Published in final edited form as:
J Autoimmun. 2014 May ; 50: 123–134. doi:10.1016/j.jaut.2014.01.034.

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

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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 dnTGFbRII 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 dnTGFbRII 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 dnTGFbRII 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*

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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.

Keywords

KLRG1^{+} CD8 cells; Primary biliary cirrhosis; T regulatory cells

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 autoanti-bodies 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 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 TGFb receptor signaling induces abnormal activation and disruptions in the balance of normal T cell subsets and their absolute numbers [6]. Notably, not all TGFb 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 1100e—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.

2.7. CD8 T cell cytotoxicity assay

CD8 T cell cytotoxicity against cholangiocytes was performed using CytoTox 96® NonRadioactive Cytotoxicity Assay Protocol (Promega) measuring lactate dehydrogenase (LDH) release of the target cells in the culture as described [14]. In brief, 100,000 mini-MACS sorted CD8$^+$ intrahepatic cells were co-cultured with a total of 10,000 murine cholangiocyte cell line [15] in T cell medium with 5% FBS. dnTGFβRII hepatic KLRG1$^+$ or naive CD8 T cells were FACS-sorted and cultured with cholangiocytes at 10:1 ratio. After 5 h incubation at 37 °C and 5% CO_2, culture supernatant was transfer to a 96 plate bottom well plate and the released LDH in the supernatant was measured with a 30-min coupled enzymatic assay; absorbance was recorded at 490 nm using a VICTOR X4 2030 Multilabel Reader (PerkinElmer). LDH release was measured in experimental, media alone, “volume correction” control, target cell maximum, target cell spontaneous and effector cell

spontaneous wells, as described by the manufacturer. The percent cytotoxicity was calculated as: % Cytotoxicity = [(Experimental Effector Spontaneous Target Spontaneous)/ (Target Maximum Target — Spontaneous)] × 100 as described by the manufacturer. NB: the above calculation makes a negative percent cytotoxicity value possible when the experimental LDH release is less than the control LDH release.

2.8. Statistics

All statistical analysis was performed using either the unpaired t test or the Manne—Whitney *U* test in GraphPad Prism 5 (version 5.01; GraphPad).

3. Results

3.1. Mice reconstituted with both dnTGFβRII and B6 bone marrow show significant protection from cholangitis compared to dnTGFβRII BMC

We addressed the issue of intrinsic versus extrinsic CD8 mediated biliary disease by constructing mixed bone marrow (mBMC) mice from allotypically variant strains. B6 CD45.1 bone marrow was mixed in equal proportions with dnTGFβRII CD45.2 bone marrow; single BMC controls included B6 (CD45.2) donors and dnTGFβRII (CD45.2) donors (Fig 1a). While recipients of dnTGFβRII donor cells developed significant disease, as expected (comparable to nonchimeric dnTGFβRII mice), mBMC recipients were protected from biliary disease, with histological scores no different from B6 recipients (Fig 1b, c). Protection from disease in the mBMC recipients was not due to differences in reconstitution efficiency since the absolute number of splenocytes was the same between single and mixed chimeric mice (Fig 1d). The B6 origin cells also did not outgrow cells of the dnTGFβRII origin, since the absolute number of spleen and liver lymphoid cells expressing CD45.1 vs. CD45.2 allotypes in the F1 recipients were comparable (Fig 1e).

3.2. The dnTGFβRII and mBMC intrahepatic cell populations show expansion of dnTGFβRII origin effector memory and terminally differentiated (KLRG1^{+} CD127^{-}) CD8 cells

To understand disease protection in the mBMC, we characterized the hepatic CD8 Tcell subsets in dnTGFβRII mice and in the BMCs. We have previously shown that dnTGFβRII CD8 cells showed an increase in hepatic CD44^{high}CD62L^{low} (ET/EMT) T cells [9]. We extended this analysis to assess whether CD44^{high}CD62L^{low} CD8 cells were further differentiated into terminally differentiated cells (KLRG1^{+} CD127^{-}, associated with antigen specific CD8 cells in the setting of persistent antigen) or effector memory (KLRG1^{-} CD127^{+}, associated in hepatic viral models with resolution of infection) [16,17]. The dnTGFβRII-origin intrahepatic cells (IHCs) have a massive increase in terminally differentiated KLRG1^{+} CD127^{-} CD44^{high}CD62L^{low} CD8 cells compared to hepatic B6 CD8^{+} T cells (Fig 2a). We used the same approach to analyze CD8 hepatic Tcells in the bone marrow chimeric mice (representative FACS gating shown in Fig 2b). As expected, the recipients of only B6 or dnTGFβRII bone marrow showed normal and increased numbers of hepatic CD8 cells, respectively (Fig 3a). In the mixed BMC recipients, however, the total number of dnTGFβRII origin CD8 T cells was significantly greater than the B6 origin CD8 T cells; these findings are comparable to the numbers found in the dnTGFβRII single BMC (Fig 3a). There were increased numbers of naïve B6-origin CD8 T cells compared to

dnTGFβRII-origin in the mBMC (Fig 3b). In contrast, in the CD8 effector/memory, central/memory, and terminally differentiated subsets the number (Fig. 3c—f) and percent (not shown) of dnTGFβRII-origin hepatic cells was also significantly greater than B6-origin cells and comparable to single dnTGFβRII BMC and non-chimeric dnTGFβRII mice. Although the number of dnTGFβRII-origin cells in effector/memory and terminally differentiated CD8 subsets were significantly increased compared to the number of B6-origin cells in the mBMC mice (Fig. 3c—f), these mice did not have significant biliary disease (Fig 1a, b). This raised the possibility that B6 regulatory cells controlled the dnTGFβRII effector CD8 cells in the mBMC.

