Toll-Like Receptor 3 Ligand Dampens Liver Inflammation by Stimulating V α 14 Invariant Natural Killer T Cells to Negatively Regulate $\gamma\delta$ T Cells

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V α 14 invariant natural killer T (V α 14iNKT) cells are at the interface between the innate and adaptive immune responses and are thus critical for providing full engagement of host defense. We investigated the role of polyriboinosinic:polycytidylic acid (poly I:C), a replication-competent viral double-stranded RNA mimic and a specific agonist that recognizes the cellular sensor Toll-like receptor 3 (TLR3), in regulating V α 14iNKT cell activation. We established for the first time that hepatic V α 14iNKT cells up-regulate TLR3 extracellularly after poly I:C treatment. Notably, activation of TLR3-expressing hepatic V α 14iNKT cells by a TLR3 ligand was suppressed by TLR3 deficiency. Our studies also revealed that V α 14iNKT cell activation in response to poly I:C administration uniquely suppressed the accumulation and activation of intrahepatic $\gamma\delta$ T cells (but not natural killer cells) by inducing apoptosis. Furthermore, we established that activated hepatic V α 14iNKT cells (via cytokines and possibly reactive oxygen species) influenced the frequency and absolute number of intrahepatic $\gamma\delta$ T cells, as evidenced by increased hepatic $\gamma\delta$ T cell accumulation in V α 14iNKT cell-deficient mice after poly I:C treatment relative to wild-type mice. Thus, hepatic V α 14iNKT cells and intrahepatic $\gamma\delta$ T cells are functionally linked on application of TLR3 agonist. Overall, our results demonstrate a novel and previously unrecognized anti-inflammatory role for activated hepatic V α 14iNKT cells in negatively regulating intrahepatic $\gamma\delta$ T cell accumulation (probably through TLR3 signaling) and thereby preventing potentially harmful activation of intrahepatic $\gamma\delta$ T cells. (*Am J Pathol* 2010, 176:1779–1789; DOI: 10.2353/ajpath.2010.090738)

V α 14 invariant natural killer T (V α 14iNKT) cells are thymic-derived innate murine T lymphocytes with significant immunoregulatory effects in cardiovascular, infectious, and autoimmune diseases as well as in tumors.^{1–10} In contrast to conventional T cells, which recognize peptide antigens presented by major histocompatibility class I and II molecules, V α 14iNKT cells respond to glycolipid antigen presented by CD1d expressed on antigen-presenting cells.^{11,12} In the last decade, several potential mechanisms underlying V α 14iNKT cell activation during immune responses have been revealed. V α 14iNKT cells are activated by lipids presented by CD1d.^{5,13} The established dogma is that the lipid tail of glycolipid antigen (including α -galactosylceramide or exogenous antigens from pathogens) is buried in CD1d, whereas the sugar head group of glycolipid antigen protrudes out of the CD1d to activate the T-cell receptor (TCR) α on the V α 14iNKT cell.^{2,13,14} After activation, V α 14iNKT cells exert multiple effects including the production of several cytokines (such as interferon [IFN]- γ , interleukin [IL]-4, and tumor necrosis factor [TNF]- α), chemokines (regulated on activation normal T cell expressed and secreted/CCL5, monocyte chemoattractant protein-1/CCL2, and macrophage inflammatory protein-1 α /CCL3)^{15–18} and cytotoxic proteins (such as tumor necrosis factor-related apoptosis-inducing ligand and Fas/Fas ligand).^{13,19} Through these mediators, activated V α 14iNKT cells can interact with and transactivate other immune cells.^{15,20,21} Thus, V α 14iNKT cells act as a “bridge” between the innate and adaptive immune systems.

V α 14iNKT cells are also activated by a TCR-independent mechanism involving Toll like receptors (TLRs). TLRs are pathogen recognition receptors that identify molecular patterns of components specific to microbes and play a critical role in initiating the innate immune

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response to microbes.²² To date, more than 10 TLRs have been reported in humans and mice, and each recognizes different microbial components.²² TLRs are located on the plasma membrane and in endosomal compartments of cells.²² Among the TLRs, TLR2, TLR4, and TLR5 recognize the bacterial signals peptidoglycan, lipopolysaccharide, and flagellin, respectively,²² whereas TLR3, TLR7, TLR8, and TLR9 play fundamental roles in detecting viral signals.^{23–26} An additional mechanism for activation of $V\alpha 14/NKT$ cells (in the absence of foreign antigen for their TCRs) is by IL-12 and/or IL-18 derived from antigen-presenting cells that have been activated via a TLR (4,7,8,9)-dependent pathway.^{13,14,27,28} The precise pathway of activation may depend on the pathogen. For example, TLR4 traditionally recognizes the bacterial signal lipopolysaccharide,²² whereas TLR7, TLR8, and TLR9 all sense viral signals.^{23–26} The potential contribution of the viral sensor TLR3 to $V\alpha 14/NKT$ cell activation has not yet been determined. Poly I:C is the specific TLR3 agonist and a replication-competent viral double-stranded RNA (dsRNA) mimic.^{23,29} dsRNA is a structure found in the genome of some viruses and is produced as a replication intermediate by viruses.^{23,29,30} Therefore, poly I:C is routinely used in experimental studies to assess the functional activity of TLR3 during immune responses.^{23,29–31}

In the present study, we evaluated the potential role of TLR3 in promoting $V\alpha 14/NKT$ cell activation by examining the response of hepatic $V\alpha 14/NKT$ cells after treatment with the TLR3 ligand, poly I:C. In addition, we assessed the functional consequences of $V\alpha 14/NKT$ cell activation on the hepatic innate immune response after poly I:C treatment. We demonstrate that a functional consequence of hepatic $V\alpha 14/NKT$ cell activation in response to poly I:C administration is the subsequent induction of apoptotic death of hepatic $\gamma\delta T$ cells. Overall, our findings demonstrate a novel role for activated hepatic $V\alpha 14/NKT$ cells in negatively regulating the recruitment, activation, and potentially harmful effector function(s) of intrahepatic $\gamma\delta T$ cells on application of the TLR3 ligand, poly I:C.

