Journal of Autoimmunity 50 (2014) 123–134

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Murine autoimmune cholangitis requires two hits: Cytotoxic KLRG1$^+$ CD8 effector cells and defective T regulatory cells

Wenting Huang [a], Kritika Kachapati [a], David Adams [a], Yuehong Wu [a], Patrick S.C. Leung [b], Guo-Xiang Yang [b], Weici Zhang [b], Aftab A. Ansari [d], Richard A. Flavell [c], M. Eric Gershwin [b], William M. Ridgway [a,*]

[a] Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine, Cincinnati, OH 45174, USA
[b] Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis, Davis, CA 95616, USA
[c] Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA
[d] Department of Pathology, Emory University School of Medicine, Atlanta, GA 30322, USA

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form
23 January 2014
Accepted 24 January 2014

Keywords:
KLRG1$^+$ CD8 cells
Primary biliary cirrhosis
T regulatory cells

ABSTRACT

Primary biliary cirrhosis (PBC) is an enigmatic disease mediated by autoimmune destruction of cholangiocytes in hepatic bile ducts. The early immunological events leading to PBC are poorly understood; clinical signs of disease occur very late in the pathological process. We have used our unique murine model of PBC in dominant-negative TGF-β receptor type II transgenic mice to delineate critical early immunopathological pathways, and previously showed that dnTGFβRII CD8 T cells transfer biliary disease. Herein we report significantly increased numbers of hepatic dnTGFβRII terminally differentiated (KLRG1$^+$) CD8 T cells, a CD8 subset previously shown to be enriched in antigen specific cells during hepatic immune response to viral infections. We performed bone marrow chimera studies to assess whether dnTGFβRII CD8 mediated disease was cell intrinsic or extrinsic. Unexpectedly, mixed (dnTGFβRII and B6) bone marrow chimeric (BMC) mice were protected from biliary disease compared to dnTGFβRII single bone marrow chimerics. To define the protective B6 cell subset, we performed adoptive transfer studies, which showed that co-transfer of B6 Tregs prevented dnTGFβRII CD8 T cell mediated cholangitis. Treg mediated disease protection was associated with significantly decreased numbers of hepatic KLRG1$^+$ CD8 T cells. In contrast, co-transfer of dnTGFβRII Tregs offered no protection, and dnTGFβRII Treg cells were functionally defective in suppressing effector CD8 T cells *in vitro* compared to wild type B6 Tregs. *In vitro* cholangiocyte cytotoxicity assays demonstrated significantly increased numbers of cytotoxic hepatic dnTGFβRII KLRG1$^+$ CD8 cells compared to B6. Protection from disease by B6 Tregs was associated with elimination of hepatic dnTGFβRII CD8 mediated cholangiocyte cytotoxicity. These results emphasize that autoimmune cholangitis requires defects in both the T effector and regulatory compartments, and that an intrinsic T cell effector defect is not sufficient to mediate autoimmune biliary disease in the setting of intact immune regulation. These results have important implications for understanding the early pathogenesis of human PBC.

1. Introduction

Primary biliary cirrhosis is an organ specific autoimmune disease in which biliary ductules are the target of autoimmune mediated destruction, resulting ultimately in cirrhosis and liver failure [1,2]. PBC is associated with a high prevalence of autoantibodies to mitochondrial antigens, most specifically pyruvate dehydrogenase PDC-E2 [3,4]. In humans, PBC pathogenesis is shrouded in mystery, since most patients develop clinical symptoms long after the initiation of the disease process. This "gap" between onset and clinical detection has frustrated efforts to

Abbreviations: dnTGFβRII, dominant negative transforming growth factor β receptor type II; PBC, primary biliary cirrhosis; BMC, bone marrow chimera; Treg, T regulatory cell; PDC-E2, pyruvate dehydrogenase E2 complex; CMT, central memory T cell; ET/EMT, effector T cell and effector memory T cell; WT, wild type; MFI, mean fluorescence intensity; IFNγ, interferon gamma; IHC, intrahepatic cell.

* Corresponding author. HPB room 356, 231 Albert Sabin Drive, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0563, USA. Tel.: +1 513 558 4701, +1 513 558 5551; fax: +1 513 558 3799.
E-mail addresses: ridgwawm@ucmail.uc.edu, wridg1@gmail.com (W. M. Ridgway).

http://dx.doi.org/10.1016/j.jaut.2014.01.034
0896-8411/

understand the early events leading to disease. The dnTGFβRII mouse was originally developed by one of us (R.A.F.) using a dominant negative type II TGFβR transgene expressed on the CD4 promoter [5] leading to transgene expression in both CD4 and CD8 cells. These cells are characterized by a significant increase in the effector and effector memory activated T cell subset ($CD44^{high}CD62L^{low}$), showing that loss of TGFβ receptor signaling induces abnormal activation and disruptions in the balance of normal T cell subsets and their absolute numbers [6]. Notably, not all TGFβ receptor signaling is lost, which allows mice to survive for almost normal lifespans (compared to complete TGFβRII knockout in both CD4 and CD8 cells, wherein mice only survive a few weeks) [7].

The dnTGFβRII mice exhibit autoimmune cholangitis; ~100% of mice develop the PDC-E2 autoantibodies seen in human PBC, and show histological destruction and damage of biliary ductules [6]. In addition, dnTGFβRII mice develop autoantibodies to gp210 and sp100, which are also considered highly specific to PBC [8]. These features establish the dnTGFβRII mice as a model of PBC. An important finding was our demonstration that adoptive transfer of minimacs purified dnTGFβRII CD4 cells led to inflammatory bowel disease but not liver pathology, while transfer of CD8 cells led to biliary disease but not inflammatory bowel disease [9]. This focused our attention on the pathological role of CD8 cells. It is known that the liver is an immune organ with high numbers of resident CD8 cells, in particular it has been suggested that the liver acts as a "graveyard" for senescent CD8 cells [10,11]. This hepatic lymphoid function suggested that non-specific effects of derangement in CD8 T cell processing in the liver could be one mechanism of biliary immune mediated disease. An alternative explanation, favored by the development of PDC-E2 specific autoantibodies in these mice, is that antigen specific T cells are actively involved in the biliary immune process.

