Research Article

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Rapid activation and hepatic recruitment of innate-like regulatory B cells after invariant NKT cell stimulation in mice

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Background & Aims: Invariant natural killer T (iNKT) cells are present within the liver and have been implicated in the development of many liver diseases. Upon activation by glycolipid ligands (including α-galactosylceramide; αGalCer), hepatic iNKT cells produce numerous cytokines and recruit both pro-inflammatory and regulatory immune cells. However, the involvement of B cells in this process is poorly defined.

Methods: Wild-type (male, C57BL/6), B cell deficient, or B cell depleted mice were injected with αGalCer or vehicle, hepatic B cell phenotype and liver injury was subsequently determined.

Results: iNKT cell activation resulted in liver injury and the rapid activation and hepatic recruitment of B cells (mainly innate-like B1 and MZ-like B cells) from the spleen and peritoneal cavity. B cells recruited to the liver produce IL-10 and TGFβ, and express cell surface CD73 (ectoenzyme which generates adenosine). B cell deficient mice developed augmented αGalCer-induced hepatitis, enhanced neutrophil recruitment and striking alterations in the hepatic cytokine milieu. αGalCer-induced hepatitis was unaltered in *IL-10*$^{-/-}$ mice, or after TGFβ neutralization, but was significantly worsened in mice treated with a CD73 inhibitor.

Conclusions: iNKT cell stimulation recruits innate-like regulatory B cells to the liver which suppress hepatic inflammation through IL-10 and TGFβ1 independent mechanisms, but involve CD73 activity. These findings highlight an important inflammation suppressing role for B cells at early time points during the development of an innate immune response within the liver, and represent a potential therapeutic target for the treatment of liver disease.

Keywords: α-galactosylceramide; Cytokine; CD20; IL-10; Co-stimulatory molecules; CD73.

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Abbreviations: NKT, natural killer T cell; α-GalCer, α-galactosylceramide; MZ-like B cell, marginal zone like B cell; ALT, alanine transaminase; Breg, regulatory B cell; TGF-β, transforming growth factor β.

Introduction

The immune system is classically divided into innate and adaptive arms. Natural killer T (NKT) cells are key components of the innate immune system [1]. NKT cells that express a highly restricted T cell receptor with an invariant α chain are called invariant NKT cells (iNKT) [1] which are relatively enriched within the liver [2,3]. iNKT cells play an important role in the pathogenesis of many liver diseases [2,4]. Activation of hepatic iNKT cells can be mediated through glycolipid ligands presented by antigen presenting cells in the context of CD1d [5]. Activated iNKT cells rapidly produce numerous cytokines which critically regulate downstream immune processes within the liver [1,2,5,6]. This iNKT cell-associated hepatic immune activation is mimicked by the administration of the glycolipid alpha galactosylceramide (αGalCer) [5,7]; an endogenous iNKT cell ligand [8]. iNKT cell activation also results in the activation and recruitment of numerous other innate (e.g. neutrophils, monocytes, NK cells; γδ T cells) and adaptive (e.g. T cells) immune cell types within the liver; which can augment or suppress hepatic immune responses [2,9–12]. This balance is critical for driving ongoing liver injury, or its resolution.

B cells are classically considered as adaptive immune cells [13] and are commonly observed within normal human liver. Numbers of B cells increase dramatically during most liver diseases [14]. However, their role in regulating liver immunity remains poorly understood. B cells possess both effector and regulatory properties which significantly impact immune responses [13,15–17]. In the mouse, B cells are broadly divided into three main subtypes [15]; follicular B cells (B2), which produce highly antigen-specific antibodies, B1 (B1a and B1b) and marginal zone (MZ) B cells which have been classified as "innate-like" B cells as a result of their ability to rapidly respond to immune challenges to produce low affinity antibodies and secrete cytokines [15,18]. In the adult mouse, B1 B cells reside mainly within the peritoneum, whereas MZ cells are found within the spleen [15,18]. During systemic immune challenge innate-like B cells can be mobilized from these sites [18]. However, their presence within the liver, and potential role in regulating hepatic immune responses have not been examined.

