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Mouse liver-specific CD8$^+$ T-cells encounter their cognate antigen and acquire capacity to destroy target hepatocytes☆

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ABSTRACT

CD8$^+$ T-cell immune response to liver antigens is often functionally diminished or absent. This may occur via deletion of these autoaggressive T-cells, through the acquisition of an anergic phenotype, or via active suppression mediated by other cell populations. We generated a double transgenic model in which mice express CD8$^+$ T-cells specific for the lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) and LCMV-NP as a hepatic neo-autoantigen, to study the immunological response of potentially liver antigen autoaggressive CD8$^+$ T-cells. Autoreactive transgenic CD8$^+$ T-cells were analyzed for functionality and cytotoxic effector status. Despite severe peripheral deletion of liver-specific CD8$^+$ T-cells, a fraction of autoreactive NP-specific CD8$^+$ T-cells accumulate in liver, resulting in hepatocyte injury and production of auto-antibodies in both male and female mice. NP-specific intrahepatic T-cells showed capacity to proliferate, produce cytokines and up-regulate activation markers. These data provide *in vivo* evidence that autoreactive CD8$^+$ T-cells are activated in the liver and developed an inflammatory process, but require additional factors to cause severe autoimmune destruction of hepatocytes. Our new model will provide a valuable tool for further exploration of the immunological response involved in inflammatory liver diseases, including autoimmune hepatitis.

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1. Introduction

In addition to metabolic functions, the liver has immunological properties playing an essential role in maintaining a delicate balance between tolerance and immunity [1,2]. The liver is exposed to endotoxins and other microbial degradation products that reach the liver via portal blood; many unique molecular mechanisms, including immune and non-immune cells, contribute to promote the development of immune tolerance. Similar mechanisms avoid hepatocytes damage from specific autoimmune process [3]. Therefore, the liver is particularly successful in the development of peripheral tolerance. However in specific circumstances, tolerance to hepatic self-proteins is broken, usually in genetically susceptible hosts, resulting in development of autoimmune hepatitis (AIH) [4].

AIH is the consequence of selective and progressive destruction of hepatic parenchyma by an inflammatory process [4]. Many cell types are involved in liver lesions characteristic of AIH, including antigen-presenting cells (APC), B lymphocytes and both CD4$^+$ and CD8$^+$ T-lymphocytes [5]. The presence of autoreactive T-cells (CD4$^+$ and CD8$^+$) in the liver and auto-antibodies (auto-Ab) production directed against various hepatic antigens, reflects the autoimmune character of the disease [6,7]. However, neither their specific contribution to the disease pathogenesis nor how they are recruited and primed in the liver has been resolved.

There are convincing arguments showing that CD8$^+$ T-cells contribute to the pathogenic autoimmune process in organ specific

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autoimmune diseases (AID) [8]. First, adoptive transfer of autoimmune disorders by $CD8^+$ T-cells in syngenic immunodeficient mice proves that these cells are essential for the initiation of autoimmunity [9]. In addition, some $CD8^+$ T-cell clones have been shown to kill specific targets without the help of $CD4^+$ T-cells [10]. Autoreactive $CD8^+$ T-cells can be detected in the peripheral blood of human patients with AID, including patients with AIH. Thus, $CD8^+$ T-cell clones specific for ASGPR or CYP2D6 from patients with AIH have the ability to produce IFN-γ cytokine and to exert cytotoxicity after recognition of CYP2D6 peptide-pulsed targets [11]. Hepatic self antigens (HAgs) are readily accessible to class I-restricted $CD8^+$ T-lymphocytes and many studies in mice suggest that autoreactive $CD8^+$ T-lymphocytes play a key role in the pathogenesis of AIH [12,13]. Transgenic (Tg) or knockout animal models have been excellent tools for dissecting pathogenesis and have provided crucial information on mechanisms responsible for the fragile and complex balance between tolerance and autoimmunity in the liver [14,15]. Moreover, knowledge of the mechanisms responsible for autoreactive T-lymphocytes activation, proliferation, clonal deletion, anergy, or ignorance in the periphery, has been enhanced by the use of TCR-Tg mouse strains [16]. Thus, several murine models have highlighted the role of HAgs-specific $CD8^+$ T-cells. However, most previous works were based on transfer of $CD8^+$ T-cells against liver-specific antigens expressed as neo-self in Tg mice [17,18]. Unfortunately, transfer of autoreactive $CD8^+$ T-cells was not sufficient to induce chronic inflammation [19]. Thus, these approaches showed severe limitations in the induction of immune-mediated hepatitis as observed in humans. Heterogeneity of T-cell clones activation *in vitro*, impact of number of transferred cells and the fact that the liver plays a robust role in peripheral tolerance could explain the disappointing results reported. To bypass this obstacle, Tg murine models have been produced to develop a spontaneous chronic progressive AIH using double Tg mice expressing a TCR carried by $CD8^+$ T-cells with specificity for a neo-self-autoAg expressed by hepatocytes [20–22]. However, these models show a central clonal deletion or a persistence of high avidity autoreactive Tg $CD8^+$ T-cells in the periphery in the absence of chronic liver inflammation. For example, double Tg mice on C57BL/6 (B6) background expressing the neo-self gag protein (FMuLVgag) from Friend virus in the thymus and liver under Albumin promoter (Alb-Gag) showed no signs of autoimmunity. Gag-specific Tg $CD8^+$ T-cells escaping central tolerance were maintained in a tolerant state in the periphery and lost the ability to proliferate and produce IL-2 in response to antigen stimulation [20]. In contrast, double Tg mice expressing influenza virus-hemagglutinin (HA) as neo-self-Ag only in the liver under hepatocyte-specific albumin promoter developed a moderate and transient form of hepatitis only in males. However, most liver-infiltrating HA-specific $CD8^+$ T-cells had an anergic status [22]. In this model, the development of a chronic T-cell-mediated liver autoimmunity on mixed genetic background (DBA2, B6 and Balb/c) could be impaired by the interaction of autoimmunity-related susceptibility loci and/or the background of these strains.

