Published in final edited form as:

Gastroenterology. 2008 June ; 134(7): 2132–2143. doi:10.1053/j.gastro.2008.02.037.

Critical Role for CD1d-Restricted Invariant NKT Cells in Stimulating Intrahepatic CD8 T-Cell Responses to Liver Antigen

DAVE SPRENGERS[*,‡], **FENNA C. M. SILLÉ**[*], **KATJA DERKOW**[§], **GURDYAL S. BESRA**[‖], **HARRY L. A. JANSSEN**[‡], **ECKART SCHOTT**[§], **MARIANNE BOES**[*,¶]

[*]Department of Dermatology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

[‡]Department of Gastroenterology and Hepatology, Erasmus MC, Erasmus University, Rotterdam, The Netherlands

[§]Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie; Charite Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin, Germany

[‖]School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[¶]Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Background & Aims: Vα14 invariant natural killer T cells (iNKT) are localized in peripheral tissues such as the liver rather than lymphoid tissues. Therefore, their role in modulating the stimulation of conventional, major histocompatibility complex (MHC)-restricted T-cell responses has remained ambiguous. We here describe a role for Vα14 iNKT cells in modulating conventional T-cell responses to antigen expressed in liver, using transferrin-mOVA (Tf-mOVA) mice.

Methods: Naïve ovalbumin-specific class I MHC-restricted T cells (OTI) were adoptively transferred into Tf-mOVA mice in the presence or absence of iNKT-cell agonist α-galactosylceramide, after which OTI T-cell priming, antigen-specific cytokine production, cytotoxic killing ability, and liver damage were analyzed.

Results: Transfer of OTI cells resulted in robust intrahepatic, antigen-specific proliferation of T cells. OTI T cells were activated in liver, and antigen-specific effector function was stimulated by coactivation of Vα14 iNKT cells using α-galactosylceramide. This stimulation was absent in CD1d$^{-/-}$Tf-mOVA mice, which lack Vα14 iNKT cells, and was prevented when interferon-γ and tumor necrosis factor-α production by Vα14 iNKT cells was blocked.

Address requests for reprints to: Department of Dermatology, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115. M.L.Boes@umcutrecht.nl; fax: (617) 525-5571.

D.S. and F.S. contributed equally to this paper; E.S. and M.B. contributed equally to this paper.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2008.02.037.

Conclusions: CD1d-restricted Vα14 iNKT cells stimulate intrahepatic CD8 T-cell effector responses to antigen expressed in liver. Our findings elucidate a previously unknown intervention point for targeted immunotherapy to autoimmune and possibly infectious liver diseases.

The initiation of adaptive immune responses generally entails the antigen-specific stimulation of naïve major histocompatibility complex (MHC)-restricted T cells by professional antigen-presenting cells (APCs), a process that requires prolonged contact between T cells and APCs. CD1d-restricted invariant natural killer T (iNKT) cells, in contrast, can secrete cytokines within only few hours of activation. In mice, the majority of CD1d-restricted iNKT cells are relatively invariant and are collectively called *Vα14 iNKT cells*.[1] Human iNKT cells mostly express a Vα24-Jα18 rearranged T-cell receptor (TCR) α chain with a Vβ11-containing TCRβ chain.[2]

Vα14 iNKT cells in mice (referred to as iNKT cells hereafter) are highly enriched in liver, in which they can represent up to 30% of lymphocytes,[3,4] and are likely to play an important role in local immune responses. Their activation, through stimulation with the glycolipid α-galactosylceramide (αGalCer), can enhance T-cell responses to soluble protein antigens by directly interacting with dendritic cells (DCs) in a CD40-dependent manner[5–7] and can enhance antitumor cytotoxicity of NK cells and $CD8^+$ T cells to inhibit metastasis to the liver.[8,9] Intrahepatic iNKT cells moreover play a protective role in the clearance of multiple pathogens such as picornavirus,[10] herpes simplex virus,[11] and *Pseudomonas earuginosa*.[12] In transgenic mouse models of hepatitis B virus (HBV) replication, Vα14 iNKT cells contribute to interferon (IFN)-α/β- and IFN-γ-dependent inhibition of viral replication,[13] and livers of hepatitis C virus (HCV) patients and patients with primary biliary cirrhosis contain large numbers of iNKT cells.[14–16] These findings support the hypothesis that iNKT cells contribute to the pathogenesis of these liver diseases. Still, the liver is an organ with paradoxical immunologic properties, functioning either as a site amendable to effective immune responses or to generation of tolerance, as appropriate.[17] We asked how intrahepatic Vα14 iNKT cells are involved in antigen-specific hepatitis whereby antigen-specific conventional CD8 T cells instigate liver injury. We here describe the role of iNKT cells in stimulating CD8 T-cell responses to antigen restricted to the liver, using a recently developed mouse model, transferrin (Tf)-mOVA mice.

Materials and Methods

Mice

Tf-mOVA,[18] CD1d-deficient,[10,19] and OTI[20] RAG1-deficient mice were maintained in a rodent barrier facility at Harvard Medical School. CD1d-deficient Tf-mOVA mice were generated by crossbreeding. All mice were on C57Bl/6J background and were used at 6 – 8 weeks of age. Studies were performed according to institutional guidelines for animal use and care. Wild-type control mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Adoptive Transfer of OTI T Cells

OTI T cells were extracted from lymph nodes and spleen of $RAG1^{-/-}$ OTI mice (>95% purity). OTI T cells (4×10^6) were injected via tail vein, with or without 100 ng αGalCer.

αGalCer was synthesized in the laboratory of Dr Gurdyal S. Besra. For proliferation measurements (at 36 hours post-transfer), OTI T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen-Molecular Probes, Carlsbad, CA).

Blocking of Lymph Node Homing or Exit

For blocking of lymph node homing, OTI T cells were preincubated with neutralizing antibody to CD62L (clone MEL14) or isotype control IgG2a (clone RTK2758, 50 μg at 4°C, 30 minutes; both Biolegend, San Diego, CA). At the time of transfer and 24 hours later, 50 μg/mouse of anti-CD62L or isotype control antibody were injected intraperitoneally (IP) into splenectomized Tf-mOVA mice. For blocking of lymph node exit, Tf-mOVA mice received FTY 720 at the time of T-cell adoptive transfer and 24 hours posttransfer (Cayman Chemical, Ann Arbor, MI; 1 mg/kg) intraperitoneally. Littermate controls received phosphate-buffered saline (PBS).

Splenectomy

Mice were anesthetized before surgery, and hair was removed at the surgical site. A small incision was made on the left side of the mouse and through the peritoneal cavity. The spleen was ligated with 5–0 Ethilon (Ethicon, Inc, Sommerville, NJ) and excised from the opening. The abdominal wall incision was closed with staples (Precise; 3M Healthcare, St Paul, MN). Experiments were performed at least 7 days postsurgery.

Isolation of Mononuclear Cell Populations From Liver, Spleen, and Lymph Nodes

After perfusion (2–5 mL PBS), the liver was removed and pressed through 70-μm mesh (BD Pharmingen, Frankin Lakes, NJ). After washing in PBS, mononuclear cells were resuspended in 33% Percoll (GE Health Care, Piscataway, NJ), overlayed onto 80% Percoll and centrifuged (20 minutes at 900*g*). Mononuclear cells were collected from the interface. After erythrocyte lysis using NH^4Cl, cells were washed twice in PBS and resuspended in PBS supplemented with 10% fetal calf serum (FCS) for flow cytometry or in RPMI medium supplemented with 5% FCS and Pen/Strep (Invitrogen-Gibco, Carlsbad, CA) for culture. Mononuclear cells were also isolated from spleen and from inguinal, axillary, mesenteric, and portal lymph nodes. Cells were prepared for flow cytometry or culture as described below.

In Vitro OTI T-Cell Stimulation With DCs

DCs used in T-cell stimulation assays were purified from mesenteric and skin-draining lymph nodes (inguinal and axillary), from spleen and from liver based on CD11c expression using MACS (Miltenyi Biotec Inc, Auburn, CA). One × 10^5 DC were incubated with 1 × 10^5 naïve OTI T cells in round-bottom 96-wells plates (BD Pharmingen), in RPMI supplemented with 5% FCS and PenStrep, for 24 hours at 37°C. As a positive control, crystalline ovalbumin (OVA) was added (10 μg/mL; Sigma-Aldrich, St Louis, MO). As a negative control, 1 × 10^5 OTI T cells were incubated without DC. After 24 hours, CD25 and CD69 expression by OTI T cells was determined by flow cytometry.

