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Non-neutralizing epitopes induce robust hepatitis C virus (HCV)-specific antibody-dependent CD56$^+$ natural killer cell responses in chronic HCV-infected patients

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Summary

Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) is of considerable interest in viral infection. However, little is known about NK-ADCC responses in chronic hepatitis C virus (HCV) infection. In this study, impaired non-specific antibody-dependent CD56$^+$ NK cell responses were observed in chronic HCV infection, as shown by decreased degranulation (extracellular CD107a expression) and interferon (IFN)-γ production in response to antibody-bound P815 cells. A peptide pool composed of epitopes recognized by anti-HCV-E1/E2 antibodies could induce pronounced HCV-specific antibody-dependent NK cell responses in sera from approximately half the chronic HCV carriers. Additionally, HCV-specific epitopes with the capacity to induce robust NK-ADCC activity were identified. Five linear NK-ADCC epitopes (aa211-aa217, aa384-aa391, aa464-aa475, aa544-aa551 and aa648-aa659 of the HCV envelope) were identified and do not overlap with putative linear neutralizing epitopes. This study revealed the dysfunctional characteristics of antibody-dependent CD56$^+$ NK cell responses in chronic HCV carriers. The key non-neutralizing NK-ADCC epitopes identified in this study may act as new targets for immunological intervention.

Keywords: antibody-dependent cellular cytotoxicity, epitope, hepatitis C virus, natural killer cell, non-neutralizing antibody

Introduction

Chronic hepatitis C virus (HCV) infection is characterized by the persistence of detectable circulating HCV RNA and anti-HCV antibodies. However, these antibodies fail to control HCV infection, as the production of neutralizing antibodies usually lags behind the evolution of HCV E1/E2 quasispecies within infected patients [1]. Some studies indicated that the extent of anti-HCV responses mediated by NK cells was associated with the outcome of HCV acute infection [2,3]. In addition to natural cytotoxicity, NK cell activity triggered by an imbalance in signals received by activating and inhibitory receptors expressed on NK cells, NK cell-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) may also be involved in immune protection in HCV infection. NK-ADCC is elicited by activation of FcγRIII (CD16) on NK cells by the Fc portion of an immunoglobulin (Ig)G antibody bound to an antigen. After engagement, the activated NK cells promptly degranulate and release perforin and granzymes, which results in the lysis of target cells. Activated NK cells also release immune modulatory cytokines such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α [4]. NK-ADCC was shown to be associated with disease progression in human immunodeficiency virus (HIV)-infected patients and simian immunodeficiency virus (SIV)-infected macaques, and was reported as a major contributor in preventing HIV infection in the RV144 clinical trial [5,6]. The induction of NK-ADCC responses has been proposed as an alternative strategy for HIV and HCV vaccine development [7]. Nattermann *et al.* demonstrated that anti-HCV E2 antibodies could mediate ADCC in approximately 50% of HCV-infected subjects, whether patients with acute, self-limited

or chronic HCV infection [8]. However, it is still unclear whether or not the capacity of antibody-dependent NK cell responses is impaired by chronic HCV infection. Also, we do not know whether the antibodies are capable of mediating NK-ADCC overlap or are completely distinct from the neutralizing antibodies produced during HCV infection.

In this study, we demonstrated that *ex-vivo* non-specific antibody-dependent $CD56^+$ NK cell responses induced by antibody-coated P815 cells were functionally impaired in chronic HCV infection. In addition, linear epitopes located in the HCV-E1/E2 protein that could mediate robust NK-ADCC were identified and compared with putative neutralizing epitopes.

Materials and methods

Study subjects

A total of 31 chronic HCV carriers and 49 healthy controls were recruited from a village in central China [9]. Chronic HCV infection was identified by anti-HCV responses and detection of HCV RNA. The clinical and laboratory characteristics of the study subjects are summarized in Table 1. Plasma HCV antibodies were detected using the Architect anti-HCV assay (Abbott GmbH & Co KG, Wiesbaden, Germany) and confirmed by the HCV-recombinant immunoblot assay (RIBA) assay (Wantai Biological Pharmacy, Beijing, China). HCV RNA was detected using the Abbott real-time HCV amplification kit (Abbott Molecular, Des Plaines, IL, USA), according to the manufacturer's instructions. None of the hepatitis C patients received any form of anti-HCV therapy, and all participants were negative for hepatitis A virus (HAV), hepatitis B virus (HBV), HIV and tuberculosis (TB). Plasma and peripheral blood mononuclear cells (PBMCs) were separated from ethylendiamine tetraacetic acid (EDTA) anti-coagulated whole blood specimens and stored at −80°C and −180°C, respectively. The study protocol was approved by the institutional review authorities of Peking University Health Science Center. Informed consent was obtained from each patient enrolled in the study.

Table 1. Characteristics of 31 chronic hepatitis C virus (HCV) carriers and 49 healthy controls

Characteristics	Chronic HCV	Healthy
Number	31	49
Female (%)*	18 (58·1)	30 (61·2)
Age (years)†	48 (62 − 33)	46 (58 − 34)
BMI†	23·2 (25·2 − 20·4)	22·8 (25·0 − 20·6)
Clinical data		
anti-HCV S/CO value†	14·12 (10·3 − 16·2)	Negative
HCV RNA (log_{10} IU/ml)†	6·43 (6·60 − 5·91)	Negative
HCV genotype		
1b	21	n.a.
2a	8	n.a.
others	0	n.a.
ALT (IU/l)†	48 (128 − 17)	28 (34 − 15)
AST (IU/l)†	44 (109 − 18)	26 (33 − 14)
Total protein (g/l)†	78·2 (85·2 − 70·6)	76·8 (82·4 − 72·4)
Albumin (g/l)†	44·0 (51·3 − 36·5)	45·6 (53·0 − 38·7)
Total bilirubin (μmol/l)†	14·1 (17·2 − 11·2)	11·8 (15·4 − 7·5)
Direct bilirubin (μmol/l)†	4·3 (5·9 − 3·0)	4.0 (5.9 − 2.7)

*Number of cases (%). †Mean (range). BMI = body mass index; n.a. = not available; S/CO = signal/cut-off; ALT = alanine aminotransferase; AST = aspartate aminotransferase.

