# Increased Degranulation of Natural Killer Cells during Acute HCV Correlates with the Magnitude of Virus-specific T cell Responses

Sandy Pelletier[1,3] (sandy.pelletier@umontreal.ca)

Christian Drouin[1,3] (cdrouin25@googlemail.com)

Nathalie Bédard[1] (nbedardca@yahoo.ca)

Salim I. Khakoo[2] (s.khakoo@imperial.ac.uk)

Julie Bruneau[1,4] (julie.bruneau@umontreal.ca)

Naglaa H. Shoukry[1,5] (naglaa.shoukry@umontreal.ca)

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Hôpital St-Luc

[2]Department of Hepatology, Division of Medicine, Imperial College, London, UK

[3]Département de microbiologie et immunologie, [4]Département de médecine familiale and

[5] Département de médecine, Université de Montréal, Montréal, QC, Canada.

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**Footnotes**

**Address Correspondence and Reprint Requests to:**
Dr. Naglaa H. Shoukry
Centre de Recherche du CHUM,
Hôpital St-Luc,
264 boul. René-Lévesque Est, Local PEA-316
Montréal (Québec) H2X 1P1
CANADA

E-mail: naglaa.shoukry@umontreal.ca

**Abbreviations:**
ELISPOT: Enzyme Linked Immunospot assay; HBV: Hepatitis B virus; HCV: Hepatitis C Virus; HIV: Human immunodeficiency virus; ICS: Intracellular Cytokine Staining; IDUs: Intravenous Drug Users; IFN-γ: Interferon gamma; PBMC: Peripheral Blood Mononuclear cells

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**Abstract**

Natural killer (NK) cells provide an early defense line against viral infections by killing infected cells and producing cytokines that inhibit viral replication. In addition, NK cells interact with dendritic cells (DCs) and this reciprocal interaction results in regulation of both innate and adaptive immune responses. Genetic studies have suggested that NK cell activity is an important determinant of HCV infectious outcome but a functional correlation was not established. We hypothesized that increased NK cell activity during acute HCV infection will correlate with spontaneous resolution. We used multiparametric flow cytometry to monitor longitudinally the phenotype and activity of NK cells in a cohort of intravenous drug users following HCV exposure. Three groups of patients were identified: acute infection with chronic evolution (n=10); acute infection followed by spontaneous resolution (n=10); and exposed un-infected individuals (n=10). We monitored the expression of several NK cell activating and inhibitory receptors as well as IFN-γ production and degranulation (CD107a). We observed decreased expression of the inhibitory NKG2A receptor on NK cells following spontaneous HCV clearance. In addition, we've observed increased NK cell degranulation during acute HCV irrespective of infectious outcome. NK cell degranulation correlated with the magnitude of HCV-specific adaptive T cell responses measured by ELISPOT. **Conclusion:** Our results demonstrate that NK cells are activated during acute HCV regardless of infection outcome. This suggests that NK cell activity is not directly implicated in HCV clearance but might play an indirect role through induction of T cell responses.

**Introduction**

The majority of individuals exposed to hepatitis C virus (HCV) develop persistent infection and chronic liver disease (1). Acute HCV is characterized by a significant delay in the onset of adaptive T cell responses despite active viral replication. This suggests a failure of innate immunity to contain viral replication and provide the necessary signals to prime efficient adaptive immunity critical to spontaneous viral clearance (2, 3). Natural killer (NK) cells are the most important effector population of the innate immune response. Two NK cell subsets can be distinguished based on their differential expression of CD56 and CD16: immunoregulatory CD3$^-$ CD56$^{bright}$CD16$^-$ and cytolytic CD3$^-$CD56$^{dim}$CD16$^+$ (4). NK cells provide an early defense line against viral infections by killing infected cells and producing cytokines that can directly inhibit viral replication and trigger the adaptive immune response. NK cells use inhibitory and activation receptors as a mean of controlling their activity. NK cells interact with dendritic cells (DCs) and this reciprocal interaction results in regulation of both innate and adaptive immune responses (5). DCs can activate NK cells by binding to NKp30 on the surface of NK cells and by secreting numerous cytokines such as IL-12 (6). In return, NK cells secrete IFN-γ and TNF-α which induce DC maturation and trigger the adaptive immune response (7). In addition, NK cells can also kill immature DCs and inhibit their capacity to prime or tolerize adaptive T cell responses (8, 9).

Two observations highlighted the potential role of NK cells during the early phase of HCV infection. First, HCV surface glycoprotein E2 can bind CD81 on the surface of NK cells and inhibit cytotoxicity and IFN-γ production (10, 11), but Yoon *et al*. have recently demonstrated that exposure of NK cells from healthy donors to in vitro-produced HCV virions did not influence their function (12). Second, genes encoding the inhibitory NK cell receptor killer-cell

immunoglobulin like receptor (KIR)2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand directly influence resolution of HCV infection in individuals homozygous for these genes (13, 14). These observations suggest that inhibition of NK function during the early phase of HCV may contribute to viral persistence.

Several groups have studied NK cells during the chronic phase of HCV infection but the results were conflicting regarding NK cell frequency, cytotoxicity, cytokine production and receptor expression (5, 15-19). This probably reflects the complexity of activation and inhibitory signals that control NK cells. Only one study has compared NK cell function in chronic HCV patients with spontaneous resolvers from a single source outbreak (15). They demonstrated that the frequency of the CD56$^{dim}$ NK cell subset was decreased in individuals with chronic HCV and that NK cells expressed higher frequency of the NKG2A/C/E receptors (15) but the activity of NK cells during acute HCV, where their role would be most prominent, and its correlation with infectious outcome was not studied.

In this study, we used multiparametric flow cytometry to monitor longitudinally the phenotypic and functional changes in NK cells from a unique cohort of intravenous drug users (IDUs) at high risk of HCV infection before and during acute HCV infections that progressed to spontaneous resolution or viral persistence. We demonstrate that NK cell degranulation and IFN-γ production are increased during acute HCV regardless of infection outcome. We also observed a decline in NKG2A expression on NK cells following spontaneous viral clearance. Finally, we show that NK cell degranulation correlates with the magnitude of the HCV-specific T cell response suggesting an indirect role for NK cells in priming adaptive immune responses.

