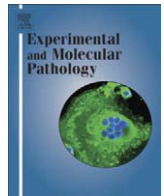




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## Characterization of the role of TCR $\gamma\delta$ in NK cell accumulation during viral liver inflammation

Tommy Gardner, Qingling Chen, Yijun Jin, Maureen N. Ajuebor\*

Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932, USA

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## ABSTRACT

Polyinosinic–polyctidylic acid (Poly I:C) is a viral RNA mimic that can induce immune responses similar to that seen during viral infection. Although poly I:C administration into mice is associated increased NK cell infiltrates in the liver, the mechanisms underlying increased hepatic NK cell accumulation in response to poly I:C administration are incompletely defined. In the current study, we have identified a novel and important role for  $\gamma\delta$ T cells in driving the accumulation and activation of NK cells in the liver during poly I:C-mediated viral liver infection. Specifically, NK cell accumulation but not activation in  $\gamma\delta$ T cell deficient mice following poly I:C administration was significantly attenuated in comparison to that seen in poly I:C-treated wildtype mice. The ability of  $\gamma\delta$ T cells to promote NK cell accumulation and activation in the liver may be virus-specific since NK cell accumulation in the liver was not altered by TCR $\gamma\delta$  deficiency following adenovirus administration.

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## Introduction

NK cell represents a unique thymus-independent lymphoid cell lineage central to innate immunity and are well suited to mediate a rapid first line of defense against most pathogens (Di Santo, 2006; Johansson et al., 2005). NK cells are abundant in the circulating blood, spleen and liver (Ajuebor et al., 2007; Di Santo, 2006). An important feature of NK cells is their ability to regulate the innate immune response by releasing soluble factors (including cytokine) systemically (into the circulation) and locally (i.e. in tissue) (Ajuebor et al., 2007; Di Santo, 2006). In addition, NK cells may also exert cytotoxic-mediated killing of a variety of target cells via Fas/FasL and TRAIL-mediated death pathways (Di Santo, 2006; Johansson et al., 2005). Several lines of evidence suggest an important role for NK cells in the pathophysiology of viral liver infections where NK cells has been shown to exert a hepatoprotective effect during murine cytomegalovirus (mCMV)-induced viral hepatitis since NK cell deficiency worsened liver injury mediated by mCMV infection (Salazar-Mather et al., 1998). Alternatively, the contribution of NK cells to viral liver infection may extend beyond their traditional role as killers of pathogens in that they can also promote liver inflammation and injury in mice in response to hepatitis B virus (Trobonjaca et al., 2002; Trobonjaca et al., 2001), adenovirus (Liu et al., 2000) and the viral RNA mimic, poly I:C (Dong et al., 2004).

The mechanisms by which activated NK cells modulate infectious diseases appear to be similar to that reported for the innate immune T cell,  $\gamma\delta$ T cell, since both innate immune cells are capable of lysing target cells and producing cytokines in response to infectious agents (Carding and Egan, 2000; Carding and Egan, 2002; Johansson et al., 2005). Hence, it has been proposed that NK and  $\gamma\delta$ T cells could have similar functional activities during an ongoing hepatic inflammatory response. For example, a previous report has demonstrated that NK cell contributes to acute viral liver inflammation and injury mediated by adenovirus (Liu et al., 2000). In addition, we recently reported that  $\gamma\delta$ T cells are also capable of initiating acute liver inflammation and injury in mice after adenovirus infection (Ajuebor et al., 2008).

Alternatively, the similarity in functional characteristics of NK and  $\gamma\delta$ T cells during viral liver injury/infection could be attributed to the fact that TCR $\gamma\delta$  (i.e. the receptor for  $\gamma\delta$ T cells) is also upregulated on a subpopulation of NK cells during viral liver injury/infection; an effect that may potentially cause both NK and  $\gamma\delta$ T cells to exert similar responses during the ongoing hepatic inflammatory process. Therefore, in the present study, we have assessed the contribution of the TCR $\gamma\delta$  to NK cell accumulation in the liver during acute viral liver inflammation.

