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# Ito Cells Are Liver-Resident Antigen-Presenting Cells for Activating T Cell Responses

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## SUMMARY

Here we identified Ito cells (hepatic stellate cells, HSC), known for storage of vitamin A and participation in hepatic fibrosis, as professional liver-resident antigen-presenting cells (APC). Ito cells efficiently presented antigens to CD1-, major histocompatibility complex (MHC)-I-, and MHC-II-restricted T cells. Ito cells presented lipid antigens to CD1-restricted T lymphocytes such as natural killer T (NKT) cells and promoted homeostatic proliferation of liver NKT cells through interleukin-15. Moreover, Ito cells presented antigenic peptides to CD8<sup>+</sup> and CD4<sup>+</sup> T cells and mediated crosspriming of CD8<sup>+</sup> T cells. Peptide-specific T cells were activated by transgenic Ito cells presenting endogenous neoantigen. Upon bacterial infection, Ito cells elicited antigen-specific T cells and mediated protection. In contrast to other liver cell types that have been implicated in induction of immunological tolerance, our data identify Ito cells as professional intrahepatic APCs activating T cells and eliciting a multitude of T cell responses specific for protein and lipid antigens.

## INTRODUCTION

Ito cells were first described as hepatic stellate cells (HSC) by the German anatomist Carl von Kupffer in 1876 and were later defined as fat-storing cells by the Japanese anatomist Toshio Ito in 1951 (Geerts, 2001). Ito cells are located in the liver in the space of Disse, which extends from endothelial cells lining the sinusoids to the hepatocyte parenchyma, and display an astral phenotype characterized by several processes (Geerts, 2001). Kupffer cells, which reflect liver-resident macrophages, and dendritic cells (DCs) represent different entities from Ito cells and are mainly located within the sinusoids (Jomantaite et al.,

2004). Although the origin of Ito cells is still a matter of vivid debate (Geerts, 2004), recent work demonstrated their development from mesenchymal origin (Baba et al., 2004). However, Ito cells are defined by expression of ectodermal neural markers such as glial fibrillary acidic protein (GFAP) (Neubauer et al., 1996). Ito cells account for 8% of liver cells and store 80% of total body retinol (vitamin A) as cytoplasmic lipid droplets (Geerts, 2001). In response to cellular stress, Ito cells transdifferentiate into myofibroblasts and produce extracellular matrix including collagen. Therefore, they have been implicated in the development of liver fibrosis and subsequent cirrhosis (Gressner et al., 2002). Moreover, Ito-derived myofibroblasts are contractile upon stimulation with vasoactive substances, suggesting a role in pathogenesis of portal hypertension (Reynaert et al., 2002).

Natural killer T (NKT) cells represent a distinct subgroup of T lymphocytes expressing an invariant T cell receptor (TCR) (Kronenberg, 2005). NKT cells recognize the endogenous lipid antigen isoglobotriaosylceramide (iGb3) (Zhou et al., 2004), multiple exogenous ligands (Mattner et al., 2005; Kinjo et al., 2005) such as mycobacterial phosphatidylinositol-mannoside (PIM) (Fischer et al., 2004), and  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) (Kawano et al., 1997) presented by CD1d molecules. The family of antigen-presenting CD1 proteins is related to MHC-I and consists of group I CD1a-c and group II CD1d on the basis of sequence homology (Moody and Porcelli, 2003). Whereas CD1a-c are expressed mainly in humans and present lipids of microbial origin to T lymphocytes, CD1d exists in both mice and humans and displays endogenous as well as bacterial lipids exclusively to NKT cells (Brigl and Brenner, 2004; Mattner et al., 2005). After lipid antigen presentation, NKT cells rapidly secrete large amounts of cytokines such as interferon- $\gamma$  (IFN $\gamma$ ) and interleukin-4 (IL-4) to perform immunoregulatory functions (Brigl and Brenner, 2004). Accordingly, NKT cells exhibit protective functions in infection (Brigl et al., 2003) and tumor models (Cui et al., 1997) and are capable of precluding autoimmune diseases such as diabetes (Hong et al., 2001) and multiple sclerosis (Miyamoto et al., 2001).

NKT cells predominate among liver lymphocytes (Crispe, 2003), so we searched for the determinants that render liver tissue such a favorable milieu for NKT cells. We identified Ito cells to be capable of inducing vigorous NKT cell responses *in vitro* and *in vivo* and promoting homeostatic proliferation of NKT cells through production of IL-15. Moreover, we found that Ito cells processed protein antigens and presented peptides to CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In an infection model with *Listeria monocytogenes*, Ito cells elicited antigen-specific T cells and protected against infection. Intrinsic production of neoantigen caused peptide-specific T cell activation by transgenic Ito cells. Thus, Ito cells are professional APCs in immunology.

## RESULTS

### Ito Cells Express CD1 Molecules and APC Surface Markers

In order to investigate the impact of Ito cells on NKT cells, we isolated Ito cells from liver of C57BL/6 mice via the pronase and collagenase perfusion method and subsequent density gradient centrifugation. In parallel, we performed experiments in the human system generating Ito cells from human liver biopsies. Figure 1A shows the star-shaped phenotype of murine and human Ito cells by phase contrast microscopy. Notably, 3 days after isolation, murine Ito cells exhibited several processes and lucent cytoplasmic vesicles indicating vitamin A-filled lipid droplets (Figure 1A). To determine purity of Ito cell preparations and absence of contaminating cells, we stained cell preparations intracellularly for GFAP, a neural marker protein that is expressed in the liver exclusively by Ito cells (Neubauer et al., 1996). Confocal microscopy revealed the filamentous distribution of GFAP in murine as well as in human Ito cells (Figure 1B). Surface expression of CD1d on stimulating cells is a prerequisite for NKT cell activation. Consequently we performed flow cytometric analysis of Ito cell-surface proteins. Indeed, Ito cells expressed CD1d molecules at high cell-surface density (Figure 1C). Moreover, murine and human Ito cells expressed the antigen-presenting molecule MHC-II and costimulatory molecules such as CD86 (B7-2), which are characteristic markers for professional APCs (Figure 1C). In contrast, murine Ito cells failed to express CD11c, which serves as a DC surface marker underlining the distinct entity of Ito cells (Figure 1C). Human Ito cells expressed group I CD1 molecules as indicated by CD1b surface staining (Figure 1C), thus far exclusively confined to DCs (Brigl and Brenner, 2004).

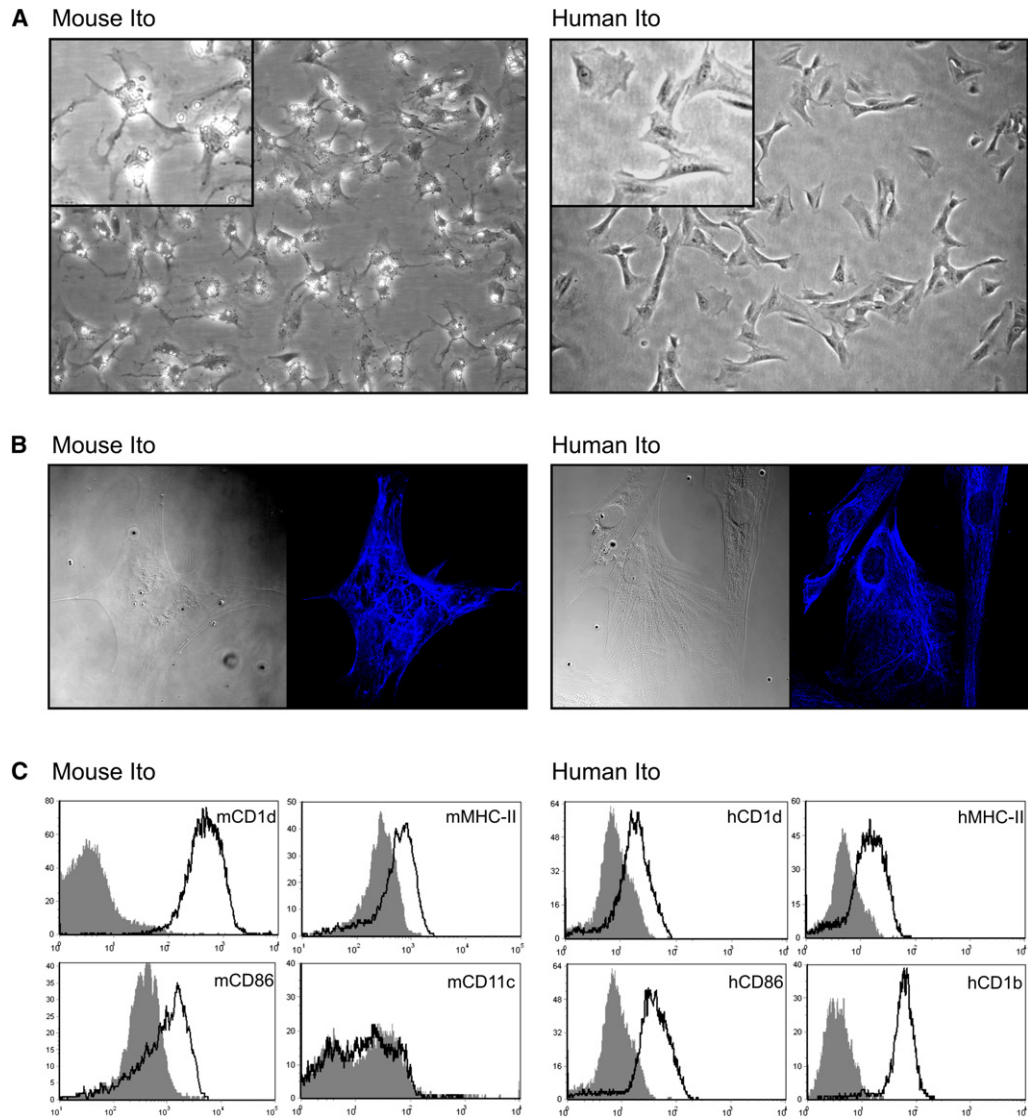
Because APCs are able to mature as indicated by upregulation of characteristic surface molecules, we analyzed Ito cells for their maturation potential. To this end, we stimulated Ito cells with IFN $\gamma$  (500 U/ml) for 3 days and analyzed surface expression of MHC-I and MHC-II (see Figure S1 in the Supplemental Data available online). Indeed, both antigen-presenting molecules were upregulated upon stimulation with IFN $\gamma$ , indicating maturation ability of Ito cells.