3.3. DnTGFβRII Tregs are defective in suppressing CD8 T effector cells in vitro

We began to address the role of Tregs in cholangitis by quantitating dnTGFβRII vs. B6 Treg numbers and function. dnTGFβRII mice actually had significantly higher numbers of hepatic $CD4^+Foxp3^+$ Tregs but no difference in the number of splenic Tregs (Fig 4a). Foxp3 MFI in both hepatic and splenic Tregs of dnTGFβRII mice was not significantly different from B6 mice (Fig 4b). There was no difference in the percent $Foxp3^+$ cells in the $CD4^+CD25^+$ subset in these strains, (~95% of $CD4^+CD25^+$ cells were $Foxp3^+$, Fig 4c), so we sorted $CD4^+CD25^+$ Tregs from both dnTGFβRII and B6 mice for an in *vitro* suppression assay against both B6 and dnTGFβRII splenic $CD4^+$ ($CD25^-$) and CD8 T effector cells (Fig 4d, left and right panels respectively). The dnTGFβRII $CD4^+CD25^-$ and CD8 T cells had significantly increased proliferation compared to B6 at the same level of CD3/CD28 stimulation (Fig 4, legend). When tested against B6 CD4 effectors, B6 Tregs were significantly more suppressive than dnTGFβRII Tregs ($P = 0.01$) although there was no difference between the Tregs in suppression of dnTGFβRII CD4 effectors (Fig 4d, left). However, the B6 Tregs were significantly more suppressive than dnTGFβRII Tregs against both B6 CD8 ($P = 0.04$) and dnTGFβRII CD8 T effectors ($P = 0.01$, Fig 4d, right). The reduced suppression by dnTGFβRII $CD4^+CD25^+$ Tregs cannot be attributed to a reduced number of $Foxp3^+$ Tregs in the $CD4^+CD25^+$ subset (Fig 4a). Thus the dnTGFβRII Tregs showed a defect in the ability to suppress CD8 (and to a lesser degree, CD4) T effector cells compared to the B6 Tregs. We next directly tested the efficacy of dnTGFβRII vs. B6 Treg function in vivo by adoptive transfer studies.

3.4. B6, but not dnTGFβRII, T regulatory cells can prevent adoptive transfer of cholangitis by dnTGFβRII CD8 cells

We co-transferred dnTGFβRII CD8 cells along with B6 or dnTGFβRII Tregs into B6 $Rag1^{-/-}$ recipients (Fig 5a). As shown in Fig 5b and c, B6 Tregs significantly suppressed the transferred disease, which was indistinguishable from transfer of B6 CD8 T cells alone. In striking contrast, co-transfer of dnTGFβRII $CD4^+CD25^+$ Tregs had no effect on the disease mediated by dnTGFβRII CD8 cells (Fig 5b, c). The B6 Treg recipients had no decrease in percentage of lymphoid cells in the liver or spleen compared to the dnTGFβRII recipients (data not shown). However, total numbers of both lymphoid cells and hepatic CD8 cells were greater in the dnTGFβRII CD8 and dnTGFβRII CD8 plus dnTGFβRII Treg recipients compared to the dnTGFβRII CD8 plus B6 Treg recipients, suggesting that the B6 Tregs decreased the proliferation or accumulation of dnTGFβRII CD8 cells (Fig 5d, e). The

percentage of CD8 T cells did not differ between the dnTGFβRII and B6 Treg recipients (data not shown).

3.5. Transfer recipients of dnTGFβRII CD8 plus B6 Tregs have decreased numbers of KLRG1$^+$CD8 cells compared to dnTGFbRII CD8 recipients

We analyzed the differentiation status of the dnTGFβRII CD8 cells in the transfer recipients; a representative FACS analysis figure is shown (Fig 6a). Mice receiving dnTGFβRII CD8 plus B6 Tregs had decreased numbers of ET/EMT CD8 cells (Fig 6c), KLRG1$^+$ CD127$^-$ CD8 cells (Fig 6d) and KLRG1$^+$ CD127$^+$ CD8 T cells (Fig 6e), and conversely increased numbers of hepatic CD8 CMT cells (Fig 6b), compared to dnTGFβRII CD8 or dnTGFβRII CD8 plus dnTGFβRII Treg recipients. These results strongly suggest that the protective effect of the B6 Tregs was associated with a decrease in the overall expansion of the dnTGFβRII CD8 T cells (resulting in significantly decreased absolute numbers of CD8 T cells, Fig 5d), an increase in CMT CD8 cell numbers, and decreased absolute numbers of CD8 differentiated effector subsets including KLRG1$^+$ CD8 cells.

3.6. Mechanism of B6 Treg protective effect: dnTGFβRII KLRG1$^+$CD8 T cells mediate cholangiocyte cytotoxicity; B6 Tregs eliminate dnTGFβRII CD8 cytotoxicity

Finally, we sought a mechanism for the B6 Treg protective effect. We tested dnTGFβRII vs. B6 intrahepatic CD8 cell mediated cytotoxicity against a cholangiocyte cell line [15]. The dnTGFβRII intrahepatic CD8 cells showed a titratable increase in cholangiocyte cytotoxicity compared to B6 CD8 (Fig 7a). Cholangiocyte cytotoxicity was largely mediated by the hepatic KLRG1$^+$ CD8 subset (Fig 7b; dnTGFβRII CD8 cell cytotoxicity: 43.9% ± 21.89%; dnTGFβRIIKLRG1$^+$ cytotoxicity: 47.0% ± 10.0%). When we isolated hepatic dnTGFβRII CD8 cells from recipients of dnTGFβRII CD8 cells plus B6 Tregs (Fig 5), however, CD8 cytotoxicity was completely eliminated (Fig 7c), and this correlated with the lack of disease in these recipients. These findings suggest that protective, wild type Tregs can correct any “intrinsic” dnTGFβRII CD8 cellular defects and act by eliminating the capacity of the KLRG1$^+$ CD8 cells to destroy cholangiocytes.

4. Discussion

These results are significant in several respects. We demonstrate here that the intrinsic molecular signaling defect of dnTGFβRII CD8 cells is insufficient to mediate disease in the presence of normal B6 cells. More specifically, B6 Tregs abrogated the pathogenicity of dnTGFβRII CD8 cells. Therefore while dnTGFβRII CD8 cells are necessary for the biliary disease, in the presence of normal (B6) Tregs no disease occurs, which indicates that dnTGFβRII Tregs are not sufficient for protection. dnTGFβRII and B6 mice have equal numbers of splenic Tregs, but the dnTGFβRII Tregs show functional defects. Intriguingly, the dnTGFβRII Tregs appear particularly defective in regulating CD8 cells. This might explain why CD8 T cells can mediate biliary disease in dnTGFβRII mice. B6 Tregs act, at least in part, by eliminating the cytotoxic capacity of KLRG1$^+$ CD8 cells, thus preventing autoimmune cholangiocyte damage. Therefore defects in both the effector and regulatory T cell subsets are necessary for the disease to progress.