Evaluation of the non-specific antibody-dependent NK cell responses by intracellular cytokine staining

A novel non-specific ADCC assay based on intracellular cytokine staining (ICS) was used to detect ADCC responses by circulating $CD56^+$ NK cells [10]. Briefly, 1×10^5 P815 cells (a mouse leukaemic cell line) were treated with medium or with a 1 : 100 dilution of polyclonal rabbit anti-mouse lymphocyte serum (Accurate Chemical and Scientific Corp., Westbury, NY, USA) for 1 h at 37°C/5% CO_2 in a volume of 200 μl of R10 medium (RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin), and then washed twice with ice-cold R10 medium; 1×10^6 peripheral blood mononuclear cells (PBMCs) were stimulated with R10 medium alone, uncoated P815 cells, antibody -coated P815 cells or phorbol myristate acetate (PMA) plus ionomycin (positive control) (Sigma-Aldrich, St Louis, MO, USA). Cells were cultured with CD107a-phycoerythrin-cyanin 5 (PE-Cy5) (clone H4A3; BD Biosciences, San Jose, CA, USA), Golgi-Stop (BD Biosciences) and brefeldin A (Sigma-Aldrich) for 6 h at 37°C/5% CO_2. After culture, PBMCs were stained with CD3-eFluor 450 (clone 17A2; eBioscience; San Diego, CA, USA), CD16-allophycocyanin (APC)-Cy7 (clone 3G8; BD Biosciences) and CD56-PE-Cy7 (clone B159; BD Biosciences). Then, cells were permeabilized using 0·25% saponin (Thermo Fisher Scientific; Waltham, MA, USA), and ICS was carried out with IFN-γ fluorescein isothiocyanate (FITC) (clone 25723.11; BD Biosciences) and TNF-α-APC (clone 6401.1111; BD Biosciences). After staining, cells were washed in phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde (PFA). All data were acquired on a BD FACS Fortessa (BD Biosciences) and analysed using FlowJo software (Treestar, Ashland, OR, USA).

NK cell purification

Untouched NK cells were enriched from PBMCs using an NK cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). In brief, NK cells were negatively isolated by depleting non-NK cells (i.e. T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes and erythroid cells) using a cocktail of biotin-conjugated antibodies, followed by streptavidin-coated microbeads. Isolation of highly pure

NK cells was achieved by depletion of magnetically labelled cells. The purity of NK cells obtained in this fashion was consistently greater than 95%. Isolated 1×10^5 NK cells were co-cultured with uncoated or 1×10^4 antibody-coated P815 cells at 37°C/5% CO_2 for 24 h, and cell-free supernatants (NK-ADCC supernatants) were collected for enzyme-linked immunosorbent assay (ELISA), as described below.

ELISA

The levels of IFN-γ, TNF-α, transforming growth factor (TGF)-β and interleukin (IL)-10 in NK-ADCC supernatants were analysed using Ready-SET-Go ELISA kits according to the manufacturer's instructions (eBioscience). The sensitivities of the ELISAs were 4 pg/ml for IFN-γ, 4 pg/ml for TNF-α, 8 pg/ml for TGF-β and 2 pg/ml for IL-10. Granzyme B was detected using a Platinum ELISA kit (eBioscience) with a sensitivity of 0·2 pg/ml.

Peptide recognition of HCV-E1/E2 antibodies

All HCV-E1/E2 antibody-specific peptides employed in this study were designed according to the sequences of published epitopes located in the HCV E1/E2 region, with slight modifications based on the sequence characteristics of HCV genotypes 1b and 2a, which are the predominant circulating genotypes in mainland China. Peptide sequences are presented in Table 2. In particular, epitopes 1 [11], 5 [12,13], 7 [14], 8 [15,16], 10 [17] and 12 [18,19] are overlapped with six putative linear neutralizing epitopes, and the other 11 epitopes (2, 3, 4, 6, 9, 11, 13, 14, 15, 16 and 17) [20–25] are targets recognized by well-characterized anti-HCV E1/E2 monoclonal antibodies. All solid-phase peptides were synthesized to 95% purity by Bio-Scientific Co. (Shanghai, China). Each peptide was dissolved to a concentration of 1 mg/ml in RPMI-1640 containing 10% dimethyl sulphoxide (DMSO), and stock solutions were diluted further in RPMI-1640 to a working concentration of 1 μg/ml.

Table 2. Peptide characteristics

Peptide no.	Sequence	Location in H77*	Protein
1	YEVRNVSGIYHVTNDCSNS	192 – 210	E1
2	SSGLYHVTNDC	197 – 207	E1
3	SIVYEAA	211 – 217	E1
4	RHWTTQGCNC	297 – 306	E1
5	SGHRMAWDMMMNWSPTT	314 – 330	E1
6	ETHVTGGS	384 – 391	E2
7	TAGLVGLLTPGA	396 – 407	E2
8	SQKIQLVNTNGSWHIN	408 – 423	E2
9	GSWHINRTALNCND	418 – 431	E2
10	SLNTGWLAGLFYQHKF	432 – 447	E2
11	DFDQGWGPISYA	464 – 475	E2
12	IVPAKSVCGPVYCFTPSPVV	496 – 515	E2
13	SGAPTYSWGA	522 – 531	E2
14	PPLGNWFG	544 – 551	E2
15	YRLWHYPCT	613 – 621	E2
16	LDAACNWTRGERCD	640 – 653	E2
17	RGERCDLEDRDR	648 – 659	E2

A total of 17 linear peptides were reported to be recognized by defined anti-hepatitis C virus (HCV) E1/E2 monoclonal antibodies. Underlined epitopes 1 [11], 5 [12,13], 7 [14], 8 [15,16], 10 [17] and 12 [18,19] are overlapped with six putative linear neutralizing epitopes, and the other 11 epitopes (2, 3, 4, 6, 9, 11, 13, 14, 15, 16 and 17) are targets recognized by well-characterized anti-HCV E1/E2 monoclonal antibodies [20–25]. *The first and final positions are numbered with reference to the consensus sequence of the polyprotein of HCV strain H77 (Genbank accession number AB009606).

