

Immune Phenotype and Function of Natural Killer and T Cells in Chronic Hepatitis C Patients Who Received a Single Dose of Anti-MicroRNA-122, RG-101

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MicroRNA-122 is an important host factor for the hepatitis C virus (HCV). Treatment with RG-101, an *N*-acetylgalactosamine-conjugated anti-microRNA-122 oligonucleotide, resulted in a significant viral load reduction in patients with chronic HCV infection. Here, we analyzed the effects of RG-101 therapy on antiviral immunity. Thirty-two chronic HCV patients infected with HCV genotypes 1, 3, and 4 received a single subcutaneous administration of RG-101 at 2 mg/kg (*n* = 14) or 4 mg/kg (*n* = 14) or received a placebo (*n* = 2/dosing group). Plasma and peripheral blood mononuclear cells were collected at multiple time points, and comprehensive immunological analyses were performed. Following RG-101 administration, HCV RNA declined in all patients (mean decline at week 2, 3.27 log₁₀ IU/mL). At week 8 HCV RNA was undetectable in 15/28 patients. Plasma interferon- γ -induced protein 10 (IP-10) levels declined significantly upon dosing with RG-101. Furthermore, the frequency of natural killer (NK) cells increased, the proportion of NK cells expressing activating receptors normalized, and NK cell interferon- γ production decreased after RG-101 dosing. Functional HCV-specific interferon- γ T-cell responses did not significantly change in patients who had undetectable HCV RNA levels by week 8 post-RG-101 injection. No increase in the magnitude of HCV-specific T-cell responses was observed at later time points, including 3 patients who were HCV RNA-negative 76 weeks postdosing. **Conclusion:** Dosing with RG-101 is associated with a restoration of NK-cell proportions and a decrease of NK cells expressing activation receptors; however, the magnitude and functionality of *ex vivo* HCV-specific T-cell responses did not increase following RG-101 injection, suggesting that NK cells, but not HCV adaptive immunity, may contribute to HCV viral control following RG-101 therapy. (HEPATOLOGY 2017;66:57-68).

Chronic hepatitis C (CHC) virus infection is a global health problem. Patients with CHC infection are at increased risk of developing liver-related complications such as hepatocellular carcinoma and cirrhosis.⁽¹⁾ Successful hepatitis C virus (HCV) treatment reduces the risk of complications and improves survival.^(2,3) Recently, direct-acting antivirals (DAAs) have become available, which can directly interfere with the HCV viral machinery. These DAAs have drastically changed the field of CHC

Abbreviations: APC, allophycocyanin; CD, cluster of differentiation; CHC, chronic hepatitis C; CMV, cytomegalovirus; DAA, direct-acting antiviral; ELISpot, Enzyme-Linked ImmunoSpot; FITC, fluorescein isothiocyanate; HCV, hepatitis C virus; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; IP-10, IFN- γ -induced protein 10; LLOQ, lower limit of quantification; miR-122, microRNA-122; NK, natural killer; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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treatment by achieving sustained virological response in a high proportion of patients.⁽⁴⁾ With such high cure rates for HCV, the next challenge of research needs to focus on vaccine development and optimizing current treatment regimens and delivering them cost-effectively.⁽⁵⁾ The cost of DAAs is considerable, and combining DAAs with compounds that have a different mechanism of action may reduce both duration and cost of treatment. An alternative therapeutic option lies in inhibition of an important host factor used by HCV, microRNA-122 (miR-122).

miR-122 is a highly conserved, liver-specific microRNA which has important functions in the regulation of cholesterol and fatty acid synthesis.⁽⁶⁾ In addition, miR-122 can bind to the HCV genome and thereby promote virus replication.^(7,8) Targeting this host factor with an antisense oligonucleotide has proven effective at inhibiting HCV across several genotypes.⁽⁹⁻¹¹⁾ A single subcutaneous dose of the *N*-acetylgalactosamine-conjugated anti-miR-122 oligonucleotide RG-101 resulted in substantial decreases in HCV RNA in all treated patients and HCV RNA negativity for at least 76 weeks in 3 patients.⁽⁹⁾

The exact mechanism of HCV inhibition by RG-101 is not yet known. As binding of miR-122 protects HCV RNA from degradation by exonucleases, blocking miR-122 could uncover the genome to these innate defense pathways.^(8,12-14) Furthermore, HCV RNA replication could be disturbed by knocking down miR-122.^(14,15) Inhibition of HCV replication may lead to natural killer

(NK) cell normalization.^(16,17) Furthermore, a reduction in viral antigen expression could restore HCV-specific T-cell responses and thereby contribute to viral clearance.

Because all former treatment options included interferon- α , an immune modulator, the effects of new therapies on the immune system are substantially different. Exogenous interferon- α treatment of patients with CHC enhanced interferon (IFN)-stimulated gene expression and activated NK cells but did not lead to recovery of the T-cell compartment.⁽¹⁸⁻²⁰⁾ The inhibition of host factor miR-122 by RG-101 acts through a different mechanism and could potentially restore antiviral immunity.

The aim of this study was to investigate whether treatment with RG-101 would change important immune effectors in CHC infection and whether restored antiviral immunity could play a role in long-term virologic impact observed in patients treated with a single dose of RG-101.

