

# IFN- $\gamma$ production by human natural killer cells in response to HCV-infected hepatoma cells is dependent on accessory cells

Shuye Zhang<sup>1,2,3,†</sup>, Banishree Saha<sup>1,†</sup>, Karen Kodys<sup>1</sup>, Gyongyi Szabo<sup>1,\*</sup>

<sup>1</sup>University of Massachusetts Medical School, Worcester, MA 10605, United States; <sup>2</sup>Shanghai Public Health Clinical Center and Institutes of Biomedical Sciences, Fudan University, Shanghai, China; <sup>3</sup>Key Laboratory of Medical Molecular Virology, Institute of Medical Microbiology, Shanghai Medical College of Fudan University, Shanghai, China

**Background & Aims:** Interferon- $\gamma$  (IFN- $\gamma$ ), a cytokine produced by activated natural killer cells (NK) and T lymphocytes, is an important regulator of innate and adaptive immunity during hepatitis C virus (HCV) infection. However, the cellular sources and mechanisms of IFN- $\gamma$  induction in HCV-infection are not fully understood.

**Methods:** We cultured normal human peripheral blood mononuclear cells (PBMCs) with different populations of immune cells and JFH-1 HCV-infected HuH7.5 (JFH-1/HuH7.5) cells.

**Results:** We found that PBMCs produced large amounts of IFN- $\gamma$  after co-culture with JFH-1/HuH7.5 cells. Using intracellular cytokine staining, we confirmed that NK cells and NKT cells (to a lesser extent) were the major IFN- $\gamma$  producers within PBMCs. Purified NK/NKT cells did not produce IFN- $\gamma$  in response to JFH-1/HuH7.5 cells and depletion of accessory (HLA-DR<sup>+</sup>) cells prevented IFN- $\gamma$  induction in PBMCs. Through selective cell depletion of dendritic cells or monocytes from PBMCs, we determined that plasmacytoid dendritic cells (pDCs) were indispensable for NK-IFN- $\gamma$  induction and the presence of monocytes was needed for maximal NK-IFN- $\gamma$  induction. We further revealed that NK-IFN- $\gamma$  induction depended on pDC-derived IFN- $\alpha$  while other IFN- $\gamma$  inducing cytokines, IL-12, and IL-18, played minimal roles. Close contact between JFH-1/HuH7.5 cells and NK cells was required for IFN- $\gamma$  production and monocyte-derived IL-15 significantly augmented IFN- $\gamma$  induction.

**Conclusions:** We discovered a novel mechanism where NK cells interact with pDCs and monocytes, efficiently producing IFN- $\gamma$  in response to HCV-infected cells. This indicates that co-operation between NK cells and accessory cells is critical for IFN- $\gamma$  production and regulation of immunity during HCV infection.

Keywords: NK; pDC; Monocyte; IFN- $\alpha$ .

Received 18 October 2012; received in revised form 17 April 2013; accepted 17 April 2013; available online 7 May 2013

\* Corresponding author. Address: University of Massachusetts Medical School, Department of Medicine, 364 Plantation Street, Worcester, MA 01605, United States. Tel.: +1 508 856 5275; fax: +1 508 856 4770.

E-mail address: gyongyi.szabo@umassmed.edu (G. Szabo).

<sup>†</sup> These authors contributed equally to this work.

**Abbreviations:** DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; IFN, interferon; ISG, interferon stimulated gene; mDC, myeloid dendritic cell; NK, natural killer; NKT, natural killer T; PAMPs, pathogen associated molecular patterns; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; pDC, plasmacytoid dendritic cell; TLR, toll-like receptor.



ELSEVIER

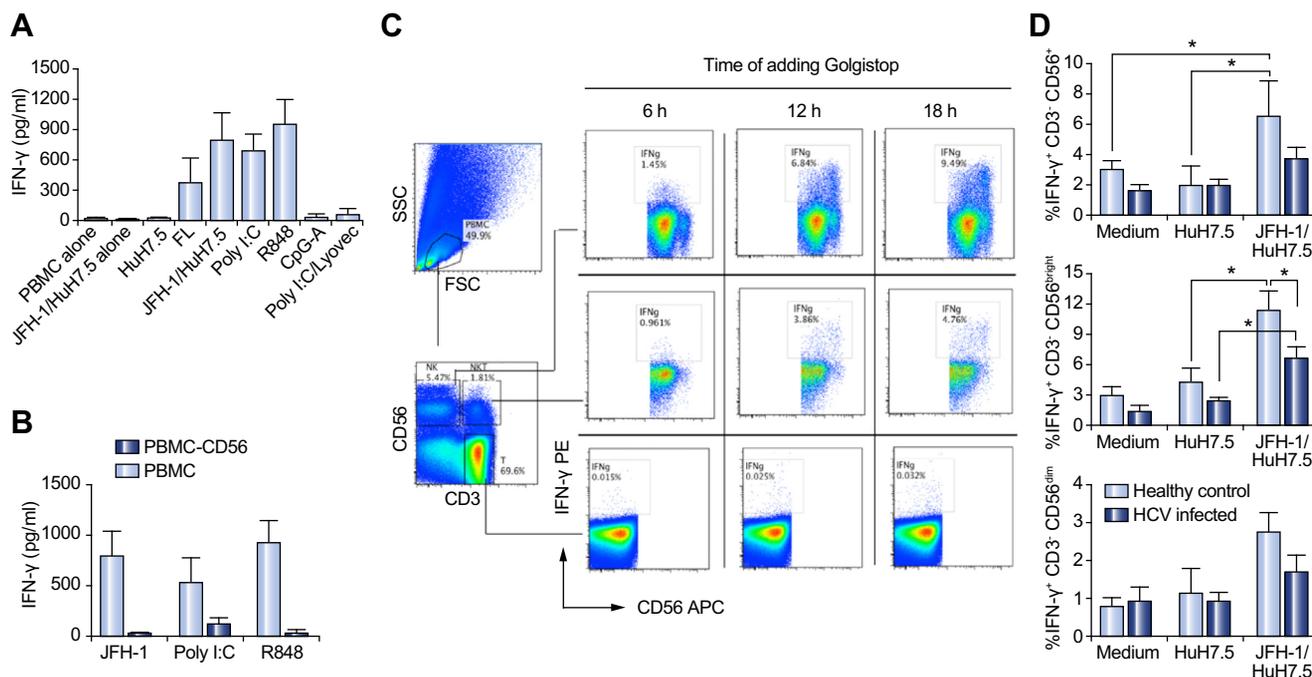
© 2013 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

## Introduction

HCV infection is a major health burden, leading to severe liver diseases in millions of people worldwide [1]. Immune responses play a crucial role in virus control and affect disease progression, although the underlying mechanism is not completely understood [2]. Recently, several studies have shown that natural killer (NK) cells, an important innate immune cell population, are involved in anti-HCV immune responses in both acute and chronic hepatitis C infection [3–7]. Increased NK cell cytotoxicity against HCV-infected cells mediated by type I IFNs has been shown by several studies [8–10], yet, it is unclear whether NK cells produce immunoregulatory IFN- $\gamma$  in response to HCV-infection.