Materials and Methods

Mice

Male C57BL/6 mice, B6129SF2/J mice, and $TLR3^{-/-}$ mice (on a B6129SF2/J background) were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of $J\alpha 18^{-/-}$ mice (on a C57BL/6 background) were kindly provided by Dr. Mitch Kronenberg (La Jolla Institute of Allergy and Immunology) with permission from Dr. M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan).³² $J\alpha 18^{-/-}$ mice were bred in a pathogen-free breeding facility at Louisiana State University Health Sciences Center–Shreveport.

Poly I:C Treatment

Poly I:C is the specific TLR3 agonist and a viral dsRNA mimic.^{23,29} dsRNA is a structure found in the genome of

some viruses and produced as a replication intermediate by viruses.^{23,29,30} For this reason, poly I:C is widely used in experimental studies to assess the functional activity of TLR3 during immune responses.^{23,29,30} In our study, mice were treated with poly I:C (30 mg/kg i.p., Sigma-Aldrich, St. Louis, MO)^{29,33,34} and manipulated at indicated time points (0, 3, 16, and 24 hours) as described below. For cytokine blocking experiments, mice received murine IFN- γ antiserum (0.5 ml/mouse i.p.)¹⁷ or control serum 16 hours before poly I:C treatment. The murine IFN- γ antiserum was generously provided by Dr. Robert Strieter (University of Virginia, Charlottesville, VA). Murine TNF monoclonal antibody (mAb) (0.2 mg, clone 1B1, R&D Systems, Minneapolis, MN) or control IgG was administered intravenously 2 hours before poly I:C treatment. To investigate the role of endogenous reactive oxygen species (ROS) in modulating hepatic innate immune cell accumulation and/or activation, a single dose of *N*-acetylcysteine (NAC) (300 mg/kg i.p., Sigma-Aldrich)³⁵ or vehicle PBS was administered simultaneously with poly I:C to C57BL/6 mice or $J\alpha 18$ knockout (KO) mice, which were then sacrificed 16 hours later.

Isolation of Hepatic Lymphocytes and Flow Cytometry

Hepatic lymphocytes were isolated using our published protocols.^{16,17} For the specific identification of hepatic $\gamma\delta T$ cells, isolated hepatic lymphocytes were preincubated with anti-mouse CD16/32 mAb (clone 2.4G2, BD Pharmingen, San Diego, CA) to block Fc γ receptors and then incubated simultaneously with fluorochrome-labeled TCR $\gamma\delta$ mAb (clone GL3, BD Pharmingen) and fluorochrome-labeled CD3 ϵ mAb (clone 145-2C11, BD Pharmingen) as we described recently.^{16,17} Three-color staining was used to assess intracellular cytokine (IFN- γ and IL-17) or intracellular active caspase 3 expression by hepatic $\gamma\delta T$ cells. In brief, fluorochrome-labeled TCR $\gamma\delta$ -CD3 $^{+}$ double-positive T cells were permeabilized with Cytofix/Cytoperm Plus (BD Pharmingen) and then stained intracellularly with either fluorochrome-labeled murine IFN- γ mAb (clone XMG1.2, BD Pharmingen),¹⁷ fluorochrome-labeled murine IL-17 mAb (clone TC11-18H10.1, BD Pharmingen),³⁶ or fluorochrome-labeled active caspase 3 mAb (clone C92-605, BD Pharmingen).^{16,37}

To determine cytokine (IFN- γ and TNF- α) or TLR3 expressions by hepatic $V\alpha 14/NKT$ cells, isolated hepatic lymphocytes were preincubated with anti-mouse CD16/32 mAb to block Fc γ receptors and then incubated simultaneously with fluorochrome-labeled TCR β mAb (clone H57-597, BD Pharmingen) and fluorochrome-labeled $V\alpha 14/NKT$ cell tetramer (CD1d-PBS57, NIH Tetramer Core Facility, Atlanta, GA).^{17,38} PBS57 is an analog of α -galactosylceramide and the CD1d/PBS57 tetramer stain $V\alpha 14/NKT$ cells comparable to the CD1d/ α -galactosylceramide tetramers.³⁸ Next, a rat anti-mouse TLR3 mAb (R&D Systems)³⁹ and phycoerythrin-labeled anti-rat IgG secondary antibody (eBioscience, San Diego, CA) were used to determine TLR3 expression on the surface of $V\alpha 14/NKT$ cell as well as within $V\alpha 14/NKT$ cells. Cyto-