How do our findings of abnormal hepatic CD8 effector and KLRG1$^+$ CD8 cells, and defective Tregs in the dnTGFβRII PBC model, relate to what is known about human PBC? It has been shown that PDC-E2 reactive, cytotoxic CD8 cells are increased in peripheral blood of PBC patients [18]. Antigen specific CD8 cytotoxic cells are also increased tenifold in PBC portal tracts [19]. Moreover, Tsuda et al. subsequently showed increased numbers of CD8$^+$ effector memory cells in PBC peripheral blood; these cells expressed increased amounts of perforin and granyzme, suggesting that they were a cytotoxic subset [20]. Our results extend these findings and suggest that human PBC hepatic CD8 populations should be studied to verify the presence of KLRG1$^+$ cytotoxic CTLs. In terms of Tregs, abnormalities have also been found in human PBC. Lan et al. found abnormalities of PBC Tregs in both peripheral blood and liver of PBC patients [21]. While they did not detect a functional defect, the ratio of CD8:Foxp3$^+$ cells was significantly higher in PBC portal tracts [21]. Bernuzzi et al. recently extended these findings by showing functional defects in novel CD8 regulatory cells [22]. Thus our findings of defective CD8 and Treg lineages have support in the human PBC literature and suggest pathways for further investigation of the human immunology.

B6 Tregs did not entirely eliminate the effects of the intrinsic signaling defects of dnTGFβRII CD8 cells. For example, B6 Tregs did not completely eliminate the activated KLRG1$^+$ CD127$^-$ EM/EMT CD8 T cells in the liver. Rather, they decreased the number of KLRG1$^+$ cells, which predominantly mediate cholangiocyte cytotoxicity. CD4$^+$CD25$^+$Foxp3$^+$ Tregs can limit CD8 T cell expansion and differentiation through modulating IL-2 homeostasis [23]. In a study investigating how human Tregs control different CD8 T cell subsets, Nikolova et al. found that Tregs can inhibit CD8 memory cell differentiation to cytokine-producing effector cells and can reduce apoptosis of CD8 memory cells compared to effector cells, partially through decreased PD-1 expression on CD8 T cells after activation [24]. A study of repopulation of CD8 T subsets under lymphopenic conditions showed CD4$^+$CD25$^+$ Tregs have minimal suppressive effect on CMT, mainly acting on naïve and ET/EMT compartments [25]. Moreover, we demonstrate that B6 Tregs were capable of completely eliminating dnTGFβRII KLRG1$^+$ CD8 mediated cholangiocyte toxicity. This raises the question of how the B6 Tregs mediate suppression compared to the dnTGFβRII Tregs. Our assay measured suppression mediated by cell:cell contact and this suggests the dnTGFβRII Tregs could have a defect in cell contact mediated suppression. Tregs constitutively express glucocorticoid-induced TNFR family-related receptor (GITR). Lan et al. showed that Tregs in human PBC have increased expression of GITR [21]. Shimuzu et al. showed that stimulation of Tregs via GITR could affect self-tolerance, so high levels of GITR could contribute to autoimmunity [26]. In addition, it has been shown that a combination of increased GITR on T cells with increased GITR ligand on APCs can cause increased resistance of CD8 cells to Treg mediated suppression [27]. The GITR—GITR ligand axis could thus potentially integrate the findings of defects in both the CD8 and Treg lineages, and represents a logical future pathway to explore to explain how the defects in cell mediated suppression seen in dnTGFβRII mice. In summary B6 Tregs in our model may act by preventing the accumulation or differentiation of effector and terminally differentiated CD8 T cells, thus preventing cholangiocyte damage mediated by

these CD8 subsets. The selective defect of dnTGFβRII Treg suppression with respect to CD8 T cells is of interest, and requires further exploration in future studies.

Finally, we show that dnTGFβRII mice, and B6.Rag1$^{-/-}$ or BMC recipients receiving dnTGFβRII cells, spontaneously accumulate enhanced numbers of hepatic KLRG1^{+} CD8 T cells. We show that dnTGFβRII KLRG1^{+} CD8 cells are cytotoxic to cholangiocytes. B6 Tregs decrease the number of KLRG1^{+} cells in transfer studies, and also prevent disease and cholangiocyte cytotoxicity. It was previously shown in the dnTGFβRII model that after stimulation with cognate antigen or via an infectious agent, splenic antigen specific KLRG1^{+} cells increase—however it was not previously shown that KLRG1^{+} cells could accumulate spontaneously, nor have hepatic KLRG1^{+} populations been previously studied [28]. KLRG1^{+} CD8 cells have been studied in models of viral immunity. Multiple studies have shown that the KLRG1^{+} CD8 subset is enriched in antigen specific cells directed towards the infectious agent [16,17,29]. Bengsh et al. showed that KLRG1^{+} cells are increased in the presence of an ongoing immune response to persistent hepatitis B virus [16]. Cush et al. showed that ~75% of cells specific for murine g-herpesviru are KLRG1^{+} cells that express IL-7 and IL-15 receptors [17]. IL-15 (in addition to IL-2) is critical for the survival of KLRG1^{+} CD8 cells, and thus modulation of IL-2, IL-15 or their receptors could affect the accumulation of these cells [30,31]. The accumulation of KLRG1^{+} cells is also driven by IL-12; mice lacking IL-12 signaling fail to develop KLRG1^{+} CD8 cells [32]. IL-12 acts to create a gradient of T-bet expression; high T-bet drives KLRG1^{+} survival [33]. So, decreased KLRG1^{+} cell numbers could reflect the actions of the Tregs to reduce the IL-12 driven autoimmune process which is critical to the dnTGFβRII model [34,35]. Excess IFNγ can also drive the expression of frankly autoreactive KLRG1^{+} CD8 T cells, as shown by the increased numbers of these cells in mice expressing the sanroquin mutation of Roquin [36]. Finally, recent studies in tumor immunology have shown that KLRG1^{+} CD8 cells are cytotoxic to tumor tissue [37—39], supporting the hypothesis that KLRG1^{+} CD8 cells may mediate hepatic autoimmunity by causing cholangiocyte cell death. This literature suggests that the increased numbers of KLRG1^{+} CD8 cells in single dnTGFβRII bone marrow chimeric mice, should be accompanied by cytokine abnormalities such as increased IL-12 and IFN-γ, which are corrected in the presence of B6 bone marrow. Furthermore, cholangiocyte apoptosis, and resultant biliary apotopes, play a major role in PBC pathogenesis [40,41]. Therefore our finding of increased cytotoxic KLRG1^{+} cells suggests we should find enhanced cholangiocyte apoptosis selectively in single dnTGFβRII mice, again corrected in the mixed chimerics. Cytokine and apoptosis abnormalities will thus be a future focus of investigation in these chimeric mice. In summary, spontaneous accumulation of large numbers of cytotoxic KLRG1^{+} CD8 cells in the dnTGFβRII model implies that they accumulate after responding to their biliary autoantigen, consistent with our recent, surprising observation that the dnTGFβRII CD8 cells must have an autoantigenic specificity to mediate disease [12]. The studies shown here and published recently strongly suggest that the dnTGFβRII defect encourages the expansion of antigen specific autoreactive T cells. This might be due to effects on either central or peripheral T cell tolerance. Since we do not yet know the identity of the relevant biliary autoantigen(s), we cannot yet directly prove this point. However, future studies will focus on the TCR repertoire in the KLRG1^{+} CD8 subset and attempt to show if this subset selectively transfer autoimmune biliary disease.