To confirm purity of Ito cell preparations, we stained cells for expression of GFAP and F4/80, a surface protein expressed by macrophages and Kupffer cells. Flow cytometric analysis revealed high expression of GFAP in  $\geq 98.5\%$  of cells in Ito cell preparations (Figure S2A, left). Cells staining positive for F4/80 were absent in Ito cell preparations (Figure S2A, right). Combination of positive staining for GFAP and negative staining for CD11c and F4/80 excluded contamination of Ito cell preparations by other liver cell types known to be APCs for activating T cell responses. In addition, we analyzed Kupffer cell phenotype in comparison to Ito cells. Kupffer cells were identified in fresh liver cell preparations by positive staining for F4/80 and were analyzed for expression of APC surface molecules. Kupffer cells highly expressed F4/80 and MHC-I. Expression of MHC-II, CD1d, and CD86 was detected only at low amounts (Figure S2B). Taken together, Ito cells display traits of APCs and highly express CD1 molecules.

### Ito Cells Induce Vigorous NKT Cell Responses

Since Ito cells expressed CD1d molecules, we examined the functional impact of Ito cells on NKT cell stimulation. To this end, we used murine Ito cells or DCs as APCs in functional T cell assays. After pulsing with the potent NKT cell antigen  $\alpha$ -GalCer, Ito cells or DCs were washed and cocultured with purified NKT cells isolated from thymus, spleen, or liver of V $\alpha$ 14-transgenic animals by negative selection as described previously (Matsuda et al., 2002). Ito cells loaded with  $\alpha$ -GalCer induced prominent proliferation of NKT cells from all three organs (Figure 2A). Addition of CD1d-blocking antibody 1 hr before coculture with T cells totally abrogated NKT cell activation. Moreover, Ito cells isolated from *Cd1d1*<sup>-/-</sup> mice failed to stimulate NKT cells after loading with lipid antigen (Figure 2A). Thus, murine Ito cells potently activated NKT cells in a CD1d-restricted fashion. In an analogous approach, we pulsed human Ito cells or DCs with  $\alpha$ -GalCer to stimulate a human V $\alpha$ 24<sup>+</sup> NKT cell line. Consistent with the findings in the murine system, lipid antigen-loaded human Ito cells activated NKT cells (Figure 2B). Blocking CD1d with a specific antibody abolished NKT cell antigen recognition (Figure 2B). Incubation of NKT cells alone with  $\alpha$ -GalCer did not induce T cell activation, excluding any impact of potentially contaminating APCs from primary T cell preparations (data not shown). In addition, we analyzed IFN $\gamma$  concentrations in cell-culture supernatants from experiments described above with ELISA. Indeed, stimulatory capacity of APCs was reflected by antigen-specific IFN $\gamma$  secretion of NKT cells (Figures S3A and S3B). Thus, both human and murine Ito cells are capable of inducing CD1d-restricted NKT cell responses.

Based on these *in vitro* findings, we questioned whether Ito cells also function as APCs *in vivo*. For this purpose, Ito cells were isolated from wild-type and *Cd1d1*<sup>-/-</sup> mice, pulsed with  $\alpha$ -GalCer, and subsequently transferred to CD1-deficient recipients. Since *Cd1d1*<sup>-/-</sup> animals lack endogenous NKT cells, we adoptively transferred CFSE-labeled NKT cells from V $\alpha$ 14-transgenic mice to



**Figure 1. Ito Cells Exhibit Traits of APCs and Express CD1d Molecules**

(A) Ito cells display a star-shaped phenotype. Phase-contrast microscopy demonstrates the morphology of murine and human Ito cell preparations. Inserts visualize lucent cytoplasmic lipid droplets at higher magnification.

(B) Ito cells are defined by the expression of GFAP. Ito cells were fixed, permeabilized, and stained with anti-GFAP. After incubation with a secondary antibody coupled to Cy5, cells were analyzed by confocal microscopy. Right panels show Cy5-emitted fluorescence intensity and left panels represent phase-contrast images of the same cells.

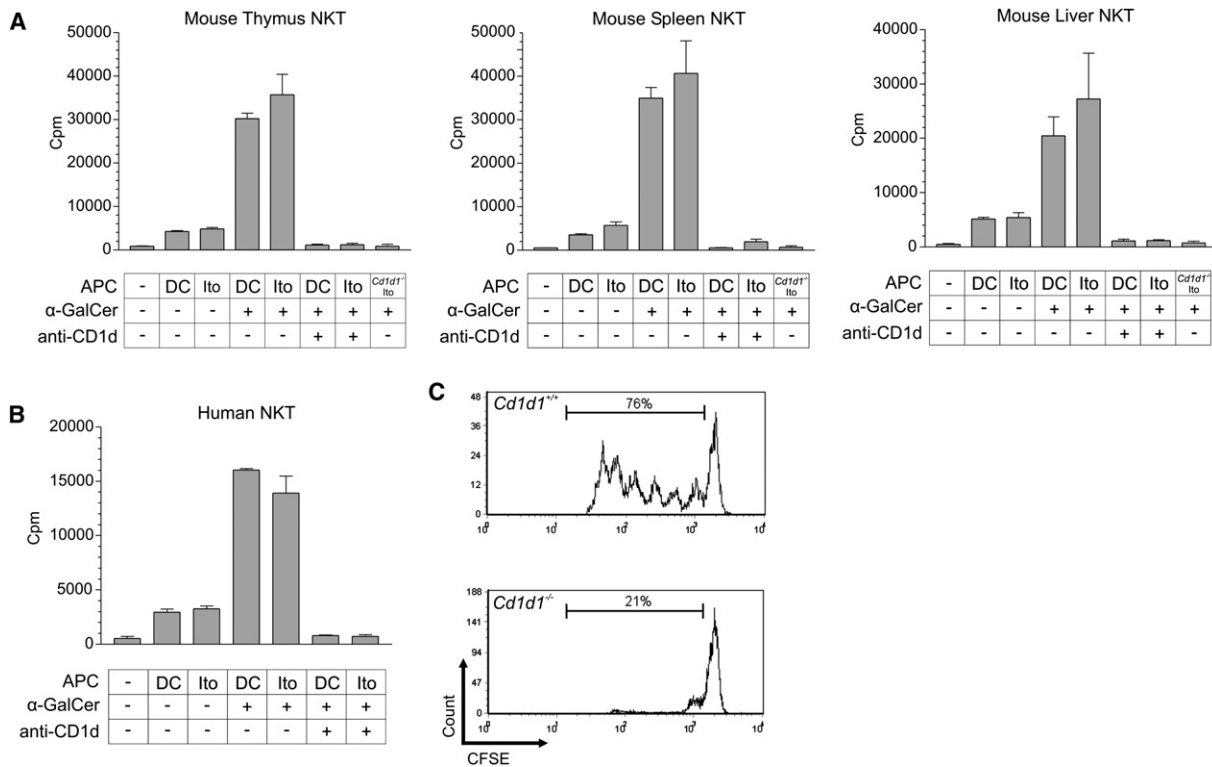
(C) Flow cytometric analysis of APC molecules expressed on Ito cells. Murine and human Ito cells were stained with antibodies against CD1d, MHC-II, and CD86. Additionally, mouse Ito cells were stained with anti-CD11c, and human Ito cells were incubated with anti-CD1b. Histograms indicate increases in fluorescence intensity resulting from specific surface molecule detection (black curves) compared to isotype controls (gray filled curves). Data are representative of at least three independent experiments with similar results.

CD1-deficient recipients in parallel with Ito cells. Because CD1-deficient recipients fail to present lipids, any effect of CD1d-restricted antigen presentation in this transfer system cannot be attributed to any other cell than donor Ito cells. 3 days after adoptive transfer, liver lymphocytes from recipient mice were isolated and NKT cells were identified with CD1d tetramers. Histograms in Figure 2C demonstrate decrease of CFSE fluorescence intensity indicating cell division of NKT cells gated on CD1d-tetramer<sup>+</sup> cells. Transfer of  $\alpha$ -GalCer-pulsed Ito cells

from wild-type mice induced antigen-specific NKT cell proliferation *in vivo*, whereas  $\alpha$ -GalCer-pulsed Ito cells from CD1-deficient animals failed to stimulate NKT cells (Figure 2C). Thus, Ito cells exhibit APC function for NKT cells *in vivo*.