Figure 1. V α 14/NKT cell deficiency promotes hepatic $\gamma\delta$ T cell accumulation and activation after poly I:C treatment. C57BL/6 wild-type (WT) and J α 18 KO mice were treated with poly I:C at the indicated time points. Next, hepatic lymphoid cells were isolated from mice. Hepatic $\gamma\delta$ T cells were identified by FACS after cell surface staining with TCR $\gamma\delta$ and CD3 mAbs (as described in *Materials and Methods*) and then gating on TCR $\gamma\delta$ /CD3 double-positive T cells. The percentage (A), absolute number (B), and representative FACS dot plot (C) of hepatic $\gamma\delta$ T cells (ie, TCR $\gamma\delta$ /CD3 double-positive T cells) are depicted. Results are presented as means $\pm$ SEM with four to six mice per group from two independent experiments. * $P \leq 0.05$ versus all other groups. D: Absolute number of intracellular IL-17 expression by hepatic $\gamma\delta$ T cells was determined by FACS (as described in *Materials and Methods*). Data are shown as means $\pm$ SEM with three to six mice per group from two separate experiments. * $P \leq 0.05$ versus vehicle-treated wild-type mice.

kine production (ie, intracellular IFN- γ and TNF) by hepatic V α 14/NKT cells was determined using fluorochrome-labeled murine IFN- γ mAb or fluorochrome-labeled murine TNF mAb (clone MP6-XT22, eBioscience) after cell permeabilization and then analysis by fluorescence-activated cell sorting (FACS). In all experiments, corresponding isotype antibodies/tetramers were used as controls. In addition, viable lymphocyte populations were gated using forward and side scatter characteristics, and data were analyzed using the FACSCalibur and FACSscan Diva software (BD Biosciences). Notably, all intracellular cytokine stainings were performed without further reactivation *in vitro*.

Statistical Analysis

All data are shown as means $\pm$ SEM. For comparisons of means between two experimental groups, a Student's unpaired *t*-test was used. Comparison among three or more experimental groups was performed using a one-way analysis of variance, followed by either Dunnett's multiple comparison test or a Newman-Keuls post hoc test. $P < 0.05$ was considered significant.

Results

V α 14iNKT Cell-Deficient Mice Exhibit Increased Hepatic $\gamma\delta$ T Cell Accumulation after Poly I:C Treatment

To evaluate the kinetics of $\gamma\delta$ T cell accumulation in the livers of C57BL/6 wild-type mice and J α 18 KO mice, flow cytometric-based techniques were used to determine the expression of cell surface markers for $\gamma\delta$ T cells (ie, TCR $\gamma\delta$ /CD3 double-positive T cells) in the livers of untreated naive mice (ie, 0 hours) and in mice treated with

poly I:C over several time points (ie, 3, 16, and 24 hours). We observed that $\gamma\delta$ T cells represent a minor population of innate immune T cells in the livers of untreated naive C57BL/6 wild-type mice (Figure 1, A and B). In addition, poly I:C treatment did not increase the frequency and absolute number of $\gamma\delta$ T cells accumulating in the livers of C57BL/6 wild-type mice at any of the time points examined relative to those in untreated naive C57BL/6 wild-type mice (Figure 1, A and B). In contrast, V α 14/NKT cell deficiency was associated with a significant increase in $\gamma\delta$ T cell accumulation in the livers of poly I:C-treated J α 18 KO mice. Specifically, the frequency ($\sim$ 2-fold) and absolute number ($>$ 2-fold) of $\gamma\delta$ T cells in the livers of poly I:C-treated J α 18 KO mice were significantly increased at 16 hours (but not at 3 or 24 hours) compared with those in corresponding C57BL/6 wild-type mice and untreated naive C57BL/6 wild-type mice (Figure 1, A and B). A representative FACS dot plot is shown in Figure 1C. Based on these results, we selected 16 hours as the optimum time point for subsequent studies.

Next, we investigated whether hepatic $\gamma\delta$ T cells displayed signs of activation. For this investigation, we determined (by FACS) intracellular expression of IFN- γ and IL-17 by hepatic $\gamma\delta$ T cells after poly I:C treatment. These cytokines (ie, IFN- γ and IL-17) are routinely secreted by activated $\gamma\delta$ T cells during inflammation.^{17,36,40} Figure 1D shows that V α 14/NKT cell deficiency induced hepatic $\gamma\delta$ T cell activation with poly I:C treatment because the absolute numbers of intracellular IL-17-expressing hepatic $\gamma\delta$ T cells were significantly increased in poly I:C-treated J α 18 KO mice (relative to vehicle-treated C57BL/6 wild-type mice). In contrast, hepatic $\gamma\delta$ T cells obtained from poly I:C-treated C57BL/6 wild-type mice did not induce a statistically significant increase in IL-17 production compared with vehicle-treated C57BL/6 wild-type mice (Figure 1D). A similar trend was seen when serum IL-17 was measured in these mice (data not shown). Notably, poly

Figure 2. Activated hepatic $V\alpha 14i$ NKT cells promote apoptotic death of intrahepatic $\gamma\delta$ T cells after poly I:C treatment. Hepatic $\gamma\delta$ T cells were identified by FACS after cell surface staining with TCR $\gamma\delta$ and CD3 mAbs. Hepatic $\gamma\delta$ T cells were fixed, permeabilized, and then stained intracellularly for active caspase 3. A representative FACS histogram (A) and absolute number (B) of intracellular active caspase 3-positive $\gamma\delta$ T cells are shown. Data are presented as means $\pm$ SEM with three to four mice per group from two independent experiments. * $P \leq 0.05$ versus poly I:C-treated wild-type (WT) mice.

