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ORIGINAL RESEARCH

Expression of Viral Antigen by the Liver Leads to Chronic Infection Through the Generation of Regulatory T Cells

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SUMMARY

Expression by the liver of a viral protein induced an immunologic tolerance mediated by interleukin-10-secreting regulatory T cells that impaired the antiviral T-cell response, leading to a chronic infection. This mechanism could be involved in the establishment of persistent infection by hepatotropic viruses.

BACKGROUND & AIMS: The constant exposure of the liver to food and bacterial antigens through the mesenteric circulation requires it to maintain tolerance while preserving the ability to mount an effective immune response against pathogens. We investigated the contribution of the liver's tolerogenic nature on the establishment of chronic viral infections.

METHODS: TTR-NP mice, which express the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) specifically in hepatocytes under control of a modified transthyretin (TTR) promoter, were infected with the Armstrong (Arm) or WE acute strains of LCMV.

RESULTS: The infection persisted for at least 147 days in TTR-NP mice. Expression of NP by the liver induced a strong peripheral tolerance against NP that was mediated by interleukin-10-secreting $CD4^+$ regulatory T cells, leading to high PD-1 (programmed death-1) expression and reduced effector function of virus-specific T cells. Despite an active immune response against LCMV, peripheral tolerance against a single viral protein was sufficient to induce T-cell exhaustion and chronic LCMV Armstrong (Arm) or WE infection by limiting the antiviral T-cell response in an otherwise immunocompetent host. Regulatory T-cell depletion of chronically infected TTR-NP mice led to functional restoration of LCMV-specific $CD4^+$ and $CD8^+$ T cell responses and viral clearance.

CONCLUSIONS: Expression of a viral antigen by hepatocytes can induce a state of peripheral tolerance mediated by regulatory T cells that can lead to the establishment of a chronic viral infection. Strategies targeting regulatory T cells in patients chronically infected with hepatotropic viruses could represent a promising approach to restore functional antiviral immunity and clear infection. *(Cell Mol Gastroenterol Hepatol 2015;1:325–341; http://dx.doi.org/10.1016/j.jcmgh.2015.02.002)*

Keywords: Chronic Infection; Hepatitis; Tolerance.

The liver is an immunoprivileged site prone to tolerance induction. For example, liver grafts are accepted without immunosuppression in several mammals,[1] and oral tolerance is abrogated when a portacaval shunt is performed.[2] The liver also has the unique ability among solid organs to activate naive $CD8^+$ T lymphocytes in an antigen-specific manner, a process that can be inefficient and lead to apoptosis through a Bim-dependant pathway.[3]

The liver is also host to several chronic infections, but infection of the liver does not inevitably lead to viral persistence. Strong innate immune responses combined with specific T-cell responses can overcome viral escape mechanisms—as in hepatitis A infection or resolved acute hepatitis B (HBV) or C virus (HCV) infections—and achieve viral clearance. However, as observed in 50% to 80% of HCV infections, pathogens can evade early innate and adaptive immune responses through high antigen loads and increased coinhibitory signaling locally in the inflamed liver, leading to T-cell exhaustion and viral persistence.[4]

After exposure, HCV reaches maximal titers several weeks before the induction of detectable humoral or cellular immune responses and the onset of liver disease; in cases where HCV titers remain relatively low, T cell responses may remain undetectable even during chronic infection.[5] Therefore, the liver may be exposed to HCV antigens in absence of strong immune responses. It has recently been shown in a model of chronic viral infection that $CD4^+$ regulatory T-cell depletion in combination with programmed-death-ligand-1 (PD-L1) blockade can significantly reduce viral titers, highlighting the importance of immune inhibitory signals in the outcome of viral infections.[6] Thus, the

Abbreviations used in this paper: **ALT, alanine aminotransferase; APC, allophycocyanin; Arm, Armstrong strain; BTLA, B and T lymphocyte attenuator; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunoassay; FACS, fluorescence-activated cell sorter; FoxP3, forkhead box P3; GP, glycoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; IP, intraperitoneal; IV, intravenous; LCMV, lymphocytic choriomeningitis virus; LIL, liver-infiltrating lymphocytes; NP, nucleoprotein; P14, $GP_{33–41}$-specific TCR transgenic; PD-1, programmed death-1; PD-L1, programmed death-ligand-1; PE, phycoerythrin; pfu, plaque-forming units; RAG, recombination-activating gene; TCR, T-cell receptor; TNF-α, tumor necrosis factor-α; TNP4, $NP_{396–404}$-specific TCR transgenic; Treg, regulatory T cell; TTR, transthyretin.**

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liver's ability to induce tolerance to locally expressed antigens could contribute to the development of chronic liver infections by altering the immunologic response against liver-expressed viral antigens.[7] Therefore, we directly assessed whether expression of a viral antigen by hepatocytes can induce a state of peripheral tolerance able to contribute to viral persistence.

We show that expression of the nucleoprotein (NP) from lymphocytic choriomeningitis virus (LCMV) specifically in hepatocytes leads to strong peripheral tolerance mediated by interleukin-10 (IL-10)-secreting $CD4^+$ forkhead box $P3^+$ ($FoxP3^+$) regulatory T cells. This allows the establishment of chronic infection by acute strains of LCMV associated with the loss of $CD4^+$ and $CD8^+$ T-cell effector function, leading to high viral titers in the liver and spleen. Depletion/silencing of $CD4^+$ regulatory T cells resulted in the progressive restoration of T-cell function, loss of PD-1 (programmed death-1) expression, and gradual viral clearance. This study demonstrates that expression of a viral antigen in the liver leads to the development of $CD4^+$ regulatory T cells able to interfere with the antiviral T-cell response, allowing the establishment of a chronic infection.