## Materials and methods

## Mice

Male C57BL6/J mice and TCR- $\delta$  deficient mice (C57BL6/J background) aged 5–7 week old were all purchased from The Jackson

\* Corresponding author. Fax: +1 318 675 4156

E-mail address: [majuebor@lsuhsc.edu](mailto:majuebor@lsuhsc.edu) (M.N. Ajuebor).

Laboratory (Bar Harbor, ME). All mice were kept in a conventional animal facility at the Louisiana State University Health Sciences Center-Shreveport and maintained under specific pathogen-free conditions. All procedures in this study were performed in accordance with institutional guidelines for animal care and use.

#### Poly I:C-induced viral liver inflammation

Endotoxin free, Poly I:C (Sigma Chem. Com, St. Louis; dose of 30 mg/kg) or vehicle (sterile PBS) was administered to mice intraperitoneally (Jiang et al., 2008; Wang et al., 2005). All mice were anesthetized with a mixture of xylazine and ketamine hydrochloride at 16 h after poly IC or vehicle administration. Livers were then perfused with ice-cold sterile PBS to remove blood elements and subsequently processed for lymphoid cell isolation using our published protocol (Ajuebor et al., 2008). An end-time point of 16 h was used in all experiments.

#### Isolation of hepatic lymphoid cells and flow cytometry

Hepatic lymphoid cells were isolated as we have recently described (Ajuebor et al., 2008). For the specific identification of NK cells, isolated hepatic lymphoid cells were preincubated with anti-mouse CD16/32 mAb (clone 2.4G2; BD Pharmingen) to block Fc $\gamma$ Rs and then stained with fluorochrome-labeled anti-NK1.1 mAb (clone PK136; BD Pharmingen) according to the manufacturer's instructions. Next, two-color staining was used to assess intracellular IFN- $\gamma$  expression by hepatic NK cells. Briefly, fluoro-chrome-labeled NK cells was permeabilized with Cytofix/Cytoperm plus (BD Pharmingen) and then stained intracellularly with fluoro-chrome-labeled murine IFN- $\gamma$  mAb (clone XMG1.2; BD Pharmingen) according to the manufacturer's instructions (Ajuebor et al., 2008).

To identify  $\gamma\delta$ T cells, isolated hepatic lymphocytes were preincubated with anti-mouse CD16/32 mAb (clone 2.4G2; BD Pharmingen) to block Fc $\gamma$ Rs and then incubated simultaneously with fluorochrome-labeled TCR $\gamma\delta$  Ab (clone GL3; BD Pharmingen) and fluorochrome-labeled CD3 $\epsilon$  mAb (clone 145-2C11; BD Pharmingen) as we have recently described (Ajuebor et al., 2008). In all experiments, corresponding isotype control antibodies were used to set analysis gates. In addition, only viable lymphocyte populations were gated using forward and side scatter characteristics and analyzed using a FACS Calibur and FACS Scan Diva software (BD Biosciences).