5. Conclusion

The dnTGFβRII model does not behave like a classic knockout model with complete loss of function of the selected gene. The effects of complete loss of dnTGFβRII signaling in CD4 and CD8 cells are drastically different than in our dnTGFβRII model, mediating death in 3—4 weeks [7]. The dnTGFβRII mouse develops a chronic disease, requiring multiple arms of the immune system, consistent with the idea that a complex disease such as PBC must involve multiple defects in the immune system. Therefore the dnTGFβRII model may actually closely mimic the pathogenic events in human PBC that impact both regulatory and effector arms of the immune system with the final effect of biliary autoimmunity. This has important implications for human PBC. First, it suggests that therapeutic approaches might be able to either decrease effector immunity (immunosuppression) or enhance regulatory immunity. Second, it raises the possibility that PBC might be a “two hit” diseaseerequiring defects in both effector and regulatory immunity [42]. Requiring at least “two hits” might explain why the disease is somewhat rare. In addition, it might explain the slow progression of disease over a long silent period: initial defects in one arm of the immune system may be insufficient to drive aggressive clinical disease. These findings raise new hypotheses to be tested in understanding and treating human PBC.

Acknowledgments

Supported by NIH grant R01 DK090019, VA grant BX000827-01A1.

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

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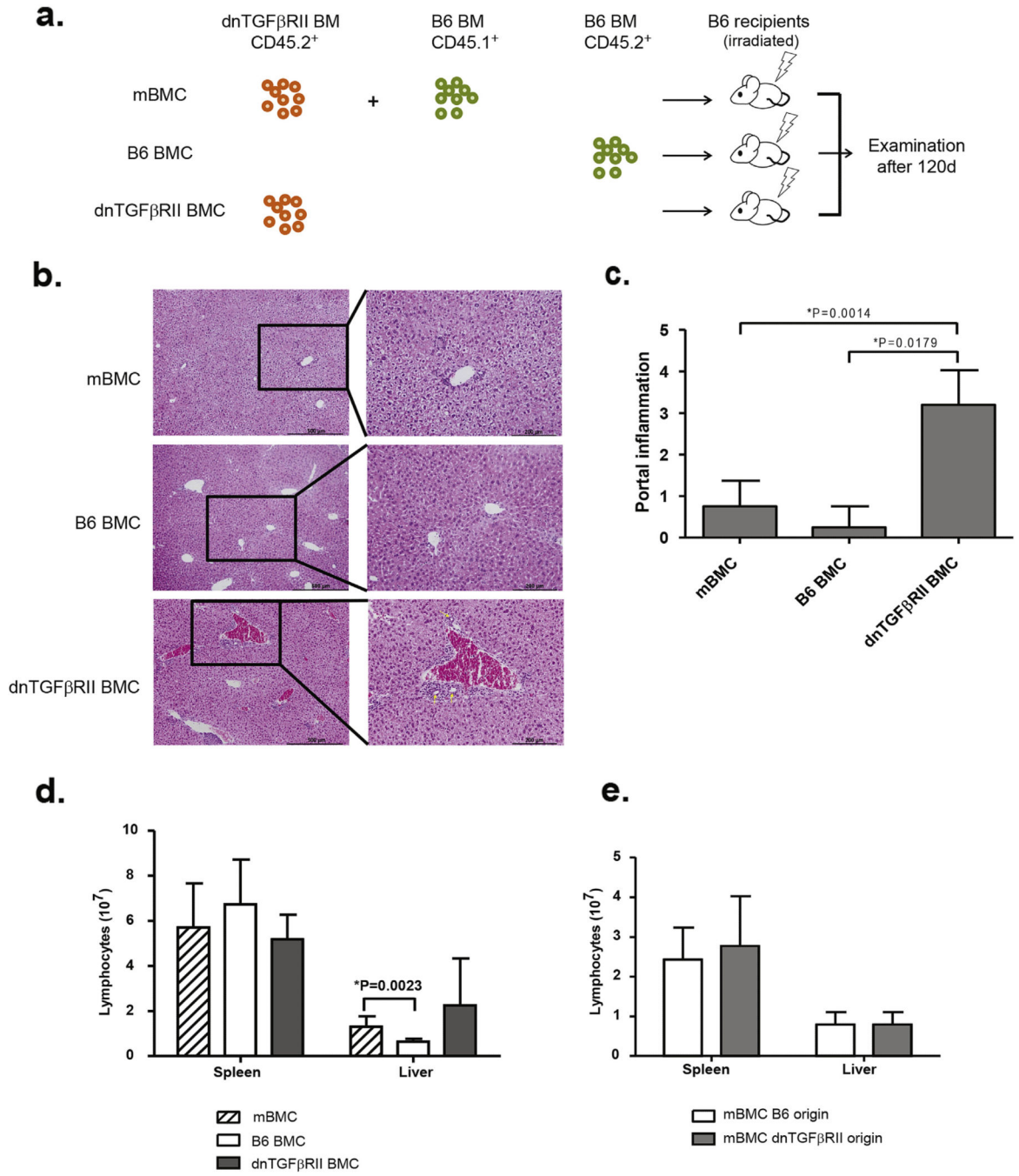

Fig. 1.