The activation of NK cells is mainly regulated by the balance of inhibitory and activating signals triggered by a series of surface receptors [11], however, NK cells can also respond to signals derived from activated accessory cells [12,13]. It has been well documented that accessory cell-derived IL-12 is crucial for NK cell-IFN- $\gamma$  production in response to various TLR agonists and infectious agents; in contrast, pDC-derived IFN- $\alpha$  increases NK cell cytotoxicity [14]. Although previous reports show that NK cells themselves do not respond to HCV virions or HCV-infected cells [15,16], recent reports revealed that human dendritic cells can be activated to produce IFNs in response to HCV-infected cells [17,18]. Based on these findings, we hypothesized that human NK cells can respond to HCV-infected cells and this might be facilitated by accessory cells.

In this study, we evaluated the roles of distinct accessory cell populations involved in NK-IFN- $\gamma$  induction and their interacting mechanisms in response to HCV-infected cells. For the first time, we showed that NK cells produced IFN- $\gamma$  in response to HCV-infected cells in a pDC- and type I IFN-dependent manner and that monocyte-derived IL-15 augmented this process. These results suggest that IFN- $\gamma$  produced by NK cells could play a crucial antiviral role during HCV-infection and NK cells are part of the immune network in response to HCV infection.



**Fig. 1. Human NK cells produce IFN- $\gamma$  in response to HCV-infected hepatoma cells.** (A) Human PBMCs ( $4 \times 10^6$ ) were co-cultured with JFH-1/HuH7.5 cells or full-length HCV replicons ( $2.5 \times 10^5$ ) or stimulated with TLR3 ligand – poly I:C (20  $\mu$ g/ml), TLR7/8 ligand – R848 (2  $\mu$ g/ml), TLR9 ligand – CpG-A (2  $\mu$ M) or RIG-I ligand poly I:C/Lyovec (1  $\mu$ g/ml) for 24 h. (B) Human PBMCs or PBMCs depleted of CD56<sup>+</sup> cells were co-cultured with JFH-1/HuH7.5 cells or stimulated with poly I:C or R848 for 24 h, IFN- $\gamma$  production in the supernatants was measured by ELISA (mean  $\pm$  SD,  $n = 3-6$ ). (C) Human PBMCs were co-cultured with JFH-1/HuH7.5 cells and Golgistop was added at different time points afterwards. 24 h after co-culture, intracellular IFN- $\gamma$  production in NK, NKT, and T cells was measured by flow cytometry. One representative data of three independent experiments is shown. (D) PBMCs and JFH-1/HuH7.5 cells were co-cultured, treated with Golgistop after 6 h, and harvested after 24 h to measure intracellular IFN- $\gamma$ . The data is presented as mean  $\pm$  SD; healthy control,  $n = 7$ ; HCV-infected,  $n = 7$ , \* $p < 0.05$ .

## Materials and methods

### Cells, replicons, and viruses

HuH7.5 cells and full-length genomic replicon cells (FL) were kindly provided by Dr. C. Rice. Transfection of *in vitro* synthesized JFH-1 RNA constructs and production of JFH-1 virus stock were performed as previously described [19]. Detailed information and protocols are described in the [Supplementary Materials and methods section](#).

### Preparation of human PBMCs, DC subsets, and co-culture experiments

PBMCs were isolated using Ficoll-Hypaque density centrifugation. Informed consent was obtained according to procedures approved by the Committee for Protection of Human Subjects in Research at the University of Massachusetts Medical School. Detailed protocols for different cell isolation and co-culture experiments are described in the [Supplementary Materials and methods section](#).

### Reagents, enzyme-linked immunosorbent assay, RNA quantification, flow cytometry, and statistical analysis

See the [Supplementary Materials and methods section](#) for more details.