#### Ito Cells Produce IL-15 for Homeostatic NKT Cell Proliferation

Interleukin-15 (IL-15) plays an essential role in NKT cell, NK cell, and memory CD8<sup>+</sup> T cell survival (Grabstein



**Figure 2. Ito Cells Induce Vigorous CD1d-Restricted NKT Cell Responses**

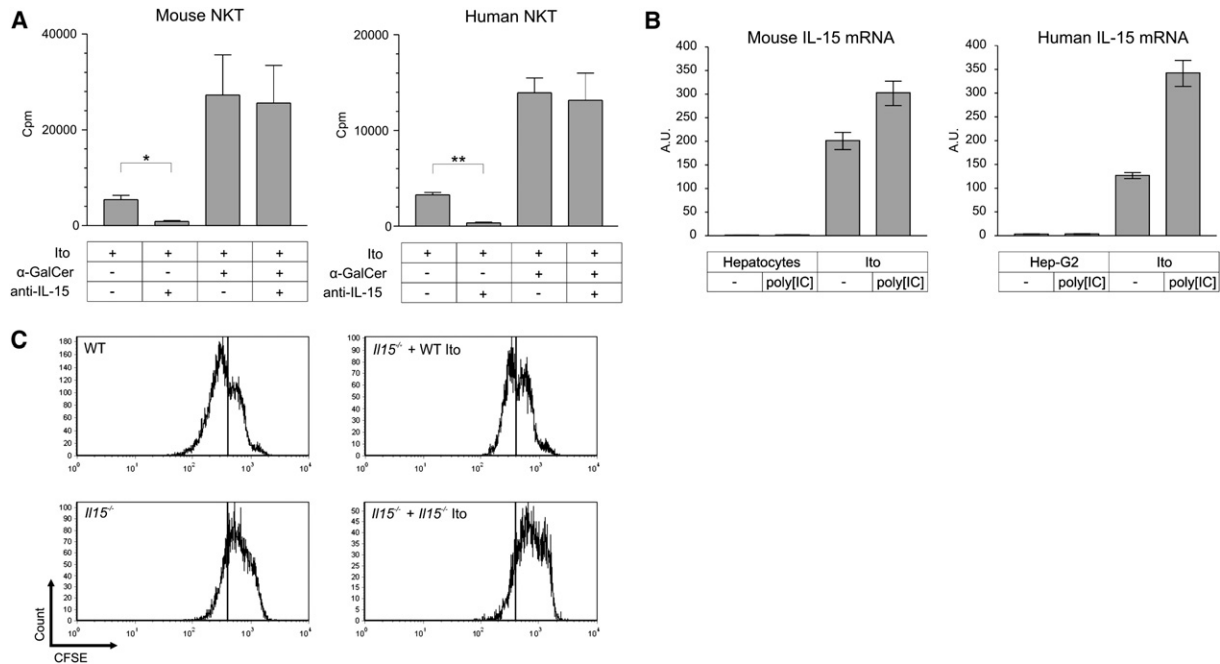
(A) Ito cells activate murine NKT cells in a CD1d-dependent fashion. Ito cells from wild-type (WT) or *Cd1d1<sup>-/-</sup>* animals or DCs were pulsed with  $\alpha$ -GalCer (1  $\mu$ g/ml) and cocultured with NKT cells from thymus, spleen, or liver from V $\alpha$ 14-transgenic mice. Additionally, Ito cells or DCs were treated with a CD1d-blocking antibody (10  $\mu$ g/ml) before addition of T cells. 2 days after stimulation, antigen-specific proliferation was determined by <sup>3</sup>H-thymidine incorporation as depicted by counts per minute (cpm) (error bars represent mean  $\pm$  SD).

(B) Human NKT cells are activated by Ito cells. Human Ito cells or DCs were incubated with  $\alpha$ -GalCer (1  $\mu$ g/ml) and exposed to a V $\alpha$ 24<sup>+</sup> human NKT cell line. In addition, Ito cells or DCs were incubated with a blocking antibody against human CD1d. 2 days after stimulation, antigen-specific proliferation was determined by <sup>3</sup>H-thymidine incorporation as depicted by counts per minute (cpm) (error bars represent mean  $\pm$  SD).

(C) Ito cells perform APC function for NKT cells in vivo. Ito cells were generated from wild-type (*Cd1d1<sup>+/+</sup>*) or *Cd1d1<sup>-/-</sup>* mice and pulsed with  $\alpha$ -GalCer for 2 hr before intravenous injection into *Cd1d1<sup>-/-</sup>* animals. In parallel, NKT cells from V $\alpha$ 14 transgenic mice were purified, labeled with CFSE, and adoptively transferred to *Cd1d1<sup>-/-</sup>* recipients. 3 days after transfer, liver lymphocytes were prepared and stained for NKT cells with CD1d tetramers. Histograms demonstrate the proliferation profiles of CFSE-labeled NKT cells gated on tetramer<sup>+</sup> cells after transfer of antigen-loaded wild-type (top) or *Cd1d1<sup>-/-</sup>* Ito cells (bottom). Graphs in (A)–(C) are representative of at least three independent experiments with similar results.

et al., 1994; Waldmann and Tagaya, 1999). Because IL-15 is critically required for NKT cell homeostasis (Matsuda et al., 2002), we analyzed the impact of IL-15 on Ito cell-mediated T cell activation. To this end, we added a neutralizing antibody against IL-15 to coculture assays of  $\alpha$ -GalCer-loaded Ito cells and NKT cells. Antigen-specific proliferation of murine NKT cells in response to  $\alpha$ -GalCer presented by Ito cells was not reduced by IL-15 neutralization (Figure 3A). Thus, IL-15 was not essential for antigen-dependent NKT cell activation. However, murine NKT cells reacted to unpulsed Ito cells with a distinct proliferative response. It is known that NKT cells are autoreactive to CD1d in the absence of nominal antigen (Brigl and Brenner, 2004). Addition of anti-IL15 to unpulsed Ito cells markedly inhibited this autoreactive NKT cell response (Figure 3A). Thus, IL-15 was critically required for Ito cell-mediated homeostatic NKT cell proliferation. These

results were also valid for the human system with human NKT cells and a neutralizing antibody against human IL-15 (Figure 3A). T cells do not produce IL-15 (Grabstein et al., 1994), so we analyzed Ito cells for IL-15 expression. To this end, we performed real-time PCR to quantify IL-15 mRNA expression amounts in murine and human Ito cells in comparison with primary mouse hepatocytes or the human hepatic cell lines HEP-G2 and HEP-3B, respectively. Moreover, cells were stimulated with the synthetic double-stranded RNA poly[IC], a known inducer of IL-15 (Matsuda et al., 2002). Quantification of IL-15 mRNA revealed that under steady-state conditions, murine and human Ito cells expressed IL-15 (Figure 3B). In contrast, primary liver cells or liver cell lines HEP-G2 (Figure 3B) and HEP-3B (data not shown) did not express IL-15. More importantly, IL-15 transcription was induced by poly[IC] stimulation in murine and human Ito cells



**Figure 3. Ito Cells Express IL-15 to Facilitate Homeostatic NKT Cell Proliferation**

(A) IL-15-dependent homeostatic proliferation of NKT cells. Murine and human Ito cells were pulsed with  $\alpha$ -GalCer (1  $\mu$ g/ml) or were left untreated before addition of NKT cells. Additionally, blocking antibodies (10  $\mu$ g/ml) to mouse or human IL-15 were added to Ito cells before coculture with NKT cells. 2 days after stimulation, NKT cell proliferation was determined by  $^3$ H-thymidine incorporation as indicated by counts per minute (cpm) (error bars represent mean  $\pm$  SD). Graphs are representative of at least three independent experiments with similar results.

(B) Quantification of IL-15 expression by Ito cells. Murine Ito cells compared to primary mouse hepatocytes as well as human Ito cells compared to the human hepatic cell line HEP-G2 were stimulated with poly[IC] for 4 hr or were left untreated. After RNA preparation, real-time PCR was performed with primers specific for IL-15 and the housekeeping gene GAPDH. Quantification of IL-15 mRNA expression standardized to GAPDH is shown as arbitrary units (A.U. = 100,000 / fold difference) (error bars represent mean  $\pm$  SD). \*p < 0.05; \*\*p < 0.01 as analyzed by Student's t test.