Reduced autoimmune biliary disease in mixed BMC mice compared to single dnTGFβRII BMC mice. (a) Schematic showing bone marrow chimera construction. (b,c) Representative histology sections (b) and mean blinded histology scores (c) from mBMC (*n* = 9), single B6 BMC (*n* = 4) and single dnTGFβRII BMC (*n* = 5). Arrows indicate ductules surrounded by lymphocytes. (d) Total lymphocyte number in spleen and liver among recipients of mBMC (*n* = 10), B6 BMC (*n* = 7) and dnTGFβRII BMC (*n* = 5) (not all mice that were studied by FACS had liver histology). The number of days after bone marrow transplantation ranged

from 41d to 158d (mice receiving dnTGFβRII often became sick and had to be sacrificed earlier). (e) Comparison of WT-origin lymphocyte number and dnTGFβRII-origin lymphocyte number in the spleen and liver of mBMC (n = 10). Error bar indicates S.D. Statistical analysis by Mann Whitney U test (c) and unpaired T test (d,e).

Fig. 2.

Flow cytometric analysis of hepatic T cells: increased KLRG1$^+$ CD8 cells in dnTGFβRII liver. (a) Representative FACS plots comparing hepatic CD8 T cell subsets between B6 and dnTGFβRII. Female B6 and dnTGFβRII aged over 120d were used for comparison. Circles indicate the KLRG1$^+$ CD8 subset. (b) Representative FACS plots of mBMC and single (B6 or dnTGFβRII) BMC controls showing the 8-color gating strategy to compare hepatic CD8 T cell subsets among different groups.

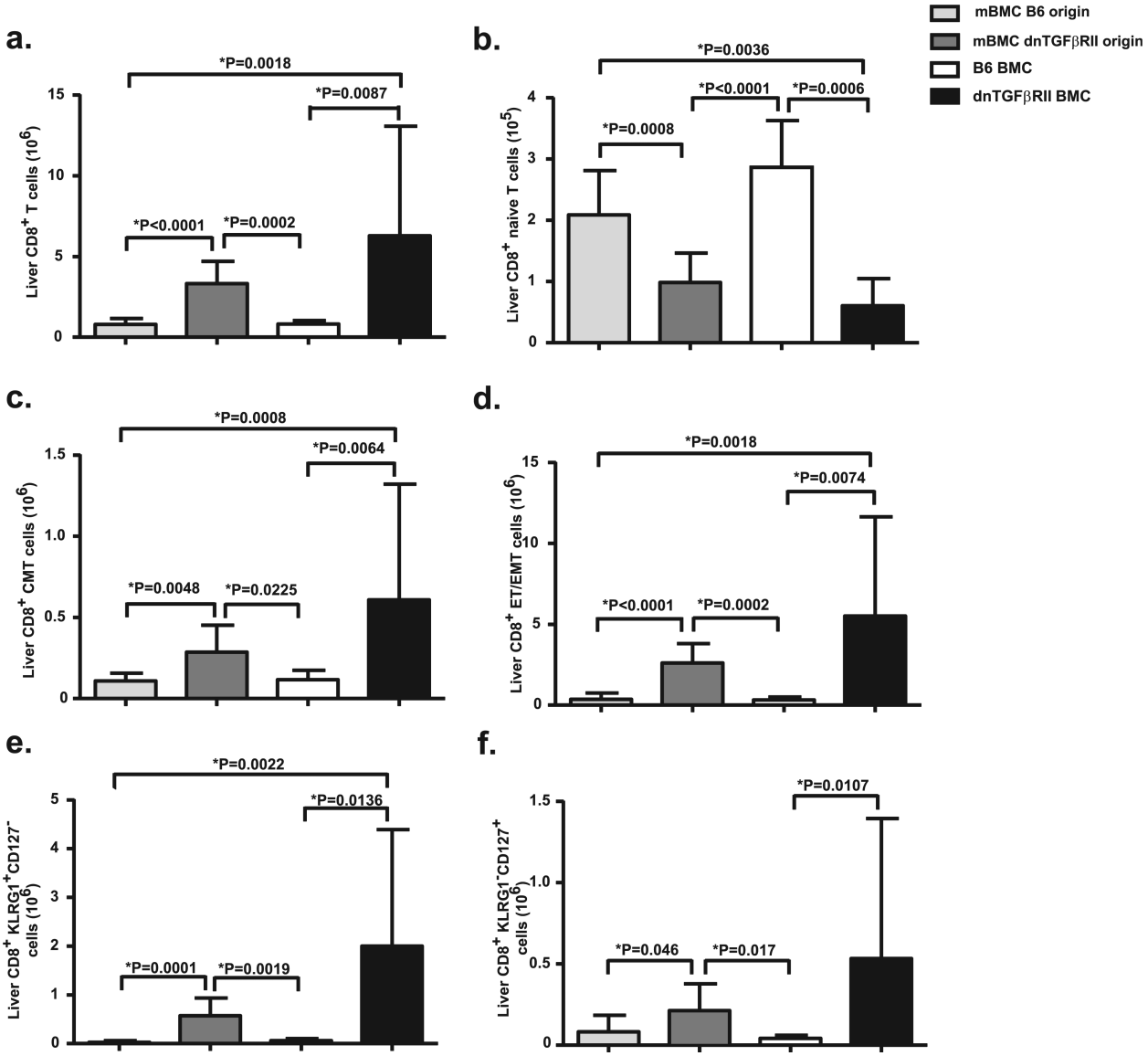

Fig. 3.

mBMC recipients have increased numbers of dnTGFβRII-origin CD8 effector cells but do not develop biliary disease. Cell numbers of total hepatic CD8 T cells (a) and hepatic CD8 T cell subsets (b—f) differentiating WT-origin and dnTGFβRII-origin cell populations in mBMC (n = 10), B6 BMC (n = 7) and dnTGFβRII BMC (n = 5) recipients. Error bar indicates S.D. Statistical analysis by unpaired T test.