## Results

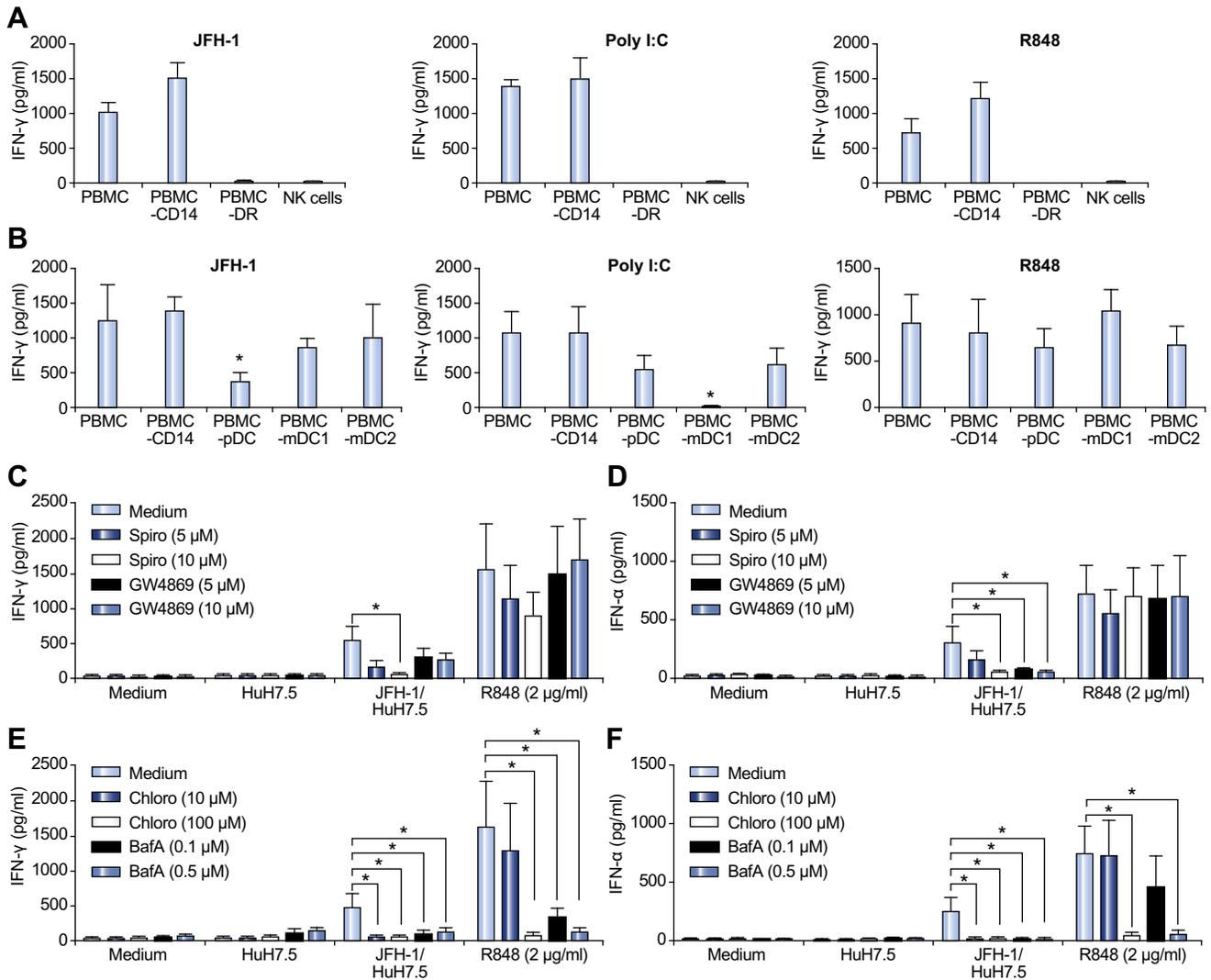
### Human NK cells produce IFN- $\gamma$ in response to HCV-infected cells

To test whether IFN- $\gamma$  was induced by human immune cells in response to HCV infection, human PBMCs were co-cultured with HCV-infected HuH7.5 hepatoma cells (JFH-1/HuH7.5) or stimu-

lated with canonical PAMPs mimicking the viral ligands. We found that a large amount of IFN- $\gamma$  was induced in PBMCs by HCV-containing full-length replicon cells (FL) or JFH-1/HuH7.5 cells and not by uninfected HuH7.5 cells or PBMCs alone (Fig. 1A). The TLR3 ligand, Poly I:C, and TLR7/8 ligand, R848, also stimulated IFN- $\gamma$  production while TLR9 ligand, CpG-A, and RIG-I ligand, poly I:C/Lyovec, did not or only weakly induced IFN- $\gamma$  production. Next, we tested whether NK cells represent IFN- $\gamma$  producers in response to HCV-infected cells, Poly I:C or R848 and found that depletion of CD56<sup>+</sup> cells (human NK and NKT marker) in PBMCs abolished IFN- $\gamma$  production in all scenarios (Fig. 1B). Furthermore, using intracellular IFN- $\gamma$  staining, we observed 10% of NK cells (CD3<sup>+</sup>CD56<sup>+</sup>) and 5% of NKT cells (CD3<sup>+</sup>CD56<sup>+</sup>) producing IFN- $\gamma$  in response to HCV-infected cells, while conventional T cells (CD3<sup>+</sup>CD56<sup>-</sup>) failed to produce IFN- $\gamma$  (Fig. 1C). Poly I:C and R848 also stimulated NK cells to produce IFN- $\gamma$ , and IFN- $\gamma$  production was more rapidly induced in response to Poly I:C and R848 than HCV-infected cells (Supplementary Fig. 1A). In addition, a small percentage of conventional T cells also responded to Poly I:C with IFN- $\gamma$  production (Supplementary Fig. 1B). These observations suggested different mechanisms responsible for IFN- $\gamma$  production in response to different stimulations.

Human NK cells consist of two major subsets: the CD56 bright, regulatory and less cytotoxic subset, and the CD56 dim, cytotoxic and less regulatory subset [20]. CD56 bright NK cells are enriched in the human liver. Here we found more IFN- $\gamma$  positivity in the CD56 bright compared to CD56 dim subset in response to HCV-infected cells, suggesting that the CD56 bright

## Research Article



**Fig. 2. Human NK cells produce IFN- $\gamma$  in response to HCV-infected hepatoma cells in an accessory cell-dependent manner.** (A) Human PBMCs, PBMCs depleted of CD14<sup>+</sup> monocytes (PBMC-CD14), PBMCs depleted of HLA-DR<sup>+</sup> cells (PBMC-DR) or purified NK cells were co-cultured with JFH-1/HuH7.5 cells or stimulated with poly I:C or R848 for 24 h, IFN- $\gamma$  production in the supernatants was measured by ELISA (mean  $\pm$  SD, n = 3). (B) Human PBMCs, PBMCs depleted of CD14<sup>+</sup> monocytes (PBMC-CD14), PBMCs depleted of pDCs (PBMC-pDC), PBMCs depleted of mDC1s (PBMC-mDC1) or PBMCs depleted of mDC2s (PBMC-mDC2) were co-cultured with JFH-1/HuH7.5 cells or stimulated with poly I:C or R848 for 24 h, IFN- $\gamma$  production in the supernatants was measured by ELISA (mean  $\pm$  SD, n = 3); \*p < 0.05 vs. other groups. (C and D) PBMCs from healthy donors were co-cultured with JFH-1/HuH7.5 cells or stimulated with R848 for 24 h in the presence or absence of exosome inhibitors as indicated. IFN- $\gamma$  and IFN- $\alpha$  production in the supernatants was measured by ELISA (mean  $\pm$  SD, n = 6, \*p < 0.05). (E and F) IFN- $\gamma$  and IFN- $\alpha$  release was measured by ELISA in the presence of endosome inhibitors, chloroquine, and bafilomycin A (mean  $\pm$  SD, n = 6, \*p < 0.05).