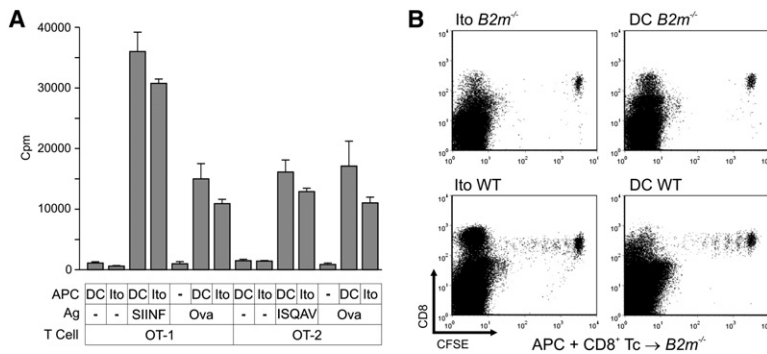
(C) Ito cell-derived IL-15 promotes homeostatic NKT cell proliferation in vivo. Ito cells from wild-type or *Il15*<sup>-/-</sup> mice were transferred to *Il15*<sup>-/-</sup> recipients. In parallel, NKT cells were isolated from V $\alpha$ 14 transgenic thymi, labeled with CFSE, and injected into wild-type or *Il15*<sup>-/-</sup> mice. 10 days after transfer, liver lymphocytes from recipient animals were isolated and stained with CD1d tetramer, and NKT cell division was analyzed by flow cytometry. Decrease of CFSE fluorescence intensity indicates proliferation of transferred NKT cells gated on CD1d-tetramer<sup>+</sup> cells.

(Figure 3B), while parenchymal liver cells did not upregulate IL-15 transcription.

To analyze the impact of Ito cells on IL-15-dependent homeostatic proliferation of NKT cells in vivo, we isolated Ito cells from wild-type and *Il15*<sup>-/-</sup> mice and transferred them to IL-15-deficient recipients. In parallel, NKT cells were purified from V $\alpha$ 14-transgenic thymi, labeled with CFSE, and injected into wild-type or IL-15-deficient mice. 10 days after transfer, liver lymphocytes from recipient animals were isolated and stained with CD1d tetramers prior to flow cytometrical analysis. As demonstrated by decrease of CFSE fluorescence intensity of CD1d-tetramer<sup>+</sup> cells, homeostatic proliferation of NKT cells was prominent in livers of WT animals, but not in *Il15*<sup>-/-</sup> mice (Figure 3C, left). Adoptive transfer of wild-type Ito cells into IL-15-deficient mice induced NKT cell division and restored homeostatic NKT cell proliferation as seen in wild-type animals. Transfer of *Il15*<sup>-/-</sup> Ito cells did not have any effect on NKT cell proliferation (Figure 3C, right). In conclusion, Ito cells express IL-15 to promote homeostatic proliferation of NKT cells.

### Ito Cells Present Peptides to CD4<sup>+</sup> and CD8<sup>+</sup> T Cells and Induce T Cell Priming In Vivo

Because we observed surface expression of MHC-I, MHC-II, and costimulatory molecules by Ito cells (Figure 1C, Figure S1), which is in accordance with earlier reports (Vinas et al., 2003; Yu et al., 2004), we aimed at elucidating the functional significance with regard to CD8<sup>+</sup> and CD4<sup>+</sup> T cell stimulation. To this end, we isolated antigen-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells from TCR-transgenic mice (OT-1 or OT-2, respectively). OT-1 or OT-2 lymphocytes were purified by positive selection of CD8<sup>+</sup> or CD4<sup>+</sup> cells, respectively, and purity of >95% of T cell preparations was confirmed by flow cytometry. Ito cells and DCs were pulsed with ovalbumin or ovalbumin-derived antigenic peptides that are specifically recognized by OT-1 or OT-2 T cells and subsequently cocultured with CD8<sup>+</sup> or CD4<sup>+</sup> T cells. CD8<sup>+</sup> T cells were specifically stimulated by Ito cells presenting the ovalbumin peptide SIINFEKL in the context of MHC-I (Figure 4A). Similarly, CD4<sup>+</sup> T cells were stimulated by the ovalbumin epitope ISQAV in the context of MHC-II (Figure 4A). Ito cells processed



bars represent mean  $\pm$  SD). Graphs are representative of at least three independent experiments with similar results.

(B) Ito cells induce T cell priming in vivo.  $1 \times 10^6$  Ito cells or DCs from wild-type or  $B2m^{-/-}$  mice were loaded with the whole ovalbumin protein before intravenous injection into  $B2m^{-/-}$  recipients. In parallel,  $5 \times 10^6$  to  $1 \times 10^7$  purified CFSE-labeled  $CD8^+$  OT-1 cells were transferred. 5 days later, liver lymphocytes were isolated and stained for CD8. Dot plots depict CFSE dilution of  $CD8^+$  cells.

the entire ovalbumin protein for antigen-specific  $CD4^+$  T cell stimulation. More importantly, Ito cells processed ovalbumin for  $CD8^+$  T cell stimulation, revealing their capacity for crosspresentation (Figure 4A). In conclusion, Ito cells efficiently present peptides to  $CD8^+$  and  $CD4^+$  T cells and process exogenous antigen for crosspriming of  $CD8^+$  T cells. Direct pulsing of  $CD4^+$  or  $CD8^+$  T cells with ovalbumin in the absence of Ito cells did not result in proliferation excluding direct antigen presentation by T cells or by contaminating APCs from primary T cell preparations (Figure 4A). In addition, we analyzed  $IFN\gamma$  concentrations in cell-culture supernatants from experiments described above by ELISA.  $CD8^+$  as well as  $CD4^+$  T cells secreted  $IFN\gamma$  in an antigen-specific manner, indicating functional differentiation of T cells upon Ito cell-mediated activation (Figure S3C). Furthermore, we asked whether  $CD8^+$  T cells activated by Ito cells develop fully competent cytotoxic function. To this end,  $^{51}Cr$ -labeled Ito cells pulsed with SIINFPEKL peptide or left untreated were incubated with OT-1 cells, and  $^{51}Cr$ -release was measured in cell-culture supernatants (Figure S3D). Indeed,  $CD8^+$  T cells activated by Ito cells developed CTL function as demonstrated by target cell lysis and subsequent  $^{51}Cr$  release.

In order to analyze the capacity of Ito cells for priming of peptide-specific T cells in vivo, we pulsed Ito cells or DCs isolated from wild-type or Beta2-microglobulin ( $B2m$ )-deficient mice with ovalbumin before intravenous injection into  $B2m^{-/-}$  recipients. Because  $B2m^{-/-}$  mice lack surface-expressed MHC-I molecules as well as  $CD8^+$  T cells, we transferred purified CFSE-labeled  $CD8^+$  OT-1 cells in parallel. 5 days after transfer, lymphocytes from liver and spleen were isolated and stained for CD8. Flow cytometric analysis indicated T cell activation after transfer of antigen-loaded Ito wild-type cells in contrast to  $B2m^{-/-}$  Ito cells (Figure 4B, left). Ito cell-induced T cell priming was comparable to the effect of DCs (Figure 4B, right). Because  $B2m$ -deficient mice lack surface-expressed MHC-I molecules for antigen presentation, any effect of MHC-I-restricted T cell activation in  $B2m$ -deficient recipients was due to priming by Ito cells. Thus, crosspresentation of antigen by other endogenous APC types can be ex-

cluded in this transfer system. In conclusion, Ito cells represent professional APCs able to present peptides to  $CD8^+$  and  $CD4^+$  T cells, to process exogenous antigen for crosspriming of  $CD8^+$  T cells, and to induce T cell priming in vivo.

#### Bacterial Antigens Are Processed by Ito Cells for Presentation to $CD8^+$ T Cells upon Infection

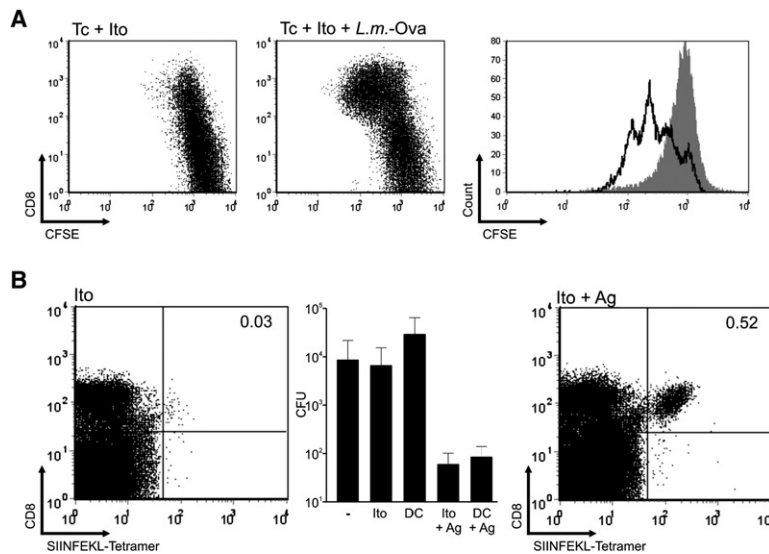
To analyze whether Ito cells induce antigen-specific  $CD8^+$  T cell responses after infection with intracellular bacteria, we incubated Ito cells with recombinant *Listeria monocytogenes* expressing the surrogate antigen ovalbumin (*L.m.-OVA*). *L. monocytogenes* is taken up by target cells through induced phagocytosis and subsequently escapes from the phagosome upon forming membrane pores by means of listeriolysin O (Beauregard et al., 1997). Then, bacterial antigens reach the cytosol for processing through the MHC-I pathway and subsequent  $CD8^+$  T cell activation. After infection of murine Ito cells with *L.m.-OVA* at a multiplicity of infection (moi) of 4 for 30 min, extracellular bacteria were killed by gentamycin. Subsequently, CFSE-labeled OT-1 cells were cocultured with infected or noninfected Ito cells. After 3 days, cells were stained with anti-CD8 and analyzed by flow cytometry. Infected Ito cells induced antigen-specific  $CD8^+$  T cell proliferation, indicated by CFSE dilution of  $CD8^+$  OT-1 cells as compared to coculture with noninfected Ito cells (Figure 5A). In conclusion, Ito cells are able to process bacterial antigens for presentation to  $CD8^+$  T cells.