I:C treatment did not induce IFN- γ production by activated hepatic $\gamma\delta$ T cells derived from C57BL/6 wild-type mice or J $\alpha 18$ KO mice (unpublished observation).

A unique feature of activated $V\alpha 14i$ NKT cells is that they promptly regulate the innate immune system by inducing the activation of natural killer (NK) cells in many model systems.^{20,41,42} Because poly I:C treatment promotes hepatic NK cell accumulation and activation,^{43,44} we also evaluated the effects of $V\alpha 14i$ NKT cell deficiency on hepatic NK cell accumulation and activation after poly I:C application. $V\alpha 14i$ NKT cell deficiency did not suppress hepatic NK cell accumulation and activation after poly I:C treatment compared with poly I:C-treated C57BL/6 wild-type mice; M. Ajuebor, unpublished observation.

$V\alpha 14i$ NKT Cell Deficiency Prevents Apoptosis of Intrahepatic $\gamma\delta$ T Cells in Response to Poly I:C Treatment

We showed in Figure 1, A and B, that $V\alpha 14i$ NKT cell deficiency was uniquely associated with increased hepatic $\gamma\delta$ T cell accumulation in response to poly I:C treatment. Next, we determined whether the lack of $\gamma\delta$ T cell accumulation in the livers of C57BL/6 wild-type mice after poly I:C application could be due to apoptotic death of hepatic $\gamma\delta$ T cells by $V\alpha 14i$ NKT cell-derived mediator(s). Moreover, viruses are capable of making immune cells more susceptible to apoptosis. We initially assessed whether $\gamma\delta$ T cells derived from the livers of poly I:C-treated C57BL/6 wild-type mice were dying by apoptosis. Active caspase 3 is a marker widely used to determine programmed cell death.^{16,37} Figure 2, A and B, depicts a significant increase in intracellular active caspase 3 expression by hepatic $\gamma\delta$ T cells obtained from poly I:C-treated C57BL/6 wild-type mice (relative to that seen in the livers of naive C57BL/6 wild-type mice), but this response was severely attenuated in J $\alpha 18$ KO mice with poly I:C treatment. These results suggest that intrahepatic $\gamma\delta$ T cells are susceptible to apoptotic death in the absence of $V\alpha 14i$ NKT cells with poly I:C treatment.

It is a well known fact that $V\alpha 14i$ NKT cells rapidly produce cytokines (including IFN- γ and TNF) after activation.^{16,18} Previous studies have documented elevated serum levels of IFN- γ and TNF in mice treated with poly I:C.^{23,33,44} Moreover, we observed that increased serum

levels of IFN- γ and TNF in C57BL/6 wild-type mice after poly I:C treatment were significantly blunted by $V\alpha 14i$ NKT cell deficiency (data not shown). More importantly, we confirmed by intracellular staining and flow cytometry that hepatic $V\alpha 14i$ NKT cells produce IFN- γ and TNF at 16 hours after poly I:C treatment (Figure 3, A and B). Despite numerous reports^{16,37,45} that hepatic $V\alpha 14i$ NKT cells subsequently die by apoptosis after activation, no $V\alpha 14i$ NKT cell loss was observed in the liver on poly I:C application. Specifically, the frequency (Figure 3C) and absolute number (data not shown) of hepatic $V\alpha 14i$ NKT cells in poly I:C-treated C57BL/6 wild-type mice were comparable to that seen in the livers of vehicle-treated C57BL/6 wild-type mice. These cytokines can promote apoptosis of immune and nonimmune cells.^{46–48} Consequently, we verified whether blockade of endogenous IFN- γ and TNF secreted by activated hepatic $V\alpha 14i$ NKT cells on poly I:C treatment could suppress the apoptosis of hepatic $\gamma\delta$ T cells. As shown in Figure 3D, pretreatment of C57BL/6 wild-type mice with IFN- γ antiserum or TNF mAb before poly I:C treatment significantly suppressed apoptotic death of intrahepatic $\gamma\delta$ T cells as depicted by reduced intracellular active caspase 3 expression relative to that seen in poly I:C-treated C57BL/6 wild-type mice given control antibody.

Effects of Endogenous ROS on Hepatic $V\alpha 14i$ NKT Cell Activation during Poly I:C Treatment

Previous studies have reported that T-cell activation is associated with increased levels of ROS.⁴⁹ Therefore, we evaluated whether endogenous ROS could regulate hepatic $V\alpha 14i$ NKT cell activation during poly I:C treatment. Treatment of C57BL/6 wild-type mice with the ROS scavenger, NAC, during poly I:C treatment significantly suppressed hepatic $V\alpha 14i$ NKT cell activation. Specifically, we found that the percentage of intracellular IFN- γ expressing $V\alpha 14i$ NKT cells in the livers of poly I:C-treated C57BL/6 wild-type mice was significantly attenuated by NAC treatment (Figure 4A). Next, we assessed the effect of impaired hepatic $V\alpha 14i$ NKT cell activation on $\gamma\delta$ T cell accumulation in the liver. Figure 4B shows that simultaneous treatment of C57BL/6 wild-type mice with NAC and