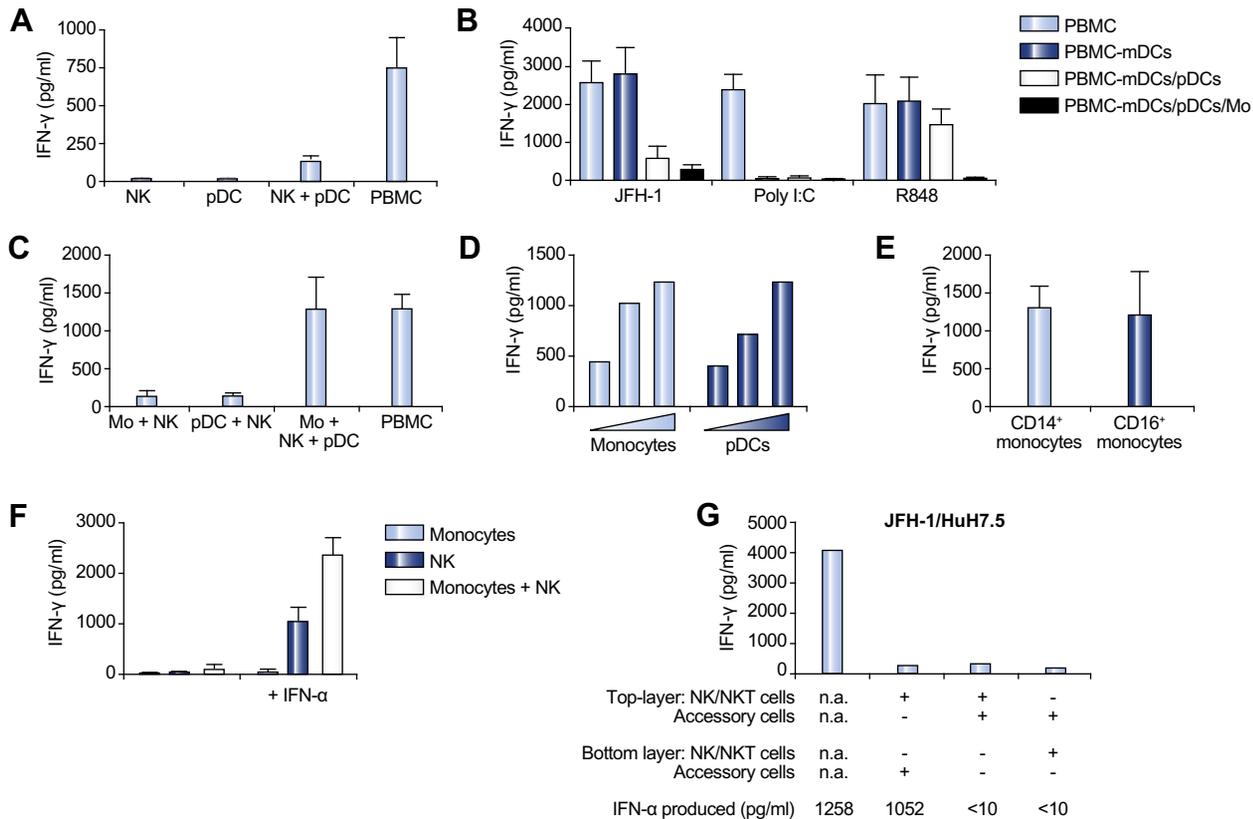
subset may play a major role in IFN- $\gamma$  production in HCV-infected livers (Fig. 1D and Supplementary Fig. 2C and D). We also observed less IFN- $\gamma$  induction by JFH-1/HuH7.5-infected hepatoma cells and in HCV-infected patients compared to healthy donors (Fig. 1D and Supplementary Fig. 2A and B).

#### Plasmacytoid dendritic cells are required for NK cell IFN- $\gamma$ production in response to HCV-infected cells

Next, we examined whether NK cells alone could produce IFN- $\gamma$  in response to HCV-infected cells. We found that purified NK cells did not produce IFN- $\gamma$  when co-cultured with JFH-1/HuH7.5 cells. Furthermore, depletion of accessory cells (HLA-DR<sup>+</sup>) from PBMCs also prevented IFN- $\gamma$  induction by HCV-infected cells (Fig. 2A),

indicating that NK-IFN- $\gamma$  production in response to HCV-infected cells totally depended on accessory cells. Likewise, IFN- $\gamma$  induction by Poly I:C and R848 required the presence of accessory cells (Fig. 2A).

To identify the indispensable accessory cell population(s) for NK-IFN- $\gamma$  production, we depleted the distinct accessory cell subsets from PBMCs to test their distinctive roles in NK-IFN- $\gamma$  induction by HCV-infected cells. We found that depletion of CD14<sup>+</sup> monocytes, CD1c<sup>+</sup> mDC1s or BDCA3<sup>+</sup> mDC2s did not prevent IFN- $\gamma$  induction in response to HCV-infected cells, while depletion of pDCs significantly reduced IFN- $\gamma$  production (Fig. 2B), suggesting that pDCs played an essential role in NK-cell activation by HCV-infected cells. We also found that CD1c<sup>+</sup> mDC1s and monocytes were involved in NK-IFN- $\gamma$  production in response to Poly



**Fig. 3. Both human pDC and monocytes are required for optimal IFN- $\gamma$  induction in NK cells in response to HCV-infected cells.** (A) Purified human NK cells, pDCs, NKs and pDCs or PBMCs were co-cultured with JFH-1/HuH7.5 cells for 24 h. (B) Human PBMCs or PBMCs depleted of accessory cell subsets as indicated, were co-cultured with JFH-1/HuH7.5 cells for 24 h. (C) Human monocytes, pDCs, and NK cells were co-cultured with JFH-1/HuH7.5 cells for 24 h. (D) Increased amounts of monocytes or pDCs were co-cultured with NKs and JFH-1/HuH7.5 cells for 24 h. (E) Purified CD14<sup>+</sup> or CD16<sup>+</sup> monocytes were co-cultured with NKs and JFH-1/HuH7.5 cells for 24 h. (F) Human monocytes and NK cells were co-cultured with JFH-1/HuH7.5 cells in the presence or absence of exogenous IFN- $\alpha$  for 24 h. Data from (A–F) is represented as mean  $\pm$  SD. (G) Accessory cells and NK cells were separated with co-cultured JFH-1/HuH7.5 cells using transwell insert as indicated, one representative data of three experiments is shown. In all experiments (A–G), IFN- $\gamma$  production was measured by ELISA 24 h after co-cultures.