Therefore, we utilized αGalCer-induced activation of iNKT cells as a robust model of hepatic innate immune activation to

identify the presence, and mechanistically delineate the role of innate-like B cells within the liver.

Materials and methods

Animals and treatments

Wild-type 8–10 week-old male C57BL/6, B cell deficient (μMT), *IL-10*$^{-/-}$ mice (Jackson Labs, Bar Harbor, Maine) and *CD1d*$^{-/-}$ mice (in house breeding colony) were used. All procedures were approved by the University of Calgary Animal Care Committee (#M10031) and performed in accordance with the guidelines of the Canadian Council on Animal Care. To specifically activate hepatic iNKT cells *in vivo* mice received a single intraperitoneal injection of αGalCer (100 μg/kg) [19]. At specified time points post-αGalCer or vehicle treatment, blood and tissue samples were collected. B cell depletion was accomplished by a single intraperitoneal injection of anti-mouse CD20 antibodies (Clone 5D2, generously provided by Genentech, Inc., San Francisco, CA) at a dose of 10 mg/kg (dose chosen based on recommendations from Genentech technical support and pilot studies performed in our laboratory) (Fig. 7G, H). Controls received isotype matched IgG.

Statistical analyses

All data are shown as mean ± standard error of the mean (SEM). For comparisons between two groups, an unpaired Student's *t* test was used. For comparisons between more than two groups an analysis of variance followed by the Student-Newman-Keuls post-hoc test was performed. When data were not normally distributed, the Mann-Whitney test was used for comparisons between two groups, and the Kruskal–Wallis test followed by Dunn's post-hoc test for comparisons between more than two groups (Graph-Pad V5, San Diego, CA). Additional experimental procedures and reagent descriptions are provided in the Supplementary Materials and methods section.

Results

Rapid activation and recruitment of B cells in the liver after iNKT cell stimulation

αGalCer administration rapidly activated hepatic B cells as reflected by an increased frequency of CD69$^+$ B cells within the liver as early as 2 h after αGalCer treatment (Fig. 1A, B). In addition, αGalCer treatment rapidly recruited B cells into the liver, with numbers of B cells significantly increasing at 16 h post-αGalCer treatment, and increasing further at 48 h post-αGalCer treatment (Fig. 1C). At 16 h post-αGalCer treatment hepatic B cells were also activated (i.e. CD69$^+$) (Fig. 1A, B). B cell activation and recruitment were prevented in iNKT cell deficient mice treated with αGalCer (Fig. 1D, E). B cells recruited into the liver post-αGalCer treatment were identified by immunohistochemistry within portal space immune cell infiltrates and hepatic sinusoids (Supplementary Fig. 3).

Innate-like B1 and MZ-like B cells are the major B cell subtypes recruited into the liver after αGalCer treatment

Major B cell subsets recruited into the liver after iNKT cell activation were phenotyped using flow cytometry. B1 B cell subsets (B1a and B1b) were identified based on cell surface expression of B220 and CD5 [20–22]. MZ and follicle (FO) B cell subsets were characterized based on differential CD21 and CD23 expression [23,24] (flow cytometry gating strategies shown in Supplementary Fig. 5). Three distinct B cell subsets were identified as being preferentially recruited into the liver post-iNKT cell activation; namely B1a B cells