Figure 3. Contribution of V α 14/NKT cell-derived cytokines to apoptosis of intrahepatic γ δ T cells after poly I:C treatment. **A** and **B**: C57BL/6 wild-type mice were treated with poly I:C or vehicle for 16 hours, and isolated hepatic V α 14/NKT cells were identified by FACS after cell surface staining with TCR β mAb and PBS57-CD1d tetramer (described in *Materials and Methods*). The cells were fixed, permeabilized, and then stained intracellularly for IFN- γ or TNF. Representative FACS histograms demonstrating IFN- γ -positive hepatic V α 14/NKT cells and TNF-positive hepatic V α 14/NKT cells are shown in **A** and **B**, respectively, from two independent experiments of four mice per group. **C**: FACS dot plot (from two independent experiments of three mice per group) depicts hepatic V α 14/NKT cell profiles from poly I:C or vehicle-treated C57BL/6 wild-type mice at the 16-hour time point. **D**: C57BL/6 wild-type mice were pretreated with murine IFN- γ antiserum, murine TNF mAb, or control antibody before poly I:C treatment, and 16 hours later isolated hepatic γ δ T cells were fixed, permeabilized, and then stained intracellularly for active caspase 3. A representative FACS histogram highlighting active caspase 3-positive hepatic γ δ T cells from two independent experiments of four mice per group is shown.

poly I:C significantly increased (twofold) γ δ T cell accumulation in the liver compared with that in poly I:C-treated C57BL/6 wild-type mice given vehicle. Notably, the frequency of intrahepatic γ δ T cells in poly I:C/NAC-treated C57BL/6 wild-type mice was comparable to that seen in the livers of poly I:C-treated V α 14/NKT cell-deficient mice (ie, J α 18 KO mice) as shown in Figure 4B. Furthermore,

concurrent treatment of V α 14/NKT cell-deficient mice with NAC and poly I:C augmented intrahepatic γ δ T cell accumulation relative to that of poly I:C-treated V α 14/NKT cell-deficient mice (Figure 4B). Taking these results together, we propose that blockade of endogenous ROS blunts hepatic V α 14/NKT cell activation in response to poly I:C application.

Figure 4. Role of endogenous ROS in hepatic V α 14/NKT cell activation on poly I:C treatment. The ROS scavenger, NAC (300 mg/kg i.p.), was administered simultaneously with poly I:C in C57BL/6 wild-type (WT) mice or J α 18 KO mice. Control mice received vehicle PBS. **A**: Hepatic V α 14/NKT cells were identified by FACS using the CD1d tetramer and TCR β mAb (see *Materials and Methods*) 16 hours after poly I:C treatment and then were stained for intracellular IFN- γ (as a marker for V α 14/NKT cell activation) after cell permeabilization. Results are presented as means $\pm$ SEM with four mice per group from two separate experiments. * $P \leq 0.05$ versus vehicle. **B**: C57BL/6 wild-type mice and J α 18 KO mice were treated concurrently with NAC/poly I:C or PBS/poly I:C for intrahepatic γ δ T cell accumulation assessment by FACS 16 hours later. Data are presented as means $\pm$ SEM with four to six mice per group from two independent studies. *** $P \leq 0.05$ versus all other groups; ** $P \leq 0.05$ versus vehicle-treated wild-type mice (no poly I:C) and poly I:C/PBS-treated wild-type mice; * $P \leq 0.05$ versus vehicle-treated wild-type mice (no poly I:C) and poly I:C/PBS-treated wild-type mice.

Figure 5. Hepatic $V\alpha 14i$ NKT cell TLR3 expression before and after poly I:C treatment. B6129SF2/J wild-type mice were treated with poly I:C or vehicle for 16 hours and extracellular and intracellular TLR3 expression by hepatic $V\alpha 14i$ NKT cells was evaluated by flow cytometry as described in *Materials and Methods*. Representative FACS histograms are shown from two independent experiments.

TLR3 Deficiency Inhibits $V\alpha 14i$ NKT Cell Activation in Response to Poly I:C Treatment

In this series of experiments, we established a critical role for TLR3 in promoting $V\alpha 14i$ NKT cell activation on poly I:C application. A representative FACS histogram in Figure 5 reveals that poly I:C treatment of B6129SF2/J wild-type mice was associated with increased (>3-fold) TLR3 expression on the surface of hepatic $V\alpha 14i$ NKT cells relative to that for vehicle-treated B6129SF2/J wild-type mice. Furthermore, we observed by FACS analysis/cell permeabilization that TLR3 was localized intracellularly in hepatic $V\alpha 14i$ NKT cells after vehicle or poly I:C treatment of B6129SF2/J wild-type mice (Figure 5). The specificity of TLR3 mAb was confirmed using peritoneal macrophages obtained from poly I:C-treated TLR3-deficient mice.⁵⁰ To ascertain the potential role of the TLR3 ligand in regulating the activation of TLR3-expressing $V\alpha 14i$ NKT cells, we used TLR3-deficient mice. Figure 6, A and B, shows that TLR3 deficiency markedly reduced the frequency and absolute number of intracellular IFN- γ -expressing hepatic $V\alpha 14i$ NKT cells after poly I:C treatment compared with those in corresponding B6129SF2/J wild-type mice. In contrast, we found that TLR3 deficiency did

not suppress the frequency and/or absolute number of TNF-producing hepatic $V\alpha 14i$ NKT cells with poly I:C treatment (Figure 6, C and D). Overall, our data provide the first demonstration of a crucial role for TLR3 in regulating the activation of TLR3-expressing $V\alpha 14i$ NKT cells after administration of the TLR3 ligand, poly I:C.