Identification of HCV E1/E2-specific NK-ADCC in chronic HCV-infected subjects

In order to confirm whether HCV membrane protein could induce an NK-ADCC response, huh7·5-based HCV replicon cells (HCV-Con I-Rep), which were transfected stably with a plasmid pNNeo/3-5 BRG (kindly donated by Dr Stanley M. Lemon, University of Texas Medical Branch at Galveston) containing HCV-1b subgenome, were cultured on 48-well plates. When cell confluence reached 80%, cells were washed three times with 1 × PBS and incubated with heat-inactivated sera from chronic HCV carriers (all were HCV-1b genotype) or healthy donors (diluted 1 : 200 in PBS) for 2 h at 37°C. After that, the plates were washed five times by PBS to remove unbinding antibodies; 1×10^5 purified NK cells from autologous individual were added to each well. After 24 h incubation, cell-free supernatants were collected for IFN-γ detection by Ready-SET-Go ELISA kit (eBioscience) with a sensitivity of 4 pg/ml.

We then tested whether HCV E1/E2 linear epitopes could induce an NK-ADCC response; 96-well ELISA plates (Nunc MaxiSorp; eBioscience) were precoated with 17 different HCV-E1/E2 epitope peptides pool (600 ng/well) and incubated with heat-inactivated sera from chronic HCV carriers or healthy donors (diluted 1 : 200 in PBS) for 2 h at 37°C. The remainder of the experimental procedure was similar to the above description. The five serum samples that induced the highest NK-ADCC were used for follow-up experiments.

Screening of HCV E1/E2-specific NK-ADCC linear epitopes

For screening of HCV E1/E2-specific NK-ADCC linear epitopes, 96-well ELISA plates (Nunc MaxiSorp; eBioscience) were precoated individually with the 17 HCV-E1E2 epitope peptides (600 ng/well) and incubated with heat-inactivated sera samples (diluted 1 : 200 in PBS) from five chronic HCV carriers with robust NK-ADCC responses (as determined in the above experiment) for 2 h at 37°C. The plates were washed five times with PBS and 1×10^5 purified NK cells from a constant healthy individual were added to each well. After 24 h, cell-free supernatants were collected for IFN-γ detection by Ready-SET-Go ELISA kit (eBioscience).

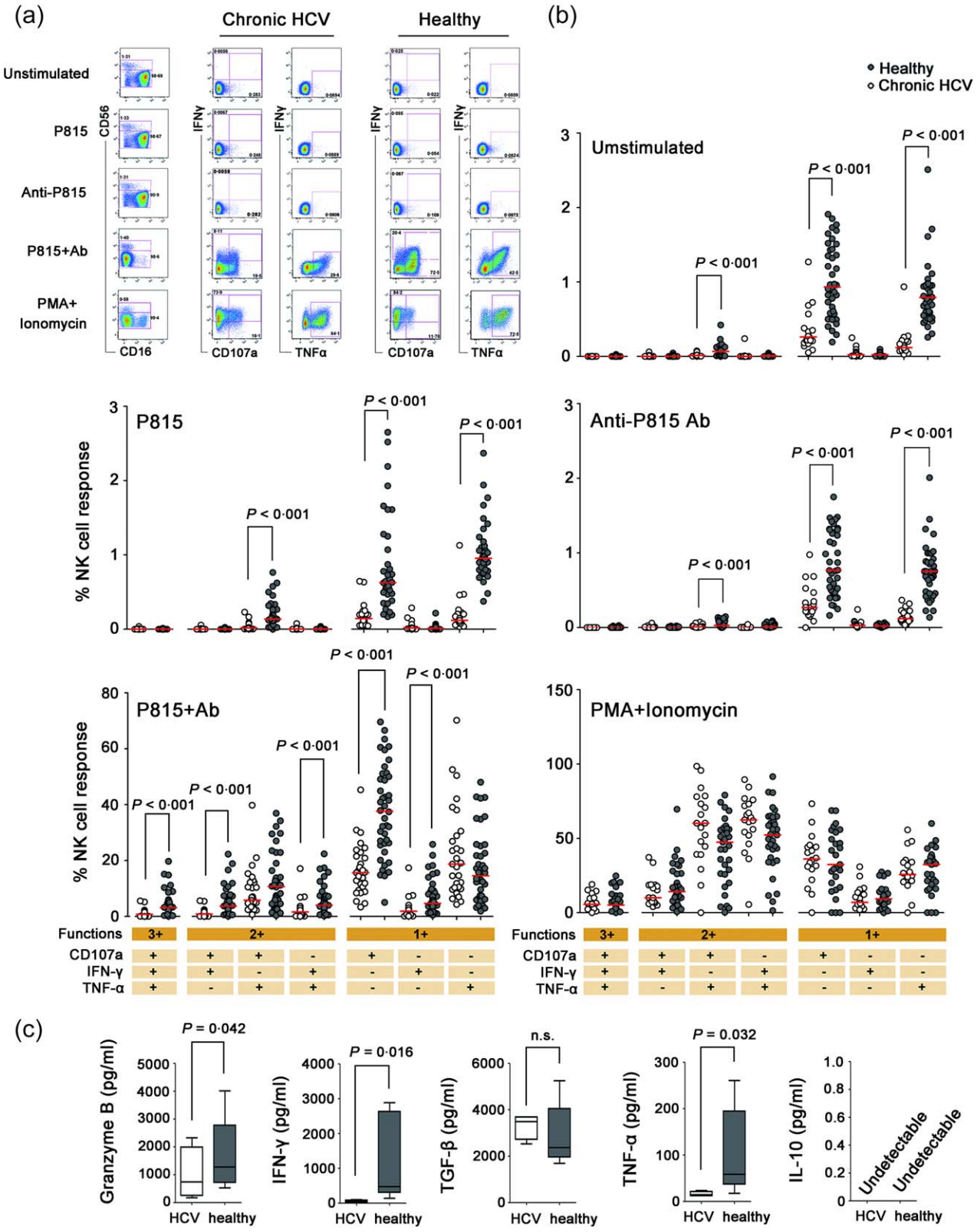
(a)
Chronic HCV
Healthy
Unstimulated
P815
Anti-P815
P815+Ab
PMA+
Ionomycin
CD56
CD16
IFNγ
CD107a
TNFα
(b)
Healthy
Chronic HCV
Umstimulated
P815
Anti-P815 Ab
P815+Ab
PMA+Ionomycin
% NK cell response
P < 0·001
Functions
3+
2+
1+
CD107a
IFN-γ
TNF-α
(c)
Granzyme B (pg/ml)
P = 0·042
IFN-γ (pg/ml)
P = 0·016
TGF-β (pg/ml)
n.s.
TNF-α (pg/ml)
P = 0.032
IL-10 (pg/ml)
Undetectable
Undetectable
HCV
healthy