To distinguish between these possibilities, we recently constructed dnTGFβRII mice with T cell repertoires confined to a specific (foreign) antigen (Ova) [12]. Unexpectedly, when the CD8 repertoire was confined to a non-hepatic antigen, biliary disease was abolished. In addition, these T cells could not transfer biliary disease, even though they still showed the characteristic T cell activation abnormalities (greatly increased $CD44^{high}$ and $CD62L^{low}$) [12]. Thus an increased population of abnormally activated CD8 cells alone is insufficient for biliary disease: an antigen specific population of T cell is likely involved in the pathogenesis.

These results left unresolved whether the biliary antigen specific, activated CD8 T cells mediated disease in an intrinsic manner (i.e. the T cells were abnormally affected by the decreased TGFβR signaling such that no external immune influences could decrease their pathogenicity) or an extrinsic manner (i.e. in addition to defects in T effector function, immune regulatory defects contributed to the disease). This distinction is important because if the disease is solely a result of an intrinsic defect of CD8 T cells, therapeutic strategies directed to enhancing immune regulation might not work. This paper studies the role of intrinsic or extrinsic factors in the pathogenesis of primary biliary cirrhosis by using BMC and T cell transfer studies with analysis of the phenotype and function of CD8 T cell and T regulatory cell subsets.

2. Materials and methods

2.1. Mice

B6 (CD45.2) mice, B6.$Rag1^{-/-}$ mice, B6.Cg-*Foxp3*^{tm2Tch}/J (hereafter, "B6.$Foxp3^{EGFP}$") mice and B6.SJL-*Ptprc*^{a} mice (hereafter referred to as B6.CD45.1) were purchased from The Jackson Laboratory. dnTGFβRII mice [6] were maintained as described previously [13]. Mice were maintained under specific pathogen-free conditions and handled in accordance with the institutional animal care guidelines of the University of Cincinnati School of Medicine.

2.2. Bone marrow chimera construction

Groups of (B6 CD45.1 × CD45.2) F1 recipient mice were irradiated with 1100–1200 rad. B6.CD45.1 and dnTGFβRII (CD45.2) mice were bone marrow donors. Mature $CD4^+$, $CD8^+$ and $CD90^+$ cells were depleted from the bone marrow cells by miniMACS (Miltenyi biotec). Mixed bone marrow chimera (mBMC) were derived by injection of a 1:1 mixture of dnTGFβRII (CD45.2) and B6.CD45.1 donor bone marrow. Single BMC chimeras received marrow cells from either dnTGFβRII (CD45.2) or B6 (CD45.2) alone. Recipient mice were given water treated with antibiotic (neomycin trisulfate salt hydrate) for 2 weeks after transfer. Recipients were harvested 120 days after bone marrow transplantation (or at the time they became ill).

2.3. Histopathology

Livers were isolated and fixed in 10% formalin, then paraffin-embedded. Samples were stained with hematoxylin and eosin, and scored blindly using microscopy. Scores were based on the severity of portal inflammation. Score 0: 0~5% of portal ducts infiltrated; score 1: 5~25%; score 3: 50~75%; and score 4: 75~100% of the liver section shows the portal duct area infiltrated by leukocytes.

2.4. CD8 and Treg co-transfer study

For transfer studies, B6.$Foxp3^{EGFP}$, B6 and dnTGFβRII mice served as donors, and B6.$Rag1^{-/-}$ mice served as recipients. 1×10^6 miniMACS enrichedB6 or dnTGFβRII splenic $CD8^+$ cells were transferred to recipients, and in some experiments 0.5×10^6 FACS-sorted splenic $CD4^+GFP^+$ cells (from B6.$Foxp3^{EGFP}$ mice) or 0.5×10^6 FACS-sorted dnTGFβRII splenic $CD4^+CD25^+$ cells were transferred into Treg co-recipients.

2.5. Flow cytometry

Flow cytometric analysis of intrahepatic cells (IHC) was performed on cells obtained by perfusion of liver with 5 mL of EGTA injected through the portal vein followed by 5 mL of Collagenase IV (Sigma–Aldrich) for 15 min. For absolute cell counts, splenocytes and IHC were counted using a hemocytometer. For surface molecule staining of conventional T cells, cells were incubated with 2.4G2 Fc block for 10 min at 4 °C followed by the indicated antibodies (from BD Biosciences, BioLegend or eBioscience). FACS was performed on LSRII or LSR-Fortessa (BD) and analyzed using FlowJo (Tree Star, version 7.6.5).

2.6. Treg suppression assay

A total of 100,000 miniMACS enriched splenic $CD8^+$ cells or FACS-sorted splenic $CD4^+CD25^-$ cells from either B6 or dnTGFβRII mice were cultured with 20,000 anti-CD3/CD28-coated beads (Invitrogen) in the presence of 50,000 FACS-sorted splenic $CD4^+CD25^+$ cell from B6 or dnTGFβRII in a criss-cross manner, along with positive and negative controls. Triplicate wells were used for each condition. The cells were cultured at 37 °C in 5% CO_2 and pulsed with 1 uCi [^{3}H] thymidine on day 3 for 16 h, then harvested and counted using a β-scintillation counter.