Discussion

$V\alpha 14i$ NKT cells provide an important link between the innate and adaptive immune systems by being able to rapidly produce both T helper (Th) 1 and Th2 cytokines, most notably IFN- γ and IL-4, after activation.^{15–18} Activated $V\alpha 14i$ NKT cells subsequently transactivate innate and adaptive immune cells, including neutrophils, NK cells, dendritic cells, macrophages, and T and B cells.^{15,20,21} Two major mechanisms for $V\alpha 14i$ NKT cell activation have been proposed. In the first model, the presentation of glycolipid antigens (such as α -galactosylceramide or exogenous antigens from pathogens) by CD1d-expressing antigen-presenting cells promptly induces activation of $V\alpha 14i$ NKT cells.^{2,13,14} The second model proposes that $V\alpha 14i$ NKT cells are indirectly acti-

Figure 6. Effects of TLR3 deficiency on hepatic $V\alpha 14i$ NKT cell cytokine production in response to poly I:C application. TLR3-deficient mice and B6129SF2/J wild-type (WT) mice were treated with poly I:C for 16 hours for the isolation and identification of hepatic $V\alpha 14i$ NKT cells by FACS. Intracellular staining was performed on hepatic $V\alpha 14i$ NKT cells (after cell permeabilization) using fluorochrome-labeled cytokine antibodies (see *Materials and Methods*) to determine the percentage and absolute numbers of both IFN- γ expressing $V\alpha 14i$ NKT cells (A and B) as well as TNF-producing $V\alpha 14i$ NKT cells (C and D). The dotted line in C and D represents the value in wild-type mice treated with vehicle. All results are presented as means $\pm$ SEM with three to four mice per group from two independent experiments. * $P \leq 0.05$ versus wild-type mice.

vated (in the absence of foreign antigen for the TCR) by IL-12 and/or IL-18 secreted by TLR (4,7,8,9)-stimulated antigen-presenting cells.^{13,27,28,51,52} Thus, TLRs serve a critical role in the initiation of innate immunity in microbes.^{13,14,27,28} Although TLR7, TLR8, and TLR9 are vital for the recognition of viruses^{23–26} and indirectly induce V α 14/NKT cell activation,^{27,51,52} the potential role of TLR3, an innate immune receptor that is also typically involved in sensing viral danger signals in mediation of V α 14/NKT cell activation remains undefined. The results presented in this study demonstrate an important role for the TLR3 ligand, poly I:C, in promoting hepatic V α 14/NKT cell activation (without prior activation by exogenous viral administration). Furthermore, we identified a novel and previously unrecognized anti-inflammatory role for activated V α 14/NKT cells in negatively regulating intrahepatic $\gamma\delta$ T cell accumulation and thereby preventing potentially harmful activation of intrahepatic $\gamma\delta$ T cells on application of poly I:C.

There is general consensus that TLR3 recognizes dsRNA (such as poly I:C), a structure found in the genome of some viruses and produced as a replication intermediate by viruses.^{23,29,30} Therefore, we assessed TLR3 expression by V α 14/NKT cells in response to poly I:C treatment. Using flow cytometric-based techniques, we observed a significant increase in TLR3 expression on the surface of V α 14/NKT cells with poly I:C treatment relative to the control. In addition, TLR3 was localized intracellularly in hepatic V α 14/NKT cells before and after poly I:C treatment. Our data are consistent with previous reports that TLR3 is expressed intracellularly and/or extracellularly by immune and nonimmune cells (including NK cells, dendritic cells, smooth muscle cells, epithelial cells, $\gamma\delta$ T cells, and mesangial cells).^{31,53–57} To our knowledge this is the first demonstration of TLR3 expression by V α 14/NKT cells. These preliminary findings of surface and intracellular TLR3 expression by V α 14/NKT cells support our pursuit of TLR3 as a potential mediator of V α 14/NKT cell activation.

Poly I:C is routinely used to trigger the TLR3 signaling pathway.^{23,29,56} After binding a dsRNA such as poly I:C, TLR3 signals through the adapter protein TRIF to activate transcription factors including nuclear factor- κ B and interferon regulatory factor 3.²³ These transcription factors induce multiple inflammatory cytokines (such as TNF- α , IFN- γ , and IL-12)^{22,23,56,58} and type I IFNs (IFN- α and IFN- β).²³ Many of the molecules regulated by TLR3 may subsequently regulate the immune response. To ascertain the functional activity of TLR3-expressing V α 14/NKT cells on poly I:C treatment, we used TLR3-deficient mice. Poly I:C treatment was associated with increased hepatic V α 14/NKT cell activation in wild-type mice as shown, by significant increases in intracellular hepatic V α 14/NKT cell IFN- γ and TNF production. These two cytokines are routinely produced by activated V α 14/NKT cells.^{1,16,59} More importantly, TLR3 deficiency markedly diminished V α 14/NKT cell activation because intracellular hepatic V α 14/NKT cell IFN- γ (but not TNF) production was almost completely suppressed in TLR3-deficient mice with poly I:C treatment compared with wild-type mice. This finding that V α 14/NKT cell intracellular TNF production was not

suppressed by TLR3 deficiency with poly I:C treatment suggests that other receptors for poly I:C (such as melanoma differentiation associated gene-5 and retinoic acid inducible gene-I) could potentially contribute to V α 14/NKT cell activation on poly I:C application. It is conceivable that TNF derived from other cells (such as macrophages) may also contribute to the apoptotic death of intrahepatic $\gamma\delta$ T cells. Nonetheless, these novel studies strongly suggest a crucial role for the TLR3 ligand, poly I:C, in promoting the activation of TLR3-expressing V α 14/NKT cells (without prior V α 14/NKT cell activation by exogenously administered virus).