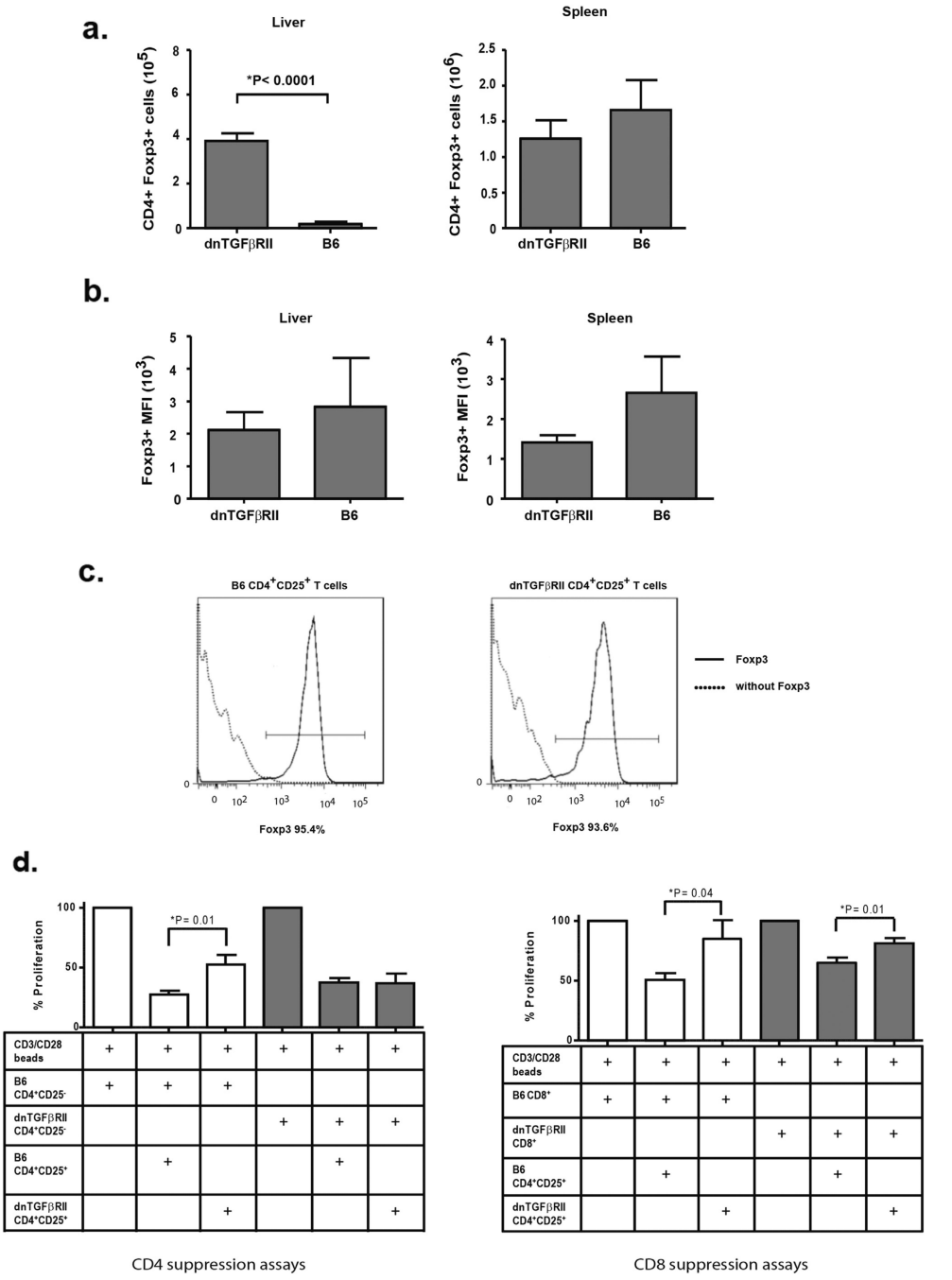

Fig. 4.

dnTGFβRII Tregs are defective in suppressing B6 and dnTGFβRII CD8 T cells. (a, b) Absolute number (a) of $CD4^+Foxp3^+$ Tregs, and (b) MFI of Foxp3 in the liver and spleen from B6 (n = 3) and dnTGFβRII (n = 3) mice. (c) Comparison of percentage of $Foxp3^+$ cells within splenic $CD4^+CD25^+$ T cells between B6 and dnTGFβRII. One representative of three experiments. (d) Proliferation of B6 (shaded white) or dnTGFβRII (shaded dark) $CD4^+CD25^-$ (left, n = 7 experiments) or $CD8^+$ (right, n = 5 experiments) cells stimulated with CD3/CD28 beads in the presence of B6 or dnTGFβRII Tregs. Suppression is shown as

a percent proliferation of effector cells alone. The mean CPMs (±SEM) of effector cells alone stimulated with CD3/CD28 beads: B6 CD4: 12,355 ± 998; B6 CD8: 12,155 ± 694; dnTGFβRII CD4: 110,736 ± 13,406, and dnTGFβRII CD8: 41,416 ± 2657. Statistical analysis by unpaired *T* test.