#### Ito Cells Mediate Protection against Infection with Intracellular Bacteria

To examine the role of Ito cells in infection in vivo, we transferred antigen-loaded (OVA) or unloaded Ito cells or DCs to recipient wild-type mice. 1 week later, animals were challenged with  $5 \times 10^4$  to  $1 \times 10^5$  *L.m.-OVA* intravenously. 48 hr after infection, spleen and liver were collected for tetramer analysis of peptide-specific T cell expansion, and bacterial load was determined by counting colony-forming units (CFU). Antigen-loaded Ito cells markedly increased the frequency of peptide-specific

**Figure 4. Ito Cells Induce T Cell Responses Specific for Protein Antigens**

(A) Ito cells present peptide to  $CD8^+$  or  $CD4^+$  T cells and process exogenous antigen for crosspriming of  $CD8^+$  T cells. Ito cells or DCs were incubated with SIINFPEKL (SIINF, 1  $\mu$ g/ml), ISQAV (1  $\mu$ g/ml), or ovalbumin (Ova, 5  $\mu$ g/ml) or were left untreated. Subsequently, antigen-pulsed Ito cells or DCs were cocultured with  $CD8^+$  T cells (OT-1) or  $CD4^+$  T cells (OT-2). Additionally, OT-1 and OT-2 T cell preparations were incubated with ovalbumin (5  $\mu$ g/ml) alone. 2 days after stimulation, antigen-specific proliferation was analyzed by  $^3H$ -thymidine incorporation as indicated by counts per minute (cpm) (error



**Figure 5. Upon Infection, Ito Cells Elicit Peptide-Specific T Cells and Mediate Protection In Vivo**

(A) Ito cells stimulate antigen-specific CD8<sup>+</sup> T cells after infection with intracellular bacteria. Ito cells were infected with *L.m.-OVA* at a moi of 4 for 30 min, and subsequently, extracellular bacteria were killed with gentamycin. CFSE-labeled OT-1 cells were added and incubated for 3 days. Cells were stained with anti-CD8 and analyzed by flow cytometry. Dot plots depict CD8 staining of CFSE-labeled OT-1 cells in the presence of infected or noninfected Ito cells. Histogram represents overlay of proliferation profiles (black line, Ito infected; gray-filled curve, Ito noninfected).

(B) In vivo, Ito cells elicit peptide-specific T cells and protect against bacterial infection. Antigen-loaded (OVA) or unloaded Ito cells or DCs were transferred to recipient mice. 1 week later, animals were intravenously infected with  $5 \times 10^4$  to  $1 \times 10^5$  *L.m.-OVA*. 48 hr after infection, spleen and liver were

collected for analysis of the frequency of SIINFEKL-specific CD8<sup>+</sup> T cells by flow cytometry and determination of bacterial burdens by counting CFU (middle panel, 4 mice per group, error bars represent mean  $\pm$  SD). Percentages of SIINFEKL-specific CD8<sup>+</sup> T cells are depicted in the quadrants of dot plots. Graphs in (A) and (B) are representative of at least three independent experiments with similar results.

CD8<sup>+</sup> T cells in spleen, as revealed by staining with SIINFEKL-loaded MHC-I tetramers (Figure 5B). In contrast to unpulsed cells, antigen-loaded Ito cells induced reduction of bacterial burden in the liver (Figure 5B). Thus, Ito cells elicit peptide-specific T cells and protect against bacterial infection in vivo.

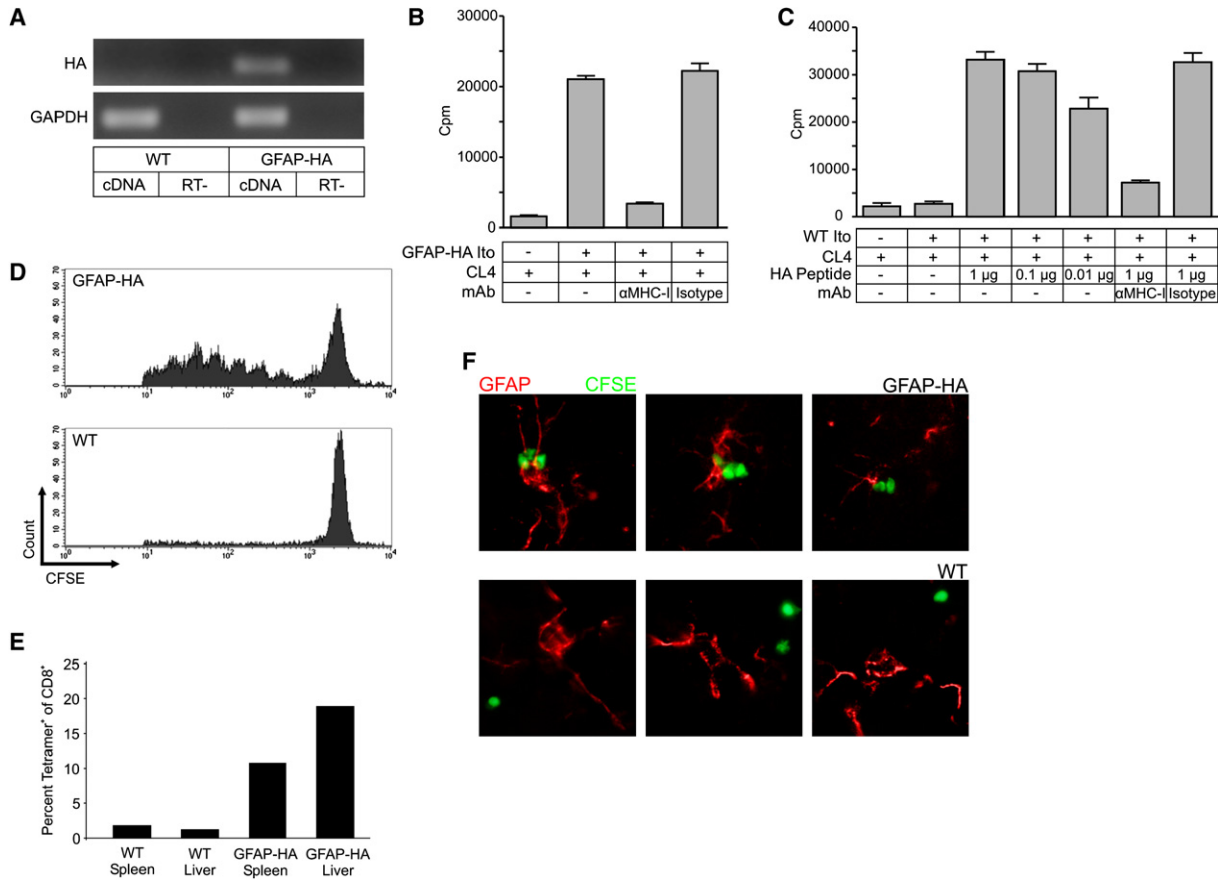
#### Transgenic Ito Cells Present Endogenous Neoantigen to Peptide-Specific T Cells

Because analyzing APC function of Ito cells relied on loading of exogenous antigen, we next addressed the question of intrinsic antigen presentation by Ito cells in vitro and in vivo. To this end, we used transgenic mice expressing the neoantigen hemagglutinin (HA) from influenza virus under the control of the GFAP promoter (GFAP-HA) (Morgan et al., 1996; Cornet et al., 2001). Because in the liver GFAP is exclusively produced by Ito cells, we isolated Ito cells from GFAP-HA transgenic mice in order to analyze HA expression. RT-PCR revealed expression of HA mRNA in Ito cells from transgenic but not from wild-type mice (Figure 6A). In order to examine the functional impact of endogenous neoantigen expression by Ito cells on T cell activation, we performed an in vitro assay with Ito cells from GFAP-HA or wild-type mice in coculture with HA-specific TCR-transgenic CD8<sup>+</sup> T cells (CL4). GFAP-HA Ito cells induced profound activation of HA-specific CD8<sup>+</sup> T cells as indicated by antigen-specific proliferation (Figure 6B). CD8<sup>+</sup> T cell activation was dependent on MHC-I-restricted antigen presentation because addition of an MHC-I blocking antibody totally abrogated the T cell response (Figure 6B). In marked contrast, Ito cells from wild-type animals did not activate HA-specific CD8<sup>+</sup> T cells (Figure 6C). However, after pulsing with different concentrations of HA peptide, wild-type Ito cells caused

MHC-I-restricted T cell activation in a dose-dependent manner (Figure 6C). In conclusion, transgenic Ito cells from GFAP-HA mice present intrinsic neoantigen for subsequent T cell activation.