Next, we investigated the functional consequences of V α 14/NKT cell activation on the hepatic innate immune response in response to poly I:C administration. A unique feature of activated V α 14/NKT cells is that they alter innate immune responses by selectively regulating the activation of NK cells in many model systems.^{15,20,21,41,42,60} Poly I:C treatment is associated with increased hepatic NK cell accumulation and activation.^{43,61} For this reason, we evaluated the effects of V α 14/NKT cell deficiency on hepatic NK accumulation and activation on poly I:C application. V α 14/NKT cell deficiency did not suppress hepatic NK cell accumulation and/or activation after poly I:C application. Interestingly, V α 14/NKT cell deficiency caused increased accumulation of intrahepatic $\gamma\delta$ T cells in J α 18 KO mice after poly I:C treatment compared with that in corresponding wild-type mice (discussed below).

$\gamma\delta$ T cells are also considered to be part of the innate immune response. $\gamma\delta$ T cells, a unique T-cell lineage that possess a distinct TCR, are composed of two glycoprotein chains called γ and δ .^{17,36,40} Although $\gamma\delta$ T cells are less widespread in tissues than in conventional $\alpha\beta$ T cells, they are found in a number of different anatomic sites, with the highest frequency in the mucosa.^{36,40} An important feature of $\gamma\delta$ T cells is to regulate the innate and adaptive immune systems by producing Th1-and/or Th2-type cytokines and more recently, IL-17.^{17,36,40} In addition, $\gamma\delta$ T cells exert cytotoxic-mediated killing of a variety of target cells via Fas/Fas ligand.^{17,36,40} Numerous studies have used $\gamma\delta$ T cell-deficient mice and/or $\gamma\delta$ T cell-depleting antibody to confirm a regulatory role for $\gamma\delta$ T cells in the pathophysiology of numerous infectious and autoimmune diseases.^{17,36,40} However, just one study⁶² has attempted to investigate whether $\gamma\delta$ T cells and V α 14/NKT cells are functionally linked *in vivo*. Specifically, Jin et al⁶² reported that either $\gamma\delta$ T cells or V α 14/NKT cells alone were ineffective in mediating allergic airway hyperresponsiveness and the synergy of both cells was required to mediate lung inflammation and injury associated with airway hyperresponsiveness. As discussed above, we observed that V α 14/NKT cell deficiency was associated with increased accumulation of intrahepatic $\gamma\delta$ T cells on poly I:C treatment. Therefore, we specifically assessed how V α 14/NKT cell deficiency may lead to increased accumulation of activated hepatic $\gamma\delta$ T cells after poly I:C treatment.

We provide the following evidence to validate the fact that $\gamma\delta$ T cells and V α 14/NKT cells are functionally linked on application of the TLR3 ligand, poly I:C. First, poly I:C treatment was associated with hepatic V α 14/NKT cell

activation as shown by elevated hepatic intracellular IFN- γ and TNF production. In addition, V α 14/NKT cell deficiency was associated with significant reductions in serum levels of IFN- γ and TNF. Our approach of using intracellular IFN- γ and/or TNF production by V α 14/NKT cells to denote activation is supported by multiple studies.^{1,16,20,42,59,63} These cytokines subsequently induced apoptosis of hepatic $\gamma\delta$ T cells because blockade of endogenous hepatic IFN- γ and TNF with specific antibodies suppressed intrahepatic $\gamma\delta$ T-cell active caspase 3 expression in wild-type mice after poly I:C treatment. Moreover, these cytokines are known to exert pro-apoptotic effects.^{46–48} Second, intrahepatic $\gamma\delta$ T cells derived from V α 14/NKT cell-deficient mice (ie, J α 18 KO mice) were resistant to apoptotic death on poly I:C application compared with corresponding wild-type mice. These results suggest that activated V α 14/NKT cells are capable of influencing the frequency (and absolute number) of intrahepatic $\gamma\delta$ T cells, as evidenced by increased $\gamma\delta$ T cell numbers in J α 18-deficient mice relative to wild-type mice in response to poly I:C application.

Last, blockade of endogenous ROS with the antioxidant, NAC, suppressed hepatic V α 14/NKT cell activation in wild-type mice after poly I:C administration. Interestingly, dampening of hepatic V α 14/NKT cell activation by blockade of ROS generation was also associated with significant increases in intrahepatic $\gamma\delta$ T cell accumulation in wild-type mice after poly I:C treatment. Notably, the frequency of intrahepatic $\gamma\delta$ T cells in NAC/poly I:C-treated wild-type mice was comparable to that seen in the livers of poly I:C-treated V α 14/NKT cell-deficient mice (ie, J α 18 KO mice). Thus, inhibiting V α 14/NKT cell activation by blockade of endogenous ROS generation subsequently promotes intrahepatic $\gamma\delta$ T cell accumulation on poly I:C application. However, our finding that concurrent treatment of V α 14/NKT cell-deficient mice with NAC and poly I:C augmented intrahepatic $\gamma\delta$ T cell accumulation relative to that of poly I:C-treated V α 14/NKT cell-deficient mice, suggests that ROS generation and V α 14/NKT cell activation are acting in parallel, rather than as part of a single pathway. Although previous studies have reported that activation of conventional T cells is