Patients and Methods

PATIENTS

We included 32 CHC patients at two sites in The Netherlands (Academic Medical Center Amsterdam and University Medical Center Groningen) (Table 1). Males and postmenopausal females with chronic HCV genotype 1, 3, or 4 infection were enrolled. Patients were treatment-naïve or had previously experienced a

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TABLE 1. Baseline Characteristics

	RG-101 (n = 28)		Placebo (n = 4)	Total
	2 mg/kg	4 mg/kg		
Total (n)	14	14	4	32
Male (n)	13	7	4	24 (75)
Age (years)	51 (46-53)	54 (48-58)	55 (52-57)	52 (49-57)
Weight (kg)	84 (71-98)	83 (69-89)	79 (74-83)	83 (72-92)
Ethnicity (n)				
Caucasian	13	12	4	29 (91)
Asian	1	1	0	2 (6)
Other	0	1	0	1 (3)
IFNL3 CC genotype (n)	3	3	2	8 (25)
IFN naïve* (n)	9	12	3	24 (75)
Baseline HCV RNA level (log ₁₀ IU/mL)	6.12 (5.78-6.75)	6.19 (5.78-6.64)	6.38 (5.83-6.85)	6.16 (5.80-6.68)
HCV genotype (n)				
1a	8	4	2	14 (44)
1b	1	1	0	2 (6)
3	4	5	1	10 (31)
4	1	4	1	6 (19)
Alanine aminotransferase level (U/L)	60 (41-139)	52 (44-61)	90 (35-232)	52 (39-124)
FibroScan (kPa)	6.8 (5.1-10.8)	7.7 (5.5-10.9)	5.1 (3.4-11.7)	7.5 (5.3-10.7)
HLA-A2 ⁺ (n)	4	5	2	11 (34)

Data are given as median (interquartile range) or as frequency (percentage).

*Naïve to IFN- α -based therapy.

virological relapse after IFN-based therapy. Patients with coinfection (hepatitis B virus or human immunodeficiency virus infection), evidence of decompensated liver disease, or a history of hepatocellular carcinoma were excluded. The study was approved by the regulatory authority and the independent ethics committee at each participating site. All patients gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki, good clinical practice guidelines, and local regulatory requirements. All authors had access to the study data and reviewed and approved the final manuscript.

STUDY

In this randomized, double-blind, placebo-controlled, phase 1b trial,⁽²¹⁾ patients received a single subcutaneous injection of RG-101, an *N*-acetylgalactosamine-conjugated oligonucleotide antagonizing miR-122 (EudraCT no. 2013-002978-49). Dosage was 2 mg/kg (n = 14) or 4 mg/kg (n = 14), and 2 patients in each group received placebo. The initial follow-up was 8 weeks after dosing. At week 8, only patients with a >2 log₁₀ decrease from baseline and a <1 log₁₀ increase in HCV RNA level from nadir were included in an extended follow-up study (n = 22, including n =

10 at 2 mg/kg and n = 12 at 4 mg/kg); of these, 15 patients (n = 6 at 2 mg/kg and n = 9 at 4 mg/kg) had HCV RNA levels below the lower limit of quantification (<LLOQ) at week 8. Patients who did not meet the above criteria were excluded from the study at week 8 (n = 10, including placebo-treated patients). The extended follow-up lasted until week 76 (Supporting Fig. S1). If patients had a virological rebound (defined as a >1 log₁₀ increase) during follow-up, a retest was performed, after which patients were excluded from the study. In the extended follow-up, rebounds occurred at week 12 (n = 8), week 16 (n = 2), week 20 (n = 1), week 28 (n = 2), week 36 (n = 2), and week 52 (n = 1); and 3 patients were lost to follow-up (Supporting Fig. S2). At week 76, 3 patients had undetectable HCV RNA levels after a single dose of RG-101. HCV RNA levels were measured using the Roche COBAS Ampli-Prep/COBAS TaqMan HCV v2.0 assay, with a reported LLOQ of 15 IU/mL.

SAMPLING

Plasma samples from CHC patients for cytokine analyses were collected at baseline, day 3, and weeks 1, 4, and 8. For comparison, healthy control plasma (n = 6) was added (not matched for age, gender, or

ethnicity). Peripheral blood mononuclear cells (PBMCs) were collected at baseline; weeks 2, 8, 20, 28, and 52; time of viral rebound or retest (between weeks 12 and 52); and end of follow-up (week 76) (Supporting Fig. S1). PBMCs from healthy blood donors were used as healthy controls (n = 13). PBMCs were separated by density gradient and cryopreserved for later analyses.

LUMINEX ANALYSES

Plasma cytokine levels were measured using a Luminex 20-plex immunoassay (Affymetrix; eBioscience, San Diego, CA). This included interleukin-12 (IL-12); chemokine (C-C motif) ligands 2, 3, and 4; cluster of differentiation 54 (CD54); IFN- γ -induced protein 10 (IP-10 or chemokine [C-X-C motif] ligand 10), granulocyte-macrophage colony-stimulating factor, IFN- α , IFN- γ , IL-1 α , IL-1 β , IL-10, IL-13, IL-17A, IL-4, IL-6, IL-8, sCD62E, sCD62P, and tumor necrosis factor- α . Plasma levels of IL-18 were measured with a DuoSet enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Plasma samples from 6 healthy controls were included in the analyses.

FLOW CYTOMETRY

PBMCs were thawed and stained for 30 minutes at 4°C with different combinations of fluorescently labeled, conjugated mouse antihuman monoclonal antibodies; CD56 BUV-395, CD27 BUV-373, human leukocyte antigen (HLA)-DR fluorescein isothiocyanate (FITC), CD3 V500, CD16 BV786, CD16 BV421, CD14 phycoerythrin (PE)-CF594, CD19 PE-CF594, CD38 PE-Cy7 (BD Biosciences, San Jose, CA), NKp46 PerCP-eFluor710, CD45RA eFluor 605NC (eBioscience) CXCR6 BV-421, CD8 BV-711, CD8 BV-785, NKp30 allophycocyanin (APC) (Biolegend, San Diego, CA), Live/Dead fixable dead cell stain RED (Invitrogen, Life Technologies, Carlsbad, CA), NKG2A PE (Beckman Coulter, Fullerton, CA), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/CD253 APC (Miltenyi Biotec, Bergisch Gladbach, Germany).

For intracellular staining, cells were fixed after surface staining, permeabilized (FoxP3/transcription factor staining buffer set; eBioscience), and stained with monoclonal antibodies; perforin FITC (BD Biosciences) Eomes PerCP-eFluor710, T-bet PE-Cy7

(eBioscience), Ki67 BV711 (Biolegend), and Granzyme B PE (Sanquin, Amsterdam, The Netherlands).