Flow Cytometry and Intracellular Cytokine Staining

T cells, iNKT cells, and DC were preincubated 5 minutes on ice with Fc-block 1:200 (clone 2.4G2; BD Pharmingen). Immunostaining was performed for 15 minutes on ice with fluorophore-conjugated antibodies (Ab) against CD11c (clone HL3; BD Biosciences), CD8 (clone 53–6.7; Abcam Inc., Boston, MA), Thy1.1 (clone OX7; Anti- genix America Inc., Huntington Station, NY), CD69 (clone H1.2F3; BD Pharmingen), CD25 (clone PC61; Biolegend), NK1.1 (clone PK136; eBiosciences, San Diego, CA), TCRβ (clone H57; eBiosciences), Vα2 (clone B20.1; BD Pharmingen), and PBS57-loaded CD1d-tetramers (NIH Tetramer Facility, Atlanta, GA). Flow cytometry analysis was performed on a FACS-Canto flow cytometer (BD Pharmingen).

For intracellular cytokine staining, OTI T cells were restimulated with SIINFEKL peptide in 96- or 48-well plates (10 μmol/L, 5 hours, 37°C) in the presence of brefeldin A (10 μg/mL; Sigma-Aldrich). Whole OVA was used as negative control (10 μg/mL; Sigma-Aldrich). For intracellular cytokine staining of iNKT cells, mice were injected intravenously (IV) with brefeldin A (250 ng/mouse) 15 minutes prior to αGalCer IV injections (100 ng/mouse). Liver iNKT cells were isolated at indicated times. Cells were stained for surface markers, fixed, permeabilized, and stained intracellular using a Cytofix/ Cytoperm kit (BD Pharmingen) and fluorophore-conjugated Ab to IFN-γ (clone XMG1.2; eBiosciences), interleukin (IL)-4 (clone 11B11; BD Pharmingen), or TNF-α (clone MP6 XT22; eBiosciences).

To assay for apoptosis, OTI T cells were extracted at days 3 and 5 after adoptive transfer in Tf-mOVA mice, and nonparenchymal cells were isolated from the liver and stained for CD8 (clone 5H10; Invitrogen-Caltag Laboratories, Carlsbad, CA) and Vα2 (clone B20.1; BD Pharmingen). For detection of caspase 3, cells were resuspended at 1×10^6 cells/mL in RPMI containing 10% FCS, 1% Pen/Strep, and 50 μmol/L β-mercaptoethanol, and 5×10^5 cells were incubated with 1 μL RED-DEVD-FMK (Red Caspase-3 Staining Kit, PromoKine, Heidelberg, Germany) for 45 minutes at 37°C.

Neutralization of IFN-γ and TNF-α Using Blocking Antibodies

Anti-IFN-γ (clone XMG1.2), anti-TNF-α (clone XT3.11), or rat IgG1 control antibody (200 μg/mL each) were IP injected at days –1, 0, 2, and 4 of IV αGalCer and OTI T-cell injection. At day 5, serum ALT levels were measured, liver pathology was scored, and OTI T cells were isolated from the liver. After restimulation with SIINFEKL, intracellular cytokines were measured.

Serum Alanine Aminotransferase Determination

Blood was collected from the tail of mice, centrifuged at 5000g for 10 minutes, and the serum was extracted. Serum alanine aminotransferase (ALT) activity was determined in serum using ALT (SGPT) reagent set (colorimetreic method by Teco Diagnostics, Anaheim, CA) according to manufacturer’s instructions.

Histology

Liver tissue was fixed in 4% formalin (pH 7.0) for at least 24 hours and embedded in paraffin. Two-micrometer-thick paraffin sections were stained with H&E and were blindly scored by a pathologist (R. T. Bronson).

Statistical Analysis

IFN-γ production by OTI T cells, their absolute cell counts, serum ALT levels, and the frequencies of iNKT and NK cells were compared using the Mann-Whitney U test (Windows; Microsoft Corp, Redmond, WA; SPSS, Chicago, IL).

Results

CD8 T-Cell Priming in Tf-mOVA Mice

The self-antigen Tf-mOVA is expressed by hepatocytes and may be presented as peptide/class I MHC complexes to CD8 T lymphocytes in antigen-draining lymphoid tissues.[21] To determine in which anatomic location(s) OTI T-cell priming occurs in Tf-mOVA mice, we extracted DCs from different tissues and performed DC/T-cell cocultures (Figure 1*A*). DC from Tf-mOVA, but not wild-type mice, activated OTI T cells, as measured by CD69 and CD25 expression at 24 hours (Figure 1*A*). DC from Tf-mOVA mesenteric lymph nodes and spleen induced most OTI cell activation, whereas DC from liver and skin-draining lymph nodes (axillary and inguinal combined) induced more modest OTI T-cell activation.

To establish whether OTI T cells are activated in naïve Tf-mOVA mice in vivo, we adoptively transferred CFSE-labeled OTI T cells and analyzed their anatomic location and proliferation status at 36 hours post-transfer (Figure 1*B*). In the Tf-mOVA mice by far, most proliferating OTI T cells were recovered from the liver, and some were detected in the spleen.

CD8 T-Cell Priming Occurs in the Liver When Lymph Node Homing or Exit Is Blocked

L-selectin (CD62L) expression of lymphocytes is critical for their homing to peripheral lymph nodes, through binding to endothelial sulfated carbohydrate ligands in the high endothelial venules.[22] Injection of neutralizing antibody to L-selectin, MEL14, blocks lymph node homing,[22] whereas spleen-directed migration is L-selectin independent. To investigate where CD8 T-cell priming occurs, we transferred CFSE-labeled OTI T cells to splenectomized Tf-mOVA mice in which lymph node homing is blocked using MEL14. At 36 hours posttransfer, most OTI T cells (>96%) were recovered from livers regardless of antibody treatment (Figure 2*A*). Additionally, we made use of the immune-modulator FTY720, which induces the migration of lymphocytes into secondary lymphoid tissues and blocks their egress into efferent lymphatics.[23] Again, the majority of OTI T cells was recovered from livers of Tf-mOVA mice (Figure 2*B*, data are shown for 6 mice/group). A fraction of the cells (30%) had proliferated in the spleen, but only very few OTI T cells were present in lymph nodes. Thus, even though in vitro DC derived from lymph nodes of Tf-mOVA mice are potent OTI T-cell activators, in vivo OTI T cells are primarily primed in the liver.

Activation of iNKT Cells by αGalCer Does Not Potentiate Proliferation of Antigen-Specific CD8 T Cells

We hypothesized that rapid secretion of cytokines by iNKT cells may stimulate intrahepatic MHC-restricted T-cell responses. We therefore injected naïve CFSE-labeled OTI T cells into Tf-mOVA mice in the presence or absence of 1 single, low dose of αGalCer (100 ng/mouse). Vα14 iNKT-cell activation by αGalCer treatment did not significantly affect the intrahepatic proliferation rate of OTI T cells nor did it affect OTI proliferation in the lymph nodes or spleen (Figure 3).

Activation of iNKT Cells by αGalCer Enhances Effector Function of Liver-Resident OVA-Specific CD8 T Cells

Proliferation of T cells does not necessarily imply acquisition of effector cell capabilities. We therefore determined whether αGalCer injection potentiates the production of IFN-γ by liver-resident OTI T cells (Figure 4*A*). Coinjecting αGalCer with OTI T cells increased the frequency of liver-resident IFN-γ-producing OTI T cells from 5.1% (3.8%–13.5%) to 15.3% (6.5%–26.6%; $P = .017$). The potentiation of OTI T-cell function was mediated by iNKT cells because αGalCer did not promote IFN-γ production by OTI T cells in CD1d$^{-/-}$Tf-mOVA mice that lack iNKT cells: 6.5% (2.1%–11.7%; $P > .05$) (Figure 4*B*). Restimulation of OTI T cells with whole OVA (10 μg) as a negative control induced background IFN-γ levels.

To investigate further whether iNKT-cell activation facilitates intrahepatic effector function of OTI T cells in Tf-mOVA mice, in vivo cytolysis assays were performed. Coinjecting αGalCer with OTI T cells caused a profound increase in specific lysis of SIINFEKL-pulsed splenocytes in the liver (Figure 4*C*).