Figure 7. A schematic model to illustrate the mechanisms of TLR3 ligand-dependent hepatic V α 14/NKT cell activation and the subsequent induction of apoptotic death of intrahepatic $\gamma\delta$ T cells on poly I:C application. **Left panel:** V α 14/NKT cells up-regulate TLR3 extracellularly on C57BL/6 wild-type (WT) mice on poly I:C treatment and activation of TLR3-expressing V α 14/NKT cells by poly I:C-induced V α 14/NKT cell cytokine production is through a signaling pathway, which could be dependent on the transcription factors nuclear factor- κ B (NF- κ B) and interferon regulatory factor-3 (IRF-3). Notably, these responses were suppressed by TLR3 deficiency. In addition, our study also revealed that the functional consequences of hepatic V α 14/NKT cell activation by the TLR3 ligand, poly I:C, is the negative regulation of $\gamma\delta$ T cells as shown by decreased accumulation of activated intrahepatic $\gamma\delta$ T cells due to apoptosis by V α 14/NKT cell-derived mediators (ie, cytokines and possibly ROS). **Right panel:** In contrast with that of C57BL/6 wild-type mice, poly I:C treatment of J α 18 KO mice was associated with lower serum levels of prototypical V α 14/NKT cell-derived cytokines (ie, TNF and IFN- γ), most likely due to a deficiency of TLR3-expressing activated hepatic V α 14/NKT cells. The functional consequence of lower cytokine levels is the suppression of apoptotic death of intrahepatic $\gamma\delta$ T cells (and thus increased survival of intrahepatic $\gamma\delta$ T cells) as shown by increased accumulation of $\gamma\delta$ T cells in the livers of poly I:C-treated J α 18 KO mice. Notably, the lack of suppression of hepatic NK cell accumulation and activation in J α 18 KO mice after poly I:C treatment may be due in part to the increased accumulation of $\gamma\delta$ T cells in the livers of these mice because we recently demonstrated that $\gamma\delta$ T cells promote NK cell accumulation in the liver after poly I:C treatment.⁴³ Our study provides the first direct evidence that intrahepatic V α 14/NKT cells and $\gamma\delta$ T cells are functionally linked via TLR3 signaling on application of poly I:C because activated intrahepatic V α 14/NKT cells negatively regulate the recruitment, activation, and potentially harmful effector function(s) of intrahepatic $\gamma\delta$ T cells on application of the TLR3 ligand, poly I:C.

associated with increased levels of ROS,⁴⁹ further studies are required to specifically determine whether activated V α 14/NKT cells are capable of directly releasing ROS after poly I:C treatment. In addition, we cannot completely exclude the potential contribution of ROS generated from other cells such as macrophages in promoting intrahepatic $\gamma\delta$ T cell accumulation in J α 18 KO mice after poly I:C treatment. Moreover, it is well recognized that macrophages are capable of generating ROS.^{64,65} Collectively, we firmly believe that we have established an important anti-inflammatory role for activated hepatic V α 14/NKT cells in negatively regulating intrahepatic $\gamma\delta$ T cell accumulation on poly I:C administration via the induction of apoptosis in a response largely dependent on endogenous cytokines and possibly ROS.

In summary, a novel concept that has been demonstrated by data derived from our study relates to the previously undefined/unrecognized biological role for activated V α 14/NKT cells in negatively regulating $\gamma\delta$ T cell accumulation and activation during the hepatic innate immune response. $\gamma\delta$ T cells are frequently activated by a variety of pathogens to induce cytokines (IL-17 and IFN- γ), which mediate inflammation and autoimmunity and help fight infection by pathogens.^{17,36,66,67} $\gamma\delta$ T cells displayed signs of activation (as shown by a preferential elevation in intracellular IL-17 but not IFN- γ expression) during V α 14/NKT cell deficiency after poly I:C application. Thus, activated $\gamma\delta$ T cells could play a key role in early defense against viral and bacterial infections and also regulate the pathology of autoimmune diseases. On the other hand, $\gamma\delta$ T cells may sustain hepatic NK cell accumulation during V α 14/NKT cell deficiency so that NK cells subsequently regulate the hepatic inflammatory response through cytokine production and death receptors as described in many model systems.^{42,68,69} In conclusion, our studies have revealed that V α 14/NKT cell activation in response to the TLR3 ligand, poly I:C, induced a complex cascade of cellular activation that uniquely involved $\gamma\delta$ T cells (but not NK cells). Specifically, we have provided the first direct evidence that V α 14/NKT cells and $\gamma\delta$ T cells are functionally linked during liver inflammation in response to the TLR3 ligand, poly I:C. Furthermore, we have highlighted a novel and previously unrecognized anti-inflammatory role for activated hepatic V α 14/NKT cells in negatively regulating $\gamma\delta$ T cell accumulation in the liver possibly via TLR3 signaling and thereby preventing potentially harmful activation of intrahepatic $\gamma\delta$ T cells (schematized in Figure 7). Our finding has important implications for the role that V α 14iNKT cell- $\gamma\delta$ T cell cross-talk may play in controlling inflammatory responses to an infection. Our study highlights a novel concept with a new avenue through which activated V α 14/NKT cells can regulate the innate immune response, and this should be explored in infectious disease models.

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