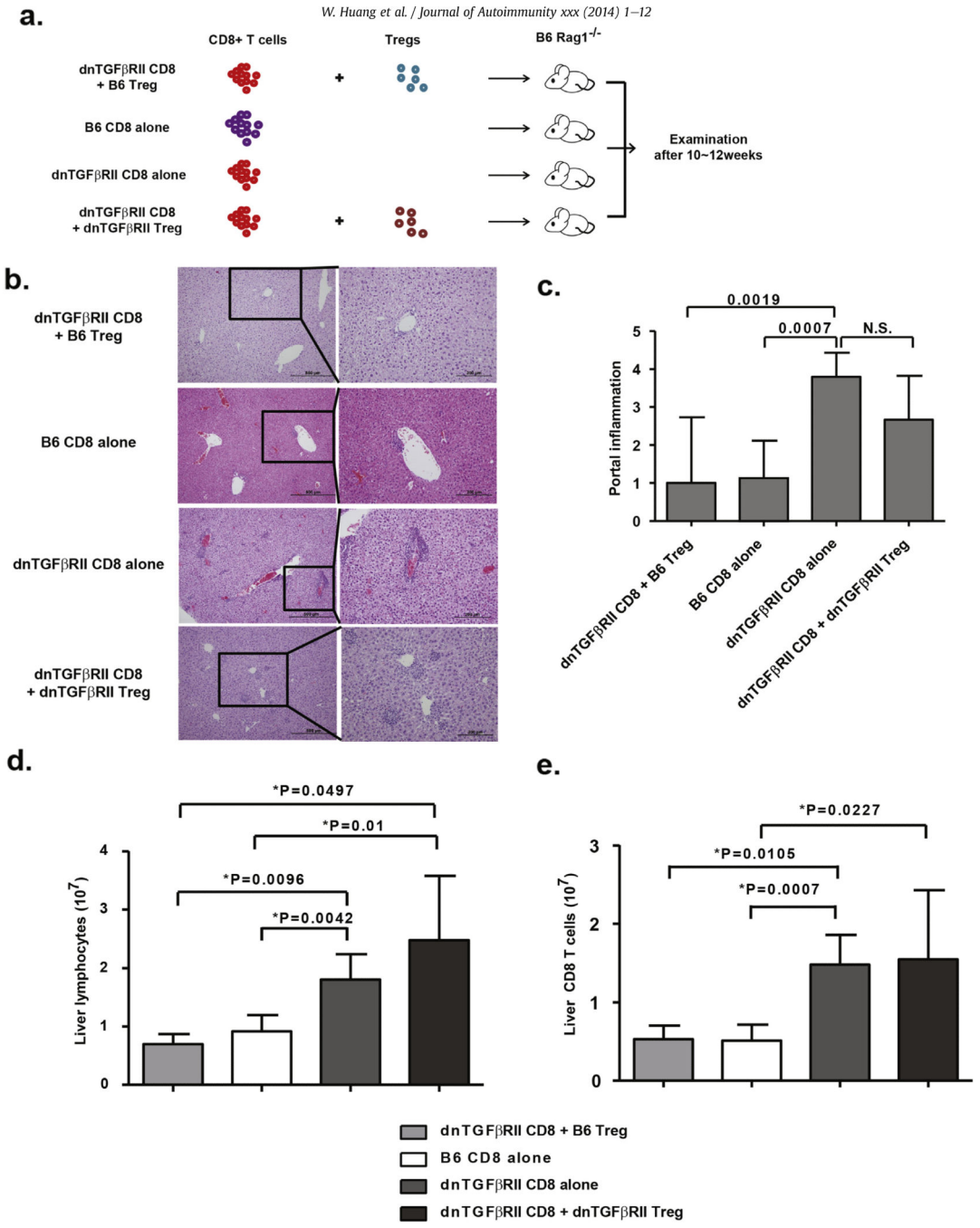

Fig. 5.

Transfer studies confirm that B6, but not dnTGFβRII Tregs can prevent adoptive transfer of autoimmune biliary disease mediated by dnTGFβRII CD8 cells. (a) Schematic showing transfer study design of B6 CD8, dnTGFβRII CD8, dnTGFβRII CD8 plus B6 Tregs or dnTGFβRII CD8 plus dnTGFβRII Tregs, as described in Methods, into B6.Rag1$^{-/-}$ recipients. (b) Representative liver histology slides at low (left) and high (right) magnification for each transfer group. (c) Mean liver histology scores among recipients of dnTGFβRII CD8 plus B6 Treg (n = 7), B6 CD8 alone (n = 8) dnTGFβRII CD8 alone (n =

10), or dnTGFβRII CD8 plus dnTGFβRII Tregs (n = 3). (d,e) The absolute number of total liver lymphocytes (d) and liver CD8 *T* cells (e) are shown for recipients transferred with dnTGFβRII CD8 and B6 Tregs (n = 3), B6 CD8 alone (n = 6) and dnTGFβRII CD8 alone (n = 4) or dnTGFβRII CD8 plus dnTGFβRII Tregs (n = 3) (not all individual mice that had liver histology slides were also studied by FACS). The experimental setup was the same as in (b). Error bar indicates S.D. Statistical analysis by Mann Whitney *U* test (c) and unpaired *T* test (d,e).