#### Statistical analysis

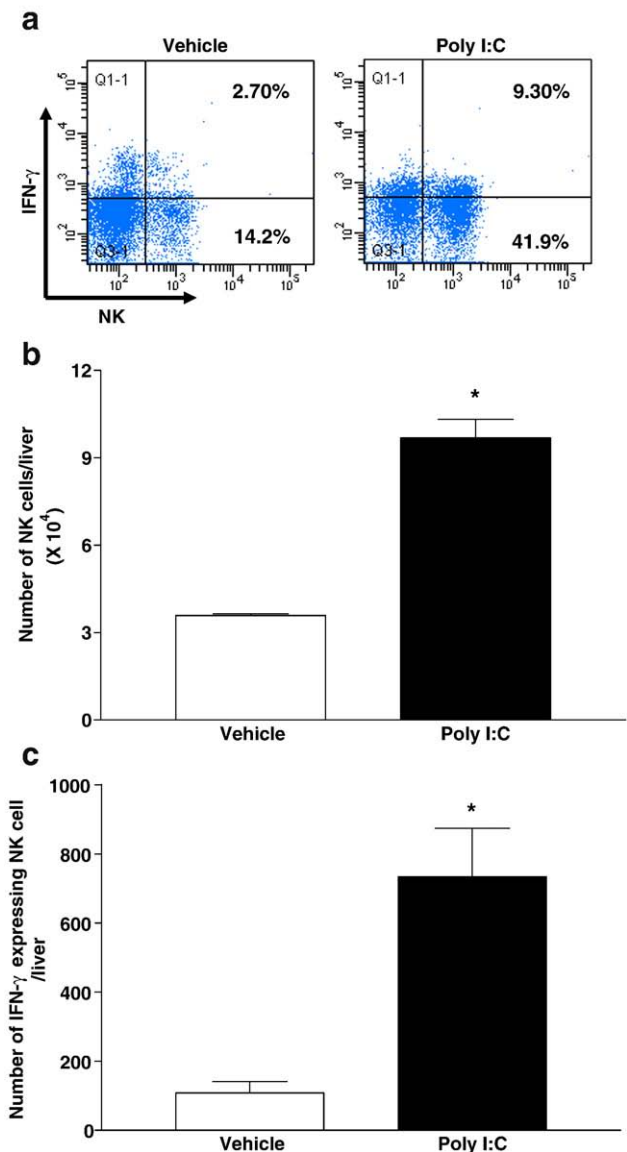
All data are shown as mean $\pm$ SEM. For comparisons of means between 2 experimental groups a Student unpaired *t* test was used. Comparison among three or more experimental groups was performed using a one-way ANOVA, followed by either Dunnett's multiple comparison test or Newman–Kuels post hoc test. A value of  $p < 0.05$  was considered significant.

#### Results and discussion

Poly I:C, a synthetic analogue of double stranded RNA (dsRNA), is widely used to mimic viral infection (Alexopoulou et al., 2001). Numerous studies have demonstrated that poly I:C can induce immune responses similar to that seen during viral infection since dsRNA is a viral gene product that is generated during the replication of many viruses (Jacobs and Langland, 1996). In the last decade, mounting evidence from animal models have established an important protective or pathogenic role for NK cells during viral liver infection mediated by HBV (Trobonjaca et al., 2002; Trobonjaca et al., 2001), mCMV (Salazar-Mather et al., 1998), adenovirus (Ajuebor et al., 2008) and poly I:C (Dong et al., 2004).

$\gamma\delta$ T cell has also been shown to exert a protective or pathogenic response in many viral diseases affecting the brain (Ponomarev and Dittel, 2005; Ponomarev et al., 2004), mucosa (Hayday et al., 2000) and liver (Ajuebor et al., 2008). Although NK and  $\gamma\delta$ T cells belong to distinct lineages, they present similar functional activities during viral infections since both innate immune cells are capable of lysing target cells and producing cytokines in response to infectious agents (Biron et al., 1999; Hayday and Tigelaar, 2003). Our study assessed the relationship between  $\gamma\delta$ T and NK cells during acute viral liver infection mediated by poly I:C.

In agreement with previous reports (Dong et al., 2004; Wang et al., 2005), we observed that poly I:C administration into mice was associated with increased NK cell accumulation in the liver (Fig. 1). Specifically, the frequency ( $\sim$ 3-fold) and absolute number ( $\sim$ 3-fold) of NK cells in the liver were significantly increased at 16 h after

