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NKT cells are important mediators of hepatic ischemia-reperfusion injury

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ABSTRACT

Introduction: IRI results from the interruption then reinstatement of an organ's blood supply, and this poses a significant problem in liver transplantation and resectional surgery.

In this paper, we explore the role T cells play in the pathogenesis of this injury.

Materials & methods: We used an in vivo murine model of warm partial hepatic IRI, genetically-modified mice, in vivo antibody depletion, adoptive cell transfer and flow cytometry to determine which lymphocyte subsets contribute to pathology. Injury was assessed by measuring serum alanine aminotransfersase (ALT) and by histological examination of liver tissue sections.

Results: The absence of T cells (CD3εKO) is associated with significant protection from injury ($p = 0.010$). Through a strategy of antibody depletion it appears that NKT cells ($p = 0.0025$), rather than conventional T (CD4 + or CD8 +) ($p = 0.11$) cells that are the key mediators of injury.

Discussion: Our results indicate that tissue-resident NKT cells, but not other lymphocyte populations are responsible for the injury in hepatic IRI. Targeting the activation of NKT cells and/or their effector apparatus would be a novel approach in protecting the liver during transplantation and resection surgery; this may allow us to expand our current criteria for surgery.

1. Introduction

Liver failure is increasing dramatically in the UK population [1]. Currently, liver transplantation is the only effective treatment for patients with end-stage disease, giving an average of 17–22 years of additional life [1–3]. Ischemia-reperfusion injury (IRI) limits access to liver transplantation and is linked to early graft failure [4–6]. Marginal organs including those from older or steatotic donors are particularly susceptible to IRI [7]. The problem of marginal organs is becoming more prevalent and results from changes in donor profile and a rise of donation after cardiac death (DCD) [8]. IRI also poses a significant challenge in the contexts of major liver resection surgery [9] and hypoxic hepatitis often seen in critically ill patients [10].

IRI results from the interruption then reinstatement of an organ's blood supply. The initial ischemic injury leads to disruption of cellular integrity with the release of Danger Associated Molecular Patterns (DAMPs). DAMPs initiate a secondary (immune-mediated) response within the liver. This immune response causes further collateral damage to cells that may have otherwise survived the primary ischemic injury [11,12]. Mice deficient in both B and T cells are protected from hepatic IRI [13–15]. We have previously demonstrated that it is T cells, not B cells (or IgM), that are the key mediators of injury [15]. This fits with work from other laboratories, identifying a variety of "key" T cells populations [16–18]. It should be noted that Caldwell et al. found CD4 + lymphocytes to be protective in their model of IRI [19]; at least in our hands, we could not attribute this protection to CD4 + Foxp3 + regulatory T cells (TReg) [20].

There is a significant enrichment of non-conventional T cells within the liver [21–23], with Natural Killer T (NKT) cells accounting for up to 30% of the resident intrahepatic lymphocytes. Hepatic NKT play an important role in hepatic immunosurveillance [24] and are linked with both immunosuppressive and pro-inflammatory responses [25]. They are capable of rapidly (within 1–2 h) producing large amounts of pro-inflammatory cytokines, including interferon-gamma (IFNγ), IL-4 and TNFα [26,27].

In this paper, we explore the role T cell subsets play in the pathogenesis of hepatic IRI.

Abbreviations: DAMPs, Danger Associated Molecular Patterns; DCD, donation after cardiac death; IFN, interferon; IgM, immunoglobulin M; IL, interleukin; IRI, ischemia-reperfusion injury; MHC, major histocompatibility complex; NK, natural killer; NKT, natural killer T cell; SEM, standard error of the mean; TCR, T cell receptor; Th, T helper cell; TReg, regulatory T Cells (CD3 + CD4 + FoxP3 +); TNF, tumour necrosis factor

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2. Materials and methods

2.1. Ethical approval & animal welfare

Following local ethical approval at the University of Edinburgh, animal work was carried out according to UK Home Office regulations (Animals Scientific Procedures Act 1986) under licenses 60/4045. Mice were housed under specific pathogen-free conditions at the University of Edinburgh. All wild type (WT), knockout and transgenic mice were age-matched male mice on a C57BL/6J background. RAG1 −/− mice lack both mature B and T cells [28]. CD3εKO mice lack mature T cells [29]. > 95% of CD4 + T-cells in OT-II mice express αβ-T Cell Receptors (TCR) recognising chicken ovalbumin [30]. IL-17RA −/− mice lack the IL-17 Receptor alpha chain and are unresponsive to both IL-17 (A-F) and IL-25 [31]. General anaesthesia (GA) was induced via inhaled isoflurane and post-operatively subcutaneous opioid analgesia (buprenorphine) was administered. Animals were sacrificed under GA by terminal exsanguination by way of cardiac puncture.

2.2. Surgical model of hepatic IRI

In this model of warm partial hepatic IRI, an atraumatic clamp was applied to the vascular pedicle supplying the left lobe for 20–50 min; the liver was then allowed to reperfuse for up to 24 h. Intraoperative core body temperature was maintained at 36 °C with a homeothermic blanket system (Harvard Apparatus, Edenbridge, UK) to minimise the masking effects of hypothermia on liver IRI [32].

The extent of any liver injury was assessed in terms of the serum level of alanine aminotransfersase (ALT), a biochemical marker of liver injury [33]. ALT was measured on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., Welwyn Garden City, UK) using a commercially available kit (Alpha Laboratories Ltd., Eastleigh, UK). ALT was correlated with the histological evidence of injury seen on sections of formalin fixed tissue stained with haematoxylin and eosin (H & E).