The activation of iNKT cells using αGalCer induces leukocyte recruitment to the liver, including NK cells, T cells, and iNKT cells.[8,24] Treatment of Tf-mOVA mice with αGalCer indeed induced a profound increase of intrahepatic leukocytes, and a large fraction consisted of NK cells and iNKT cells (Figure 5*A*). However, αGalCer treatment did not result in a significant increase in intrahepatic OTI T cells: coinjection of αGalCer changed the median OTI T-cell number in Tf-mOVA livers from 1.8×10^6 to 1.6×10^6 (day 5 post-transfer). From αGalCer-coinjected CD1d$^{-/-}$Tf-mOVA mice, we retrieved 1.4×10^6 intrahepatic OTI T cells ($P > .05$) (Figure 5*B*). Does αGalCer treatment induce apoptosis of recruited OTI cells? Only a small fraction of OTI T cells in livers of mice that did not receive αGalCer was undergoing apoptosis, which was only slightly increased after αGalCer treatment. Apoptotic OTI T cells were characterized by Vα2 costaining for active caspase 3 or the probe DEVD-FMK (Figure 5*C*). Taken together, in Tf-mOVA mice, iNKT-cell activation by αGalCer does not stimulate proliferation or liver-directed migration of antigen-specific CD8 T cells. Instead, iNKT-cell activation facilitates the intrahepatic effector function of OTI T cells.

Activation of iNKT Cells May Promote Hepatitis Induced by OTI T Cells

To determine whether iNKT-cell activation promotes liver damage in Tf-mOVA mice, we analyzed serum levels of ALT at 5 days post-OTI transfer in the presence or absence of

αGalCer (Figure 6*A*). Transfer of OTI T cells induced mild hepatitis (median ALT level, 37.5 IU/mL). Injection of αGalCer alone caused a modest increase of serum ALT levels in Tf-mOVA and WT mice, which is most likely due to nonspecific liver damage after activation of iNKT cells.[25] Coinjection of both factors in Tf-mOVA mice increased the median serum ALT level (57 IU/L; $P > .05$). Furthermore, severe hepatitis (>5× upper limit of normal) only occurred in Tf-mOVA mice that received both OTI and αGalCer. Analysis of liver histology revealed that activation of OTI T cells by transfer into Tf-mOVA mice induced severe hepatitis by day 5, regardless of αGalCer presence (Figure 6*B*). Treatment with αGalCer in the absence of OTI T cells caused only mild inflammation.

Neutralization of TNF-α and IFN-γ Function Inhibits αGalCer-Mediated Potentiation of Intrahepatic Antigen-Specific CD8 T Cells

Treatment with αGalCer induces iNKT cells to secrete TNF-α and IFN-γ.[26,27] Indeed, shortly after αGalCer injection, intrahepatic iNKT cells produced TNF-α and IFN-γ (Figure 7*A*). Is potentiation of intrahepatic OTI T-cell function by αGalCer injection mediated by TNF-α and IFN-γ? Antibody-mediated neutralization of both cytokines significantly reduced the frequency of intrahepatic IFN-γ-producing OTI T cells compared with levels observed using isotype control Ab (Figure 7*B*). Interestingly, only anti-TNF-α Ab and not anti-IFN-γ Ab pretreatment could prevent liver damage after transfer of OTI T cells + αGalCer as measured by increased serum ALT levels (Figure 7*B*) and liver damage (Figure 7*C*).

Discussion

We here describe the role of Vα14 iNKT cells in stimulating intrahepatic antigen-specific CD8 T-cell responses using a recently established mouse model that allows for study of intrahepatic immune responses in vivo.[18] The presence of the antigen on Tf-mOVA hepatocytes led to retention of antigen-specific CD8 T cells in the liver. These intrahepatic CD8 T cells were fully functional because they produced IFN-γ after brief restimulation with SIINFEKL, for induction of hepatitis in vivo. Using a different transgenic mouse model in which alloantigen was present within both liver and lymph nodes, it was recently suggested that the site of primary T-cell activation determines their functional faith: CD8 T cells activated in the periphery were able to cause liver injury, whereas intrahepatically activated T cells exhibited defective function.[28] As recently suggested by Derkow et al in their study describing the Tf-mOVA model,[18] we here show that, whereas Tf-mOVA antigen is only expressed in hepatocytes,[18] DCs in lymphoid organs presented this antigen to OTI T cells (Figure 1*A*). However, when lymph node homing and exit were blocked, the large majority of transferred CD8 T cells was recovered from livers. Thus, even though the antigen is present in the periphery, in Tf-mOVA mice, OVA-specific CD8 T cells are most likely primed in the liver, and this is in accordance with previous studies.[28–30]

Several studies have investigated the role of iNKT cells in intrahepatic immunity. αGalCer-activated iNKT cells can cause extensive liver damage[25] and inhibited tumor metastasis to the liver,[8,31] which involved the activation of tumor nonspecific CD8 T cells.[8,31] Very little was known, however, about whether iNKT cells mediate antigen-specific hepatitis whereby

antigen-specific conventional T cells instigate liver injury. Here, we demonstrate that activation of CD1d-restricted iNKT cells with αGalCer facilitates the effector function of intrahepatic antigen-specific CD8 T cells. Activation of iNKT cells tripled the frequency of intrahepatic IFN-γ-producing OTI T cells and increased their cytolytic capacity, contributing to liver damage.

αGalCer-mediated iNKT-cell activation did not appear to increase intrahepatic effector function of OTI T cells by promoting their proliferation or influx from the periphery. Neither could we demonstrate a switch in polarization phenotype of OTI T cells as measured by IL-4 production: regardless of αGalCer coinjection, only very few (<1%) intrahepatic OTI T cells produced IL-4 (data not shown). Instead, iNKT-cell activation influences the cytolytic ability and IFN-γ production of intrahepatic mOVA-specific CD8 T cells primed in the liver. Because αGalCer did not promote mOVA-specific IFN-γ production in CD1d$^{-/-}$mOVA mice, its enhancement of OTI T-cell effector function is CD1d dependent.

Surface expression of CD1d in liver is detected on hepatocytes,[32] DCs,[32] Kupffer cells,[33] and Ito cells.[34] Display by DCs of both CD1d/αGalCer and specific peptide-loaded class I MHC molecules can trigger iNKT cells to enhance T-cell responses to soluble antigen.[6] Kupffer cells and hepatic stellate cells are additional candidate cells that may mediate the CD8 T-cell stimulation we report here because these cells are potent Vα14 iNKT-cell activators.[33,34]

We show that αGalCer induces iNKT cells to produce TNF-α and IFN-γ. The production of these cytokines by iNKT cells can be crucial in mediating antimetastatic effects of αGalCer treatment.[26,27,35] In our study, pre-treatment with neutralizing antibodies to either anti-TNF-α or anti-IFN-γ reduced the frequency of IFN-γ-producing OTI T cells in liver to the levels observed in the absence of αGalCer. Therefore, both cytokines appear to be potent effectors of αGalCer-mediated stimulation of intrahepatic CD8 T cells, either directly or indirectly by stimulating surrounding lymphocytes such as NK cells.[8,31] It is yet unclear whether iNKT cell-derived cytokines stimulate intrahepatic CD8 T cells directly or through stimulation of antigen presentation by APCs such as Kupffer cells, Ito cells, and DCs. Importantly, anti-TNF-α treatment, but not anti-IFN-γ treatment, prevented serum ALT elevations and liver damage in all animals. This is in accordance with previous studies that suggest that activated iNKT cells use the production of IFN-γ and TNF-α as different effector tools, whereby especially the latter plays an essential role in the hepatic injury induced by αGalCer.[27,36]

The observed stimulatory effect of iNKT-cell activation on the intrahepatic antigen-specific immune response suggests the possibility to use αGalCer in immune-modulatory therapy for human conditions characterized by insufficient Th1 responses in the liver. αGalCer-mediated iNKT-cell activation may stimulate weak virus-specific immune responses generally observed in patients with chronic HBV and HCV infection (reviewed in Bertoletti and Ferrari[37]). However, as shown by others previously,[8,24,25] and also shown here, αGalCer-mediated iNKT-cell activation can cause liver injury. Moreover, use of especially repeated dosages of αGalCer somehow causes redirection of the immune response toward T helper cell 2 rather than T helper cell 1 reactivity (reviewed in Van Kaer[38]). Therefore,

several obstacles need to be overcome before αGalCer can be used as treatment to stimulate inadequate T helper cell 1 immune responses in human liver.