To further analyze the liver-specific autoimmune response and the role of the liver in peripheral tolerance, the double Tg model presented in this study was generated directly on B6 background to avoid the impact of genetic heterogeneity on T-cell development and tolerance breakdown. This model shows specific signs of autoimmunity (cellular and humoral immune responses) and develops mild liver inflammation in both male and female mice. As such the model reported here should be of unique value for dissecting the molecular basis for the liver's role in the balance between tolerance- and immune-mediated autoimmune liver injury.

2. Methods

2.1. Cells

The T $CD8^+$ NP18 clone, which has previously been shown to have specific reactivity to amino acids 396–404 of the nucleoprotein (NP) from lymphocytic choriomeningitis virus (LCMV), is restricted by the MHC class I H-$2D^b$ molecule [23]. Mice thymocytes, splenocytes, PBMC and lymph node cells were prepared according to standard protocols with minor modifications and cultured in RPMI + Glutamax medium (Invitrogen), 10% SBF, 5×10^{-5} M 2-ME, 100 U/mL penicillin G and 100 μg/mL streptomycin.

2.2. Transgenic mice generation

To determine TCR-α and -β gene usage, synthesized cDNA from total RNA of T $CD8^+$ NP18 clone was amplified with a panel of Vα and Vβ specific primers as described in Ref. [24] and PCR amplification products were subcloned and sequenced. TCR transgenes were constructed by subcloning PCR amplified regions encoding rearranged Vα8.5Jα5.1 and Vβ12D2Jβ2.3 domains into pT-Vα and pT-Vβ TCR-transgenic vectors, respectively [25]. The TCR chain expression of both constructs was confirmed by transient transfection into 58 α^-/β^- T-cell hybridomas and RT-PCR. Their ability to respond to $NP_{396-404}$ peptide was confirmed using an IL-2 production assay. Transgenic constructs coding for the functional VJα and VDJβ rearrangements of NP18 clone were co-injected in fertilized eggs from C57BL/6 (H-2^b) mice.

Genomic DNA from founder mice and their offspring was screened for the presence of TCRα and TCRβ chains DNA by PCR amplification, using specific primers. TCR chains expression was proved by RT-PCR amplification of total RNA from Tg splenocytes. Two transgenic (Tg) TCR mouse lines (TNP4 and TNP5) were kept and only TNP5 was used in further experiments in this study. The double transgenic TNP5/TTR-NP mice line was obtained after breeding of TCR-transgenic mice (TNP5/B6) with the TTR-NP/B6 mice [26]. All transgenic mice used for this study were bred in the animal facility and were kept under specific pathogen-free conditions. All animal experiments were performed according to national and institutional guidelines.

2.3. Flow cytometry

Thymocytes, splenocytes, PBMC or lymph node cells (1×10^6) were labeled with fluorescence-conjugated antibodies specific for TCRβ chain, CD4, CD8, CD25, CD69, CD44 or CD122. All antibodies were purchased from eBioscience, except the Extravidin-PE (Sigma). The H2-D^b-$NP_{396-404}$ tetramer (NP-tet) was produced as previously described in Ref. [27] and was used to stain NP-specific effector cells. Four-color flow cytometry was performed on a FACScalibur (BD Biosciences), and data were analyzed using Cell Quest Pro software (BD Biosciences).

2.4. Transgenic CTLs functionality

Proliferation assays were performed on splenocytes from Tg mice. Briefly, cells were stained with 5 μM CFSE (eBioscience), then 1×10^6 cells per well were seeded in a 24-wells plate. Cells were stimulated with 10 μg/mL of the cognate $NP_{396-404}$ peptide (FQPQNGQFI) or CMVpp65 (NLVPMVATV) irrelevant peptide as specificity control. Intrahepatic lymphocytes were isolated as described in Ref. [28] and combined with syngeneic splenocytes loaded with specific peptides. Unstimulated and PMA stimulated controls were included in all experiments. Cells were harvested

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