Fig. 1. Impaired $CD56^+$ natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) responses in chronic hepatitis C virus (HCV) carriers. (a) Gating strategies for CD16, CD107a, interferon (IFN)-γ and tumour necrosis factor (TNF)-α on $CD3^-CD56^+$ NK cells stimulated with medium alone (unstimulated), P815 cells (P815), anti-P815 antibody (anti-P815), antibody-coated P815 cells (P815 + antibody) and phorbol myristate acetate (PMA) plus ionomycin. Representative results from one chronic HCV-infected patient and one healthy individual are shown. (b) Degranulation (CD107a) and cytokine secretion (IFN-γ and TNF-α) in $CD56^+$ NK cells on unstimulated condition or activated with antibody-coated P815 cells in 31 chronic HCV-infected patients and 49 healthy controls. The percentages of single-, double- and triple-positive $CD56^+$ NK cells are shown, and the red bars indicate the median values. Comparisons between groups were performed using the Mann–Whitney *U*-test. (c) Secreted cytokines were quantified in NK-ADCC supernatants by enzyme-linked immunosorbent assay (ELISA). Purified NK cells from HCV carriers ($n = 5$) and healthy donors ($n = 5$) were stimulated with antibody-coated P815 cells for 24 h, and supernatants were collected to test the levels of granzyme B, IFN-γ, TNF-α, transforming growth factor (TGF)-β and IL-10 by ELISA. Data are shown as the median cytokine concentration and the interquartile range. Statistical analyses were performed by non-parametric *t*-test. All *P*-values are two-tailed and were considered significant when less than 0·05. [Colour figure can be viewed at wileyonlinelibrary.com].

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Comparisons between groups were performed using the Mann–Whitney *U*-test or a non-parametric *t*-test, as necessary. Spearman's correlation test was used to evaluate correlations between groups. All *P*-values were two-tailed and considered significant when less than 0·05.

Results

Non-specific antibody-dependent NK cell responses were impaired significantly in chronic HCV infection

To investigate the characteristics of non-specific antibody-dependent NK cell responses, PBMCs were stimulated with antibody-coated P815 cells and the expression of CD107a, TNF-α and IFN-γ was detected by flow cytometry. As shown in Supporting information, Fig. S1a, $CD56^+$ NK cells were gated from live $CD3^-CD56^+$ lymphocytes. Compared to NK cells stimulated with uncoated P815 cells, CD16 expression decreased dramatically on NK cells stimulated with antibody-coated P815 cells, while the expression of CD107a, IFN-γ and TNF-α increased significantly, indicating the induction of antibody-dependent NK cell responses (Fig. 1a). Samples incubated with PMA plus ionomycin were used as positive controls.

Subsequently, antibody-dependent NK cell responses in 31 chronic HCV carriers was characterized and compared with healthy controls. As shown in Fig. 1b, the frequency of NK cells expressing CD107a or IFN-γ after stimulation with antibody-coated P815 cells was reduced significantly in chronic HCV carriers compared with healthy controls (each $P < 0{\cdot}001$). After stimulation with antibody-coated P815 cells, CD107a/IFN-γ/TNF-α triple-positive NK cells and IFN-γ/TNF-α or CD107a/IFN-γ double-positive NK cells were reduced dramatically in HCV-infected individuals compared to healthy controls (each $P < 0{\cdot}001$). These results indicated that antibody-dependent NK cell responses were impaired in chronic HCV infection, although the frequency of total $CD56^+$ NK cells in circulating lymphocytes was increased in chronic HCV carriers (Supporting information, Fig. S1b, $P = 0{\cdot}046$). The reason might ascribed partially to the impaired spontaneous cytotoxity of NK cells, as the frequency of CD107a, TNF-α– and CD107a/TNF-α-positive NK cells were decreased significantly in chronic HCV infection when cultured with media alone or anti-P815 antibody alone ($P < 0{\cdot}001$, Fig. 1b).

To verify the results of the ICS, purified NK cells (five subjects per group) were stimulated with antibody-coated P815 cells for 24 h and culture supernatants were harvested for cytokine detection. Granzyme B, IFN-γ, TNF-α, TGF-β and IL-10 levels were determined by ELISA and compared between chronic HCV carriers and healthy controls. Similar to the results by ICS, levels of granzyme B ($P = 0{\cdot}042$), IFN-γ ($P = 0{\cdot}016$) and TNF-α ($P = 0{\cdot}032$) were significantly lower in supernatants from HCV-infected carriers than in healthy controls, while there was no difference in TGF-β levels (Fig. 1c). In addition, IL-10 production was undetectable in supernatants from either HCV-infected subjects or healthy individuals (Fig. 1c).

NK-ADCC responses were higher in chronic HCV carriers with abnormal serum alanine aminotransferase (ALT)

We then identified the relationship between ALT level and NK-ADCC response; 31 chronic HCV carriers were separated into ALT normal ($ALT \leq 40$ IU/l) and abnormal groups ($ALT > 40$ IU/l). As shown in Fig. 2, the percentage of $CD107a^+$ $CD56^+$ NK cells was significantly higher in patients with abnormal ALT than patients with normal ALT ($P = 0{\cdot}039$). A similar trend was observed for the percentage of IFN-γ-producing NK cells, although it did not reach a significant difference ($P = 0{\cdot}082$, Fig. 2). It is possible that the stronger NK-ADCC response induced more HCV-infected target cell lysis and resulted in increase of ALT production. However, no correlations between NK-ADCC responses and HCV loads or non-invasive markers of fibrosis (APRI/FIB-4) were found in the study (data not shown).