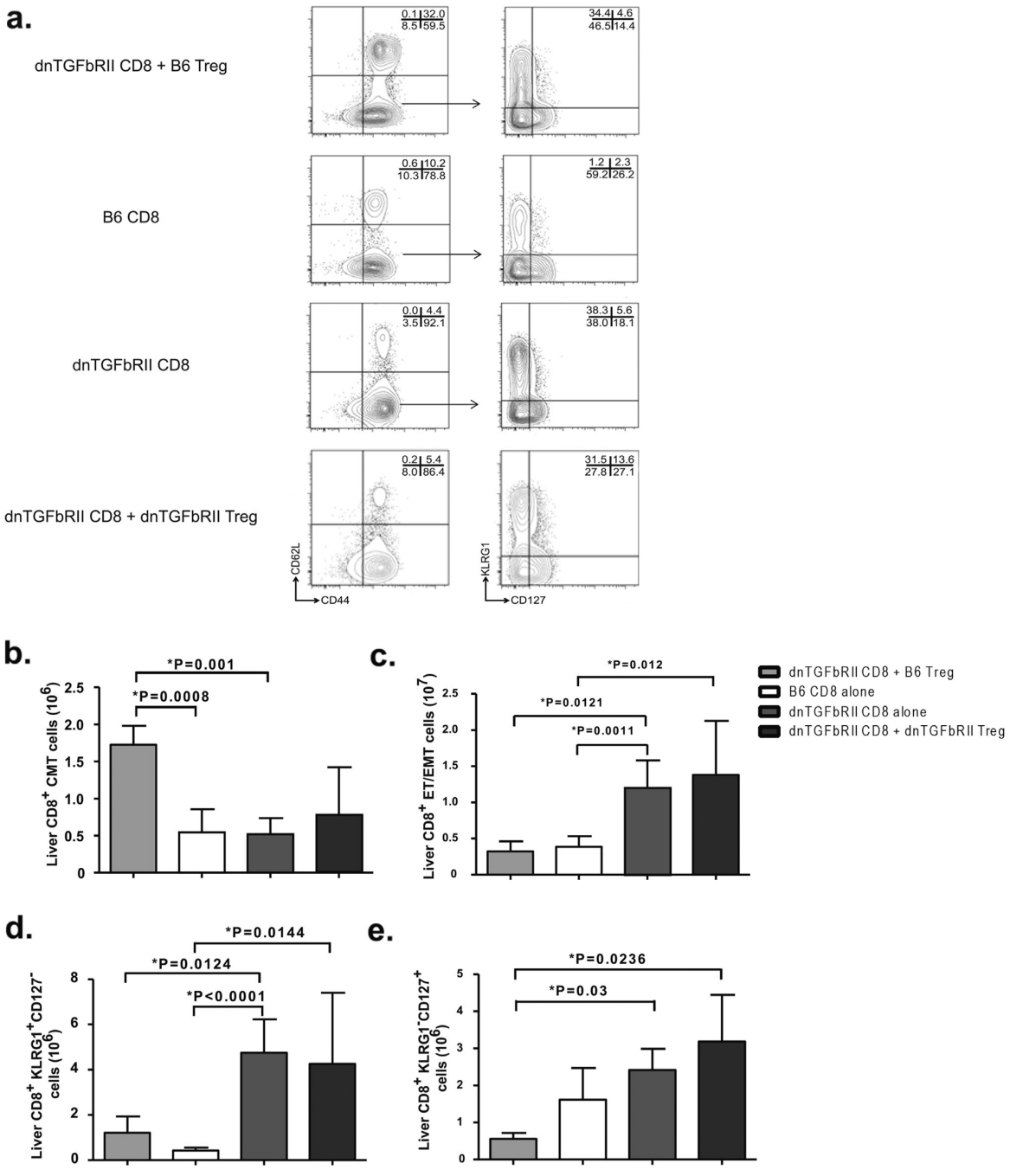

Fig. 6.

B6 Tregs reduce the total number of CD8 cells and KLRG1$^+$ CD8 cells while preventing biliary disease. (a) Representative FACS plots showing intrahepatic lymphocyte CD8 T cell subsets from recipients transferred with dnTGFβRII CD8 T cells and B6 Tregs (n = 3), B6 CD8 T cells alone (n = 6) dnTGFβRII CD8 T cells alone (n = 4) or dnTGFβRII CD8 plus dnTGFβRII Tregs (n = 3), as described in Methods. The absolute number of CMT (b), ET/EMT (c), terminal effector cell (d) and effector memory cells (e) are shown. Error bars indicate S.D. Statistical analysis by unpaired T test.

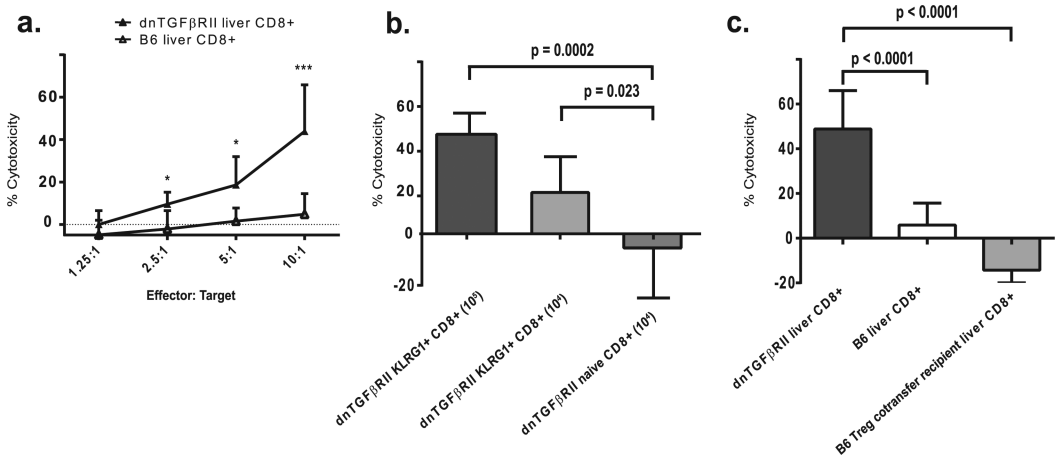

Fig. 7.

Intrahepatic dnTGFβRII KLRG1^{+} CD8 cells are cytotoxic to cholangiocytes; B6 Tregs eliminate dnTGFβRII mediated cholangiocyte toxicity. (a) MiniMACS-sorted liver CD8 T cells from dnTGFβRII or B6 were co-cultured with immortalized murine cholangiocyte cells (mCL) at titrated ratios; *n* = 3/group. Killing assay was performed as described in methods. Error bars are shown only above the curve. *: P < 0.05; ***p < 0.001 by unpaired T test. (b) CD8^{+}, CD44^{high}CD62L^{low} CD127^{-} KLRG1^{+} cells were sorted from dnTGFβRII liver and cultured with murine cholangiocyte cells at a ratio of 100,000(CD8) to 10,000 (mCL) (left column) or 10,000 (CD8) to 1000 (mCL) (middle column) while 10,000 naïve (CD8^{+} CD44^{low} CD62L^{high}) dnTGFβRII cells were cultured with 1000 mCL (right column). Killing assay was then performed as above; experiment performed *n* = 3 for each group. (c) MiniMACS-sorted liver CD8 T cells (100,000 cells/well) from 1) dnTGFβRII, 2) B6 or 3) B6.Rag1$^{-/-}$ mice reconstituted with dnTGFβRII CD8 T cells and B6 Tregs (as in Fig. 5) were co-cultured with mCL at 10:1 ratio. *n* = 3~4/group. Error bar indicates S.D. Statistical analysis by unpaired *T* test.