I:C and R848 stimulation, respectively (Fig. 2B and Supplementary Fig. 3), suggesting that a unique mechanism, different from Poly I:C and R848 induced NK activation, was involved in NK-IFN- $\gamma$  induction in response to HCV-infected cells.

HCV-RNA containing exosomes from HCV-infected cells may activate pDC to produce IFN- $\alpha$  [21]. To test whether this process was also required for NK-IFN- $\gamma$  production, we studied the effects of two structurally unrelated neutral sphingomyelinase inhibitors (spiroepoxide and GW4869) that inhibit exosome release. As shown in Fig. 2C, spiroepoxide significantly reduced IFN- $\gamma$  secretion in co-cultures of PBMCs with JFH-1/HuH7.5. GW4869 also lowered the IFN- $\gamma$  levels but to a lower extent. Both spiroepoxide and GW4869 significantly reduced IFN- $\alpha$  secretion in a dose dependent manner (Fig. 2D). Neither compound inhibited IFN- $\alpha$  nor IFN- $\gamma$  production mediated by R-848.

HCV-infected cells activate pDCs by triggering TLR7 [17,18]. Hence, we treated the co-cultures of PBMC and JFH-1/HuH7.5 with endosome acidification inhibitors, chloroquine and bafilomycin A. As shown in Fig. 2E and F, we observed that both inhibitors abolished the IFN- $\alpha$  and IFN- $\gamma$  induction in the co-cultures or R-848 treated PBMCs, indicating that TLR-7/8 and endosome pathways played essential roles in triggering NK cells to produce IFN- $\gamma$ .

#### Monocytes are required for optimal IFN- $\gamma$ induction in response to HCV-infected cells

To further evaluate the requirement of HCV-induced IFN- $\gamma$  induction in NK cells and the role of pDC in this process, we tested co-cultures of isolated pDCs and NK cells with HCV-infected HuH7.5 cells. However, isolated pDCs plus NK cells only produced minimal IFN- $\gamma$  in response to HCV-infected cells compared to PBMCs (Fig. 3A), suggesting the necessity for other cell populations for optimal NK-IFN- $\gamma$  induction. We further found that depletion of mDCs did not affect NK-IFN- $\gamma$  induction; however, depletion of both pDCs and monocytes abolished NK cell-IFN- $\gamma$  induction (Fig. 3B), suggesting the involvement of both pDCs and monocytes in the optimal IFN- $\gamma$  induction in response to HCV-infected cells. We also confirmed that mDCs and monocytes were necessary for NK-IFN- $\gamma$  induction by Poly I:C and R848, respectively (Fig. 3B).

To evaluate the importance of monocytes, we isolated CD14<sup>+</sup> monocytes, pDCs, and NK cells, then co-cultured them with JFH-1/HuH7.5 cells. We found that monocytes-NK or pDCs-NK co-cultures produced minimal IFN- $\gamma$ , while a mixture of monocytes, pDCs, and NK cells synergistically resulted in comparable levels of IFN- $\gamma$  as PBMCs in response to HCV-infected cells

## Research Article

(Fig. 3C). We further showed a dose-dependent increase in NK-IFN- $\gamma$  induction with the increasing number of monocytes or pDCs in co-cultures (Fig. 3D). Finally, we tested the role of both CD14<sup>+</sup> monocytes and CD16<sup>+</sup> monocytes and found that both cell populations comparably enhanced NK-IFN- $\gamma$  production (Fig. 3E). Since the type I IFN was produced by pDCs in co-cultures, we examined whether the dependence on pDC could be replaced by exogenous type I IFN. We found that adding recombinant IFN- $\alpha$ 2a to co-cultures of NK cells and HCV-infected HuH7.5 cells induced IFN- $\gamma$  production although at a relatively low level (Fig. 3F), while the presence of monocytes in co-cultures plus exogenous IFN- $\alpha$  synergistically increased NK-IFN- $\gamma$  production (Fig. 3F). By contrast, type I IFN and monocytes did not affect Poly I:C induced NK-IFN- $\gamma$  production (Supplementary Fig. 4). We also determined that cell-cell contact between NK cells and HCV-infected cells was required for IFN- $\gamma$  induction in co-cultures. Separation between NK cells, accessory cells, and HCV-infected HuH7.5 cells by transwell disrupted NK-IFN- $\gamma$  production even in the presence of type I IFN production (Fig. 3G).

### *Mechanisms involved in NK-IFN- $\gamma$ induction in response to HCV-infected cells*

To search for the underlying mechanism of IFN- $\gamma$  induction, we studied the potential cytokine involvement in NK-IFN- $\gamma$  production in co-cultures, including the IFN- $\gamma$  inducing cytokines, IL-12 and IL-18, and the pDC specific cytokine, IFN- $\alpha$ . We found substantial IFN- $\alpha$  induction and minimal induction of IL-12 and IL-18 in co-cultures of human PBMCs and JFH-1/HuH7.5 cells (Fig. 4A and data not shown), indicating the involvement of IFN- $\alpha$  but not IL-12 or IL-18 in NK-IFN- $\gamma$  induction in response to HCV-infected cells. We further showed that anti-IL12 blocking antibody did not affect IFN- $\gamma$  induction while anti-IFNAR blocking antibody significantly reduced IFN- $\gamma$  induction in co-cultures (Fig. 4B), confirming the essential role of type I IFN but not IL-12 in NK-IFN- $\gamma$  induction in response to HCV-infected cells. In contrast, IL-12 was substantially induced by Poly I:C stimulation and anti-IL-12 blocking antibody completely prevented NK-IFN- $\gamma$  production in response to Poly I:C, while R848 induced IFN- $\gamma$  production only partially depended on IL-12 (Fig. 4B).

Since cytokines, IL-12 and IL-18 were not involved in NK-IFN- $\gamma$  induction by HCV-infected cells; pDCs and monocytes may assist NK activation via IL-15 that can be induced by IFN- $\alpha$  on antigen presenting cells and activate proximal NK cells through a trans-presentation way [22,23]. We found here that monocytes expressed higher levels of maturation markers, CD80, CD86, and also IL-15R $\alpha$  in co-cultures with HCV-infected cells, indicating that monocytes were activated in co-cultures and may use IL-15 to signal NK-IFN- $\gamma$  production (Supplementary Fig. 5). Indeed, when we measured *IL-15* transcript levels in monocytes in the presence of JFH-1/HuH7.5 or IFN- $\alpha$ , they were elevated in both conditions (Fig. 4C and D).