In order to investigate presentation of intrinsic antigen by Ito cells in vivo, we conducted adoptive transfers of CFSE-labeled HA-specific CD8<sup>+</sup> T cells to GFAP-HA transgenic or wild-type recipient mice. 3 days after transfer, liver lymphocytes were purified for subsequent CD8 staining and measurement of proliferation profiles by flow cytometry. Transfer of HA-specific CD8<sup>+</sup> T cells (CL4) to GFAP-HA recipient mice caused activation of lymphocytes from liver, as indicated by decrease of CFSE fluorescence intensity (Figure 6D, top). This activation can already be observed as early as 40 hr after transfer (data not shown). Unspecific activation of T cells was excluded by CL4 transfer to wild-type recipients showing lack of CD8<sup>+</sup> T cell proliferation in hepatic tissue (Figure 6D, bottom). Staining of lymphocytes from recipient mice by HA-loaded MHC-I-tetramers revealed increased frequency of HA-specific T cells from GFAP-HA transgenic livers when compared to wild-type as well as transgenic spleen, indicating preferential accumulation of peptide-specific T cells in GFAP-HA liver (Figure 6E). In parallel, we analyzed liver sections of recipient mice for the anatomical distribution of transferred CD8<sup>+</sup> T cells in relation to HA-presenting Ito cells in situ. After staining endogenous Ito cells for the liver-specific Ito marker molecule GFAP, fluorescence microscopy demonstrated clustering of CFSE<sup>+</sup> CD8<sup>+</sup> T cells in association with Ito cells when HA-specific T cells were transferred to GFAP-HA transgenic but not to wild-type mice (Figure 6F). Taken together, endogenous Ito cells present intrinsic antigen for subsequent T cell activation in vitro and in vivo.





**Figure 6. Transgenic Ito Cells Present Endogenous Neoantigen**

(A) Ito cells from GFAP-HA transgenic mice express hemagglutinin (HA). Total RNA was isolated from GFAP-HA transgenic (GFAP-HA) or wild-type (WT) Ito cells and analyzed by RT-PCR for HA expression. Depicted are PCR products amplified with HA- and GAPDH-specific primers. Reverse transcription (cDNA), RT minus control (RT).

(B and C) Transgenic Ito cells present intrinsic antigen to peptide-specific T cells *in vitro*.

(B) Ito cells from GFAP-HA mice were cocultured with HA-specific CD8<sup>+</sup> T cells (CL4).

(C) In parallel, Ito cells from wild-type mice were cocultured with CL4 T cells. Peptide pulsing of Ito cells was performed with different concentrations of HA peptide before coculture with T cells.

(B and C) Blocking antibodies to MHC-I (20 µg/ml) or isotype controls were added before incubation. 2 days after stimulation, antigen-specific proliferation was analyzed by <sup>3</sup>H-thymidine incorporation as indicated by counts per minute (cpm) (error bars represent mean ± SD).

(D) HA-specific T cells from liver show activation in GFAP-HA mice *in vivo*. HA-specific CD8<sup>+</sup> T cells from CL4-TCR transgenic spleen were purified, CFSE labeled, and intravenously transferred to GFAP-HA transgenic (GFAP-HA, top) or wild-type (WT, bottom) mice. 3 days after transfer, liver lymphocytes were purified and stained for CD8 prior to flow cytometric analysis. Histograms depict CFSE dilution gated on CD8<sup>+</sup> cells.

(E) HA-specific T cells preferentially accumulate in GFAP-HA liver. In parallel to (D), lymphocytes from liver and spleen were stained with HA-peptide-loaded MHC-I tetramers and anti-CD8. Depicted are percentages of tetramer<sup>+</sup> among CD8<sup>+</sup> T cells in spleens and livers of wild-type and GFAP-HA mice.

(F) Antigen-specific T cells cluster around HA-presenting Ito cells *in situ*. As per an analogous experimental set-up as under (D), livers of T cell recipient animals were excised, and cryosections were stained for GFAP prior to fluorescence microscopy analysis. Overlays of CFSE<sup>+</sup> HA-specific T cells and GFAP-expressing Ito cells are shown for GFAP-HA transgenic (GFAP-HA) and wild-type (WT) livers. Graphs in (A)–(F) are representative of at least two independent experiments with similar results.

## DISCUSSION

It has been shown recently that liver is an efficient and self-sufficient priming site for naive CD8<sup>+</sup> T cells independently of secondary lymphoid organs or bone marrow-derived APCs (Klein and Crispe, 2006). However, in these elegant studies, the cellular basis of intrahepatic antigen presentation has not been addressed. In earlier reports, several liver cell types have been implicated in induction of T

cell tolerance (Crispe, 2003). In contrast, our data identify Ito cells as professional intrahepatic APCs activating T cells and eliciting a multitude of T cell responses specific for protein and lipid antigens.

Proof of APC function of Ito cells relies on purity of both Ito cell and T cell preparations to exclude T cell activation by contaminating APCs. Purity of Ito cell preparations was determined as ≥98.5% by a combination of positive GFAP staining (Neubauer et al., 1996) and negative

CD11c and F4/80 staining excluding the presence of additional APCs known to elicit T cell responses. Functional impact of potentially contaminating APCs in primary T cell preparations was excluded by lack of activation when T cells were stimulated with antigen alone in the absence of Ito cells.

With regard to group II CD1 antigen presentation, we demonstrate vigorous NKT cell activation by human and murine Ito cells in a CD1d-restricted manner. Ito cells performed professional APC functions for NKT cells comparable to those of DCs in vitro and induced NKT cell proliferation in vivo when transferred from wild-type into *Cd1d1*<sup>-/-</sup> mice, excluding contribution of any other APC in recipient mice. NKT cells account for up to 50% of liver lymphocytes; however, the factors rendering liver tissue a preferential location for NKT cells remained elusive (Kronenberg and Gapin, 2002). Different liver cell types capable of presenting the surrogate antigen  $\alpha$ -GalCer have been implicated in NKT cell activation (Bezbradica et al., 2005; Schmiege et al., 2005). It has been shown recently that >90% depletion of Kupffer cells reduced the percentage of IFN $\gamma$ - or IL-4-producing liver NKT cells when  $\alpha$ -GalCer was injected 2 hr before cytokine measurement (Schmiege et al., 2005). In this work, depletion of Kupffer cells by injection of clodronate-liposomes has not been monitored for its effect on phagocytic Ito cells. Thus, decreased numbers of responsive NKT cells cannot be attributed solely to depletion of Kupffer cells. Furthermore, the NKT cell restriction element CD1d is expressed only at very low amounts on Kupffer cells compared to Ito cells, and hence Ito cells appear superior for CD1d-mediated antigen presentation (see Supplemental Data).

IL-15 is a crucial cytokine in survival of NKT cells, as indicated by lack of NKT cells in IL-15 and IL-15 receptor-deficient animals (Kennedy et al., 2000; Lodolce et al., 1998). Our data demonstrate that hepatic Ito cells produce IL-15, facilitating homeostatic NKT cell proliferation. Thus, Ito cells induce both CD1d-restricted, antigen-dependent and homeostatic, IL-15-dependent NKT cell proliferation. Although we do not exclude contribution of other liver cell types to NKT cell homeostasis, our findings shed light on the enigma of NKT cell abundance in the liver with the Ito cell providing the crucial environment for NKT cell expansion.

In addition to group II CD1 molecules, human Ito cells expressed CD1b molecules at high surface density. So far, surface expression of group I CD1 molecules has been exclusively confined to DCs (Brigl and Brenner, 2004). Moreover, Ito cells vigorously stimulated T cell clones specifically recognizing the lipid lipoarabinomannan (LAM) when pulsed with sonicate from *Mycobacterium leprae* in a CD1b-restricted fashion (see Supplemental Data). Thus, human Ito cells present lipids of microbial origin to T cells, demonstrating functional significance of CD1b expression on Ito cells comparable to DCs.

Expression of APC markers by Ito cells and presentation of peptide antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells demonstrate that Ito cells are capable of stimulating protein-

specific T cells. Transfer of antigen-loaded Ito cells and CD8<sup>+</sup> T cells into B2m-deficient mice provided a model that lacks endogenous MHC-I-restricted antigen presentation. Activation of peptide-specific T cells in recipient B2m-deficient mice excluded crosspresentation of antigen by other endogenous APCs and demonstrated profound priming capacity of Ito cells in vivo. Moreover, Ito cells processed exogenous protein antigens for subsequent activation of CD8<sup>+</sup> T cells, a mechanism referred to as crosspresentation characterizing professional APC function (Ackerman and Cresswell, 2004; Winau et al., 2005a). Thus, Ito cells can mediate surveillance of the extracellular space for antigens by transporting proteins into the MHC-II as well as the MHC-I pathway for broad activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

To investigate APC functions of Ito cells in infection immunology, we analyzed the impact of Ito cells upon infection with the intracellular pathogen *L. monocytogenes* (Pamer, 2004). In vitro, Ito cells processed bacterial antigens for subsequent MHC-I presentation and CD8<sup>+</sup> T cell activation. In vivo, Ito cells elicited peptide-specific T cells and mediated protection against infection with *L. monocytogenes* comparable to the effect of DCs. The majority of pathogens targeting hepatocytes have to cross the perisinusoidal space of Disse, so Ito cells adjacent to hepatocytes are located at a privileged site to sense pathogens attacking liver parenchyma.