**Material and Methods**

**Study subjects and clinical follow-up**. HCV acutely infected subjects were recruited among high-risk IDUs participating in the Montreal Acute HepC cohort study (HEPCO)(20), the methadone treatment and the Hepatology clinics at St-Luc hospital of the Centre Hospitalier de l'Université de Montréal (CHUM). This study was approved by the institutional ethics committee (Protocols # SL05.014 and SL05.025) and conducted according to the Declaration of Helsinki. All participants signed informed consent upon enrolment. Acute HCV infection was defined as detection of positive HCV RNA and/or HCV antibodies following a previous negative test in the past 6 months, or positive HCV RNA with concomitant negative HCV antibodies tests. Duration of infection was defined as the time (in weeks) post detection of viremia (PDV). Spontaneous viral resolution or persistent infection was defined as the absence or presence of HCV RNA at 12 weeks post enrolment. Exposed un-infected are IDUs who have admitted sharing a needle or injection materials with an HCV infected individual but remained HCV RNA and HCV antibody negative. In this study, three time points were analyzed for each patient representing three phases of HCV infection: Pre-infection baseline, acute HCV and follow-up. Baseline was defined as time before HCV infection or reported needle sharing for exposed un-infected (range: –3 to –27 weeks; mean –15 weeks). Samples were available for 7 chronic patients, 4 spontaneous resolvers and 5 exposed un-infected. Acute HCV was defined as 0-14 weeks (mean 4 weeks) PDV or needle sharing for exposed un-infected. Samples were available for all patients except 2 exposed un-infected. When more than one time point was studied during the acute phase, the time point with the highest CD107a frequency was chosen. This is because CD107a up-regulation in response to stimulation was the most consistent activation marker for all patients studied. The follow-up time point was defined as 19-65 weeks (mean 41 weeks)

PDV or needle sharing for exposed un-infected. Samples were available for all patients except for one chronic patient and two spontaneous resolvers that were lost to follow-up. All patients tested negative for human immunodeficiency virus (HIV) and hepatitis B virus (HBV), and were either uneligible or refused IFN therapy.

**HCV RNA testing and quantification.** Qualitative HCV RNA tests were performed using an automated COBAS AmpliPrep/COBAS Amplicor HCV test, version 2.0 (sensitivity, 50 IU/ml) (Roche Molecular Systems, Inc., Branchburg, NJ). HCV genotyping was done using standard sequencing for the NS5B region, and was performed by the Laboratoire de Santé Publique du Québec (Ste Anne-de-Bellevue, QC, Canada) as part of the clinical follow-up of patients. Additional HCV RNA quantification was performed using an in-house quantitative real-time reverse transcription-PCR assay as previously described (21).

**Flow cytometry antibodies and reagents.** Directly conjugated antibodies against the following surface molecules were used: CD3-Pacific Blue (clone UCHT1), CD16-allophycocyanin (APC)-Cy7 (clone 3G8) or CD16-APC-H7 (clone 3G8), CD56- phycoerythryin (PE)-Cy7 (clone B159), CD107a-PE-Cy5 (clone H4A3), CD158a-fluorescein isothiocyanate (FITC) (clone HP-3E4), CD158a-PE (clone HP-3E4), CD158b-FITC (clone CH-L), CD161-PE-Cy5 (clone DX12), NKB1-FITC (clone DX9), NKG2D-APC (clone 1D11), and NKp30-Alexa 647 (clone P30-15) (all from BD Biosciences, San Jose, CA, USA); CD69-PE-Texas Red (ECD) (clone TP1-55-3), and NKp44-PE (clone Z231) (both from Beckman Coulter, Marseille, France); NKB1-Alexa 700 (clone DX9) (from Biolegend, San Diego, CA, USA); NKG2A-PE (clone #131411) (from R&D Systems, Minneapolis, MN, USA). The following intracellular antibodies were used: IFN-γ-APC (clone B27) (from BD Biosciences). Live cells were identified using an Aqua Live/Dead fixable dead cell Stain Kit (Molecular Probes, Eugene, OR, USA) according to the

manufacturer's protocol. We've used four phenotypic panels and one functional panel. "Fluorescence minus one" control stains were used to determine background levels of staining. Multiparameter flow cytometry was performed using a standard BD LSR II instrument equipped with blue (488 nm), red (633 nm), and violet (405 nm) lasers (BD Biosciences,) to systematically perform seven-color staining using FACSDiva software (BD Biosciences). Compensation was performed with single fluorochromes and BD CompBeads (BD Biosciences). Data files were analyzed using FlowJo software, version 8.6.3 for Mac (Tree Star, Inc., Ashland, OR).

**Multiparametric phenotypic characterization of NK cells.** All flow cytometry assays were performed on cryopreserved samples. For phenotypic analysis, $1\text{-}2 \times 10^6$ peripheral blood mononuclear cells (PBMCs) were stained with surface antibodies for 30 min at 4°C and washed twice in fluorescence-activated cell sorting (FACS) buffer (1× phosphate-buffered saline [PBS], 1% fetal bovine serum [FBS], 0.02% NaN3), and fixed in FACS Fix buffer (1× PBS, 1% formaldehyde).

**Intracellular cytokine staining (ICS) and CD107a degranulation assay.** $2 \times 10^6$ PBMCs were incubated with anti-CD107a antibody and either culture media as a negative control or K562 leukemia target cell line (ATCC, Manassas, VA, USA) at 37°C in R-10 medium (RPMI medium [Invitrogen, Carlsbad, CA, USA] supplemented with 10% FBS). Following 1 h of stimulation, 10 μg/ml of brefeldin A (Sigma-Aldrich, St-Louis, MO, USA) and 6 μg/ml of monensin sodium salt (Sigma-Aldrich) were added, and cells were incubated for a total of 6 h. Cells were washed with FACS buffer, stained for viability and cell surface antigens, then permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience). Cells were then stained with anti-IFN-γ antibody for 30 min, washed twice in BD Perm/Wash buffer (BD Biosciences), and fixed in FACS fix buffer. For analysis, cells were gated on viable CD3$^-$ CD56$^{birght}$ CD16$^-$ and CD3$^-$

$CD56^{dim}CD16^{+/lo}$ NK cells (Figure 1A). Percent specific expression is calculated as the background-adjusted function in the presence or absence of target cell line.