### *IFN- $\gamma$ produced by NK cells induces potential antiviral immune responses*

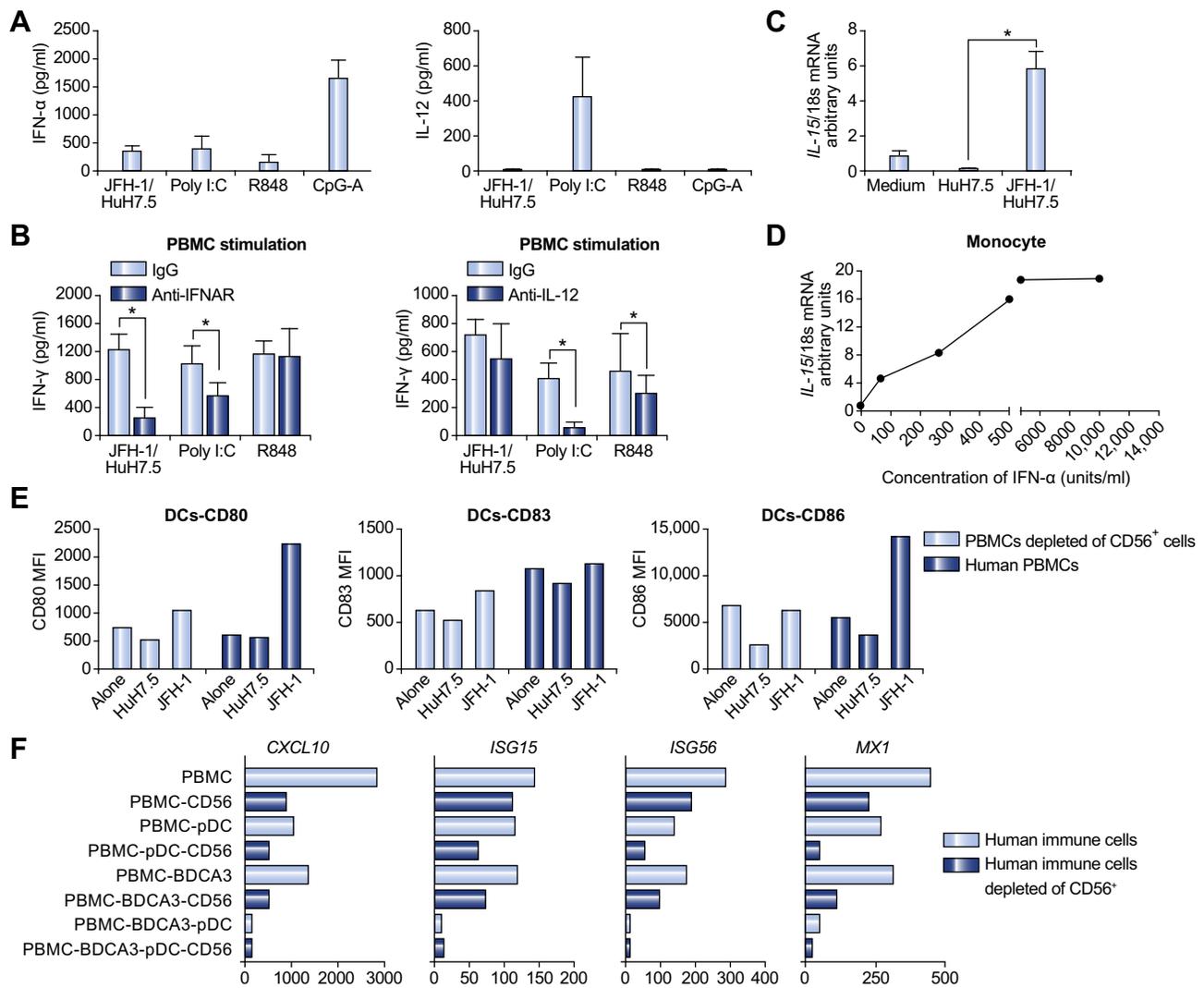
Finally, we studied the antiviral functions of NK-produced IFN- $\gamma$ . We found that depletion of NK/NKT cells from PBMCs not only resulted in significantly lower expression of co-stimulatory molecules, such as CD80 and CD86, on dendritic cells in response to HCV-infected cells (Fig. 4E), but also led to decreased induction of

ISGs expression in co-cultures, including *CXCL10*, *ISG15*, *ISG56*, and *MX1* (Fig. 4F). Finally, consistent with previous reports, we showed that in the presence of pDCs, NK cells induced massive cell death of HCV-infected HuH7.5 cells (Supplementary Fig. 6), probably through the TRAIL-apoptotic pathway. Based on these data, here we try to build a novel model reflecting the cell interaction mechanism leading to NK-IFN- $\gamma$  production in response to HCV infection, where pDC derived or exogenous IFN- $\alpha$  sensitized NK cells actively recognize HCV-infected hepatocytes and produce IFN- $\gamma$  in response, while monocytic cells, such as monocytes or liver Kupffer cells, synergistically enhance IFN- $\gamma$  induction through an IL-15 mediated mechanism (Supplementary Fig. 7). IFN- $\gamma$  from NK cells has important immunoregulatory roles in enhancing the antiviral status in HCV-infected hepatocytes and maturation of antigen presenting cell populations.

## Discussion

Recent reports showed increased NK cytotoxicity induced by type I IFN pathway during HCV-infection or after IFN- $\alpha$  based therapy. Type I IFN activated NK cells were found to induce apoptosis of HCV-infected hepatoma cells through a TRAIL-triggered cell death pathway [3,4,6,8–10]. However, it is still unclear whether another important aspect of NK cells, IFN- $\gamma$  production, is induced and whether NK cell-derived cytokines play any roles in response to hepatitis C infection [24]. Here using co-cultures of human immune cells and JFH-1 infected hepatoma cells, we revealed a novel mechanism in which NK cells produced IFN- $\gamma$  in response to HCV-infected cells through a pDC-type I IFN dependent mechanism. We also demonstrated that the optimal NK-IFN- $\gamma$  production depended on the presence of monocytes. We further showed that NK cell-derived IFN- $\gamma$  had a synergistic effect in inducing interferon stimulated genes (ISGs) expression and maturation of dendritic cells (DCs) in response to HCV-infected cells. Our results strongly suggest that NK cells and IFN- $\gamma$  play an active role in the orchestration of the innate immune activation in addition to their increased cytotoxicity during HCV-infection.