In conclusion, functional antigen-specific CD8 T cells can be activated in the liver. Stimulation of iNKT cells with 1 single low dose of αGalCer facilitates the intrahepatic effector function of these antigen-specific T cells. Activation of iNKT cells may stimulate effector function of antigen-specific CD8 T cells that are primed in the liver, leading to hepatitis and subsequent liver damage. Thus, cross talk between CD1d-restricted Vα14 iNKT cells and APCs in the liver may be critically involved in controlling intrahepatic MHC-restricted T-cell responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the Harvard Skin Disease Research Center (to M.B.); by The Netherlands Organization for Scientific Research (NWO; Vidi grant to H.J. and Veni grant to M.B.); by the Boehringer Ingelheim Fonds (to F.S.); by the Deutsche Forschungsgemeinschaft (to E.S.); by a Personal Research Chair from Mr James Bardrick (to G.S.B.), as a former Lister Institute-Jenner Research Fellow, the Medical Research Council, and The Wellcome Trust; and by the Foundation Trust Funds Erasmus University Rotterdam (to D.S.).

The authors thank the Boes laboratory for helpful discussions and Teresa Bianchi also for tail vein injections; Dr Mark Exley for providing CD1d$^{-/-}$ mice; and The NIH Tetramer Facility for supplying the CD1d tetramers.

Abbreviations used in this paper:

αGalCer	α-galactosylceramide
APC	antigen-presenting cell
CFSE	carboxyfluorescein diacetate succinimidyl ester
DC	dendritic cell
OVA	ovalbumin
iNKT cell	invariant natural killer T cell
Tf	transferrin

References

1. Brossay L, Burdin N, Tangri S, et al. Antigen-presenting function of mouse CD1: one molecule with two different kinds of antigenic ligands. Immunol Rev 1998;163:139–150. [PubMed: 9700507]
2. Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. Nat Rev Immunol 2002;2:557–568. [PubMed: 12154375]
3. Matsuda JL, Naidenko OV, Gapin L, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. J Exp Med 2000;192:741–754. [PubMed: 10974039]
4. Bendelac A, Rivera MN, Park SH, et al. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol 1997;15:535–562. [PubMed: 9143699]

5. Carnaud C, Lee D, Donnars O, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J Immunol 1999;163:4647–4650. [PubMed: 10528160]
6. Hermans IF, Silk JD, Gileadi U, et al. NKT cells enhance CD4+ and CD8+ T-cell responses to soluble antigen in vivo through direct interaction with dendritic cells. J Immunol 2003;171:5140–5147. [PubMed: 14607913]
7. Fujii S, Shimizu K, Smith C, et al. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T-cell immunity to a coadministered protein. J Exp Med 2003;198:267–279. [PubMed: 12874260]
8. Nakagawa R, Nagafune I, Tazunoki Y, et al. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice. J Immunol 2001;166:6578–6584. [PubMed: 11359810]
9. Smyth MJ, Crowe NY, Pellicci DG, et al. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. Blood 2002;99:1259–1266. [PubMed: 11830474]
10. Exley MA, Bigley NJ, Cheng O, et al. CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. J Leukoc Biol 2001;69:713–718. [PubMed: 11358978]
11. Grubor-Bauk B, Simmons A, Mayrhofer G, et al. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V α 14-J α 281 TCR. J Immunol 2003;170:1430–1434. [PubMed: 12538704]
12. Nieuwenhuis EE, Neurath MF, Corazza N, et al. Disruption of T helper 2-immune responses in Epstein-Barr virus-induced gene 3-deficient mice. Proc Natl Acad Sci U S A 2002;99:16951–16956.
13. Kakimi K, Lane TE, Chisari FV, et al. Cutting edge: inhibition of hepatitis B virus replication by activated NK T cells does not require inflammatory cell recruitment to the liver. J Immunol 2001;167:6701–6705. [PubMed: 11739482]
14. Kita H, Naidenko OV, Kronenberg M, et al. Quantitation and phenotypic analysis of natural killer T cells in primary biliary cirrhosis using a human CD1d tetramer. Gastroenterology 2002;123:1031–1043. [PubMed: 12360465]
15. Lucas M, Gadola S, Meier U, et al. Frequency and phenotype of circulating Vα24/Vβ11 double-positive natural killer T cells during hepatitis C virus infection. J Virol 2003;77:2251–2257. [PubMed: 12525661]
16. Nuti S, Rosa D, Valiante NM, et al. Dynamics of intra-hepatic lymphocytes in chronic hepatitis C: enrichment for Vα24+ T cells and rapid elimination of effector cells by apoptosis. Eur J Immunol 1998;28:3448–3455. [PubMed: 9842887]
17. Crispe IN, Giannandrea M, Klein I, et al. Cellular and molecular mechanisms of liver tolerance. Immunol Rev 2006;213:101–118. [PubMed: 16972899]
18. Derkow K, Loddenkemper C, Mintern J, et al. Differential priming of CD8 and CD4 T cells in animal models of autoimmune hepatitis and cholangitis. Hepatology 2007;46:1155–1165. [PubMed: 17657820]
19. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 2005;23:127–159. [PubMed: 15771568]
20. Hogquist KA, Jameson SC, Heath WR, et al. T-cell receptor antagonist peptides induce positive selection. Cell 1994;76:17–27. [PubMed: 8287475]
21. Lanzavecchia A, Sallusto F. Regulation of T-cell immunity by dendritic cells. Cell 2001;106:263–266. [PubMed: 11509174]
22. Gallatin WM, Weissman IL, Butcher EC. A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 1983;304:30–34. [PubMed: 6866086]
23. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004;427:355–360. [PubMed: 14737169]

24. Osman Y, Kawamura T, Naito T, et al. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. Eur J Immunol 2000;30:1919–1928. [PubMed: 10940881]
25. Fujii H, Seki S, Kobayashi S. A murine model of NKT cell-mediated liver injury induced by α-galactosylceramide/d-galactosamine. Virchows Arch 2005;446:663–673. [PubMed: 15906084]
26. Inui T, Nakagawa R, Ohkura S, et al. Age-associated augmentation of the synthetic ligand-mediated function of mouse NK1.1 ag(+) T cells: their cytokine production and hepatotoxicity in vivo and in vitro. J Immunol 2002;169:6127–6132. [PubMed: 12444115]
27. Inui T, Nakashima H, Habu Y, et al. Neutralization of tumor necrosis factor abrogates hepatic failure induced by α-galactosylceramide without attenuating its antitumor effect in aged mice. J Hepatol 2005;43:670–678. [PubMed: 15922476]
28. Bowen DG, Zen M, Holz L, et al. The site of primary T-cell activation is a determinant of the balance between intrahepatic tolerance and immunity. J Clin Invest 2004;114:701–712. [PubMed: 15343389]
29. Klein I, Gassel HJ, Crispe IN. Cytotoxic T-cell response following mouse liver transplantation is independent of the initial site of T-cell priming. Transplant Proc 2006;38:3241–3243. [PubMed: 17175235]
30. Wuensch SA, Pierce RH, Crispe IN. Local intrahepatic CD8+ T-cell activation by a non-self-antigen results in full functional differentiation. J Immunol 2006;177:1689–1697. [PubMed: 16849478]
31. Nakagawa R, Inui T, Nagafune I, et al. Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by α-galactosylceramide. J Immunol 2004;172:6550–6557. [PubMed: 15153469]
32. Trobonjaca Z, Leithauser F, Moller P, et al. Activating immunity in the liver. I. Liver dendritic cells (but not hepatocytes) are potent activators of IFN-γ release by liver NKT cells. J Immunol 2001;167:1413–1422. [PubMed: 11466360]
33. Schmieg J, Yang G, Franck RW, et al. Glycolipid presentation to natural killer T cells differs in an organ-dependent fashion. Proc Natl Acad Sci U S A 2005;102:1127–1132. [PubMed: 15644449]
34. Winau F, Hegasy G, Weiskirchen R, et al. Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity 2007;26:117–129. [PubMed: 17239632]
35. Hayakawa Y, Takeda K, Yagita H, et al. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. Eur J Immunol 2001;31:1720–1727. [PubMed: 11385616]
36. Biburger M, Tiegs G. α-Galactosylceramide-induced liver injury in mice is mediated by TNF-α but independent of Kupffer cells. J Immunol 2005;175:1540–1550. [PubMed: 16034092]
37. Bertoletti A, Ferrari C. Kinetics of the immune response during HBV and HCV infection. Hepatology 2003;38:4–13. [PubMed: 12829979]
38. Van Kaer L α-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. Nat Rev Immunol 2005;5:31–42. [PubMed: 15630427]