Materials and Methods

Mice and Viruses

Transthyretin-nucleoprotein (TTR-NP) transgenic mice[8] (8- to 12-week-old females) expressing LCMV NP specifically in hepatocytes (kindly provided by F. Alvarez, CHU Sainte-Justine, Montreal, Canada) or control C57BL/6 (B6) mice (8- to 12-week-old females) (Charles River, Montreal, Canada) were infected with either acute strains LCMV-Arm (200 plaque-forming units [pfu] intraperitoneally [IP]) or LCMV-WE (200 pfu intravenously [IV]). Hemizygous TTR-NP1/0 mice were generated as a F1 cross between homozygous TTR-NP and C57BL/6 mice. The $NP_{396–404}$-specific T-cell receptor (TCR) transgenic TNP4 mice[9] were kindly provided by F. Alvarez (CHU Sainte-Justine). The glycoprotein $GP_{33–41}$-specific TCR transgenic P14 mice were kindly provided by P. Ohashi (Princess Margaret Cancer Centre, Toronto, Canada). The recombination-activating gene–nucleoprotein (RAG-NP) mice were obtained by crossing TTR-NP and RAG-1 mice (kindly provided by C Daniel, INRS-Institut Armand-Frappier, Laval, Quebec, Canada). The RAG-1 phenotype was assessed by flow cytometry, and NP expression was monitored by polymerase chain reaction, as previously described elsewhere.[8]

LCMV-WE and Armstrong strains were obtained from R.M. Zinkernagel at the Institute of Experimental Immunology (Zurich, Switzerland). LCMV titration was performed via a standard focus-forming assay. All experiments were performed under protocols approved by the INRS Institutional Committee for Animal Care and following guidelines published by the Canadian Council on Animal Care.

Lymphocyte Isolation From Liver and Spleen

Livers were perfused via the portal vein with RPMI 1640 (Thermo Fisher Scientific, Mississauga, ON, Canada) and removed. The livers and spleens were finely minced in RPMI 1640, passed through a 100-gauge steel mesh, and centrifuged at 400*g* for 5 minutes at 4°C. Cells contained in the supernatant were washed 3 times with RPMI 1640/5% fetal calf serum before being centrifuged on a Percoll (GE Healthcare Canada, Mississauga, ON, Canada) gradient to purify lymphocytes.[10]

Flow Cytometry

For the flow cytometry analysis, isolated cells were washed, resuspended in phosphate-buffered saline containing 5% fetal calf serum (fluorescence-activated cell sorter [FACS] buffer), and incubated with directly conjugated primary antibodies for 30 minutes at 4°C. Cells were then washed and resuspended in 200 μL FACS buffer containing 1% formaldehyde. Class I tetramer staining was performed using phycoerythrin (PE)-coupled $NP_{396–404}$ and $GP_{33–41}$ H2-D^b-restricted tetramers for 30 minutes at 37°C in FACS buffer followed by surface staining. Anti-CD25 allophycocyanin (APC) was purchased from eBioscience (San Diego, CA). Anti-CD45 PE/CF594 was purchased from BD Biosciences (San Jose, CA). Anti-PD-1 fluorescein isothiocyanate and anti-PD-1 allophycocyanin (APC), anti-CD4 APC/Cy7, anti-CD4 fluorescein isothiocyanate, anti-CD8 PE/Cy7, anti-CD62L Alexa Fluor 700, anti-CD44 PercP/Cy5.5, anti-CD3 APC, interferon-γ (IFN-γ) PE, tumor necrosis factor-α (TNF-α) APC, and B and T lymphocyte attenuator (BTLA) Alexa Fluor 647 were purchased from BioLegend (San Diego, CA). Intracellular FoxP3 staining was performed using PE-coupled anti-mouse/rat FoxP3 antibody (clone FJK-16s) and fixation/permeabilization buffer optimized for staining of mouse cells with FJK-16s monoclonal antibodies (eBioscience). Intracellular staining of Helios was performed using Alexa Fluor 647 coupled anti-mouse Helios antibody (clone 22F6) (eBioscience) and using the fixation/permeabilization buffer optimized for staining of mouse cells with FoxP3 (FJK-16s) monoclonal antibodies (eBioscience). Ki-67 protein intracellular staining was performed using anti-mouse/rat Ki-67 efluor450 conjugated antibody (clone SolA15) (eBioscience). Class II tetramer staining (NIH Tetramer Core Facility, Atlanta, GA) (PE-labeled H2-IA^b $GP_{31–45}$, $GP_{66–77}$, $NP_{309–328}$, or control H2-IA^b hCLIP) was performed at 37°C for 3 hours (2 μg/mL) in FACS buffer. The cells were then washed in FACS buffer, surface stained (CD3, CD4, CD8, CD44, CD62L, CD25, and 7-AAD viability stain) (eBioscience and BioLegend), and fixed. Samples were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Intracellular Cytokine Staining

Intracellular cytokine staining was performed using isolated lymphocytes stimulated for 5 hours in the presence of 10 U/mL IL-2 and Brefeldin A (10 μg/mL) and one of $GP_{33–41}$, $NP_{396–404}$, $GP_{61–80}$ peptide, or $NP_{311–325}$ (5 μg/mL). Cells were stained for surface and viability markers as described earlier, and then they were fixed and permeabilized for intracellular staining using fixation and permeabilization buffers from BioLegend. Cells were then