downstream signaling in the Nrf2 pathway
- Tregs from patients demonstrated a higher baseline ROS production, lipid peroxidation and apoptosis rate as compared to healthy controls (HCs)
- Tregs from patients exhibited higher cell acidification and oxygen consumption after oligomycin injection, indicative of mitochondrial uncoupling
- We also demonstrate activation of Nox2 in patient Tregs, as a mechanism for the intracellular ROS production and mitochondrial dysfunction

6 ACKNOWLEDGEMENTS

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