Non-specific antibody-dependent NK cell responses were correlated with the loss of CD16 on NK cells

We found that the potency of the antibody-dependent NK cell responses (reflected in the frequency of $CD107a^+$ and IFN-γ^+ NK cells) correlated directly with decreased

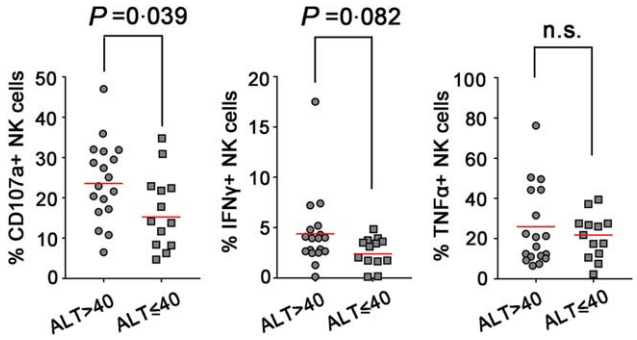

Fig. 2. Natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) responses were higher in chronic hepatitis C virus (HCV) carriers with abnormal serum alanine aminotransferase (ALT). Thirty-one chronic HCV carriers were divided into two groups according to serum ALT level (higher or not than 40 IU/l). The percentage of CD107a, interferon (IFN)-γ and tumour necrosis factor (TNF)-α-positive NK cells were compared between the ALT normal group (ALT ≤ 40 IU/l) and the abnormal group (ALT > 40 IU/l). Statistical analyses were performed by non-parametric t test. All *P*-values were two-tailed and considered significant when lower than 0·05. [Colour figure can be viewed at wileyonlinelibrary.com].

expression of FcγRIIIa (CD16) on NK cells in both chronic HCV patients and controls [for CD107a, $P < 0{\cdot}001$ for chronic HCV patients and $P = 0{\cdot}038$ for healthy controls (Fig. 3a); for IFN-γ, $P < 0{\cdot}001$ for chronic HCV patients and $P = 0{\cdot}009$ for healthy controls (Fig. 3b)]. Additionally, the cytotoxicity receptors NKp46 ($P = 0{\cdot}006$ for total NK and $CD56^{dim}$ subset) and NKG2D ($P < 0{\cdot}001$ for total, $CD56^{dim}$ and $CD56^{bright}$ NK cells) were down-regulated on NK cells in chronic HCV carriers (Supporting information, Fig. S1c) [26–28].

$CD56^{dim}$ NK cells, but not $CD56^{bright}$ NK cells, contribute mainly to NK-ADCC activation

In order to determine which NK cell subsets, $CD56^{dim}$ or $CD56^{bright}$, hold the main capacity to induced NK-ADCC activation, we compared the three functions (CD107a/IFN-γ/TNF-α) profile between $CD56^{dim}$ and $CD56^{bright}$ subsets activation following stimulation with antibody-coated P815 cells. The result showed that three single functions, triple and double combinations of functions, were all higher in the activated $CD56^{dim}$ NK cells (all $P < 0{\cdot}001$) (Fig. 4), indicating that the $CD56^{dim}$ subset contributed mainly to NK-ADCC activation.

Identification of HCV-specific antibody-dependent NK cell responses in chronic HCV-infected subjects *in vitro*

To study whether HCV membrane protein could induce an NK-ADCC response, huh7·5-based HCV replicon cells (HCV-Con I-Rep) were used to bind anti-HCV sera to activate autologous NK cells. The result showed that significantly higher IFN-γ concentrations were detected in the group

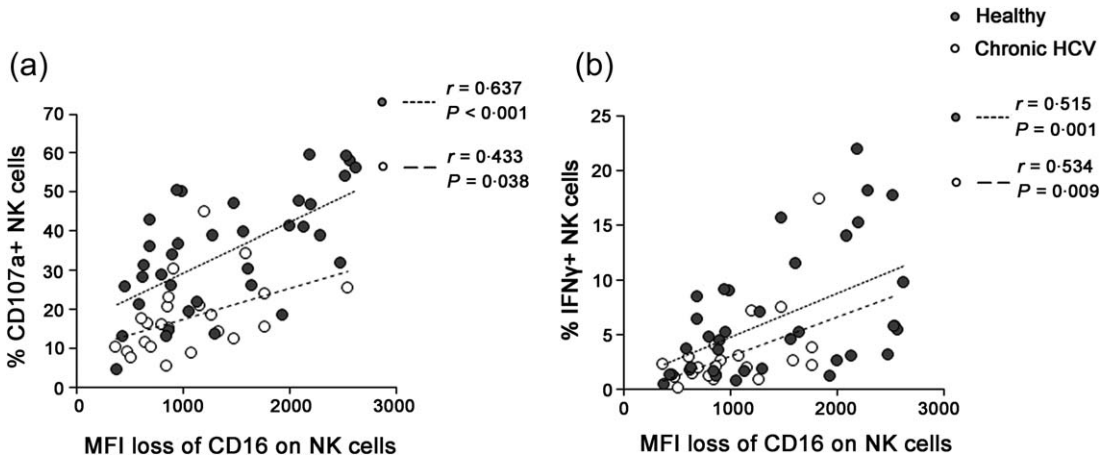

Fig. 3. Non-specific antibody-dependent natural killer (NK) cell responses were correlated with the loss of CD16 on NK cells. the potency of the antibody-dependent NK cell responses were calculated as the frequencies of $CD107a^+$ (a) or interferon (IFN)-γ^+ (b) NK cells incubating with antibody-bound P815 cells minus the percentage of these cells incubating with P815 cells alone. The Pearson's correlation test was used to evaluate correlations between groups. Loss of $CD16^+$ cells on $CD56^+$ NK cells indicated CD16 mean fluorescence intensity (MFI) of these cells stimulated with P815 cells alone minus CD16 MFI following stimulation with antibody-bound P815 cells. All *P*-values are two-tailed and were considered significant when less than 0·05.