Ex vivo CD8⁺ T-cell frequencies were determined in HLA-A2-positive patients (n = 11 patients: genotypes 1a, n = 6; 3a, n = 3; 4a, n = 1; and 4d, n = 1) by HLA class I multimers. Multimers were loaded with dominant epitopes NS3₁₀₇₃₋₁₀₈₁ (CINGVCWTV; APC) (Sanquin), NS4₁₄₀₆₋₁₄₁₅ (KLVALGINAV) (PE), and NS5B₂₅₉₄₋₂₆₀₂ (ALYDVVTKL) (FITC) (Immudex, Copenhagen, Denmark). Gating was based on the background level observed in healthy non-HCV-infected controls (Supporting Fig. S3). As control, we stained for cytomegalovirus (CMV)-pp65₄₉₅₋₅₀₄ (NLVPMVATV) and Epstein-Barr virus BMLF-1₂₅₉₋₂₆₇ (GLCTLVAML) tetramers (Sanquin). All measurements were performed on an LSR Fortessa cytometer (BD Biosciences) and analyzed by FlowJoMacV9.7.5 software.

NK-CELL FUNCTION

Patients were selected when samples were available for day 1 as well as week 8 (n = 13), and healthy blood donors (n = 13) were added as controls. PBMCs were thawed and incubated overnight with IL-12 and IL-15 or no stimulus in the presence of CD107a FITC (eBioscience). For the last 3 hours, monensin and brefeldin A were added, after which cells were stained with live/dead stain, CD3 V500, and CD56 BUV-395 as above. After fixing the cells, intracellular staining was performed (IFN- γ BV421, tumor necrosis factor- α AF700, macrophage inflammatory protein-1 β Pe-Cy7; BD Biosciences), and measurements were done on an LSR Fortessa. The proportion of IL-12/IL-15 activated cells was calculated by subtracting the number of positive cells in the unstimulated condition.

IFN- γ -ENZYMELINKED IMMUNOSPOT ASSAYS

IFN- γ -Enzyme-Linked ImmunoSpot (ELISpot) assays were performed *ex vivo* in duplicate at 2×10^5 PBMCs/well. Thawed PBMCs were rested overnight (37°C + CO₂) and stimulated with panels of 15-mer peptides that overlapped by 11 amino acids corresponding to HCV genotype 1a, 1b, 3a, or 4a (described in Barnes et al.⁽²²⁾ and Kelly et al.⁽²³⁾). Patient PBMCs were stimulated with the panel of peptides that matched their own viral genotype and subtype (where possible). Peptides were arranged into

10 pools corresponding to core, E1, E2, P7&NS2, NS3p, NS3h, NS4, NS5A, NS5B I, and NS5B II. Each peptide was used at a final concentration of 3 $\mu\text{g}/\text{mL}$. Internal controls were dimethyl sulfoxide (Sigma-Aldrich, UK) as a negative control and concanavalin A (Sigma-Aldrich) as a positive control. Other antigens used were a pool of major histocompatibility complex class 1–restricted epitopes of influenza A, Epstein-Barr virus, and CMV (BEI Resources, Manassas, VA) and a lysate of CMV-infected cells (Virusys Corp, Taneytown, MD). Spot-forming units were calculated per 10^6 PBMCs and background levels subtracted. Positive responses were defined as (1) the mean of responses to a pool minus background being greater than 48 spot-forming units/ 10^6 PBMCs and (2) the mean of responses to a well exceeding 3 times the background. The background level of 48 spot-forming units/ 10^6 PBMCs/pool was determined previously in 74 healthy controls, which was the mean + 3 standard deviations.⁽²²⁾

STATISTICAL ANALYSES

For differences between groups, the *t* test (normal distribution) or Mann-Whitney test (non-normal distribution) was used. For longitudinal analysis in individual patients, the Wilcoxon signed rank test was used. $P < 0.05$ was considered statistically significant. GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA) was used for analyses.

Results

IP-10 LEVELS DECREASE IN PATIENTS DOSED WITH RG-101

HCV RNA levels declined in all patients dosed with RG-101 ($n = 28$). At week 4, the mean reduction was 4.1 \log_{10} IU/mL in patients dosed with 2 mg/kg and 4.8 \log_{10} IU/mL in patients dosed with 4 mg/kg compared to 0.0 \log_{10} IU/mL in placebo-treated patients (Fig. 1A). Plasma IP-10 and IL-18 levels were significantly higher in CHC patients at baseline compared to healthy controls (for IP-10, median 58.1 pg/mL and 19.3 pg/mL, $P < 0.0001$; for IL-18, 322.1 pg/mL and 140.9 pg/mL, $P < 0.0001$; Fig. 1B), and no significant differences were observed within the three groups of CHC patients at baseline (placebo, 2 mg/kg, and 4 mg/kg; data not shown).

IP-10 levels decreased significantly in patients dosed with RG-101 (Fig. 1C). At week 1, the median

decline in IP-10 levels was 23.9 pg/mL in 2 mg/kg–dosed patients, 42.3 pg/mL in 4 mg/kg–treated patients ($P = 0.02$ and $P = 0.007$ compared to placebo, respectively), and 1.4 pg/mL in placebo-treated patients. IL-18 levels, however, increased after dosing with RG-101 (Supporting Fig. S4). Of 28 patients, 15 had undetectable HCV RNA 8 weeks postdosing, and IP-10 levels significantly decreased in both of these groups (Fig. 1D). However, IL-18 levels only significantly increased in the group of patients who had HCV RNA levels $<\text{LLOQ}$ at week 8 (Fig. 1D). None of the other measured cytokines or chemokines showed a significant change upon dosing with RG-101, including IFN- γ , IFN- α , tumor necrosis factor- α , and IL12p70 (Supporting Fig. S4).