**IFN-γ ELISPOT.** 96-well polyvinylidene diflouride-backed microtiter plates (Millipore, Bedford, MA, USA) were pre-wet with 35% ethanol (15 μl/well) for one minute, washed with PBS and coated overnight at 4°C with 100 μl/well (3 μg/ml) of anti-IFN-γ capture mAb (BD Biosciences). Plates were washed with PBS and blocked for 2 hours at 37°C with 200 μl/well of R-10 medium. Cryo-preserved PBMCs were thawed quickly in a 37°C water bath and washed in R-10 medium. $2 \times 10^5$ PBMCs/well were stimulated in duplicate with the various peptides pools at a final concentration of 5 μg/ml of each peptide in AIM-V-HS medium (AIM-V complete medium [Invitrogen], 2% Human Serum AB [Wisent, Saint-Bruno, QC, Canada]) for 36 h at 37°C, 5% $CO_2$. Patients were stimulated with 11 peptide pools spanning the entire HCV polyprotein and corresponding to HCV genotype 1a (H77 sequence) or genotype 3a (K3a/650 sequence), according to patient's infecting HCV genotype. Patients infected with other HCV genotypes as well as exposed un-infected were stimulated with peptides corresponding to HCV genotype 1a (H77 sequence). Peptides were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA, USA). At the end of the incubation period, plates were washed with PBS-T (PBS, 0.05% Tween-20) then incubated with biotinylated anti-IFN-γ antibody (clone 250 4S.B3) (BD Biosciences) at 0.5 μg/ml in PBS/0.5% BSA for 2 h at room temperature. Plates were washed and incubated with Streptavidin-Alkaline Phosphatase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) (1:1000) in PBS/0.5% BSA for 1 hour at room temperature. Spots were developed using Alkaline Phosphatase conjugate substrate kit (Bio-Rad Laboratories) for 5 minutes and stopped using tap water. Spots were counted using an Immunospot Analyzer Instrument (Cellular

Technology Ltd [CTL], Shaker Heights, OH, USA). PBMCs incubated with media alone served as negative controls. Positive controls were: PMA (phorbol myristate acetate [Sigma-Aldrich]) 50 ng/ml − Ionomycin (Sigma-Aldrich) 1 µg/ml and a control peptide pool CEF (NIH AIDS Research and Reference Reagent Program, Germantown, MD, USA). Specific Spot forming cells (SFC) were calculated as (mean number of spots in test wells-mean number of spots in media control wells) and normalized to $SFC/10^6$ PBMCs. A response was scored positive if greater than 50 $SFC/10^6$ PBMCs.

**HLA and KIR typing**. HLA typing was performed by the core facility of the Fonds de la Recherche en Santé du Québec (FRSQ) AIDS and Infectious Disease Network, Montreal, QC, Canada, using standard sequence-specific primer-PCR high-resolution techniques as previously described (22). KIR typing was performed by using the Lifematch T typing kit (Tepnel, Stamford, CT) on a Luminex platform.

**Statistical analysis.** Comparisons between patient groups with HCV chronic evolution, spontaneous resolution and exposed un-infected, during baseline, acute and follow-up phases of infection, were evaluated by 2-way ANOVA (repeated measures). Data were analyzed with SigmaStat 3.5 for Windows (Systat Software, Inc., Chicago, IL, USA). Comparisons between HCV chronic evolution, spontaneous resolution, exposed un-infected and healthy donors were evaluated by 1-way ANOVA. Correlations were evaluated by Pearson's test if data passed normality test or by Spearman's test if data did not pass normality. Comparisons between groups of patients bearing certain KIR and HLA genes were evaluated by the two-tailed $t$ test for independent samples, or 1-way ANOVA if more than 2 groups. Data were analyzed with GraphPad Prism 5.02 for Windows (GraphPad Software, San Diego, CA, USA).

**Results**

**Acute HCV infection is associated with increased NK cell degranulation and IFN-γ production.** Ten patients with acute HCV followed by chronic evolution (chronics), ten patients with acute resolving HCV, and ten exposed un-infected were identified among IDUs at high risk for HCV infection as described in Materials and Methods. Patients' demographics and characteristics are listed in Table 1. We monitored longitudinally the frequency of the different NK cell subsets in these patients. NK cell gating strategy by flow cytometry is shown in Figure 1A. Although the frequency of the $CD56^{dim}CD16^{+}$ NK cell subset was decreased in all patient groups as compared to healthy donors (Figure 1B), it did not change significantly within each group relative to the phase of HCV infection. Similarly, no changes were observed in the frequency of $CD56^{bright}CD16^{-}$ NK cell subset (Figure 1C).

NK cell function was evaluated by measuring IFN-γ production by ICS and CD107a expression, a degranulation marker that is indicative of the cytotoxicity of NK cells (23) in response to co-culture with the classical NK cell target K562 cells. Representative staining and gating strategy are presented in supplementary Figure S1. The frequency of NK cells single positive for either CD107a or IFN-γ or double positive for both functions was evaluated. We observed an increase in the frequency of degranulating cells ($CD107a^{+}IFN-\gamma^{-}$) in both $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cell subsets during the acute phase in HCV-infected patients with either chronic evolution or spontaneous resolution as compared to baseline and/or follow-up (Figures 2A and 2B). Similar results were observed in comparison to exposed un-infected and healthy donors. Increased degranulation was not only observed for the mean of the groups but also for the majority of individual patients with HCV chronic evolution (Figures 2C and 2D) and spontaneous resolution (Figures 2E and 2F). The frequency of IFN-γ producing cells ($CD107a^{-}$

IFN-$\gamma^+$) by both NK cell subsets was also increased during the acute phase in patients with chronic evolution only. However, the frequency of these cells remained significantly lower in chronics and spontanous resolvers at all time points compared to healthy donors (Figures 3A and 3B). The exposed uninfected group exhibited an intermediate level of IFN-$\gamma$ production suggesting that opioid usage may have an influence on production of cytokines by NK cells. Increased frequency of IFN-$\gamma$ producing cells was not only observed for the mean of the group but also for the majority of patients with HCV chronic evolution (Figures 3C and 3D). Finally, as illustrated in Figures 3E and 3F, the frequency of NK cells positive for both functions (CD107a$^+$IFN-$\gamma^+$) was decreased in all 3 groups of patients compared to healthy donors during the follow-up phase. This was only observed in the CD56$^{\text{bright}}$CD16$^-$ NK cell subset (Figure 3E) but not within the CD56$^{\text{dim}}$CD16+ NK cell subset remained unchanged over time in all groups and was comparable to the frequencies observed in healthy donors. Altogether, these results demonstrate that NK cells are activated during acute HCV infection regardless of infection outcome. These data also suggest that opioid usage may influence the cytokine producing capacity of NK cells.