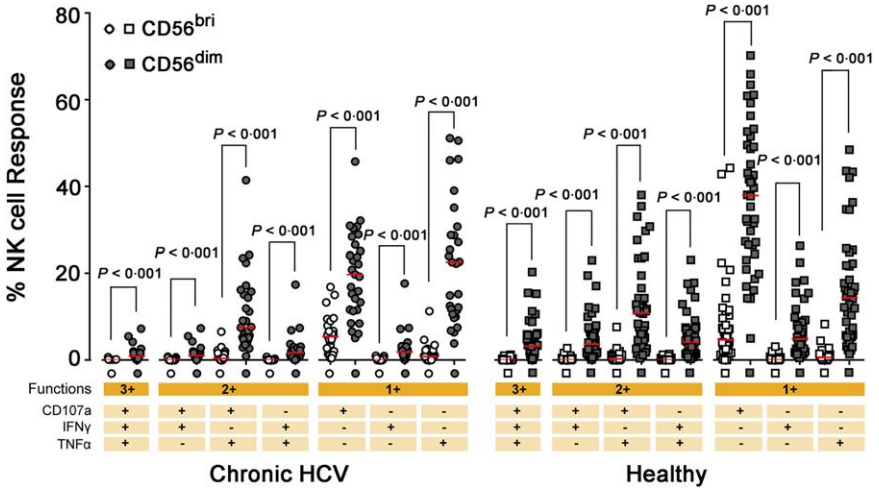

Fig. 4. Comparision of natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) responses mediated by $CD56^{dim}$ NK cells and $CD56^{bri}$ NK subset. The functional profile of $CD56^{dim}$ and $CD56^{bri}$ subsets stimulated with antibody-coated p815 cells was shown. The percentages of CD107a/interferon (IFN)-γ/tumour necrosis factor (TNF)-α single-, double- or triple-positive NK cells in the respective subset were shown and the red bar indicated median value. Comparisons between groups were performed using Mann–Whitney *U*-test. All *P*-values were two-tailed and considered significant when lower than 0·05. [Colour figure can be viewed at wileyonlinelibrary.com].

bound with positive anti-HCV sera than the group inoculated with healthy sera ($P < 0{\cdot}001$) (Fig. 5a). To confirm HCV-specific antibody-dependent NK cell responses, 17 linear peptides (Table 2) recognized by defined anti-HCV E1/E2 monoclonal antibodies were pooled and used to bind anti-HCV sera to activate autologous NK cells. As shown in Fig. 5b, the peptide pool induced HCV-specific NK-ADCC responses successfully in 18 of 31 (58·1%) patients.

Screening of linear epitopes capable of mediating robust antibody-dependent NK responses

To determine the specific epitopes capable of mediating robust antibody-dependent NK responses, each of the 17 peptides was bound to sera from HCV carriers with robust antibody-dependent NK responses (as determined in Fig. 5b), and the antigen–antibody complexes were used to activate purified NK cells from two different healthy donors (donors A and B) (with the exception that case 271 was tested only against donor A due to the limited volume of serum available). As shown in Fig. 6, peptides were bound to five different HCV sera (HCV-1b for cases 37 and 121 and HCV-2a for cases 204, 152 and 271). Among the 17 peptides, peptides 3, 6, 11, 14 and 17 induced higher NK responses than the other 12 peptides in both healthy donors, suggesting the conservation of specific NK-ADCC epitopes.

As shown in Table 2 and Fig. 7, epitopes aa192–202 and 313–326 in the E1 region, epitope aa396-407 in the HVR1 region and epitopes aa412–423 and aa496–515 in the E2

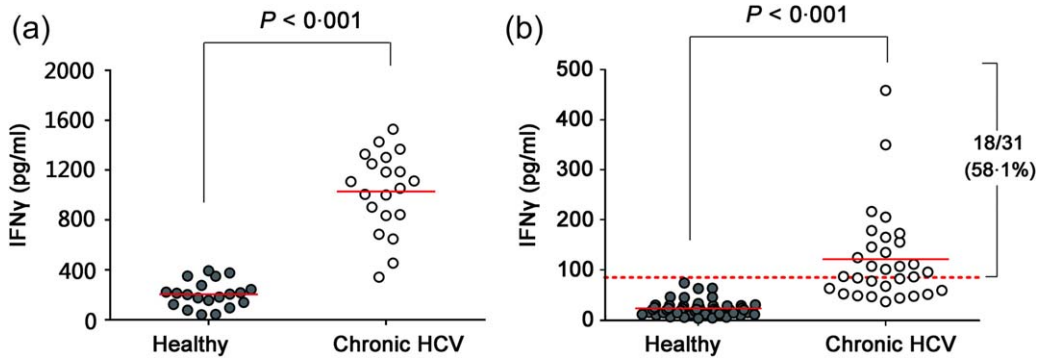

Fig. 5. Identification of hepatitis C virus (HCV)-specific natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) functions in chronic HCV-infected subjects *in vitro*. (a) Huh7·5-based HCV replicon cells (HCV-Con-Rep) were cultured on 48-well plate to 80% confluence and were incubated with heat-inactivated sera from 20 chronic HCV carriers (all were HCV-1b genotype) or 20 healthy donors to form antigen–antibody complexes. Purified autologous NK cells were used as effector cells. Interferon (IFN)-γ levels in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). (b) Seventeen peptides representing epitopes known to be recognized by anti-HCV antibodies were pooled and precoated onto 96-well plates. Serum samples from 31 HCV patients and 49 healthy individuals were added subsequently. Purified autologous NK cells were used as effector cells and then IFN-γ in the supernatants were detected. The dotted red line indicates the average IFN-γ level of the healthy controls plus three times the standard deviation (mean ± 3 s.d.). Comparisons between groups were performed using the Mann–Whitney *U*-test. All *P*-values are two-tailed and were considered significant when less than 0·05. [Colour figure can be viewed at wileyonlinelibrary.com].