TRAIL EXPRESSION DECREASES ON CD56^{bright} NK CELLS

At baseline, CHC patients and healthy controls had comparable frequencies of NK cells in the blood (median 7.4% and 7.0%, respectively, $P = 0.71$; Fig. 2A; full gating in Supporting Fig. S5A). In patients dosed with RG-101, the proportion of NK cells increased significantly from baseline 7.4% to median 10.1% ($P = 0.02$) at week 8 and 10.3% at follow-up week 12–28 (which included patients with viral rebound defined as a >1 log increase in viral load from nadir between weeks 12 and 28 and patients with HCV RNA $<\text{LLOQ}$ at week 28; $n = 5$, $P = 0.02$). In placebo-treated patients, no changes were observed in NK-cell proportions during follow-up (Fig. 2A). Furthermore, no changes in lymphocyte count were observed in the blood (Supporting Fig. S5B). Patients who had HCV RNA levels $<\text{LLOQ}$ at week 8 had significantly lower proportions of NK cells compared to patients who had HCV RNA $>\text{LLOQ}$ at week 8 (8.4% and 13.1% respectively, $P = 0.01$; Fig. 2A). NK cells can be divided into two subsets, depending on their expression of CD56: CD56^{bright} and CD56^{dim} NK cells (Fig. 2B). Upon dosing, CD56^{dim} NK cells significantly increased from median 91.7% to 94.8% at weeks 12–28 ($P = 0.0007$), whereas CD56^{bright} NK cells decreased (median 8.3% at baseline and 4.8% at follow-up week 12–28, $P = 0.001$; Fig. 2B). Expression of TRAIL on CD56^{bright} NK cells was significantly up-regulated in CHC patients at baseline compared to healthy controls (median 13.3% and 7.3% of CD56^{bright} NK cells, respectively, $P = 0.001$; Fig. 2C). TRAIL expression significantly decreased as soon as week 2 in patients upon dosing

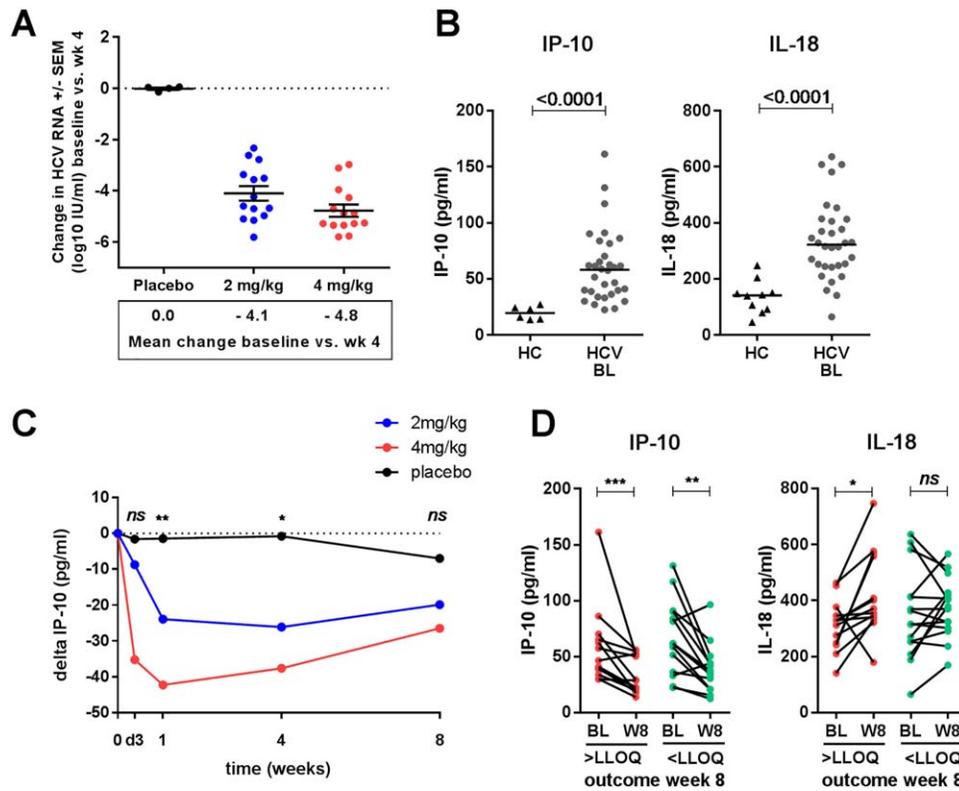


FIG. 1. (A) Mean change in HCV RNA levels between baseline and week 4 in patients dosed with placebo (black dots) or with RG-101 at 2 mg/kg (blue dots) and 4 mg/kg (red dots). (B) Plasma IP-10 and IL-18 levels in healthy controls and CHC patients at baseline; bars indicate median. (C) Change in plasma IP-10 levels (median) in patients dosed with placebo (black dots) or with RG-101 at 2 mg/kg (blue dots) and 4 mg/kg (red dots); level of significant difference indicated between RG-101-dosed (2 mg/kg and 4 mg/kg) and placebo-dosed patients. (D) Plasma IP-10 and IL-18 levels at baseline and week 8 in patients with HCV RNA levels <LLOQ and >LLOQ at week 8. Statistical testing: Mann-Whitney test (B,C) and Wilcoxon matched pairs test (D). **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Abbreviations: BL, baseline; HC, healthy control; ns, not significant; SEM, standard error of the mean.

with RG-101 (from median 13.3% at baseline to 6.4% of CD56^{bright} NK cells at week 2, *P* < 0.0001; Fig. 2C,D). No changes in TRAIL expression were observed in placebo-treated patients (not shown). No difference in TRAIL expression was observed in patients who had HCV RNA levels <LLOQ versus patients who had HCV RNA levels >LLOQ at week 8 (Fig. 2C, green and red dots).