**Longitudinal phenotypic analysis for the expression of activation and inhibitory receptors by NK cells during acute HCV.** We next monitored the longitudinal changes in expression of the activation marker (CD69), the NK cell activating receptors (NKG2D, NKp30 and NKp44) and the NK cell inhibitory receptors (CD161, NKG2A, KIR2DL1/S1, KIR2DL2/L3/S2 and KIR3DL1) by NK cells directly *ex-vivo* following HCV exposure. Representative staining for each marker are presented in supplementary Figures S2 and S3. We observed no significant difference in the expression of NKG2D, NKp30, NKp44, KIR2DL1/S1 and KIR2DL2/L3/S2

between the different groups of patients or between the different HCV infection phases studied (data not shown). However, as illustrated in Figure 4A, we observed decreased expression of the activation marker CD69 on CD56$^{dim}$CD16$^+$ NK cells in chronics and spontaneous resolvers at all time points studied as compared to healthy donors and exposed uninfected. The frequency of the inhibitory receptor KIR3DL1$^+$ expressing CD56$^{dim}$CD16$^+$ NK cells was also decreased during follow-up compared to the acute phase for patients with HCV chronic evolution (Figure 4B). Similarly, it was also decreased during the acute and follow-up phases compared to baseline for patients with spontaneous resolution (Figure 4B). Analysis of the frequency of NKG2A$^+$ NK cells revealed decreased expression of this receptor by both NK cell subsets following HCV viral clearance (Figure 4C and 4D). Finally, we observed decreased frequency of CD161$^+$ CD56$^{dim}$CD16$^+$ NK cells during baseline, acute and follow-up phases in patients with chronic evolution compared to exposed un-infected and healthy donors (Figure 4E). These results reflect the complexity of the inhibitory and activating signals governing NK cell activity. They suggest that decreased expression of NKG2A might be an important mechanism contributing to HCV spontaneous clearance or limiting HCV induced inflammation, and that lower expression of CD161 might be involved in chronic evolution.

**Correlation between NK cell activity and expression of different NK cell receptors**. Given the observed increase in NK cell function and differential perturbations in the expression of activation and inhibitory receptors between patient groups or infection time points, we tested whether expression of a specific receptor or family of receptors correlates with NK cell activity and can be used as a surrogate marker of innate immune activation irrespective of infectious outcome. As demonstrated in Table 2, a correlation was established between expression of all of

the NK cell receptors studied except for KIR2DL2/L3/S2, and frequency of activated NK cells measured by the frequency of CD107a$^+$IFN-$\gamma^-$, CD107a$^-$IFN-$\gamma^+$ or CD107a$^+$IFN-$\gamma^+$ in each NK cell subset. Figure 5 shows the correlation with the frequency of CD69$^+$ CD56$^{dim}$CD16$^+$ NK cells and frequency of NKG2A CD56$^{dim}$CD16$^+$ NK cells, as two representative examples. All patient groups (chronics, spontaneous resolvers, exposed un-infected and healthy donors) were plotted on the same graphic. As illustrated in Figure 5A, CD69 expression negatively correlated with CD107a$^+$IFN-$\gamma^-$, but positively correlated with CD107a$^-$IFN-$\gamma^+$ and with CD107a$^+$IFN-$\gamma^+$ (Figures 5B and 5C). In addition, NKG2A expression positively correlated with CD107a$^+$IFN-$\gamma^-$ and CD107a$^+$IFN-$\gamma^+$ (Figures 5D-F). Despite the correlations established with several NK cell receptors, NK cell degranulation or IFN-$\gamma$ production did not preferentially correlate with activating or inhibitory receptors. This underscores further the delicate ablance between activating and inhibitory signals leading to activation of NK cells.

**NK cell degranulation correlates with the magnitude of HCV-specific adaptive T cell responses but not viral load.** NK cells can interact with DCs and this cross-talk between these two cell types regulates both the innate and adaptive immune response (5). We sought to determine whether NK cell activity can affect the induction of adaptive immune responses irrespective of infectious outcome. We examined the correlation between NK cell activity measured by degranulation or cytokine production and the magnitude of the HCV-specific T cell response during acute HCV. T cell responses were measured by IFN-$\gamma$ secretion in response to overlapping HCV peptides spanning the entire HCV polyprotein using the enzyme linked immunospot (ELISPOT) assay. Results for patients with HCV chronic evolution and spontaneous resolution are shown in Figure 6. In the exposed un-infected group, only 1 out of

the 10 patients had a detectable ELISPOT response above background levels and was not considered in this analysis. We observed a positive correlation between the frequency of CD107a$^+$IFN-$\gamma^-$ CD56$^{dim}$CD16$^+$ NK cells and the magnitude of the HCV-specific T cell response (Figure 6A and 6B). In contrast, we observed a negative correlation between the frequency of CD107a$^-$IFN-$\gamma^+$ NK cells and the HCV-specific T cell response but the data did not reach statistical significance (p = 0.079, r = -0.40 for CD56$^{dim}$CD16$^+$ and CD56$^{bright}$CD16$^-$ NK cells; Figure 6C and 6D). Finally, there was no correlation between frequency of CD107a$^+$IFN-$\gamma^+$ NK cells and ELISPOT data (Figure 6E and 6F). These correlations remained unchanged even after excluding one outlier patient (spontaneous resolver) with a T cell response of $\cong$4000 spot forming cells (SFC)/million PBMCs (data not shown). These results demonstrate a dichotomy between the two NK cell functions and demonstrate that NK cell degranulation but not IFN-$\gamma$ production is a more critical parameter in the cross-talk between innate and adaptive immunity at least during acute HCV.

To determine if NK cell activity affected HCV plasma viral load, HCV RNA viral load was quantified by real-time RT-PCR in plasma of patients with HCV chronic evolution and spontaneous resolution during the acute phase. No correlation was established between the highest viral load and NK cell degranulation or IFN-$\gamma$ production (data not shown). It is important to note that in the majority of spontaneously resolved patients, HCV RNA was detectable by the sensitive qualitative PCR assay but was below the level of quantification in the quantitative HCV-RNA assay.

**NK cells from KIR2DL3 and HLA-C1 homozygous patients do not exhibit increased degranulation or IFN-$\gamma$ production.** Genetic studies have demonstrated that patients who are

homozygous for KIR2DL3 and its ligand HLA-C1 have a better chance of clearing HCV infection (13, 14). This suggested that NK cells expressing KIR2DL3 are more easily activated because of the weak affinity between this inhibitory receptor and its ligand (24). In order to test this hypothesis, we have performed KIR and HLA-C typing in 9 patients with chronic evolution, 9 with spontaneous resolution and 10 exposed un-infected. However, given the limited number of patients homozygous for both the KIR2DL3 and HLA-C1 loci we could not correlate NK cell activity with this protective KIR/HLA-C haplotype (data not shown).

**Discussion**

We have analyzed for the first time the longitudinal evolution of the NK cells phenotype and function during acute HCV infections in a unique cohort of IDUs with either spontaneous resolution or chronic evolution. Furthermore, we have examined NK cell activity in a cohort of HCV-exposed but un-infected high risk IDUs. Although, HCV exposure is self reported and might not be completely accurate, this group served as an important control for the influence of drug use on the activity of NK cells. A variable pattern of expression of activating and inhibitor receptors was observed reflecting the complex signals involved in activation of NK cells. We demonstrated that NKG2A expression is down modulated following spontaneous viral clearance suggesting an important role for this receptor. Furthermore, we've demonstrated increased NK cell activity during acute HCV as demonstrated by increased degranulation and IFN-$\gamma$ production. NK cell degranulation correlated with the magnitude of HCV-specific adaptive T cell responses.