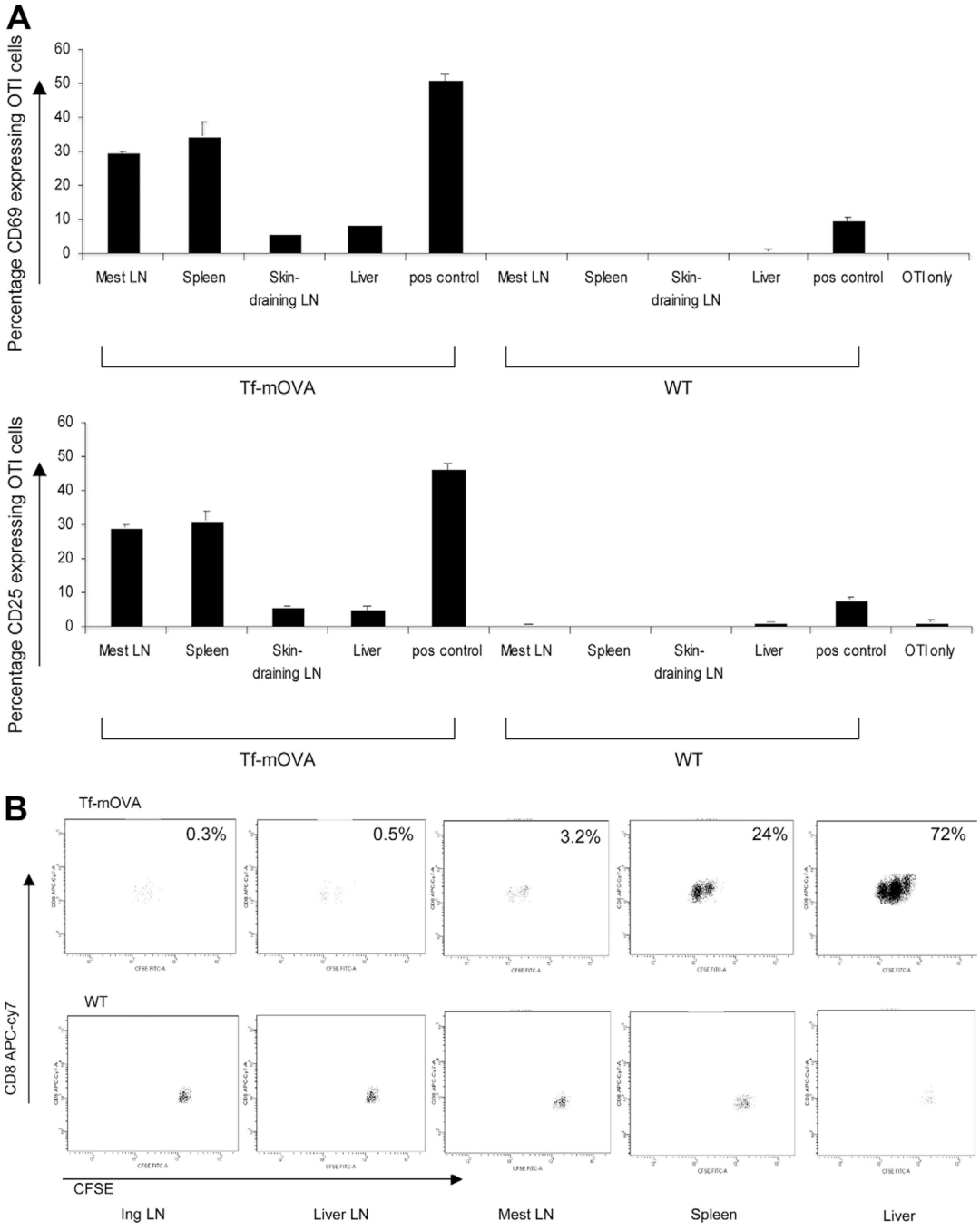

Figure 1.

Activation of OTI T cells in a mouse expressing mOVA as liver-antigen. (*A*) Analysis of OTI T cell activation in vitro. OTI T cells were cultured with DCs from Tf-mOVA mice or wild-type mice and were analyzed for activation markers at 24 hours. Tf-mOVA DCs but not wild-type DCs from liver, spleen, and lymph nodes were able to activate OTI T cells in the absence of added OVA antigen, as measured by up-regulation of CD69 and CD25. Data shown of a representative experiment of 3 experiments using 4 mice per experiment. (*B*) Analysis of OTI T-cell activation in vivo. CFSE-labeled OTI T cells were transferred

into wild-type or Tf-mOVA mice (4×10^6 T cells/mouse) and analyzed for OTI T-cell homing and proliferation in lymph nodes, spleen, and liver at 36 hours. In the Tf-mOVA mice (*top panels*), most proliferating OTI T cells were recovered from liver, whereas few OTI T cells were found in mesenteric (*Mest LN*) inguinal (*Ing LN*), and the liver-draining portal lymph node (*Liver LN*). *Numbers* depicted in the *upper right corner* of each *dot plot* reflect the percentage of OTI T cells present of OTI T cells recovered from all compartments combined. In WT mice (*bottom panels*), OTI T cells did not proliferate and were mostly recovered from the lymph nodes. Data shown are from a representative experiment of 5 experiments performed with 4 mice each.

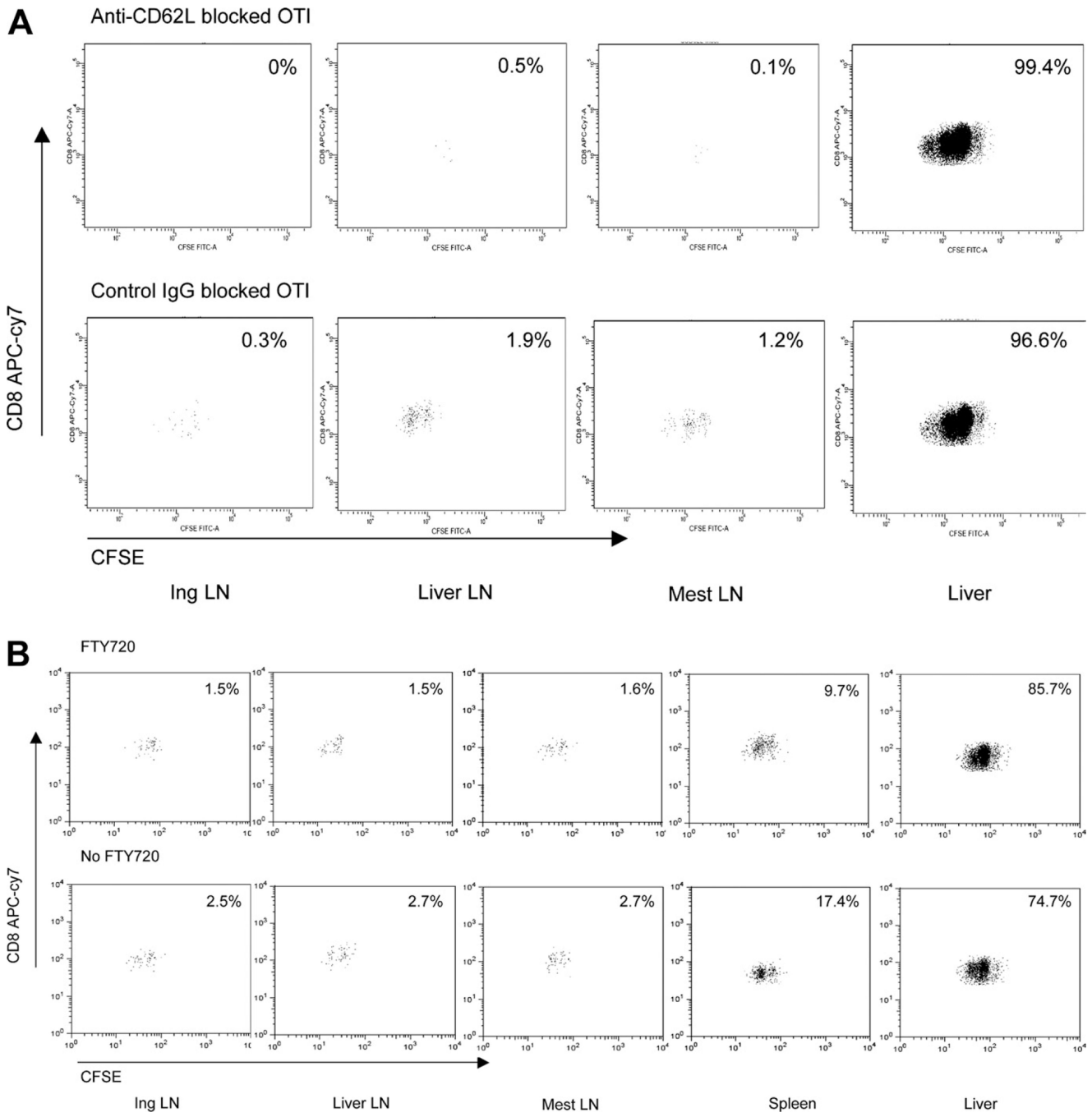

Figure 2.