DECREASED EXPRESSION OF NK CELL-ACTIVATION RECEPTORS

In CHC patients, expression of Fcγ receptor CD16 on NK cells was significantly higher compared to healthy controls (median 80.8% and 74.9%, respectively, *P* = 0.04), and this decreased after dosing with RG-101 (80.8% at baseline to 77.0% at week 8, *P* = 0.0019; Fig. 3A). Furthermore, expression of

activating receptors NKp30 and NKp46 on NK cells decreased in patients who were dosed with RG-101 (NKp30, 75.0% at baseline to 62.8% at week 8, *P* = 0.01; and NKp46, 74% at baseline and 67.2% at week 8, *P* = 0.01; Fig. 3B,C). Expression of T-box transcription factors T-bet and Eomes decreased upon dosing with RG-101 (from 94.7% at baseline to 89.2% at week 8 for T-bet, *P* = 0.0003, and from 74.6% to 60.8% for Eomes, *P* = 0.001; Fig. 3D,E), whereas NK-cell IFN-γ production significantly decreased (Supporting Fig. S6). Expression of the cell cycle marker Ki67 in CHC patients was 5.9% at baseline and significantly decreased at week 2 (3.8%, *P* < 0.0001) and week 8 (4.9%, *P* = 0.02) (Fig. 3F). Other markers expressed on NK cells showed minor or no changes (Supporting Fig. S7). Changes in T-bet, Eomes, and Ki67 expression were also observed in the CD8⁺ T-cell and CD56⁺ NK T-cell compartments

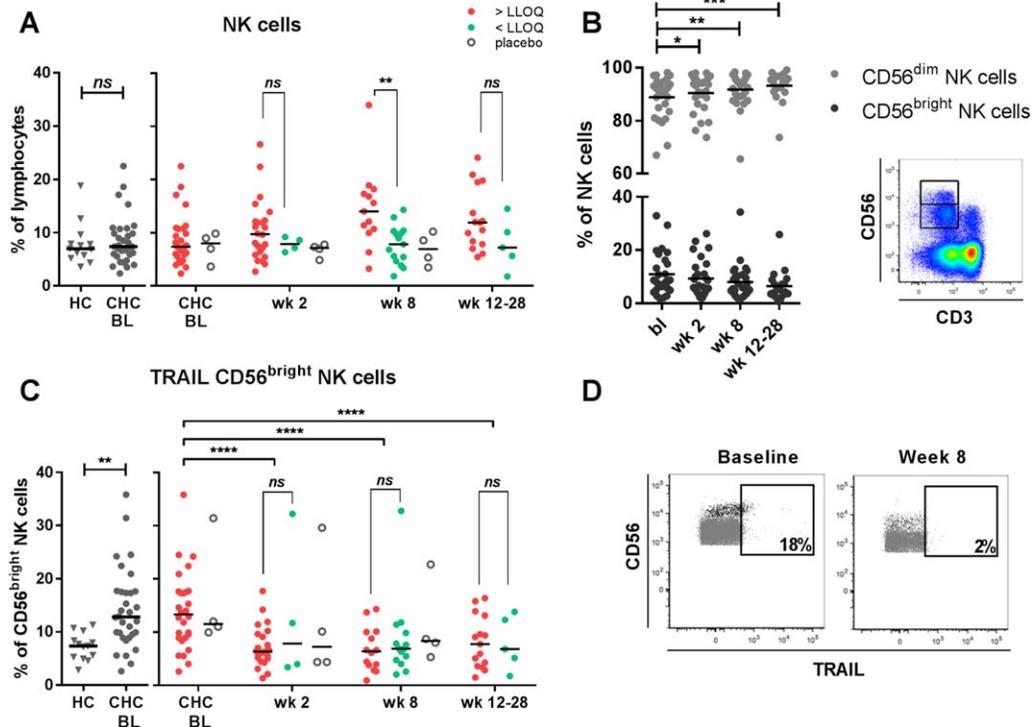


FIG. 2. (A) Proportion of NK cells as a percentage of total lymphocytes in healthy controls and CHC patients dosed with RG-101 at baseline and weeks 2, 8, and 12-28 (including patients with viral rebound between weeks 12 and 28 and patients with HCV RNA <LLOQ at week 28). Data are grouped in patients who are HCV RNA–positive (in red) or have HCV RNA levels <LLOQ (in green), as well as in patients receiving placebo (in gray); bars indicate median. (B) Proportion of CD56^{bright} and CD56^{dim} cells as a percentage of total NK cells in RG-101-dosed patients at baseline and weeks 2, 8, and 12-28; bars indicate median. Representative dot plot showing NK-cell gating in total lymphocyte population. CD56^{bright} and CD56^{dim} NK-cell gates are shown. (C) Percentage of TRAIL⁺ cells within CD56^{bright} NK cells in healthy controls and patients dosed with RG-101; bars indicate median. The same groups as in (A) are used. (D) Representative dot plot showing TRAIL gating; percentages of TRAIL⁺ cells within the CD56^{bright} NK-cell population (black) are shown. CD56^{dim} NK cells are shown in gray for comparison. Statistical testing: Mann-Whitney test (A,C) and Wilcoxon matched pairs test (B,C). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001. Abbreviations: BL/bl, baseline; HC, healthy control; ns, not significant.

(Supporting Fig. S8). No differences were observed between patients with HCV RNA levels <LLOQ and patients with HCV RNA levels >LLOQ at week 8 (Fig. 3A-F).

HCV-SPECIFIC T-CELL RESPONSES DID NOT CHANGE IN PATIENTS DOSED WITH RG-101

Next, we investigated whether HCV-specific T cells play a role in the (long-term) viral load reduction after RG-101 dosing. The frequency of HCV-specific T cells was measured in all HLA-A2⁺ patients (*n* = 11, including 2 placebo-treated patients). Baseline *ex vivo* HCV-specific CD8⁺ T-cell frequencies were low

in CHC patients (median 0.009%, range 0%-0.21%; Fig. 4A) and did not change upon dosing with RG-101 (median 0.008%, range 0%-0.19% at week 2 and median 0.007%, range 0%-0.05% at week 8; *P* = not significant) or in patients who received placebo (Supporting Fig. S9A). Similarly, CMV-specific and Epstein-Barr virus-specific T-cell proportions did not change upon dosing with RG-101 (Supporting Fig. S9B). The functional capacity of HCV-specific T cells, as measured by IFN- γ -ELISpot, did not change significantly in patients who were treated with RG-101 compared to placebo (Fig. 4B). Baseline IFN- γ T-cell responses were low in patients with CHC (with 8/32 patients having positive responses), and there was no difference in the baseline T-cell responses of patients who had HCV RNA levels <LLOQ and patients who were HCV RNA–positive at week 8 (*P* = 0.56)