Similar to previous reports that have studied NK cell function during chronic HCV (15, 16, 18), we demonstrate that CD56$^{dim}$CD16$^{+}$ NK cells are significantly depleted in HCV infected patients

as compared to healthy donors and irrespective of acute infection outcome. Depletion of this NK cell subset could result from persistent activation and their transformation into activated CD56^{bright} cells (25). However, we did not observe any increase in the frequency of CD56^{bright} NK cells or in the ratio of CD56^{dim}/CD56^{bright} in any of the patient groups or phases studied (data not shown). In addition, more recent studies suggest that CD56^{bright} NK cells differentiate into CD56^{dim} NK cells (26, 27). Another possibility is reduced generation or survival of CD56^{dim} NK cells. Enhanced NK cell migration from the blood to the liver could also explain this depletion although several studies have demonstrated that total NK cell numbers in the liver are normal (28) or even decreased (29, 30) during HCV infection.

We demonstrate that NK cell degranulation is increased during acute HCV compared to baseline or follow-up irrespective of acute HCV outcome towards chronic evolution or spontaneous resolution. We observe an increase in IFN-$\gamma$ production by NK cells during acute HCV in the group with chronic evolution only but not in the others. However, we observe that NK cells from all HCV infected individuals degranulated more but produced less IFN-$\gamma$ as compared to healthy donors and exposed uninfected. These results demonstrate that NK cells are activated during acute HCV infection but suggest a dichotomy or dissociation between NK cell degranulation and cytokine production functions. Indeed, recent studies have demonstrated that NK cells in chronic HCV patients are polarized towards cytotoxicity (31, 32) and that this polarization is likely due to in vivo stimulation by IFN-$\alpha$.

Reduced NK cell IFN-$\gamma$ production in HCV-infected patients could be explained by two mechanisms. First, HCV surface glycoprotein E2 was shown to bind to CD81 on the surface of NK cells and inhibit cytotoxicity and IFN-$\gamma$ production (10, 11). Although an interesting possibility, it would not explain the low IFN-$\gamma$ production during baseline, and another study by

Yoon *et al*.(12) has recently demonstrated that exposure of NK cells from healthy donors to *in vitro*-produced HCV virions did not influence their function. Another possible explanation for low IFN-γ production by NK cells, in this particular cohort, is opioid abuse. It has been reported that heroin abuse can reduce NK cell activity (33, 34). Drug abuse in our cohort of HCV-exposed IDUs could account for low IFN-γ production during baseline. Interestingly, exposed un-infected IDUs exhibit an intermediate level of IFN-γ production that is not significantly different from either HCV-infected IDUs or normal donors suggesting that drugs may influence NK cell activity but are not likely to be the only factor.

Expression of the early activation marker CD69 was decreased on NK cells from HCV-infected patients with chronic evolution and spontaneous resolution but intermediate on exposed uninfected individuals. This decrease was observed during baseline, acute and follow-up phases, basically reflecting the same pattern as IFN-γ production. Indeed, CD69 expression positively correlated with $CD107a^-IFN\text{-}\gamma^+$ and $CD107a^+IFN\text{-}\gamma^+$ but negatively correlated with $CD107a^+IFN\text{-}\gamma^-$. The fact that HCV E2 glycoprotein has also been shown to decrease CD25 expression(10), another activation marker, on NK cells, further supports a direct inhibitory *in vivo* mechanism by HCV viral particles.

We demonstrate normal expression of the inhibitory receptor NKG2A in HCV chronic patients. This is in contrast with other studies that demonstrated that NKG2A is upregulated on NK cells during chronic HCV (5, 17) and that it correlates with increased necro-inflammatory score in the liver (35) and increased IL-10 production by NK cells (36). It is possible that this is only a question of timing and is related to the development of chronic liver disease as most of the patients in our cohort were followed for less than a year and had not developed any serious liver disease or inflammation as compared to more than 20 years of chronic infection in other studies

(15). Interestingly, we have also demonstrated that expression of NKG2A on NK cells is down modulated following spontaneous viral clearance. This probably reflects a shut-off mechanism to guard against liver inflammation and the development of immune suppressive IL-10 NK cells. Alternatively, it was demonstrated that HCV infected hepatocytes express enhanced levels of HLA-E and that the HLA-A2 restricted epitope HCV core AA (35-44) stabilizes HLA-E expression (17). Since NKG2A binds to HLA-E, enhanced expression of NKG2A by NK cells from chronic HCV patients resulted in reduced cytolysis of HLA-E expressing hepatocytes (17). Reduced expression of NKG2A by spontaneous resolvers in our study could be a mechanism for these patients to escape cytotoxic impairment induced by HLA-E expressing infected hepatocytes.

We further demonstrate that NK cell degranulation and IFN-γ production correlate with the expression of several NK cell activating and inhibitory receptors. Despite the multiple correlations established, NK cell degranulation or IFN-γ production did not preferentially correlate with a particular subset of activating or inhibitory receptors. Interestingly, some receptors positively correlated with IFN-γ production but negatively correlated with degranulation. These results further underscore the dichotomy between NK cell degranulation and IFN-γ production and highlight the complex activating and inhibitory receptors that dictate NK cell activity.

We demonstrate that NK cell degranulation, a surrogate measure of cytotoxicity (23), correlated with the magnitude of the HCV-specific T cell response measured by ELISPOT. It is possible that killing of HCV-infected hepatocytes by NK cells during acute HCV would decrease viral load and allow for the development of an efficient adaptive immune response. However, this possibility is limited since NK cell activity did not correlate with viral load. It is more likely that

destruction of HCV-infected hepatocytes by NK cells facilitates antigen transfer to the lymph nodes and priming of HCV-specific CD4+ and CD8+ T cells. Indeed, a recent study in mice demonstrated that NK cell-mediated killing of target cells triggers antigen-specific CD8$^+$ and CD4$^+$ T cell-mediated and humoral responses (37). In addition to killing infected and cancerous cells, NK cells can also kill immature DCs (8, 9). Killing of immature DCs presenting HCV antigens could limit the development of tolerized adaptive T cell responses and favor the development of antiviral T cells, a process known as DC editing (38).

We observed a trend towards negative correlation between NK cell IFN-γ production and the magnitude of the HCV-specific T cell response but this correlation did not reach statistical significance. This was a surprising result as IFN-γ is important for inducing the maturation of DCs. However, it was shown that production of TNF-α by NK cells was more potent than IFN-γ at inducing DC maturation (7). Correlation between production of TNF-α by NK cells and magnitude of HCV-specific T cell responses will need further investigation.