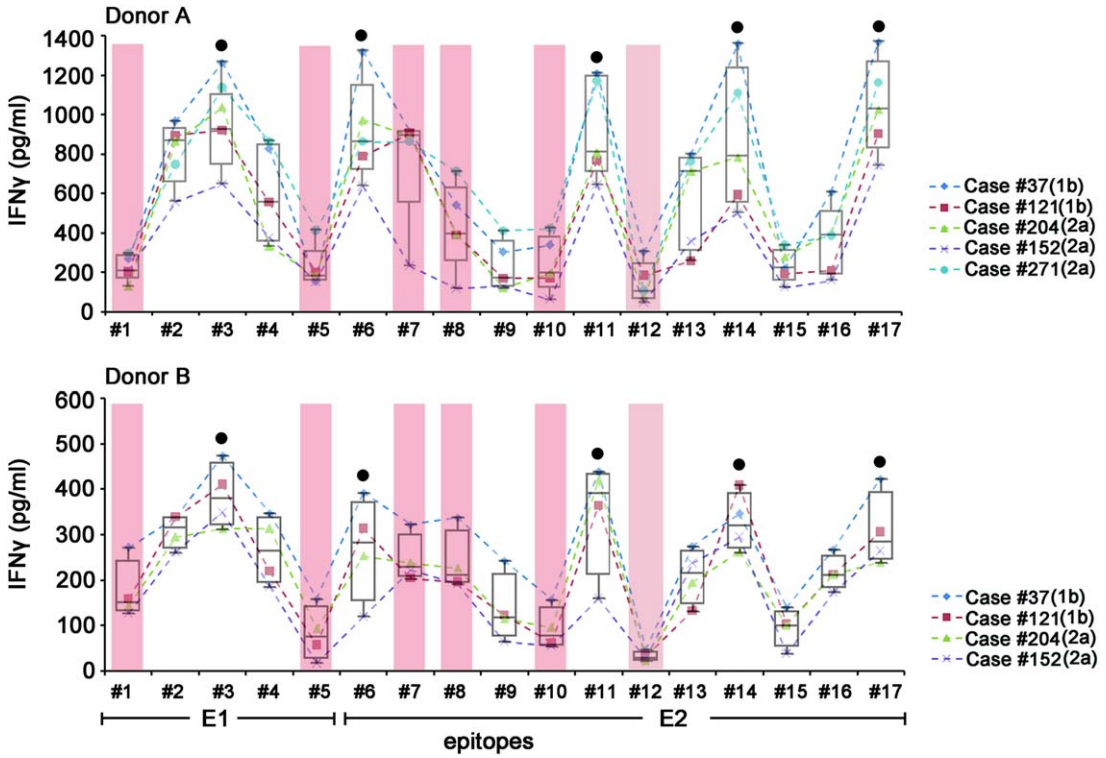

Fig. 6. Screening of linear epitopes within the hepatitis C virus (HCV) E1/E2 region for their ability to induce natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC). Individual synthetic HCV-E1E2 peptides were precoated onto 96-well plates and incubated with serum samples from five different chronic HCV carriers (genotype 1b for 37 and 121; genotype 2a for 204, 152 and 271). Purified NK cells from two healthy donors (donor A for the upper panel and donor B for the lower panel) were used as effector cells. Interferon (IFN)-γ levels in cell-free supernatants were determined by enzyme-linked immunosorbent assay (ELISA). HCV patient no. 271 was not tested with donor B NK cells due to the limited amount of serum available. Peptides 3, 6, 11, 14 and 17 (indicated by black dots) induced higher NK-ADCC responses than the other 12 peptides in both donors. Six putative neutralizing epitopes overlapped with peptides 1, 5, 7, 8, 10 and 12 are indicated by pink columns. The median and the interquartile ranges are shown for each peptide. [Colour figure can be viewed at wileyonlinelibrary.com].

region are putative linear neutralizing epitopes. In addition, epitope aa434–446 can be recognized by some antibodies and is also a potential neutralizing epitope [29]. Among the 17 HCV E1/E2-derived peptides tested in this study, 1, 5, 7, 8, 10 and 12 overlap with the putative linear neutralizing epitopes. None of the NK-ADCC peptides (3, 6, 11, 14 and 17) overlapped with those putative neutralizing epitopes. These data indicate that NK-ADCC epitopes may be distinct from neutralizing epitopes.

Discussion

Because NK cells are enriched in human liver [30,31] and NK-related immune factors can predict viral clearance after acute HCV infection [3,31–33], NK cell-mediated immunity is thought to play a pivotal role in host defence against HCV infection. Recently, Grebely *et al.* demonstrated that spontaneous clearance of HCV occurred most often after the induction of an anti-HCV humoral immune response, indicating that antibody-mediated immune responses such as NK-ADCC might contribute to the spontaneous clearance of HCV [34]. Unlike neutralizing antibodies, which directly prevent viral infection, NK-ADCC antibodies eliminate HCV-infected cells by triggering the release of cytotoxic substances from NK cells, including granzyme, perforin and cytokines [35]. Whether or not NK-ADCC and NK cell natural cytotoxicity are impaired in chronic HCV infection is still controversial. NK natural cytotoxicity has been reported to be diminished [30,36], unaffected or even increased [33,37–40] in chronic HCV infection. However, little is known regarding antibody-dependent NK cell responses in chronic HCV infection. Liu *et al.* reported impaired non-specific NK-ADCC in HIV-1 infection using P815 cells (a mouse mastocytoma cell line) as target cells, which avoids the interference caused by activation of killer activation receptors (KARs) or killer inhibitory receptors (KIRs) on NK cells [41]. Alter *et al.* found that the percentage of NK cells expressing CD107a after activation with plate-coated P815-antibody complex was decreased in chronic HCV patients compared to healthy controls, but a statistical difference was not found due to the limited number of cases examined [3].