To study intrinsic antigen presentation by Ito cells, we extended our experiments with transgenic mice expressing the neoantigen hemagglutinin under the GFAP promoter (Morgan et al., 1996), which in the liver is exclusively active in Ito cells (Neubauer et al., 1996). GFAP-HA transgenic Ito cells express hemagglutinin for presentation to antigen-specific T cells. In vivo HA-specific T cells isolated from liver tissue of GFAP-HA mice were activated as indicated by antigen-specific T cell proliferation. Moreover, HA-specific T cells accumulated in GFAP-HA transgenic liver when compared to spleen. Although we cannot formally exclude stimulation of HA-specific T cells in extrahepatic tissue of GFAP-HA mice, histological analysis of liver revealed clustering of HA-specific T cells around HA-presenting Ito cells. Taken together, our data indicate that endogenous Ito cells present intrinsic antigen to T lymphocytes in situ.

Different cell types have been implicated in intrahepatic antigen presentation, namely liver sinusoidal endothelial cells (LSEC), Kupffer cells, and hepatocytes. LSECs possibly can present exogenous antigens, but induce T cell tolerance (Katz et al., 2004; Knolle and Gerken, 2000; Limmer et al., 2000). Kupffer cells have been shown to be capable of presenting antigens, but are markedly less efficient than splenic macrophages (Roland et al., 1994). Furthermore, Kupffer cells appear to be negative regulators of T cell activation by paracrine factors in a complex interplay with LSECs (Knolle and Gerken, 2000). Hepatocytes can induce short-term proliferation of CD8<sup>+</sup> T cells but lead to premature T cell death, possibly caused by a lack of costimulatory molecules such as CD86 (Bertolino et al., 1999, 2001). Our data qualify Ito cells as APCs

equally efficient as DCs in eliciting T cell responses. In contrast to Kupffer cells, which are located within the sinusoidal compartment, Ito cells are localized in the perisinusoidal space of Disse adjacent to hepatocytes (Geerts, 2001). Although LSECs separate the space of Disse from the sinusoidal space, this barrier is incomplete because it lacks a basement membrane, does not have tight junctions, and underlies dynamic changes by the formation of fenestrae and sieve plates (Braet and Wisse, 2002). Since lymphocytes can be found in the liver parenchyma, diapedesis and transit of cells through the space of Disse demonstrates that the sinusoidal barrier is penetrable. Thus, Ito cells may function as professional APCs for activation or restimulation of T cells contacting sub-endothelial compartments either under physiological conditions through incomplete compartment separation or under pathological conditions when this barrier is disintegrated. In this context, Ito cells appear to be located at a privileged site and may act as resident sentinels of liver parenchyma.

So far, Ito cells were known for storage of retinol (vitamin A), which is critical for immune function and maintenance of gut integrity (Stephensen, 2001). Moreover, it has been demonstrated that T cells stimulated in the presence of the retinol metabolite retinoic acid upregulate gut homing receptors  $\alpha 4\beta 7$  and CCR9 (Iwata et al., 2004). DC-derived retinoic acid increased T cell tropism to small intestine, whereas vitamin A deficiency resulted in depletion of T cells from intestinal lamina propria. Ito cells store up to 80% of total body retinol, so it is tempting to speculate on a dual role for Ito cells in both antigen presentation and regulation of T cell tropism.

Ito cells have also been located in extrahepatic tissues including pancreas, intestine, and lung (Nagy et al., 1997). Assuming that extrahepatic Ito cells perform antigen-presenting functions comparable to that of intrahepatic Ito cells shown here, we propose that Ito cells represent tissue-resident professional APCs, which elicit T cell responses and serve as target cells in situ within the scope of a systemic Ito network.

## EXPERIMENTAL PROCEDURES

### Experimental Animals and Cell Lines

6- to 8-week-old C57BL/6, Balb/c, OT-1, OT-2,  $V\alpha 14$ -J $\alpha 18$  transgenic,  $Cd1d1^{-/-}$ ,  $B2m^{-/-}$ ,  $Il15^{-/-}$ , GFAP-HA transgenic, and CL4 TCR transgenic mice were bred under specific pathogen-free conditions. OT-1 and OT-2 breeder mice were obtained from Jackson Laboratories.  $V\alpha 14$ -J $\alpha 18$  transgenic and  $Cd1d1^{-/-}$  breeders were kindly provided by A. Bendelac, Princeton. Human NKT cell lines were kindly provided by E. Scotet and M. Bonneville, Nantes. In brief, peripheral blood lymphocytes from healthy human donors were sorted for  $V\alpha 24^{+}$  cells and were subsequently expanded with PHA, IL-2, and irradiated feeder cells. The CD1b-restricted T cell line LCD4.7 reactive with LAM from *M. leprae* was described previously (Sieling et al., 2000). Human hepatocellular carcinoma cell lines HEP-G2 and HEP-3B were from ATCC. All material was used in accordance with the local ethics commission.

### Preparation of Ito Cells

Ito cells were prepared from liver of C57BL/6, Balb/c,  $Cd1d1^{-/-}$ ,  $Il15^{-/-}$ , and GFAP-HA transgenic mice. In brief, the abdominal cavity

of anesthetized animals was opened to cannulate the portal vein. Subsequently, liver was perfused for 5 min with solution SC-1, 4 min with solution SC-2, 4 min with 0.5 mg/ml pronase solution, and 4 min with 0.25 mg/ml collagenase solution at a flow rate of 6 ml/min (Kristensen et al., 2000). After perfusion, tissue was further digested in 120 ml SC-2 solution supplemented with 50 mg collagenase, 50 mg pronase, and 2 mg DNase. After digestion, the cell suspension was passed through a nylon mesh and centrifuged at 400  $\times$  g for 10 min. Subsequently, cells were purified through 8% Nycodenz (Axis-Shield PoC) gradient centrifugation. Ito cells were grown in DMEM medium (GIBCO-BRL) containing 5% FCS for 3 days before experiments were performed. Cells were recovered by mild trypsinization or detachment via Accutase (PAA, Austria). Human Ito cells were obtained from liver specimens of patients undergoing liver needle biopsy for diagnosis of hepatic diseases. Material was used in accordance with the local ethics commission. Surface marker expression and stimulatory capacity varied between Ito cells from different donors. Ito cells were established by outgrowth from explants placed in Opti-MEM-1 (GIBCO-BRL) containing 5% FCS and 5% pooled human AB serum. Cells were recovered by mild trypsinization or detachment via Accutase and used for experiments between passages 3 and 6. Purity of Ito cell preparations was >98.5% as confirmed by positive staining for GFAP, a specific marker for hepatic Ito cells, and negative staining for CD11c and F4/80. Mouse Kupffer cell preparations were purchased from Dominion Pharmakine.

### Antibodies, Antigens, and Chemicals

We used antibodies against murine CD86 (GL1) and CD11c (N418) from Becton Dickinson (BD), MHCII (TIB120) and F4/80 (HB-198) from ATCC, and CD1d (20H2) kindly provided by A. Bendelac, Princeton. Antibodies to human CD1b (MT-101), CD1d (CD1d24), HLA-DR (L243), CD86 (FUN-1), and CD40 (5C3) were purchased from BD. Blocking antibodies against human CD1b (BCD1b) and CD1c (21A3) were purified from hybridoma supernatants. Polyclonal antibodies to human and mouse IL-15 were purchased from Abcam. Antibody to mouse CD8 $\alpha$  (YTS 169.4) was purified from hybridoma supernatants with protein G-sepharose and conjugated with Cy5 according to standard protocols. Antibody cocktail to human and murine GFAP (4A11, 1B4, 2E1) was from BD. All fluorochrome-conjugated secondary antibodies and isotype controls were purchased from BD. Listeria antibody was from ATCC (43251). We used a mouse monoclonal antibody against HA (clone 37.38). For intracellular staining, Cytofix/Cytoperm and Perm/Wash reagents from BD were used. Synthetic peptides SIINFEKL (OVA<sub>257-264</sub>), and ISQAVHAAHAEINEAGR (ISQAV, OVA<sub>323-339</sub>) were purchased from Jerini BioTools. HA-peptide (512-520) was purchased from Neosystem Laboratory. Ovalbumin, phytohemagglutinin (PHA), and poly[IC] were purchased from Sigma.  $\alpha$ -GalCer was kindly provided by Pharmaceutical Research Laboratories, Kirin Brewery. Mouse recombinant IFN $\gamma$  was purchased from Strathmann Biotech. Mycobacterial lipids were obtained from *M. leprae* as described previously (Sieling et al., 2000).

### Generation of DCs

For mouse DCs, bone marrow cells were prepared from femora of C57BL/6 mice and plated in cell-culture dishes in complete DMEM medium at 37°C for 2 hr. Nonadherent cells were collected and cultured in medium in the presence of GM-CSF (20 ng/ml, Strathmann) for 10 days. For human DCs, monocytes from peripheral blood were isolated by Ficoll gradient. Cells were plated in cell-culture dishes in PBS for 2 hr and washed, and adherent cells were cultured in complete RPMI medium in the presence of recombinant IL-4 and GM-CSF (200 U/ml; Strathmann) for 6 days.