It was reported that genes encoding the inhibitory NK cell receptor KIR2DL3 and its HLA-C1 ligand directly influence resolution of HCV infection in individuals homozygous for these genes (13). Because of the limited number of patients homozygous for KIR2DL3 and HLA-C1, it was impossible to establish a statistically significant correlation between these genes and increased NK cell activity. In addition, the heterogeneous population studied, composed of HCV-infected patients with chronic evolution or spontaneous resolution, and exposed un-infected, may have also influenced our results. Studying NK cell activity in a much larger and homogenous group of HCV infected individuals will be essential to elucidate this point in future studies.

In conclusion, we propose that NK cells are activated during acute HCV regardless of infection outcome. This suggests that NK cell activity might not be directly implicated in HCV clearance.

However, NK cells may play an indirect role through enhanced transfer of HCV antigens to lymph nodes and induction of T cell responses. Because of the well described NK-DC cross-talk, it is also tempting to speculate that NK cells might edit DCs presenting HCV antigens by inducing DC maturation or killing immature DCs with tolerogenic potential and thus favor the generation of an optimal antiviral T cell response.

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**Figure Legends**

**Figure 1.  No change in frequency of CD56$^{dim}$CD16$^{+}$ NK cells following HCV-exposure**.  A) Strategy for gating on the two NK cell subsets by flow cytometry: viable lymphocytes CD3$^{-}$ CD56$^{dim}$CD16$^{+}$ and CD3$^{-}$CD56$^{bright}$CD16$^{-}$.  Frequency of B) CD3$^{-}$CD56$^{dim}$CD16$^{+}$ NK cells and C) CD3$^{-}$CD56$^{bright}$CD16$^{-}$ NK cells was determined *ex-vivo* in patients with HCV chronic evolution (•), HCV spontaneous resolution (Δ), exposed un-infected (■) and healthy donors (○).  The acute phase of HCV infection is represented by the shaded area.  Mean is represented by a horizontal bar.  *p < 0.05;  **p < 0.01;  ***p < 0.001.  2-way ANOVA (repeated measures) or 1-way ANOVA (comparison with healthy donors).

**Figure 2.   Acute HCV infection is associated with increased NK cell degranulation regardless of infection outcome.** Degranulation was measured by CD107a surface staining in patients with HCV chronic evolution (•), HCV spontaneous resolution (Δ), exposed un-infected (■) and healthy donors (○).  PBMCs were co-incubated with or without K562 target cells. Background expression of CD107a was subtracted from expression with target cells.   A) Frequency of CD107a$^{+}$IFN-γ$^{-}$ cells gated on CD56$^{bright}$CD16$^{-}$ NK cells and B) CD56$^{dim}$CD16$^{+}$ NK cells.   C) Frequency of CD107a$^{+}$IFN-γ$^{-}$ cells in chronic patients (•) only gated on CD56$^{bright}$CD16$^{-}$ NK cells and D) CD56$^{dim}$CD16$^{+}$ NK cells.  For each individual patient, different infection time points are joined by a line.  E) Frequency of CD107a$^{+}$IFN-γ$^{-}$ cells in spontaneous resolvers (Δ) only gated on CD56$^{bright}$CD16$^{-}$ NK cells and F) CD56$^{dim}$CD16$^{+}$ NK cells.   The acute phase of HCV infection is represented by the shaded area.  Mean is represented by a horizontal bar.  *p < 0.05;  **p < 0.01;  ***p < 0.001.  2-way ANOVA (repeated measures) or 1-way ANOVA (comparison with healthy donors).

**Figure 3. Acute HCV infection is associated with increased IFN-γ production by NK cells in chronically evolving infections.** Cytokine production was measured by intracellular IFN-γ in patients with HCV chronic evolution (•), HCV spontaneous resolution (Δ), exposed un-infected (■) and healthy donors (○). PBMCs were co-incubated with or without K562 target cells. Background expression of IFN-γ was subtracted from expression with target cells. A) Frequency of CD107a$^-$IFN-γ$^+$ cells gated on CD56$^{bright}$CD16$^-$ NK cells and B) CD56$^{dim}$CD16$^+$ NK cells. C) Frequency of CD107a$^-$IFN-γ$^+$ cells in chronic patients (•) only gated on CD56$^{bright}$CD16$^-$ NK cells and D) CD56$^{dim}$CD16$^+$ NK cells. E) Frequency of CD107a$^+$IFN-γ$^+$ cells gated on CD56$^{bright}$CD16$^-$ NK cells and F) CD56$^{dim}$CD16$^+$ NK cells. The acute phase of HCV infection is represented by the shaded area. Mean is represented by a horizontal bar. *p < 0.05; **p < 0.01; ***p < 0.001. 2-way ANOVA (repeated measures) or 1-way ANOVA (comparison with healthy donors).

**Figure 4. Expression of activation markers, activating receptors and inhibitory receptors by NK cells.** Expression of phenotypic markers by NK cells was determined *ex-vivo* in patients with HCV chronic evolution (•), HCV spontaneous resolution (Δ), exposed un-infected (■) and healthy donors (○). A) Frequency of CD69$^+$ cells gated on CD56$^{dim}$CD16$^+$ NK cells. B) Frequency of KIR3DL1$^+$ cells gated on CD56$^{dim}$CD16$^+$ NK cells. C) Frequency of NKG2A$^+$ cells gated on CD56$^{bright}$CD16$^-$ and D) CD56$^{dim}$CD16$^+$ NK cells. E) Frequency of CD161$^+$ cells gated on CD56$^{dim}$CD16$^+$ NK cells. The acute phase of HCV infection is represented by the shaded area. Mean is represented by a horizontal bar. *p < 0.05; **p < 0.01; ***p < 0.001. 2-way ANOVA (repeated measures) or 1-way ANOVA (comparison with healthy donors).

**Figure 5. Correlation between NK cell degranulation, IFN-γ production and expression of phenotypic markers.** Results for NK cell degranulation and IFN-γ production shown in Figures

2 and 3 were plotted against results for expression of phenotypic markers shown in Figure 4 to establish a correlation. Patients with HCV chronic evolution, HCV spontaneous resolution and exposed un-infected during baseline, acute and follow-up, as well as healthy donors are shown. A) Correlation between frequency of $CD69^+$ and $CD107a^+IFN\text{-}\gamma^-$, B) $CD107a^-IFN\text{-}\gamma^+$ and C) $CD107a^+IFN\text{-}\gamma^+$ cells gated on $CD56^{dim}CD16^+$ NK cells. D) Correlation between frequency of $NKG2A^+$ and $CD107a^+IFN\text{-}\gamma^-$, E) $CD107a^-IFN\text{-}\gamma^+$ and F) $CD107a^+IFN\text{-}\gamma^+$ cells gated on $CD56^{dim}CD16^+$ NK cells. Correlation determined with Spearman or Pearson test based on the normality of data.