2.3. In vivo antibody depletion

2.3.1. CD4 and CD8 depletion

We have previously used monoclonal antibodies YTS169.4.2.1 and YTS191.1.1.2 in vivo to deplete CD8 + and CD4 + cells respectively [34]. Mice were given an intraperitoneal (i.p.) injection of 50μg CD8 depleting antibody (YTS 169.4.2.1) or 100μg of CD4 depleting antibody (YTS 191.1.1.2) or isotype control (AFRC MAC 51) made up to 150 μl in PBS at days -6 and days -2 prior to surgery; antibodies were sourced commercially (Immunosolv, Edinburgh, UK). This regimen gave 99% depletion of CD8 + T cells in the spleen and liver using YTS 169.4.2.1 and 91% of CD4 + T cells using YTS 191.1.1.2 (data not shown); this is consistent with that established in the literature [34,35].

2.3.2. NK and NKT depletion

PK136 (BioXcell, West Lebanon, USA) was used to deplete NK1.1 + cells. On a C57BL/6 background this depletes both NK and NKT cells [36,37]. Mice were given an i.p. injection of either 100μg PK136 or mouse IgG2a isotype control (C1.18.4, BioXcell) 48 h pre-operatively. This gave a depletion of 91% $CD3_{int}NK1.1+$ cells (data not shown) in line with that described in the literature [36–38].

NK cells were selectively depleted by anti-asialo GM1 antibody [39]. Mice received i.p. injection of 30 μl reconstituted anti-asialo GM1 antibody (Wako, Neuss, Germany) or an equivalent volume of rabbit serum (Sigma-Aldrich) at day -1 relative to surgery. Similar to reports in the literature [38,40,41], this regimen gave a depletion of over 90% NK cells (data not shown).

2.4. Flow cytometry

Single cell preparations from the liver were generated by a combination of mechanical disruption (GentleMACS, Miltenyi Biotec, Bisley, UK) and enzymatic digestion (2 mg/ml Collagenase D, Roche). These were then passed through a 70 μm filter and centrifuged at 50*g* for 5 min to remove debris and hepatocytes. Red cells were lysed (Red Cell Lysis Buffer, Sigma-Aldrich, Poole, UK). Immune cells were isolated by positive selection using a CD45 + MicroBead AutoMACS separation (Miltenyi Biotec, Bisley, UK) and then stained with a fixable Live-Dead marker (Life Technologies, Paisley, UK) and a multi-colour panel of antibodies, including CD3, CD4, CD8, CD19 and NK1.1 (Biolegend, San Diego, USA). NKT cells were also identified using PBS-57 tetramers, an analogue of alpha-galactosylceramide developed by Dr. Paul Savage (The NIH Tetramer Facility, Emory University, Atlanta, USA) and complexed to CD1d tetramers [42].

Samples were then run on a BD LSR II Fortessa (BD Biosciences, Oxford, UK) and analysed with FlowJo software (Tree Star, Ashland, USA). T cells were defined as CD3 + CD19 − cells and NKT cells as CD3intNK1.1 + (or CD3 + Tetramer +) cells, within a forward-side scatter defined lymphocyte gate (Supplemental Fig. 1).

2.5. Adoptive transfer

Isolation of lymphocytes from spleen was performed by mechanical disaggregation through a 40 μm filter. Cells were transferred either as mixed populations (e.g. “splenic lymphocytes”), or after purification with AutoMACS CD4 + MicroBeads, using previously published cells transfer protocols [43,44].

2.6. Immunohistochemistry

Tissue from the models of murine liver injury was fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid (Sigma-Aldrich)). Sections were deparaffinised and rehydrated before endogenous peroxidase and avidin/biotin activity were quenched, prior to incubating with a rat monoclonal anti-mouse Ly6g antibody (Ab25377, Abcam, Cambridge, UK) at a dilution of 1 in 100. Slides were subsequently incubated at room temperature with polyclonal rabbit anti-rat biotinylated secondary antibody (E0468, DAKO, Ely, UK) at 1 in 400 dilution for 40 min. Sections were then developed with VectaStain RTU Elite (Vector Laboratories, Peterborough, UK) followed by diaminobenzidine (DAB125, Spring Biosciences, Pleasanton, UK), before being counterstained.

2.7. Statistical analysis

Groups were analysed with the aid of Prism 5 for Mac OSX (Graphpad Software, La Jolla, USA); specific statistical methods are referred to in the results section. All values in graphs represent mean ± standard error of the mean (SEM) unless stated otherwise.

3. Results

3.1. T cells play a central role in the secondary immune-mediated injury

RAG1 −/− mice are deficient in both mature B and T cells and were significantly protected from experimental hepatic IRI across a range of ischemic injuries (Fig. 1). There was significant protection in RAG1 −/− mice up to a point where the observed injury in RAG1 −/− and WT converged; this corresponded to complete ischemic necrosis within this model. T cell deficient (CD3εKO) mice were also significantly protected from injury (Fig. 1).

WT and RAG1 −/− mice underwent 20–50 min of warm left lobe hepatic ischemia and were reperfused for 24 h. There was significant protection in RAG1 −/− mice (which lack IgM, T and B cells) compared to WT controls (Kruskal-Wallis $p = 0.0058$, $n > 3$ per time-point). We had previously shown this was not as a result of B cells (or IgM) [15]. Mice lacking T cells (CD3εKO) or WT controls underwent