NK cell activity is regulated through two major ways: first, the balance between numerous inhibitory and activating receptors on the NK cell surface and second, the cross-talk with other cells, especially with dendritic cells [11]. Although it is tempting to speculate that NK cells respond to HCV virions or HCV-infected cells directly, our results do not support this hypothesis. Consistently, earlier reports even showed that NK cell activity was compromised after exposure to HCV virions or HCV-infected cells [15,16,25]. Here we show for the first time that NK cells respond to HCV-infected cells and produce IFN- $\gamma$  requiring the presence of accessory cells. A cross-talk between NK cells and dendritic cells has been recognized in many studies, especially in response to PAMPs or infections [12]. One canonical cross-talk mechanism repeatedly corroborated in different models is that increased NK cytotoxicity depends on pDC-derived type I IFN while increased NK-IFN- $\gamma$  production depends on mDC-derived IL-12 [14]. However, different from this paradigm, we show novel evidence that increased NK-IFN- $\gamma$  induction by HCV-infected cells depends on pDCs and type I IFN and not on mDCs and IL-12. We found that human PBMCs produced all three types of IFNs and minimal inflammatory cytokine production, including IL-12 and IL-18. Indeed, neutralizing anti-IL-12 antibody or depletion



**Fig. 4.** IFN- $\alpha$  but not IL-12 is involved in IFN- $\gamma$  induction in NK cells in response to HCV-infected cells. (A) Human PBMCs were co-cultured with JFH-1/HuH7.5 cells or stimulated with poly I:C, R848 and CpG-A for 24 h. IFN- $\alpha$  and IL-12 production was measured by ELISA. (B) Human PBMCs were co-cultured with JFH-1/HuH7.5 cells or stimulated with poly I:C or R848 in the presence or absence of anti-IFNAR or anti-IL-12 antibody. IFN- $\gamma$  production was measured by ELISA (mean  $\pm$  SD,  $n = 3$ ), \* $p < 0.05$ . (E) Human PBMCs or PBMCs depleted of CD56<sup>+</sup> cells were co-cultured with JFH1/HuH7.5 cells for 24 h. Co-cultured PBMCs were collected and expression of co-stimulatory molecules, CD80, CD83, and CD86, on DCs (Lin1- HLA-DR<sup>+</sup> population) was analyzed by flow cytometry. One representative data of two experiments is shown. (F) Human immune cells or their counterparts depleted of CD56<sup>+</sup> cells were co-cultured with JFH-1/HuH7.5 cells for 12 h. Co-cultured cells were collected and ISGs (CXCL10, ISG15, ISG56, and MX1) expression levels were measured by real time PCR. One representative data of two experiments is shown.

of mDCs failed to prevent NK-IFN- $\gamma$  induction in response to HCV-infected cells in our experiments, while neutralizing anti-IFNAR antibody or depletion of pDCs significantly decreased NK-IFN- $\gamma$  production. While we identified a novel role of pDCs in NK-IFN- $\gamma$  production, we also demonstrated that pDCs can be fully replaced by exogenous IFN- $\alpha$  treatment, indicating that the close contact between pDCs and NK cells was not necessary. In contrast, a close contact between accessory cells and NK cells was indispensable for optimal IFN- $\gamma$  induction in response to poly I:C or R848. Our finding of increased human NK-IFN- $\gamma$  production by type I IFN was unexpected as exogenous IFN- $\alpha$  is insufficient to induce IFN- $\gamma$  from NK cells even in the presence of poly I:C and R848, suggesting the existence of an unknown activating

mechanism by type I IFN-activated NK cells in response to HCV-infected cells. Our results suggest that monocytes or mDCs fulfill this unique function.

In our experiments, IFN- $\alpha$ -treated NK cells produced only low levels of IFN- $\gamma$  in response to HCV-infected cells, while optimal IFN- $\gamma$  induction required both pDC-derived type I IFNs and interaction with monocytes. We discovered that monocytes are involved in maximal NK-IFN- $\gamma$  induction in response to HCV virus infection. Although the exact mechanism needs to be further elucidated, monocytes expressed higher levels of maturation markers, CD80, CD86, and also IL-15R $\alpha$  in co-cultures with HCV-infected cells, so they might boost NK-IFN- $\gamma$  production through the IL-15 pathway. This finding has important implications

## Research Article

during hepatitis C infection. Based on our findings, we propose a model where in the HCV-infected liver, human dendritic cells first recognize HCV-infected hepatocytes and produce type I IFNs which not only increase NK cell cytotoxicity but also sensitize IFN- $\gamma$  induction in NK cells by HCV-infected cells (Supplementary Fig. 7). The large number of NK cells and Kupffer cells in the human liver [26,28] allows ample interactions between IFN- $\alpha$  sensitized NK cells and “liver-resident macrophages”, Kupffer cells, in the local liver environment to produce IFN- $\gamma$  in response to hepatitis C infection. Most likely the same mechanism will also function during IFN- $\alpha$  therapy for chronic-HCV infected patients and account for at least some of the anti-viral effects mediated by PegIFN- $\alpha$ . The limitation of our study is that it characterized functions of circulating NK cells that should be carefully interpreted regarding the function of NK cells in the HCV-infected liver.

Our novel findings reveal a sequential type I IFN and type II induction from human immune cells in response to hepatitis C infection. We propose that the subsequent NK derived type II IFN production plays a role in maintaining, amplifying, and supplementing the initial type I IFN response. As reported previously, we confirmed that type I IFN and type II IFN have synergistic effects in inducing expressions of many interferon stimulating genes in HCV-infected hepatoma cells [27]. More importantly, although type I IFN generally has a stronger anti-viral effect than type II IFN, type II IFN plays an important role in regulation of immune responses [28]. We noticed that depletion of NK cells prevented maturation of dendritic cells in response to HCV-infected cells, probably through IFN- $\gamma$ , recapitulating their well recognized immunoregulatory effects on antigen presenting cells.