**Figure 6. NK cell degranulation correlates with HCV-specific T cell adaptive immune response.** NK cell degranulation and IFN-γ production were determined in patients with HCV chronic evolution and HCV spontaneous resolution during the acute phase of HCV as shown in Figures 2 and 3. The magnitude of the HCV-specific T cell response approximately 2 months later in these same patients was determined by IFN-γ secretion in response to overlapping HCV peptides spanning the entire HCV polyprotein using the ELISPOT assay. A) Correlation between HCV-specific T cell response and frequency of $CD107a^+IFN\text{-}\gamma^-$ cells gated on $CD56^{bright}CD16^+$ NK cells, B) frequency of $CD107a^+IFN\text{-}\gamma^-$ cells gated on $CD56^{dim}CD16^-$ NK cells, C) frequency of $CD107a^-IFN\text{-}\gamma^+$ cells gated on $CD56^{bright}CD16^+$ NK cells, D) frequency of $CD107a^-IFN\text{-}\gamma^+$ cells gated on $CD56^{dim}CD16^-$ NK cells, E) frequency of $CD107a^+IFN\text{-}\gamma^+$ cells gated on $CD56^{bright}CD16^+$ NK cells, F) frequency of $CD107a^+IFN\text{-}\gamma^+$ cells gated on $CD56^{dim}CD16^-$ NK cells. Correlation determined with Spearman test.

**Supplementary Figures Legends**

**Supplementary Figure S1. Representative flow cytometry staining for CD107a and IFN-γ.**

Degranulation was measured by CD107a surface staining and cytokine production was measured

by intracellular IFN-γ. PBMCs were co-incubated with (bottom row) or without (top row) K562

target cells. CD107a$^+$ and IFN-γ$^+$ cells were gated on both NK cell subsets: CD56$^{bright}$CD16$^-$

(left column) and CD56$^{dim}$CD16$^{+/lo}$ (right column).

**Supplementary Figure S2. Representative flow cytometry staining for activation markers**

**and activating receptors.** One activation marker (CD69) and three activating receptors

(NKp44, NKp30 and NKG2D) were examined *ex-vivo* in both NK cell subsets: CD56$^{bright}$CD16$^-$

(top row) and CD56$^{dim}$CD16$^{+/lo}$ (bottom row).

**Supplementary Figure S3. Representative flow cytometry staining for inhibitory receptors.**

Five inhibitory receptors (NKG2A, CD161, KIR2DL1/S1, KIR2DL2/L3/S2 and KIR3DL1) were

examined *ex-vivo* in both NK cell subsets: CD56$^{bright}$CD16$^-$ (top row) and CD56$^{dim}$CD16$^{+/lo}$

(bottom row).

**Table 1. Demographics and Characteristics of patients and donors.**

|  | n | Gender (% M) | Age (median yr) | Race (caucasien) | Genotype 1/2/3 |
|---|---|---|---|---|---|
| Acute HCV ->chronic evolution | 10 | 90% | 31 | 8/10 | 1/0/8 |
| Acute HCV ->spontaneous resolution | 10 | 60% | 29 | 7/10 | 1/2/4 |
| HCV Exposed un-infected | 10 | 70% | 39 | 9/10 | N/A |
| Healthy donors | 10 | 60% | 31 | 7/10 | N/A |

**TABLE 2. Correlation between NK cell cytotoxicity, IFN-g production and expression of phenotypic markers**

| | $CD107a^+IFN\text{-}\gamma^-$ | | $CD107a^-IFN\text{-}\gamma^+$ | | $CD107a^+IFN\text{-}\gamma^+$ | |
|---|---|---|---|---|---|---|
| | $CD56^{dim}CD16^+$ | $CD56^{bright}CD16^-$ | $CD56^{dim}CD16^+$ | $CD56^{bright}CD16^-$ | $CD56^{dim}CD16^+$ | $CD56^{bright}CD16^-$ |
| **Activation marker** | | | | | | |
| **CD69** | p = 0.0051 | p = ns | p = 0.0001 | p = 0.035 | p = 0.011 | p < 0.0001 |
| | r = -0.32 | | r = 0.43 | r = 0.24 | r = 0.29 | r = 0.46 |
| **Activation receptors** | | | | | | |
| **NKG2D** | p = 0.0007 | p < 0.0001 | p = ns | p = ns | p = ns | p = ns |
| | r = 0.43 | r = 0.50 | | | | |
| **NKp30** | p = ns | p = 0.002 | p = ns | p = ns | p = ns | p = 0.0098 |
| | | r = 0.48 | | | | r = 0.35 |
| **NKp44** | p = ns | p = ns | p = ns | p = ns | p = 0.0355 | p = 0.0376 |
| | | | | | r = 0.24 | r = 0.24 |
| **Inhibitory receptors** | | | | | | |
| **CD161** | p = 0.0022 | p = 0.014 | p = 0.0022 | p = 0.011 | p = ns | p = 0.0478 |
| | r = -0.34 | r = -0.27 | r = 0.34 | r = 0.28 | | r = 0.22 |
| **KIR2DL1/S1** | p = 0.0036 | p = ns | p = ns | p = 0.016 | p = ns | p = 0.0359 |
| | r = -0.33 | | | r = 0.27 | | r = 0.24 |
| **KIR2DL2/L3/S2** | p = ns | p = ns | p = ns | p = ns | p = ns | p = ns |
| **KIR3DL1** | p = ns | p = ns | p = ns | p = ns | p = 0.0298 | p = ns |
| | | | | | r = 0.26 | |
| **NKG2A** | p = 0.002 | p = ns | p = ns | p = ns | p = 0.0004 | p = 0.0286 |
| | r = 0.34 | | | | r = 0.39 | r = 0.25 |