CD8 T-cell priming is unaffected when lymph node homing or exit is blocked. (*A*) To block lymph node homing of T cells, splenectomized Tf-mOVA mice were treated with MEL14 antibody (anti-CD62L) or isotype control antibody (control IgG) and injected with 4×10^6 CFSE-labeled OTI T cells that were preincubated with MEL14 or control antibody. The *percentage* in the *upper right corner* of each *dot plot* reflects the fraction of OTI T cells present in liver, inguinal (*Ing LN*) and mesenteric (*Mest LN*) lymph nodes, and the liver-draining portal lymph node (*Liver LN*) of the total OTI T cells recovered. Data shown

are from a representative experiment of 3 experiments with 4 mice each. (*B*) To block lymph node exit of T cells, Tf-mOVA mice were treated with FTY720 and injected with 4 × 10^6 CFSE-labeled OTI T cells. The *percentage* in the *upper right corner* of each *dot plot* reflects the fraction of OTI T cells present in each organ, as described above. Data shown are from a representative experiment of 3 experiments with 4 mice each. A control experiment showing the functionality of the FTY720 reagent is shown in Supplemental Data S1 (see Supplemental Data S1 online at www.gastrojournal.org).

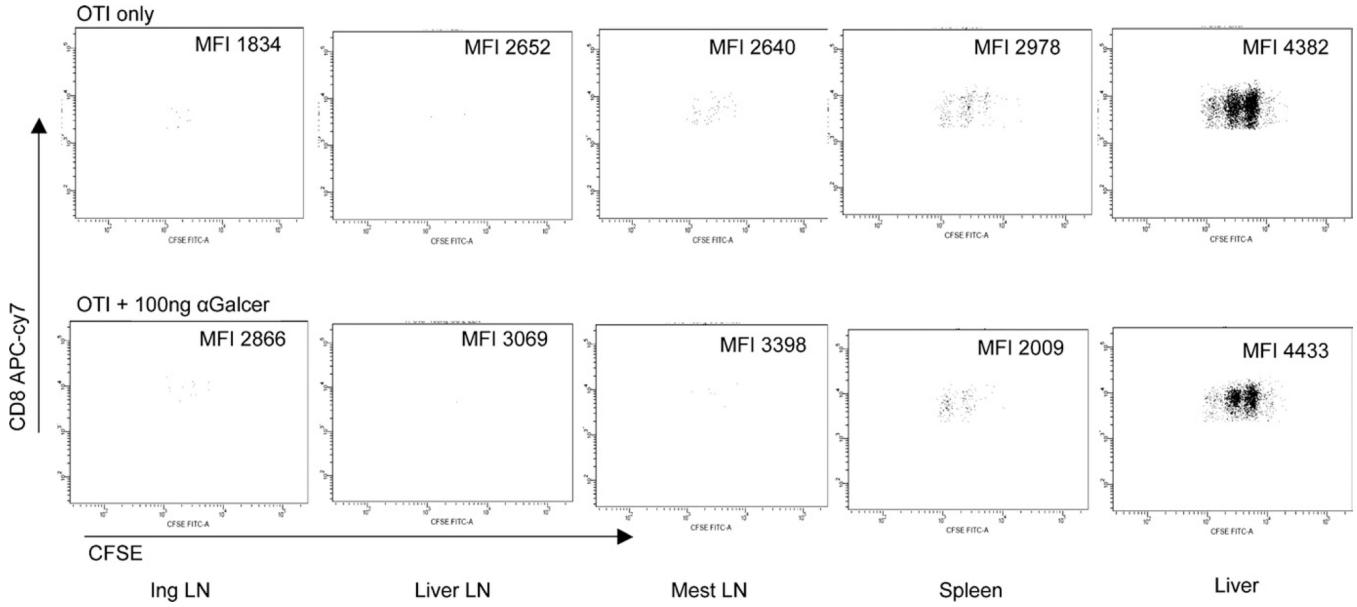

Figure 3.

OTI T-cell proliferation in Tf-mOVA mice is unaffected by activation of iNKT cells using αGalCer. CFSE-labeled OTI T cells were injected into Tf-mOVA mice in the presence (*top panels*) or absence (*bottom panels*) of αGalCer. The *number* in the top right corner of each *dot plot* reflects the CFSE mean fluorescence intensity (MFI) of OTI T cells recovered from the organs indicated below each plot at 36 hours after transfer. Data shown are from a representative experiment of 5 experiments with 4 mice each.

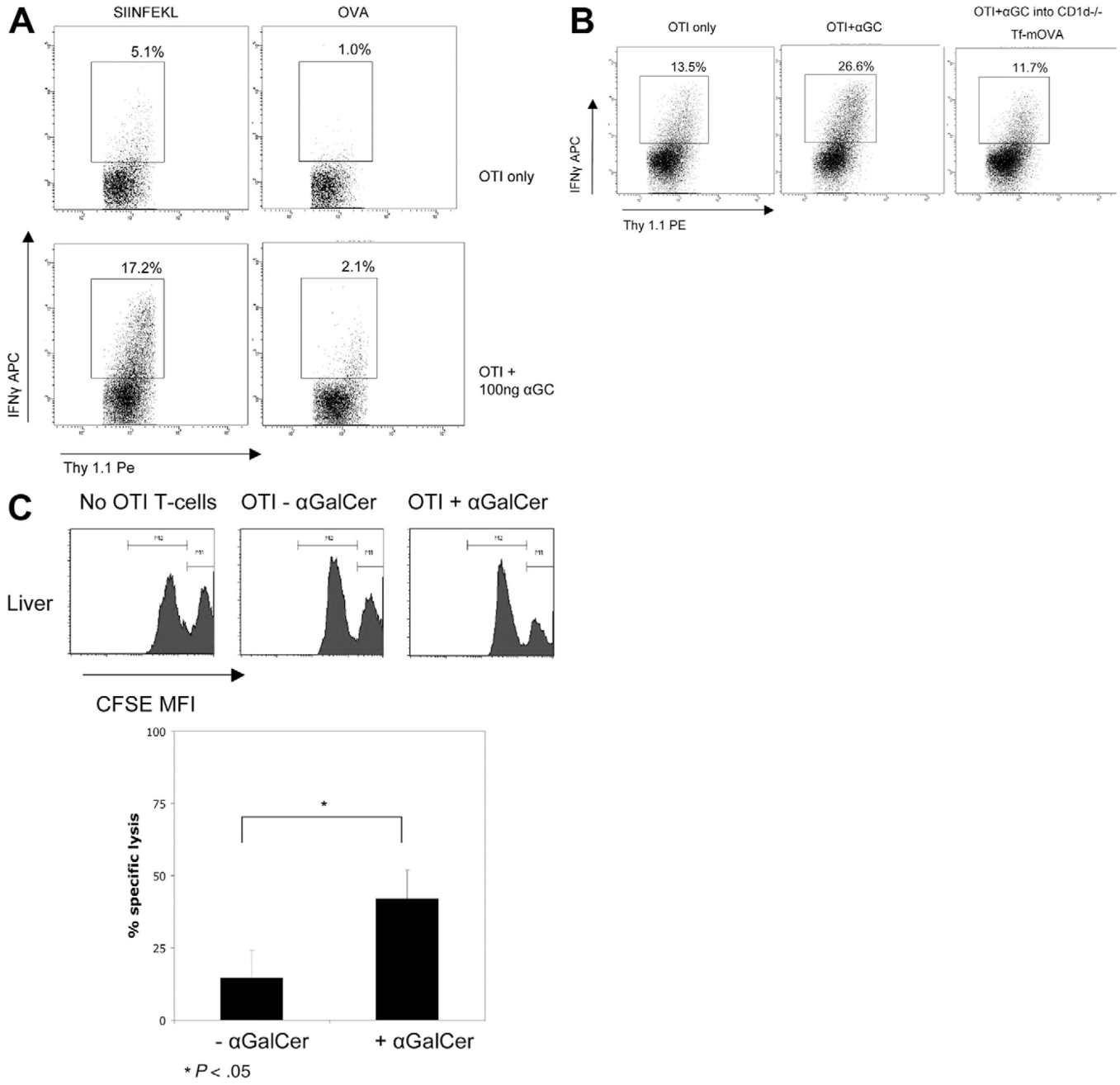

Figure 4.