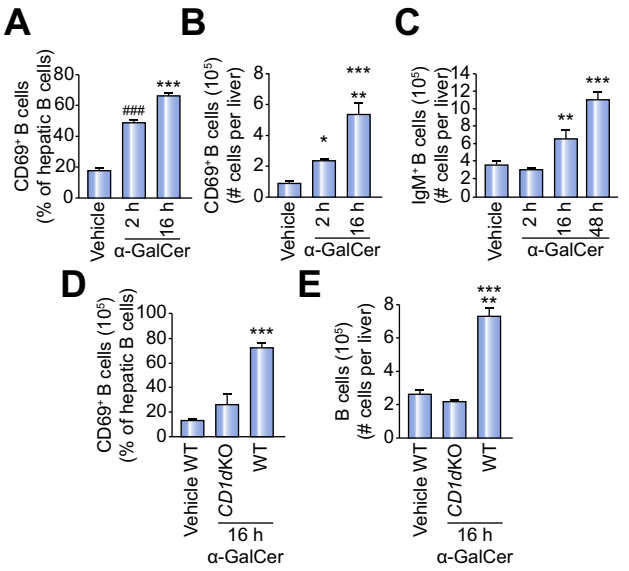

Fig. 1. Hepatic iNKT cell activation causes the early recruitment and activation of B cells in the liver. αGalCer treatment activates hepatic B cells (i.e. CD69$^+$) at 2 and 16 h post-αGalCer treatment (A, B). For (A) $^{***}p \leqslant 0.001$ 48 h *vs.* all other groups; $^{###}p \leqslant 0.001$ 16 h *vs.* vehicle group, and for (B) $^{***}p \leqslant 0.001$ 48 h *vs.* vehicle group. $^{**}p \leqslant 0.01$ 48 h *vs.* 16 h group; $^{*}p \leqslant 0.05$ 16 h *vs.* vehicle group (n = 4–7 mice/group). (C) Total hepatic B cell numbers/liver are increased in αGalCer–treated mice. $^{**}p \leqslant 0.01$ 16 h *vs.* vehicle and 2 h groups; $^{***}p \leqslant 0.001$ 48 h *vs.* all other groups (n = 5–8 mice/group). (D, E) αGalCer-induced B cell activation (CD69$^+$) and hepatic recruitment are prevented in iNKT cell deficient (*CD1d* KO) mice. $^{***}p \leqslant 0.001$ and $^{**}p \leqslant 0.01$ *vs.* other groups (n = 3–4 mice/group).

(IgM$^+$B220$^{neg\text{-}low}$CD5$^+$), B1b B cells (IgM$^+$ B220$^{neg\text{-}low}$CD5$^-$), and MZ-like B cells (IgM$^+$ B220$^+$CD23$^{neg\text{-}low}$CD21$^+$). Numbers of hepatic B1a, B1b, and MZ-like B cells were significantly increased post-αGalCer treatment (Fig. 2A–C). However, αGalCer treatment did not alter the hepatic FO B cell sub population (identified as IgM$^+$ B220$^+$ CD23^{Hi} CD21$^+$; Fig. 2D).

The spleen is the major source of MZ-like B cells, and the peritoneal cavity is the major source of B1 B cells, recruited into the liver after αGalCer treatment

Splenectomy prevented the recruitment of MZ-like B cells, and partially attenuated B1a B cell recruitment, but did not alter B1b cell recruitment into the liver post-iNKT cell activation (Fig. 3A–C). B1 cells are found mainly within the peritoneum [25,26], and both B1a and B1b B cells were readily identified within the peritoneal cavity (Fig. 3D, E). Moreover, αGalCer treatment significantly reduced peritoneal B1b, but not B1a B cell numbers (Fig. 3D, E); consistent with B1b cells being recruited from the peritoneal cavity into the liver post-iNKT cell activation.

B cells recruited to the liver express a diverse array of chemokine receptors

The chemokine receptors most relevant to hepatic B cell recruitment during tissue inflammation are not completely understood. αGalCer treatment resulted in significant hepatic recruitment of B cells expressing an array of chemokine receptors, including those suggested as being important for B cell transendothelial migration within the human liver [14] (Fig. 4). These findings suggest that chemokine regulation of B cell migration into the