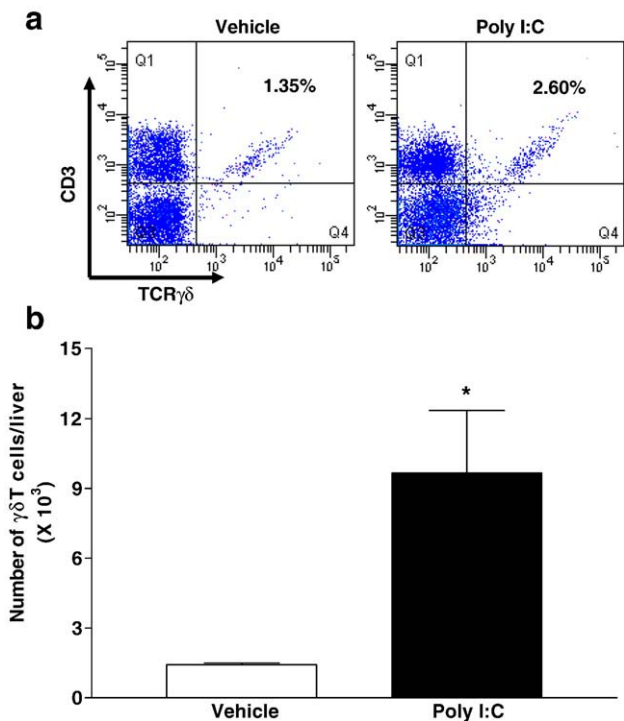


**Fig. 1.** Hepatic NK cell recruitment and activation in poly I:C-treated mice. (a) A representative FACS dot plot demonstrating NK cell accumulation (lower right quadrant) intracellular IFN- $\gamma$  expression by isolated hepatic NK cells (upper right quadrant) after poly I:C or vehicle administration. (b) Absolute number of NK cell accumulation in the liver of poly I:C or vehicle-treated mice. Data are presented as mean $\pm$ SEM;  $n = 3-4$  mice per group; \* $P \leq 0.05$  versus vehicle. (c) Absolute number of IFN- $\gamma$  expressing hepatic NK cells after poly I:C or vehicle treatment. Values are presented as mean $\pm$ SEM;  $n = 3-4$  mice per group; \* $P \leq 0.05$  versus vehicle.

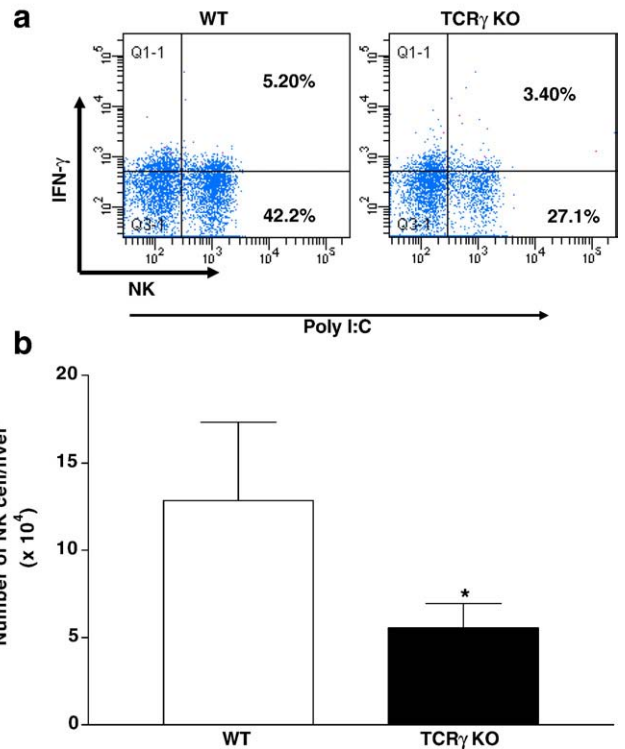
poly I:C administration when compared to vehicle-treated mice (Figs. 1a, b). We also confirmed that previous findings (Dong et al., 2004; Wang et al., 2005) that poly I:C activates hepatic NK cell since the percentage (Fig. 1a) and absolute number (Fig. 1c) of intracellular IFN- $\gamma$  expression by isolated hepatic NK cells were increased in response to poly I:C treatment relative to vehicle.

Next, we used flow cytometry-based techniques to determine whether poly I:C administration into mice was also associated with increased  $\gamma\delta$ T cell [identified as TCR $\gamma\delta$ -CD3(+) double positive T cells] infiltrates in the liver. Interestingly, we observed that  $\gamma\delta$ T cells constitute a minor population of T cell in the liver of vehicle-treated mice (Fig. 2). With poly I:C administration, the frequency of  $\gamma\delta$ T cells in the liver was modestly but significantly increased (~2-fold) at 16 h when compared to vehicle-treated mice (Fig. 2a). Furthermore, the absolute number  $\gamma\delta$ T cells in the liver after poly I:C administration was significantly increased (~7-fold) over vehicle-treated controls (Fig. 2b). Although poly I:C has been reported to cause liver injury in mice (Dong et al., 2004), we did not see observe liver damage in our study since serum alanine transaminase (ALT) level in poly I:C-treated mice was similar to that seen in vehicle-treated mice (data not shown). ALT is widely used as a biochemical marker for liver damage (Ajuebor et al., 2005; Ajuebor et al., 2008; Dong et al., 2004).