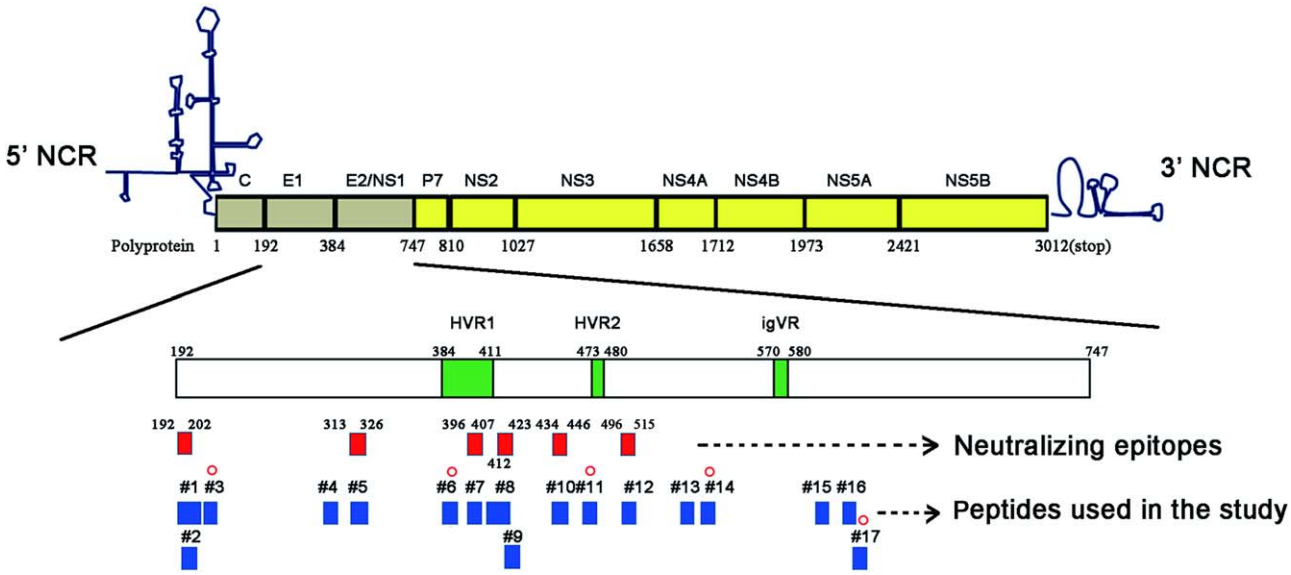

Fig. 7. Distribution of five defined linear natural killer-mediated antibody-dependent cellular cytotoxicity (NK-ADCC) epitopes on hepatitis C virus (HCV)-E1/E2. The position of HVR1 (aa384-aa411), HVR2 (aa473-aa480) and igVR (aa570-aa580) in the open reading frame (ORF) of E1/E2 protein (aa192–aa747) are shown by green rectangles. Six putative neutralizing epitopes (aa192–aa202, aa313–aa326, aa396–407, aa412–aa423, aa434–aa446, and aa496–515) are indicated by red rectangles. The 17 linear epitope peptides used in this study are indicated by blue rectangles. NK-ADCC-specific epitopes (3, 6, 11, 14 and 17) identified in this study are marked with red circles. HVR = hypervariable region; igVR = intergenotypic variable region; NCR = non-coding region. [Colour figure can be viewed at wileyonlinelibrary.com].

In the present study, we found that the capacity of circulating NK cells to degranulate and produce IFN-γ following engagement of CD16 with antibody-bound P815 cells was impaired significantly in chronic HCV carriers. Impaired spontaneous cytotoxicity of NK cells and altered NKp46 and CD16 expression might contribute separately to the deficient NK-ADCC responses in chronic HCV patients. NK cell-mediated ADCC was reported to be decreased in individuals with persistent HIV infection, but increased in HIV elite controllers [42]. However, it is hard to evaluate HCV-specific NK-ADCC in HCV resolvers due to the rapid decay of HCV-E1/E2 antibody responses in these individuals [43]. Consistent with this, we have tested non-specific antibody-dependent NK cell responses induced by HCV E1/E2 peptides in HCV spontaneous resolvers within 5 − 10 years after HCV clearance, and no NK-ADCC responses were found in these patients (data not shown).

In addition, five linear epitopes (3, 6, 11, 14 and 17) that could induce robust antibody-dependent NK cell responses were identified. These epitopes did not overlap with putative linear neutralizing epitopes except for 17, which is located in 648 − 659 of the E2 region and overlaps partially with a conformational epitope that has been reported to induce a broad neutralizing activity towards diverse HCV genotypes in a small animal model [44]. Overall, this observation indicates that the antibodies induced by neutralizing epitopes of HCV E1/E2 interfere with the ability of viruses to recognize susceptible host cells, but have little ability to induce NK cell-mediated ADCC.

Although we have identified five potential NK-ADCC epitopes in the present study, their ability to mediate NK-ADCC responses against different HCV genotypes remains to be determined. Also, whether $CD56^{-}CD16^{+}$ NK cells could mediate ADCC and whether their functions were impaired are still undefined. In conclusion, our results may be applicable in the development of novel immunological intervention strategies designed to elicit robust NK-ADCC responses against HCV infection.

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Disclosure

None.

Author contributions

L. L., M. J., J. W. and X. F. performed the experiments, L. Z. and Z. X. analysed data, H. L. edited the manuscript, T. S. designed the study, L. L. and M. J. wrote the paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Distribution and phenotypical patterns of circulating $CD56^{+}$ natural killer (NK) cells in chronic hepatitis C virus (HCV) carriers. (a) Gating strategies for $CD56^{bright}$ and $CD56^{dim}$ NK subsets in peripheral blood mononuclear cells (PBMCs). (b) The frequencies of total $CD56^{+}$ NK cells within PBMCs (b) and the frequencies of $CD56^{bright}$ and $CD56^{dim}$ NK cells within total $CD56^{+}$ NK cells (c) were calculated. Statistical analyses were performed by non-parametric *t*-test. All *P*-values are two-tailed and were considered significant when less than 0·05. (c) The percentages of total $CD56^{+}$ NK cells, $CD56^{bright}$ and $CD56^{dim}$ NK subsets expressing CD158, CD161, NKG2A, NKp46, NKG2C and NKG2D were determined by flow cytometry and are shown as the medians and interquartile ranges. Statistical analyses were performed by non-parametric *t*-test. All *P*-values are two-tailed and were considered significant when lower than 0·05.