Activation of Vα14 iNKT-cell using αGalCer potentiates mOVA-specific effector function of OTI T cells. (*A*) Five days after OTI T-cell transfer ± αGalCer into Tf-mOVA mice, the percentage of liver-derived IFN-γ-producing OTI T cells was determined by intracellular cytokine staining. Data shown are of a representative experiment, of 6 experiments with 4 mice each. (*B*) Treatment with αGalCer does not increase the percentage liver-derived IFN-γ-producing OTI cells isolated from CD1d$^{-/-}$Tf-mOVA mice lacking CD1d-restricted iNKT cells. Data shown are of 1 representative experiment of 3 performed with 6 mice each. (*C*) For in vivo cytolysis assays, 4 million OTI T cells ± αGalCer were transferred

into Tf-mOVA mice at day 0. Lysis of SIINFEKL-loaded splenocytes by OTI T cells in the liver was measured at day 3. Antigen-specific cytolysis was calculated as described in the Materials and Methods section. *Top*: CFSE staining of representative experiment. *Bottom*: medians ± SD from 6 mice per group.

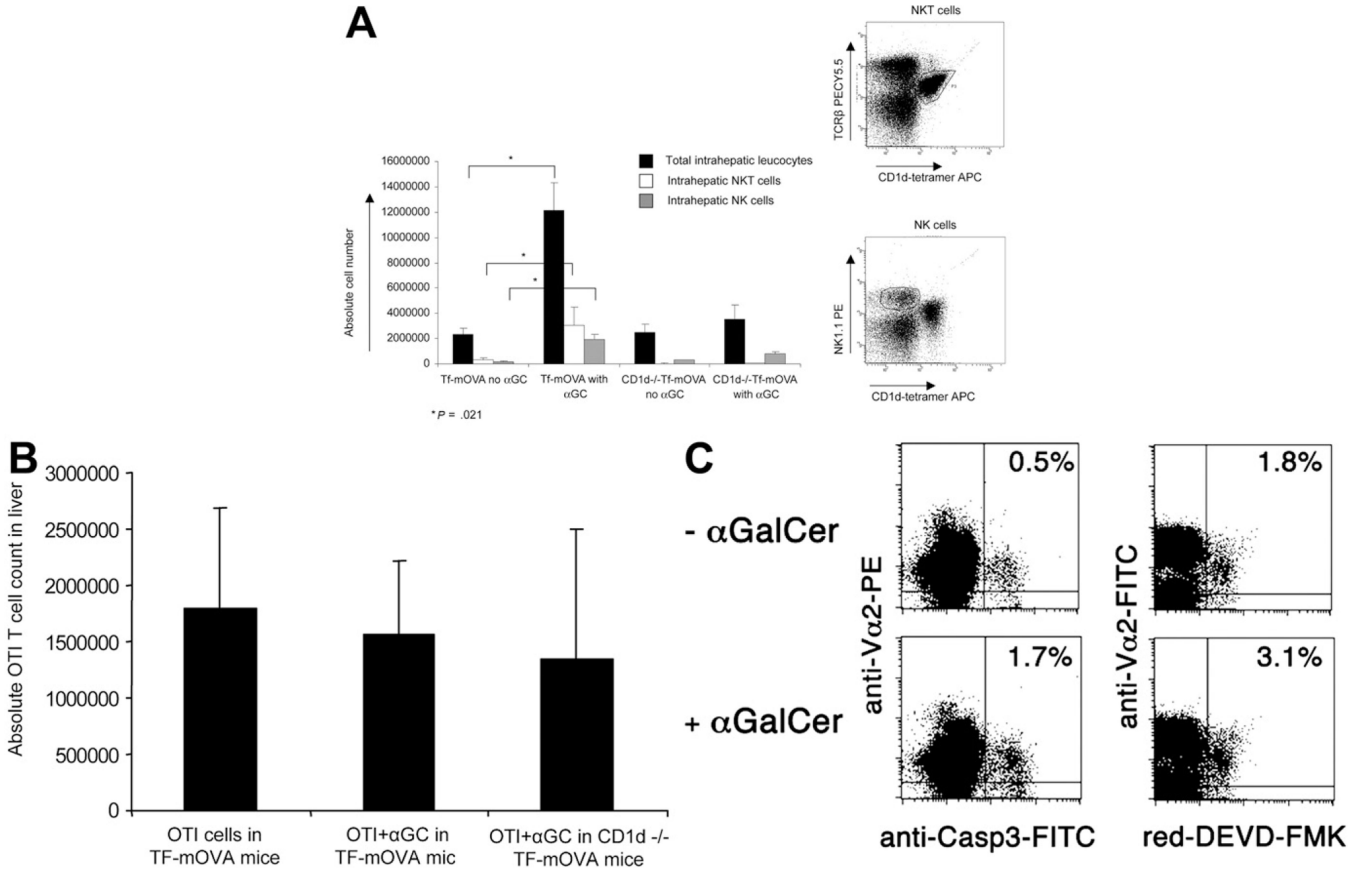

Figure 5.

iNKT-cell-mediated increase in leukocyte numbers in livers of Tf-mOVA mice after transfer of OTI T cells ± αGalCer. (*A*) α-GalCer was injected in Tf-mOVA and CD1d$^{-/-}$Tf-mOVA mice, and, 5 days later, the absolute numbers of total lymphocytes, Vα14 iNKT cells (TCRβ^{+} and CD1d-tetramer^{+}; gated population in *top right panel*), and NK cells (NK1.1^{+} and CD1d-tetramer^{-}; gated population in *bottom right panel*) in the liver were compared with their counterparts in the livers of mice that had not received αGalCer. Data shown are medians ± SD of 4 mice per group. α-GalCer treatment induced a significant influx of Vα14 iNKT cells and NK cells ($P = .021$), which was absent in CD1d$^{-/-}$Tf-mOVA mice. (*B*) OTI cells ± αGalCer were transferred into Tf-mOVA or CD1d$^{-/-}$Tf-mOVA mice, and, 5 days later, their absolute intrahepatic cell counts were determined. Shown are medians ± SD of 6 mice per group. (*C*) OTI cells ± αGalCer were transferred into Tf-mOVA mice, and, at day 3, the frequencies of intrahepatic apoptotic OTI cells were determined by analyzing caspase-3 activity. OTI cells were defined as CD8^{+} Vα2^{+} cells, and the *dot plots* show representative data of 3 experiments, after gating on the CD8^{+} cell population. At day 5 after transfer of OTI T cells in the presence or absence of αGalCer, caspase-3 activity was minimal and no significant difference was observed (data not shown).