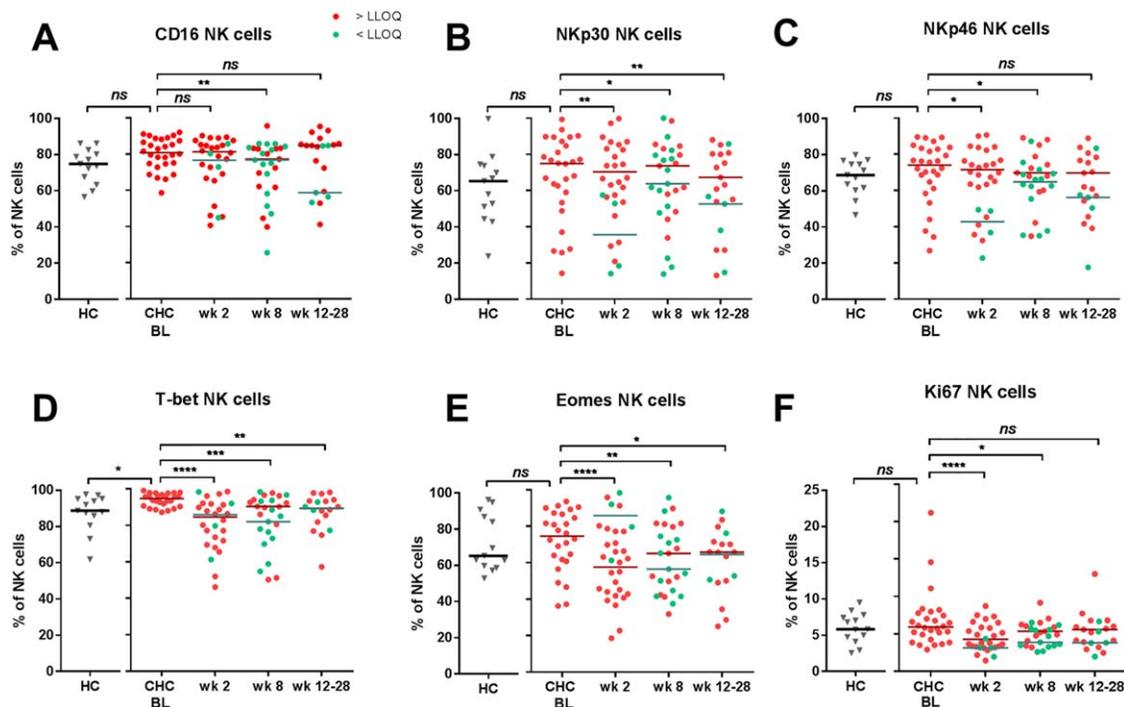


FIG. 3. Proportion of (A) CD16, (B) NKp30, (C) NKp46, (D) T-bet, (E) Eomes, and (F) Ki67 in healthy controls as well as in CHC patients dosed with RG-101 ($n = 28$) at baseline and at weeks 2, 8, and 12-28 (including patients with viral rebound between weeks 12 and 28 and patients with HCV RNA $<$ LLOQ at week 28); bars indicate median. Patients who have HCV RNA levels $<$ LLOQ are shown as green dots, and patients with HCV RNA levels $>$ LLOQ are as red dots. Statistical testing: Mann-Whitney test (A-F, healthy control versus CHC) and Wilcoxon matched pairs test (A-F). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Abbreviations: BL, baseline; HC, healthy control; ns, not significant.

(Fig. 4C). Furthermore, no changes were observed in IFN- γ T-cell responses between baseline and week 8 in patients who had HCV RNA levels $<$ LLOQ at week 8 ($P = 0.13$) or HCV RNA levels $>$ LLOQ at week 8 ($P = 0.88$) (Fig. 4D).

Three patients were tested HCV RNA-negative up to 76 weeks after dosing with RG-101. In addition, 5 patients had a late viral rebound at week 28 ($n = 2$), week 36 ($n = 2$), and week 52 ($n = 1$). In none of these patients was an increase in HCV-specific T-cell responses observed (Fig. 5).

Discussion

In this unique phase 1b study we assessed the effects of dosing with the *N*-acetylgalactosamine-conjugated anti-miR-122 oligonucleotide RG-101 on antiviral immunity in CHC patients in humans. We show that, after a single monotherapy dose of RG-101, as a consequence of HCV RNA decline, IP-10 levels decrease,

NK-cell proportions increase, and expression of NK cell-activation receptors decreases. Furthermore, NK-cell IFN- γ production significantly decreased, and no restoration of *ex vivo* HCV-specific T-cell functionality was observed after viral load decline or after long-term HCV RNA negativity.

In CHC patients, continuous immune activation is demonstrated by elevated levels of IFN-stimulated genes, such as IP-10, and viral load suppression during DAA treatment is associated with a decrease in IP-10 levels.^(16,17,24) In patients who have viral relapse, IP-10 levels have been shown to increase again.⁽²⁴⁾ In our study, IP-10 levels decreased in all patients who were dosed with RG-101, irrespective of their treatment outcome. We did not observe a subsequent increase in IP-10 levels in patients who had experienced a viral rebound; however, because patients were not followed after rebound, IP-10 levels may have increased at a later time. Alternatively, RG-101 could induce other mechanisms leading to IP-10 decrease, irrespective of viral load decline.⁽²⁵⁾ In *in vitro* studies, miR-122 has