Finally, although our results suggest that TLR3 and TLR8 ligands can partially reproduce IFN- $\gamma$  induction in PBMCs in the presence of HCV-infected hepatoma cells, the mechanisms seem to be different from those involved in HCV-induced NK cell activation. Our data indicate that the accessory cell requirement was different in support of a TLR3- or TLR8-induced NK-IFN- $\gamma$  response where the presence of mDC1 supported poly I:C-induced and monocytes supported R848-induced IFN- $\gamma$  production, while pDC derived IFN- $\alpha$  and monocytes together were required for HCV-induced IFN- $\gamma$  production. However, it remains to be determined whether the combination of these or related events contributes to IFN- $\gamma$  induction by HCV-infected cells in PBMCs.

In conclusion, we show a unique mechanism where human NK cells actively produce IFN- $\gamma$  in response to HCV-infected cells through a type I IFN and monocytes dependent pathway. Our results suggest that in addition to cytotoxicity, NK cells are actively involved in anti-HCV immunity during hepatitis C infection via production of IFN- $\gamma$ .

### Financial support

This work was supported by NIH grant R37AA014372 (GS).

### Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

The underlying research reported in the study was funded by the NIH Institutes of Health.

### Acknowledgements

The authors thank Drs Charles M.Rice, Takaji Wakita, and Christoph Seeger for kindly providing reagents.

### Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2013.04.022>.

### References

- [1] Thomas D, Zoulim F. New challenges in viral hepatitis. *Gut* 2012;61:i1-i5.
- [2] Lemon SM. Induction and evasion of innate antiviral responses by hepatitis C virus. *J Biol Chem* 2010;285:22741-22747.
- [3] Ahlenstiel G, Edlich B, Hogdal LJ, Rotman Y, Nouredin M, Feld JJ, et al. Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. *Gastroenterology* 2011;141:1231-1239, 1239 e1231-e1232.
- [4] Amadei B, Urbani S, Cazaly A, Fiscaro P, Zerbini A, Ahmed P, et al. Activation of natural killer cells during acute infection with hepatitis C virus. *Gastroenterology* 2010;138:1536-1545.
- [5] Edlich B, Ahlenstiel G, Azpiroz AZ, Stoltzfus J, Nouredin M, Serti E, et al. Early changes in interferon signaling define natural killer cell response and refractoriness to interferon-based therapy of hepatitis C patients. *Hepatology* 2012;55:39-48.
- [6] Oliviero B, Varchetta S, Paudice E, Michelone G, Zaramella M, Mavilio D, et al. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology* 2009;137:1151-1160, 1160 e1151-e1157.
- [7] Dessouki O, Kamiya Y, Nagahama H, Tanaka M, Suzu S, Sasaki Y, et al. Chronic hepatitis C viral infection reduces NK cell frequency and suppresses cytokine secretion: reversion by anti-viral treatment. *Biochem Biophys Res Commun* 2010;393:331-337.
- [8] Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon- $\alpha$ -dependent manner. *Gastroenterology* 2010;138:325-335, e321-e322.
- [9] Golden-Mason L, Cox AL, Randall JA, Cheng L, Rosen HR. Increased natural killer cell cytotoxicity and Nkp30 expression protects against hepatitis C virus infection in high-risk individuals and inhibits replication in vitro. *Hepatology* 2010;52:1581-1589.
- [10] Pelletier S, Drouin C, Bedard N, Khakoo SI, Bruneau J, Shoukry NH. Increased degranulation of natural killer cells during acute HCV correlates with the magnitude of virus-specific T cell responses. *J Hepatol* 2010;53:805-816.
- [11] Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* 2011;331:44-49.
- [12] Barreira da Silva R, Munz C. Natural killer cell activation by dendritic cells: balancing inhibitory and activating signals. *Cell Mol Life Sci* 2011;68:3505-3518.
- [13] Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat Rev Immunol* 2007;7:279-291.
- [14] Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol* 2005;174:727-734.
- [15] Yoon JC, Lim JB, Park JH, Lee JM. Cell-to-cell contact with hepatitis C virus-infected cells reduces functional capacity of natural killer cells. *J Virol* 2011;85:12557-12569.
- [16] Yoon JC, Shiina M, Ahlenstiel G, Rehmann B. Natural killer cell function is intact after direct exposure to infectious hepatitis C virions. *Hepatology* 2009;49:12-21.

- [17] Takahashi K, Asabe S, Wieland S, Garaigorta U, Gastaminza P, Isogawa M, et al. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. *Proc Natl Acad Sci U S A* 2010;107:7431–7436.
- [18] Zhang S, Kodys K, Babcock GJ, Szabo G. CD81/CD9 tetraspanins aid plasmacytoid dendritic cells in recognition of HCV-infected cells and induction of IFN $\alpha$ . *Hepatology* 2012. <http://dx.doi.org/10.1002/hep.25827>.
- [19] Chang S, Kodys K, Szabo G. Impaired expression and function of toll-like receptor 7 in hepatitis C virus infection in human hepatoma cells. *Hepatology* 2010;51:35–42.
- [20] Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* 2009;126:458–465.
- [21] Dreux M, Garaigorta U, Boyd B, Decembre E, Chung J, Whitten-Bauer C, et al. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe* 2012;12:558–570.
- [22] Carson WE, Ross ME, Baiocchi RA, Marien MJ, Boiani N, Grabstein K, et al. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J Clin Invest* 1995;96:2578–2582.
- [23] Yamaji K, Nabeshima S, Murata M, Chong Y, Furusyo N, Ikematsu H, et al. Interferon-alpha/beta upregulate IL-15 expression in vitro and in vivo: analysis in human hepatocellular carcinoma cell lines and in chronic hepatitis C patients during interferon-alpha/beta treatment. *Cancer Immunol Immunother* 2006;55:394–403.
- [24] Shoukry NH, Pelletier S, Chang KM. A view to natural killer cells in hepatitis C. *Gastroenterology* 2011;141:1144–1148.
- [25] Crotta S, Brazzoli M, Piccoli D, Valiante NM, Wack A. Hepatitis C virions subvert natural killer cell activation to generate a cytokine environment permissive for infection. *J Hepatol* 2010;52:183–190.
- [26] Protzer U, Maini MK, Knolle PA. Living in the liver: hepatic infections. *Nat Rev Immunol* 2012;12:201–213.
- [27] Okuse C, Rinaudo JA, Farrar K, Wells F, Korba BE. Enhancement of antiviral activity against hepatitis C virus in vitro by interferon combination therapy. *Antiviral Res* 2005;65:23–34.
- [28] Zhang SY, Boisson-Dupuis S, Chappier A, Yang K, Bustamante J, Puel A, et al. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. *Immunol Rev* 2008;226:29–40.