# Figure 1

**A.**



**B.** CD56^{dim}CD16^+

**C** CD56^{bright}CD16^-



Legend:
- ● Chronic
- △ Resolver
- ■ Exposed un-infected
- ○ Healthy donor

# Figure 2

# Figure 3

# Figure 4

**A.**

**CD56$^{dim}$CD16$^+$ CD69$^+$**



**B.**

**CD56$^{dim}$CD16$^+$ KIR3DL1$^+$**



**C.**

**CD56$^{bright}$CD16$^-$ NKG2A$^+$**



**D.**

**CD56$^{dim}$CD16$^+$ NKG2A$^+$**



**E.**

**CD56$^{dim}$CD16$^+$ CD161$^+$**
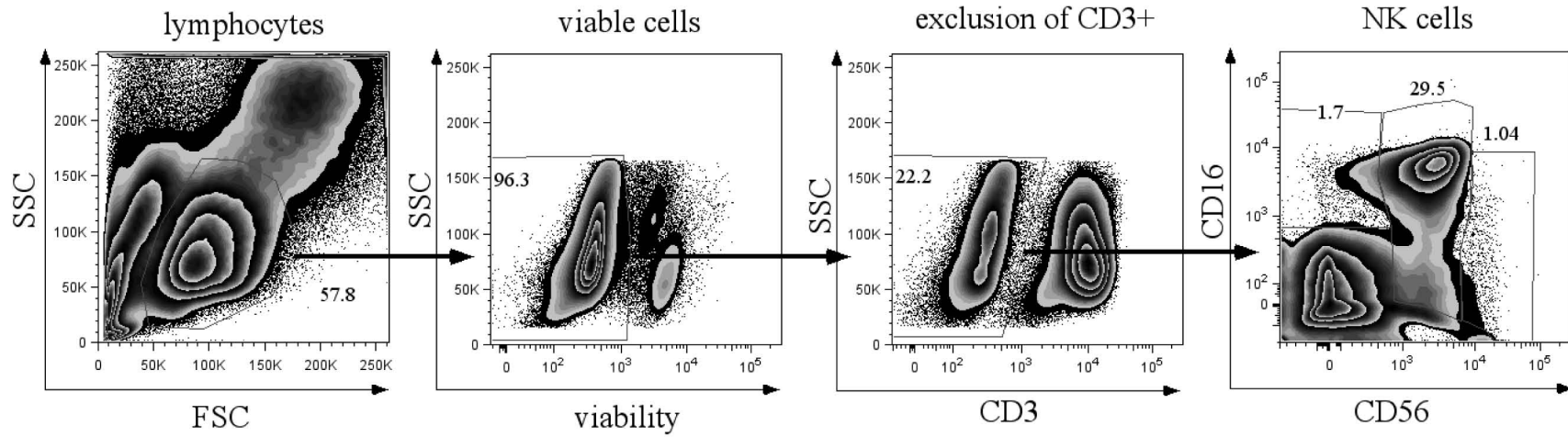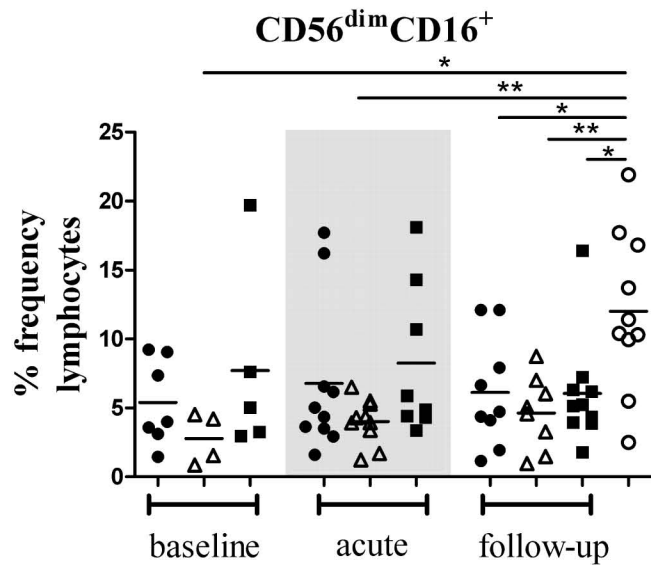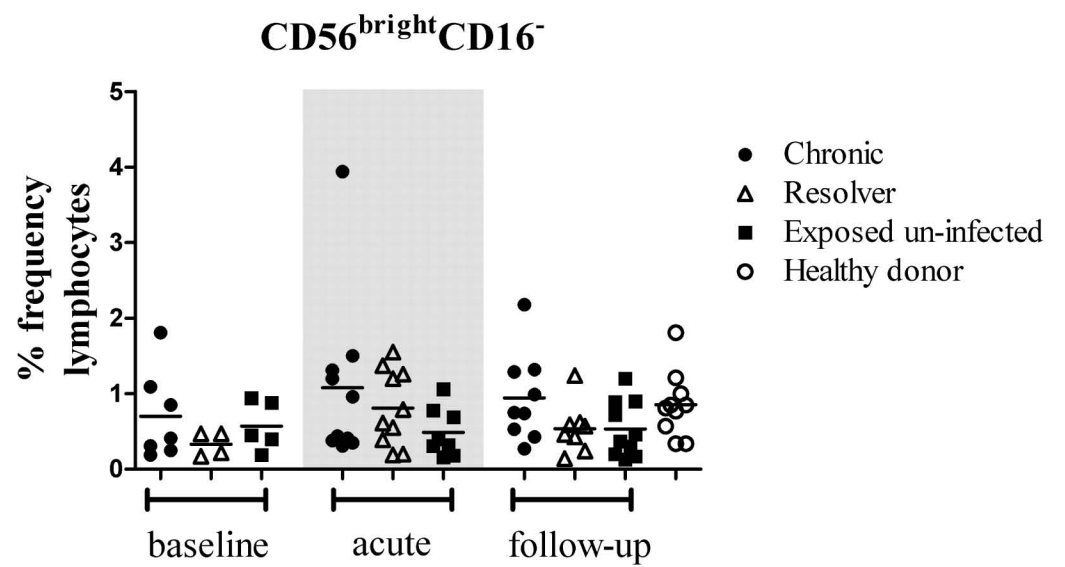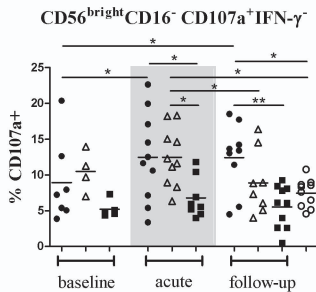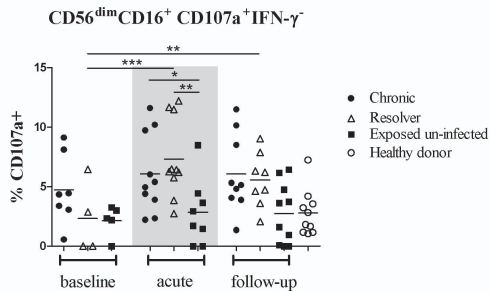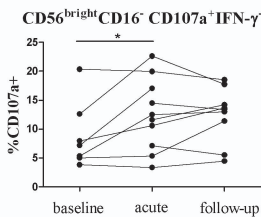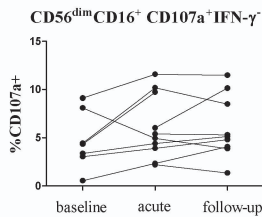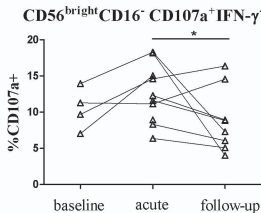
# Figure 5

# Figure 6

# Figure S1

Figure S2

Figure S3