Next, we determined the potential mechanisms that may promote the accumulation of NK cells in the liver following poly I:C administration. In the first mechanism, we investigated whether a potential crosstalk between  $\gamma\delta$ T and NK cells may underlie increased accumulation of hepatic NK cells after poly I:C administration. Indeed, we demonstrate a previously unrecognized important role for the  $\gamma\delta$ T cells in driving the accumulation of NK cells in the liver during poly I:C-mediated viral liver infection since hepatic NK cells accumulation in  $\gamma\delta$ T cell deficient mice (i.e. TCR- $\delta$  KO mice) following poly I:C administration was significantly attenuated in comparison to that seen in the liver of poly I:C-treated wildtype



**Fig. 2.**  $\gamma\delta$ T cell accumulation in the liver after poly I:C treatment. Hepatic lymphoid cells were isolated at the indicated times, stained with specific fluorochrome-labeled TCR $\gamma\delta$  and CD3 mAbs and then analyzed by flow cytometry to reveal the percentages (a; representative FACS dot plot) and absolute numbers (b) of  $\gamma\delta$ T cells (i.e. TCR $\gamma\delta$ -CD3 double positive T cells) per liver. Data are presented as mean $\pm$ SEM;  $n=3-4$  mice per group; \* $P\leq 0.05$  versus vehicle.



**Fig. 3.** Effects of TCR $\gamma\delta$  deficiency on hepatic NK cell accumulation in response to poly I:C administration. (a) A representative FACS dot plot demonstrating reduced NK cell accumulation (lower right quadrant) in the liver of TCR $\gamma\delta$  deficient mice after poly I:C administration relative to that seen in poly I:C-treated wildtype (WT) mice. (b) Absolute number of NK cell accumulation in the liver of poly I:C treated WT or TCR $\gamma\delta$  deficient mice. Data are presented as mean $\pm$ SEM;  $n=3-4$  mice per group; \* $P\leq 0.05$  versus WT.

mice (Fig. 3). Interestingly, NK cell activation (in terms of NK cell intracellular IFN- $\gamma$  expression) was not significantly suppressed by TCR- $\delta$  deficiency (MNA, unpublished observation).

Recent studies demonstrate that mouse NK cell express TCR $\gamma\delta$  transcripts during development (Stewart et al., 2007; Veinotte et al., 2006) and infection (Emoto et al., 2001). In the second and final mechanism, we speculated that the reduction in hepatic NK cell accumulation by TCR $\gamma\delta$  deficiency could be attributed to a reduction in the subpopulation of hepatic NK cells that may have upregulated the TCR $\gamma\delta$  during poly I:C-mediated viral liver infection. However, in contrast to published reports that murine NK cell express TCR $\gamma\delta$  transcripts during development (Stewart et al., 2007; Veinotte et al., 2006) and infection (Emoto et al., 2001), we found by flow cytometric analysis that the TCR $\gamma\delta$  was not upregulated extracellularly or intracellularly by hepatic NK cells following poly I:C administration (MNA, unpublished observation). It has been previously demonstrated that NK cell accumulation in the liver following poly I:C administration may be attributed to IL-12 produced by activated Kupffer cells (Dong et al., 2004). Therefore, it is possible that TCR $\gamma\delta$  deficiency could potentially suppress hepatic IL-12 levels; an effect that could lead to reduced NK cell accumulation in the liver of poly I:C-treated  $\gamma\delta$ T cell deficient mice.

In summary, our study characterized a key mechanism that governed the accumulation of NK cells in the liver of poly I:C infected mice. We showed an important role for  $\gamma\delta$ T cells in promoting the accumulation of NK cells in the liver during viral liver infection mediated by poly I:C. It is noteworthy that the ability of  $\gamma\delta$ T cells to promote NK cell accumulation in the liver may be virus-specific. Our hypothesis is based on our recent observation that NK cell accumulation in the liver is not altered by TCR $\gamma\delta$  deficiency following adenovirus administration (Ajuebor et al., 2008).

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