### Phase-Contrast and Confocal Microscopy

Phase-contrast microscopy was performed on Ito cells examined in culture flasks with a Leica DM IRBE microscope. For confocal analysis, Ito cells were grown on multichambered slides (Nalgene Nunc) for 2 days before fixation with 4% paraformaldehyde overnight.

Subsequently, cells were permeabilized with PBS containing 0.1% TritonX-100 for 5 min, and unspecific antibody binding was blocked with PBS containing 5% FCS, 1% Fc-receptor block, and 0.05% Tween20. Subsequently, Ito cells were incubated with antibodies for 30 min and washed five times with PBS. Slides were embedded with Immumount (Dako) and sealed with coverslips. Staining was visualized with a Leica TCS-NT confocal microscope.

#### FACS Analysis

Ito cells were detached from culture flasks by mild trypsinization or treatment with Accutase and resuspended in FACS buffer (PBS, 1% FCS, 0.05% Na<sub>3</sub>N). Cells were incubated with antibodies described above at 4°C for 30 min. Subsequently, Ito cells were washed in FACS buffer and analyzed with a FACSCalibur flow cytometer (BD).

#### T Cell Assays

Thymus and spleen NKT cells were isolated from V $\alpha$ 14-J $\alpha$ 18 transgenic mice. Organs were excised and single-cell suspensions were prepared with a sieve before erythrolysis and resuspension of cells in complete medium. To obtain hepatic NKT cells, liver was perfused with PBS through the portal vein, removed, and homogenized with a mesh. Cell suspensions were washed with medium and centrifuged at 50 × g for 2 min, and the supernatants were collected. This step was repeated four times. Cells from pooled supernatants were further purified by a 40%/70% Percoll gradient. NKT cells were negatively selected by depletion of CD8<sup>+</sup> T cells with anti-CD8 microbeads and separation on MACS columns (Miltenyi Biotec) as described previously (Matsuda et al., 2002). OT-1, OT-2, and CL4 lymphocytes were prepared from mesenteric lymph nodes or spleen of OT-1, OT-2, or CL4 mice, respectively. T cells were positively selected with anti-CD4 or anti-CD8 microbeads and separated on MACS columns. Purity of cell isolations used was >95%. For T cell proliferation assays, 2.5 × 10<sup>4</sup> Ito cells were seeded in triplicates in 96-well round-bottom plates. Antigens and blocking antibodies were added as indicated 1 hr before addition of T cells at the following concentrations: 1 μg/ml  $\alpha$ -GalCer, 10 μg/ml anti-IL-15, 10 μg/ml anti-CD1d, 1 μg/ml SIINFEKL, 1 μg/ml ISQAVHAAHAHAINEAGR, 5 μg/ml ovalbumin, 1 μg/ml PHA, and 0.1–1 μg/ml HA peptide. NKT, OT-1/OT-2, or CL4 cells were added at a concentration of 5 × 10<sup>4</sup> to 1 × 10<sup>5</sup> cells per well in a final volume of 200 μl complete DMEM medium and plates were incubated at 37°C and 5% CO<sub>2</sub>. On day 2, supernatant aliquots were collected for measurement of IFN $\gamma$  and cells were pulsed with 0.5 μCi/well <sup>3</sup>H-thymidine (Amersham). 18 hr later, cells were collected for radioactivity measurement on a Top Count scintillation counter (Packard). Mouse and human IFN $\gamma$  ELISA (Biosource International) was performed according to the manufacturers' protocol. <sup>51</sup>Cr-release cytotoxicity assay was performed as described before (Winau et al., 2005b).

#### RT-PCR

For IL-15 and HA mRNA quantification, total RNA was isolated with the High Pure RNA Extraction Kit (Roche) or Trizol (Invitrogen) followed by DNase digestion (Roche). cDNA was synthesized with the SuperScript III First-Strand Synthesis System and random hexamer primers (Invitrogen). RT-PCR was performed in triplicates with the ABI PRISM SDS 7000 system (Applied Biosystems). Mouse (m) and human (h) IL-15-, HA-, and GAPDH-specific cDNA fragments were amplified with the SYBR Green I Reaction Mix (Roche) and primers hGAPDH-For2 (GGA GTC AAC GGA TTT GGT CGT), hGAPDH-Rev2 (ACG GTG CCA TGG AAT TTG C), hIL-15-For2 (TCA CCC CAGT TGC AAA GTA ACA), hIL-15-Rev2 (TTC CTC ACA TTC TTT GCA TCC A), mGAPDH-For1 (AGC CTC GTC CCG TAG ACA AAA), mGAPDH-Rev1 (TGG CAA CAA TCT CCA CTT TGC), mL-15-For1 (GAG GAA TAC ATC CAT CTC GTG C), mL-15-Rev1 (CCT ACA CTG ACA CAG CCC AAA A), HA-For2 (AAA GAA AGC TCA TGG CCC AA), and HA-Rev2 (TCT CCG TCA GCC ATA GCA AAT). Threshold cycle values C<sub>T</sub> for target- and GAPDH-specific fragment amplification were determined with ABI PRISM SDS7000 software, and delta-C<sub>T</sub> values were calculated. Fold difference (FD) in expression amounts between

IL-15 and GAPDH was calculated according to the formula  $FD = 2^{-(\Delta C_T)}$ . Relative quantification of IL-15 expression standardized to GAPDH is expressed as arbitrary units (A.U. = 100,000 / FD). RT-PCR products were resolved on 3% agarose gels.

#### Adoptive Transfer of Ito, NKT, and CD8<sup>+</sup> T Cells

1 × 10<sup>6</sup> Ito cells generated from wild-type or *Cd1d1*<sup>-/-</sup> mice were incubated with 1 μg/ml  $\alpha$ -GalCer for 2 hr. After extensive washing, Ito cells were injected into the lateral tail vein of *Cd1d1*<sup>-/-</sup> recipients. NKT cells were purified from thymus of V $\alpha$ 14-J $\alpha$ 18 transgenic animals prior to labeling with 5 μM CFSE (Molecular Probes) in PBS for 5 min at RT. Subsequently, NKT cells were washed, resuspended in PBS, and intravenously transferred to *Cd1d1*<sup>-/-</sup> recipient mice. 3 days after transfer, NKT cells from liver were isolated and stained with CD1d tetramers prior to analysis of CFSE dilution by flow cytometry. TCR transgenic CD8<sup>+</sup> T cells were positively selected from spleen of CL4 mice by CD8 microbeads. After CFSE labeling, 5 × 10<sup>6</sup> CL4 cells were intravenously injected into GFAP-HA or Balb/c mice. 3 days after transfer, CL4 cells were isolated from liver and spleen, stained for CD8, and analyzed by flow cytometry. OT-1 cells were prepared from mesenteric lymph nodes of OT-1 mice. After CFSE labeling, 5 × 10<sup>6</sup> to 1 × 10<sup>7</sup> OT-1 cells were intravenously injected into *B2m*<sup>-/-</sup> animals.

#### Tetramer Analysis

Tetramers were constructed and loaded with antigens as described (Matsuda et al., 2000). For flow cytometric analysis, 1 × 10<sup>6</sup> cells were incubated for 15 min at 4°C with polyclonal rat antibody, anti-CD16/CD32, and streptavidin (Molecular Probes) in PBS containing 0.5% BSA and 0.01% sodium azide. After incubation, cells were stained for 60 min at 4°C with PE-conjugated  $\alpha$ -GalCer-loaded CD1d-tetramers, SIINFEKL-, or HA-peptide-loaded MHC-I tetramers. Subsequently, cells were washed with PBS including 0.5% BSA and 0.01% sodium azide. Propidium iodide was added shortly prior to FACS analysis.

#### Bacterial Infection and CFU

*Listeria monocytogenes* strain EGD expressing ovalbumin (*L.m.*-OVA) was grown overnight in tryptic soy broth (TSB), washed in PBS, aliquoted in PBS/10% glycerol, and stored at -80°C. Then, aliquots were thawed and bacterial titers were determined by plating serial dilutions on TSB agar plates. For in vitro infection, Ito cells were seeded in 96-well plates at a concentration of 1 × 10<sup>4</sup> cells/well in 200 μl DMEM and were infected with *L.m.*-OVA at a moi of 4. After 30 min, adherent and extracellular bacteria were killed with gentamycin (5 μg/ml). Ito cells were incubated for 24 hr, and CFSE-labeled OT-1 lymphocytes were added at a concentration of 1 × 10<sup>5</sup>/well. After 3 days, cells were stained with CD8 mAb and proliferation was analyzed by FACS. For in vivo infection, listeria (5 × 10<sup>4</sup> to 1 × 10<sup>5</sup>) were injected in a volume of 200 μl PBS into the lateral tail veins of mice 7 days after adoptive transfer of antigen-loaded (OVA) or unloaded Ito cells or DCs. Liver and spleen were homogenized 48 hr after infection in PBS for determination of bacterial burdens. Serial dilutions of homogenates were plated on TSB agar plates and colonies were counted after incubation at 37°C overnight.

#### Statistical Analysis

Student's t test was used for analysis of individual columns.

#### Supplemental Data

Four Supplemental Figures can be found with this article online at <http://www.immunity.com/cgi/content/full/26/1/117/DC1/>.

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