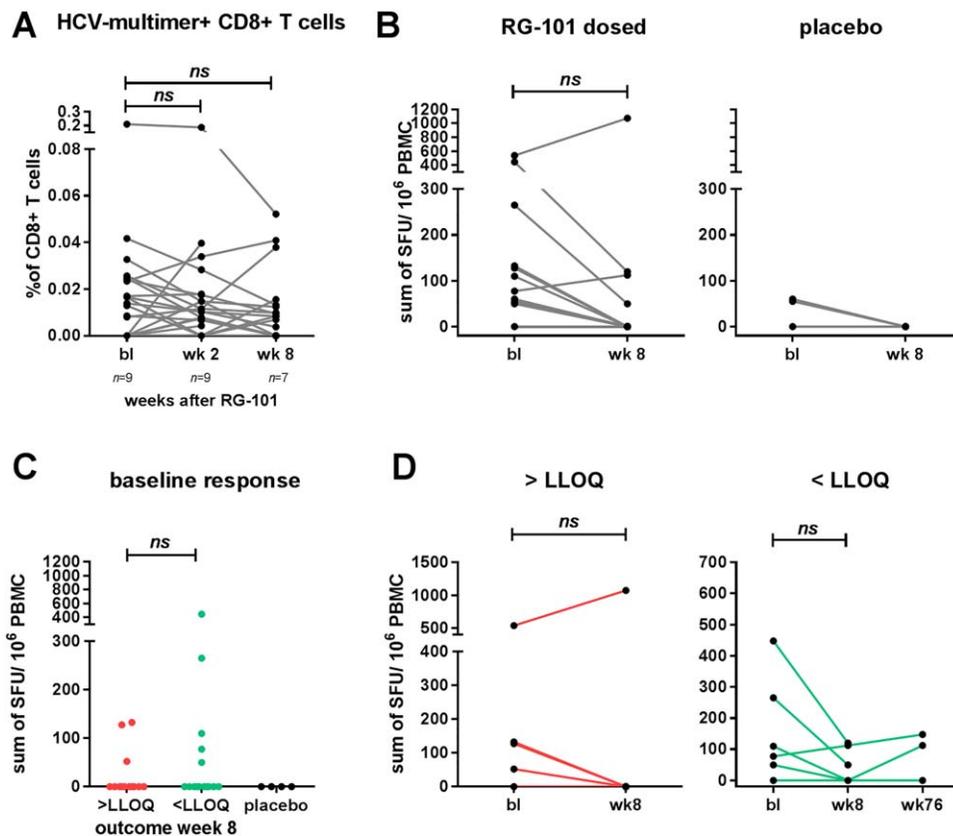


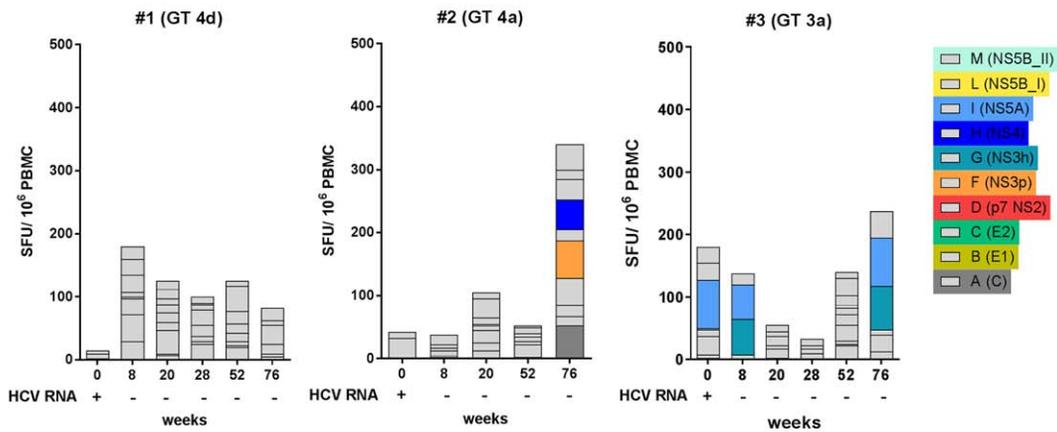
FIG. 4. (A) *Ex vivo* multimer-positive cells as a proportion of total CD8⁺ T cells in RG-101-dosed patients at baseline and weeks 2 and 8 (including $n = 2$ HCV RNA <LLOQ, excluding $n = 2$ due to missed visit). Each dot represents one epitope; a positive response was defined as more than 5 events with a lower threshold of 10,000 measured CD8⁺ T cells; bars indicate median. (B) ELISpot responses at baseline and week 8 in patients dosed with RG-101 (left) and placebo (right). Patients' PBMCs were stimulated *ex vivo* with genotype-specific HCV-peptide pools. (C) Baseline ELISpot responses in patients dosed with RG-101 with HCV RNA levels >LLOQ (red, left) and <LLOQ (green, right) at week 8 and in patients receiving placebo. (D) IFN- γ -ELISpot responses in patients dosed with RG-101 who had HCV RNA levels >LLOQ (in red) and <LLOQ (in green). Statistical testing: Mann-Whitney test (C) and Wilcoxon matched pairs test (A,B,D). Abbreviations: bl, baseline; ns, not significant; SFU, spot-forming unit.

been shown to have proinflammatory effects, which can be attenuated by inhibition with anti-miRNA-122.⁽²⁶⁾ No other measured cytokines or chemokines were altered in the plasma of patients dosed with RG-101, which is consistent with preclinical data showing that RG-101 does not elicit undesirable systemic immune responses.⁽²⁷⁾

NK cells play an important role in HCV infection. NK cells significantly increased in frequency in the blood of patients after dosing with RG-101. Interestingly, at week 8, the frequency of NK cells was higher in patients who were HCV RNA-positive compared to patients who had HCV RNA levels <LLOQ. Possibly, a rebound in viral load with a subsequent rise in IL-18 levels in these patients could have led to NK-cell expansion. After RG-101 dosing, CD56^{bright} NK

cells decreased, while CD56^{dim} NK cells increased, similar to data from DAA-treated patients.⁽¹⁷⁾ TRAIL is an important ligand expressed by NK cells which can induce apoptosis in target cells through the TRAIL receptor (TRAIL-R1 or TRAIL-R2). Target cells include virally infected hepatocytes, which have increased TRAIL receptor expression in CHC infection.⁽²⁸⁾ Furthermore, TRAIL expression on NK cells is up-regulated in HCV-infected patients^(29,30) and has been shown to decrease upon viral load reduction by DAA treatment.^(16,17) We observed a similar decrease in TRAIL expression on CD56^{bright} NK cells after a single dose of RG-101. In addition, expression of the Fc γ receptor CD16 as well as the cell cycle marker Ki67 decreased in patients dosed with RG-101, suggesting a reduction of the activated NK-cell

long term HCV RNA negative (week 76)