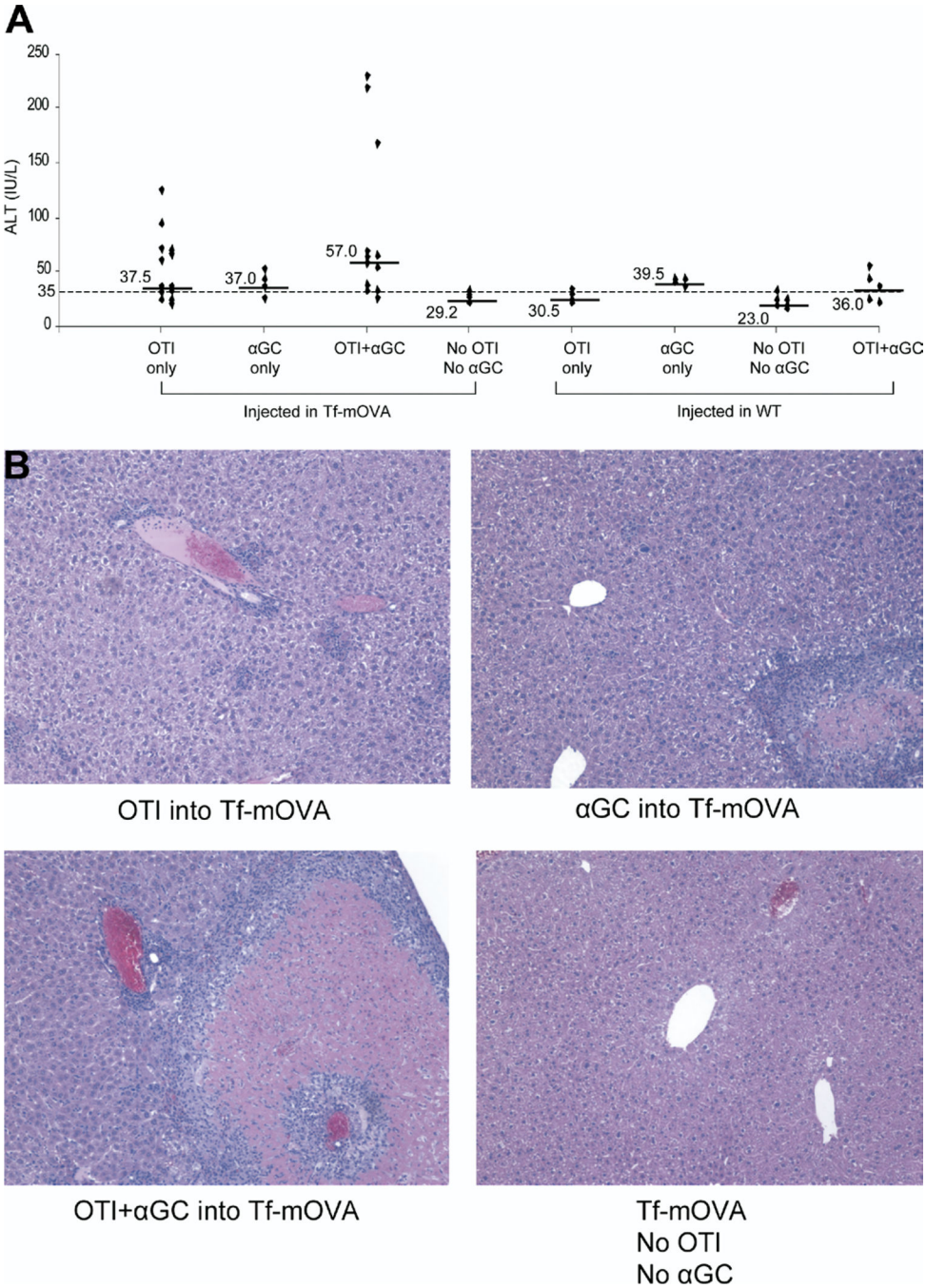

Figure 6.

Analysis of liver damage induced by transfer of OTI T cells in Tf-mOVA mice. (*A*) Analysis of serum ALT levels 5 days after the transfer of 4×10^6 OTI T cells in the presence or absence of αGalCer in Tf-mOVA and wild-type mice. The *dotted line* represents the upper limit of normal (35 IU/L). The *dots* represent ALT levels of individual mice, and the *small lines* with the corresponding *numbers* represent the median ALT level of a population. (*B*) Analysis of liver histology by H&E staining at 5 days posttransfer of OTI cells ± αGalCer. The *photographs* shown are representative images of liver tissue obtained from 3 mice per

group. In Tf-mOVA mice that received OTI T cells ± αGalCer, hepatitis was induced that involved mixed inflammatory cell infiltrates especially in the portal areas. α-GalCer without OTI cells caused intrahepatic infarction but only mild inflammation. In Tf-mOVA mice that received neither OTI cells nor αGalCer, no hepatitis or infarction was observed.

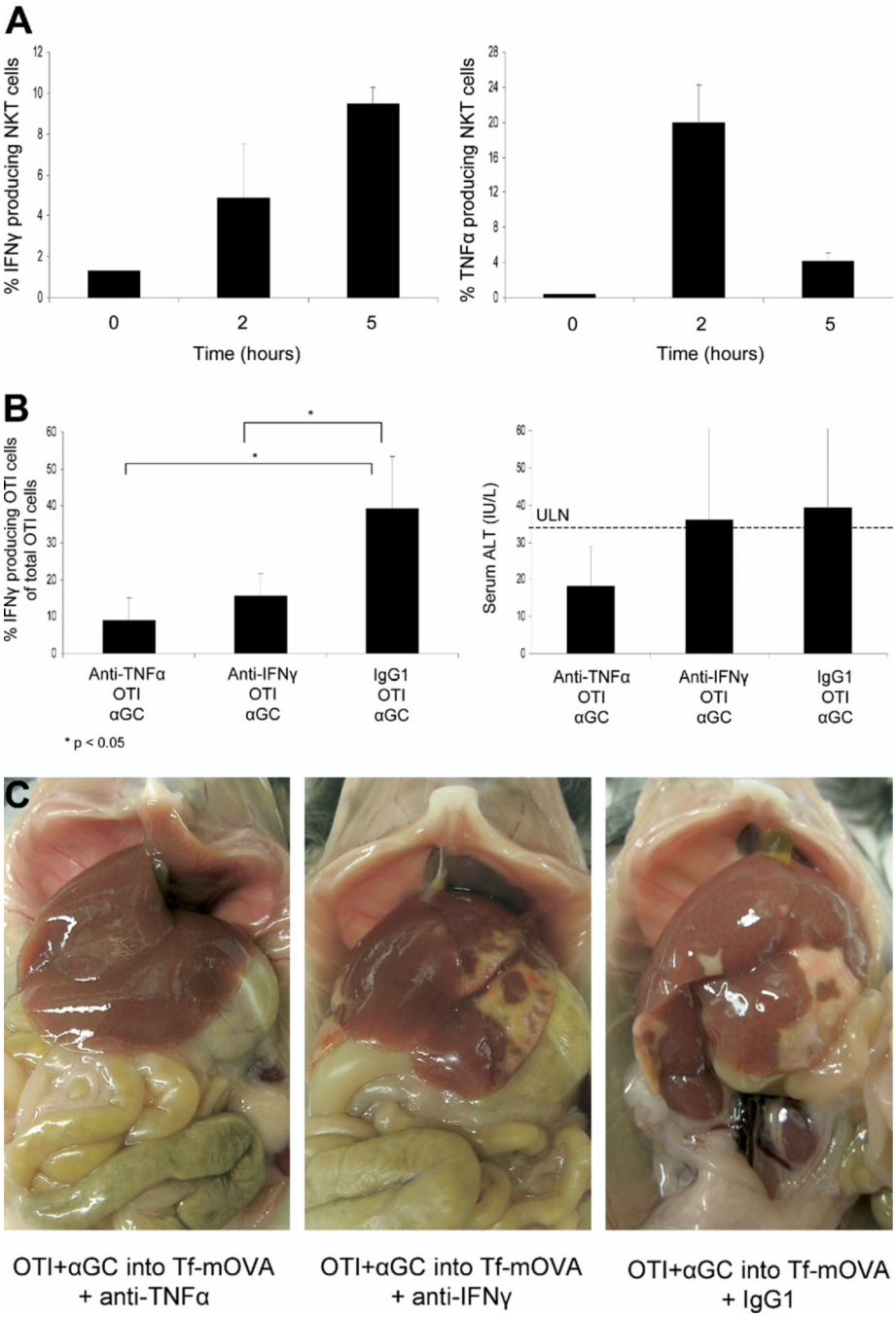

Figure 7.

Pretreatment with neutralizing antibodies to TNF-α and IFN-γ inhibits αGalCer-mediated potentiation of effector function of liver-resident OTI T cells. (*A*) Wild-type mice were treated with αGalCer, and liver-derived Vα14 iNKT cells were analyzed for production of TNF-α and IFN-γ by intracellular cytokine staining at 2 and 5 hours. Shown are medians ± SD of 10 mice per group. (*B*) *Left panel.* Tf-mOVA mice were pretreated with anti-TNF-α, anti-IFN-γ, and isotype control Ab prior to transfer of OTI cells + αGalCer, and, 5 days later, the percentage of liver-derived IFN-γ-producing OTI T cells was determined

by intracellular cytokine staining. Shown are medians ± SD of 4 mice per group of 1 representative experiment of 2 performed. *Right panel.* Additionally, in these mice, serum ALT levels were determined. (ULN, upper limit of normal). (*C*) Livers of mice were scored blindly by 2 individuals for the appearance of liver damage, as visible macroscopically. Shown are representative images of mice treated with anti-TNF-α, anti-IFN-γ, and isotype control Ab, as indicated (6 mice for each group).