HCV RNA negative ≥ week 20

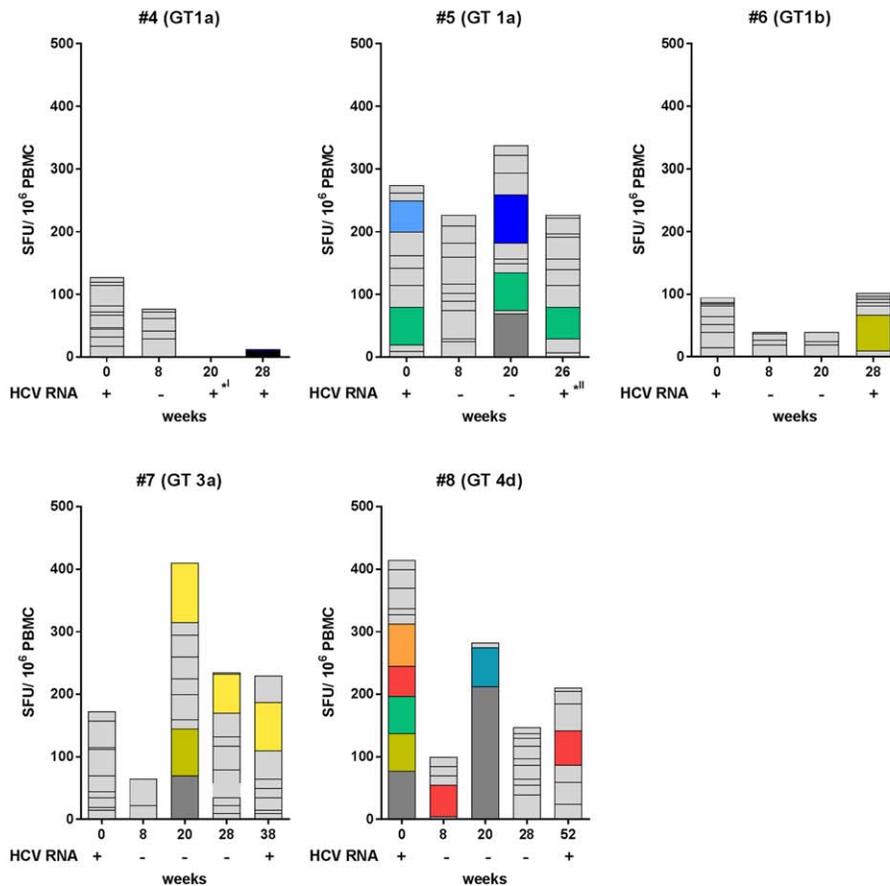


FIG. 5. IFN- γ -ELISpot responses in patients who were HCV RNA–negative for 76 weeks (upper graphs, patients 1-3) as well in patients who had HCV RNA levels <LLOQ for 20 or more weeks (lower graphs, patients 4-8). Responses considered above background (>48) are in color, and the other responses are in gray. *I Patient 4 had a <1 log₁₀ increase in HCV RNA at week 20 (PBMC missing) and was negative again at week 24. Rebound was at week 28. *II Patient 5 had a >1 log₁₀ increase in HCV RNA at week 24 and a <1 log₁₀ increase in HCV RNA at retest (week 26, last available PBMCs), and rebound was at week 36. Abbreviations: GT, genotype; SFU, spot-forming unit.

phenotype. Other markers also indicated decreased NK-cell activation upon RG-101 dosing, including NKp30 and NKp46, which have been shown to decrease upon DAA treatment,⁽¹⁷⁾ and T-bet.⁽³¹⁾ As some of these changes in NK-cell markers showed similar changes on bulk CD8⁺ T cells and CD56⁺ NK T cells, the observed changes in the NK-cell compartment might be the result of an overall decrease in immune activation upon a decline in HCV viral load. However, as miR-122 has been implicated in immune regulation, direct effects of miR-122 inhibition could also contribute to the changes in NK cells observed.^(25,26,32,33)

Chronic CHC infection leads to dysfunctional (“exhausted”) HCV-specific CD8⁺ T cells. In patients successfully treated with IFN-free therapy, a restoration of the proliferative capacity of these HCV-specific CD8⁺ T cells has been observed.⁽³⁴⁾ However, whether the function of these HCV-specific CD8⁺ T cells also improves is unknown, as is whether restoration of HCV immunity could contribute to HCV viral control. We therefore analyzed HCV-specific CD8⁺ T cells in RG-101-dosed patients, and we did not observe an increase in the *ex vivo* magnitude of HCV-specific CD8⁺ T cells as measured by HLA-A2-restricted multimers. To overcome the limitation of only measuring three specificities with multimers, which could be not optimally genotype-matched⁽²³⁾ or could have undergone viral escape, we subsequently analyzed T-cell functionality by stimulating with overlapping peptide pools and assessing IFN- γ production in ELISpot assays. This also allowed us to measure the functionality of both CD8⁺ and CD4⁺ HCV-specific T cells. The magnitude of HCV-specific T-cell responses did not change in patients who were HCV RNA-negative at week 8, suggesting that T cells do not contribute to HCV RNA decline after RG-101 dosing. In line with this, we observed no specific change in HCV-specific T-cell responses in patients who were long-term HCV RNA-negative, suggesting that other mechanisms play a role in the long-term viral load reduction after RG-101 dosing.

In conclusion, one dose of anti-miR-122 RG-101 led to a decrease in HCV RNA levels in all patients and sustained virological response >76 weeks in 3 patients. Dosing with RG-101 does not elicit systemic immune activation. A decrease in IP-10 levels and normalization of NK-cell phenotype were observed, which are likely the result of HCV RNA decline. Lastly, our data suggest that HCV-specific T-cell recovery does not play a role in the decline or long-term negativity of HCV RNA in patients dosed with RG-101.

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