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Frequency, Private Specificity, and Cross-Reactivity of Preexisting Hepatitis C Virus (HCV)-Specific CD8$^+$ T Cells in HCV-Seronegative Individuals: Implications for Vaccine Responses

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ABSTRACT

T cell responses play a critical role in controlling or clearing viruses. Therefore, strategies to prevent or treat infections include boosting T cell responses. T cells specific for various pathogens have been reported in unexposed individuals and an influence of such cells on the response toward vaccines is conceivable. However, little is known about their frequency, repertoire, and impact on vaccination. We performed a detailed characterization of CD8$^+$ T cells specific to a hepatitis C virus (HCV) epitope (NS3-1073) in 121 HCV-seronegative individuals. We show that *in vitro* HCV NS3-1073-specific CD8$^+$ T cell responses were rather abundantly detectable in one-third of HCV-seronegative individuals irrespective of risk factors for HCV exposure. *Ex vivo*, these NS3-1073-specific CD8$^+$ T cells were found to be both naive and memory cells. Importantly, recognition of various peptides derived from unrelated viruses by NS3-1073-specific CD8$^+$ T cells showed a considerable degree of T cell cross-reactivity, suggesting that they might in part originate from previous heterologous infections. Finally, we further provide evidence that preexisting NS3-1073-specific CD8$^+$ T cells can impact the T cell response toward peptide vaccination. Healthy, vaccinated individuals who showed an *in vitro* response toward NS3-1073 already before vaccination displayed a more vigorous and earlier response toward the vaccine.

IMPORTANCE

Preventive and therapeutic vaccines are being developed for many viral infections and often aim on inducing T cell responses. Despite effective antiviral drugs against HCV, there is still a need for a preventive vaccine. However, the responses to vaccines can be highly variable among different individuals. Preexisting T cells in unexposed individuals could be one reason that helps to explain the variable T cell responses to vaccines. Based on our findings, we suggest that HCV CD8$^+$ T cells are abundant in HCV-seronegative individuals but that their repertoire is highly diverse due to the involvement of both naive precursors and cross-reactive memory cells of different specificities, which can influence the response to vaccines. The data may emphasize the need to personalize immune-based therapies based on the individual's T cell repertoire that is present before the immune intervention.

Boosting T cell responses is one strategy to prevent or treat infections, including hepatitis C virus (HCV) infection. A vigorous and broad CD8$^+$ T cell response has been correlated with spontaneous clearance of acute HCV infection (1–3) and is therefore suggested to be one important target for vaccine concepts (4). The breadth of the T cell response, as well as the structure of the T cell receptor (TCR), is important for the recognition of structurally similar epitopes, e.g., from viral variants which may prevent viral escape (5–8). Several clinical trials of T cell inducing vaccines have been conducted not only for HCV and some ongoing approaches have shown promising T cell-inducing capacity (4, 9, 10). However, different vaccine receivers usually react to the vaccination with diverse T cell response magnitudes. The reasons for this variability of the immune response to vaccines can be the individual genetic background or the available T cell repertoire responding to the vaccine. We sought here to investigate the role of the preexisting CD8$^+$ T cell repertoire to an immunodominant HCV-specific major histocompatibility complex class I (MHC-I)-restricted epitope (NS3-1073), which was included in a HCV peptide vaccine (11) and primarily define its frequency in a large cohort of HCV-seronegative individuals (HCV-SNs).

There have been an increasing number of reports showing that

Received 2 March 2015 **Accepted** 22 May 2015

Accepted manuscript posted online 3 June 2015

Citation Zhang S, Bakshi RK, Suneetha PV, Fytili P, Antunes DA, Vieira GF, Jacobs R, Klade CS, Manns MP, Kraft ARM, Wedemeyer H, Schlaphoff V, Cornberg M. 2015. Frequency, private specificity, and cross-reactivity of preexisting hepatitis C virus (HCV)-specific CD8$^+$ T cells in HCV-seronegative individuals: implications for vaccine responses. J Virol 89:8304–8317. doi:10.1128/JVI.00539-15.

Editor: S. Perlman

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.00539-15.

doi:10.1128/JVI.00539-15

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TABLE 1 Summary of number samples from HCV-SN individuals included[a]

Sample group	Abbreviation	No. of samples				
			Ex vivo		*In vitro*	
		Total	Analyzed (multimer staining)	Analyzed (enrichment)	Analyzed	Responder (%)
Risk-free	RF	23	15	0	23	9 (39.1)
Healthy blood donor	HBD	66	56	17	54	17 (31.5)
Potentially exposed	PE	32	10	3	32	12 (37.5)
Sum	SN	121	81	20	109	38 (34.8)

[a] The total numbers of samples from HCV-SN individuals included in this study are summarized. The numbers of samples used for *ex vivo* phenotyping and *in vitro* stimulation with NS3-1073 peptide, as well as the numbers of *in vitro* responders, are given. The numbers of samples refer to all individuals included and are also divided into the three different risk groups for HCV exposure (risk-free, healthy blood donors, and potentially exposed individuals).

different virus specific T cells can be detected in seronegative individuals (12, 13). These viruses include, e.g., HIV, herpes simplex virus (HSV), and also HCV (13–16). Different reasons for the presence of HCV-specific T cells, including low-level exposure to HCV without seroconversion, the presence of naive precursor T cells, and memory T cell cross-reactivity, have been under debate. It has been shown that low-level HCV exposure is able to prime T cell responses without apparent seroconversion, which happens more often in health care workers, sexual partners of hepatitis C patients, and intravenous drug users (17, 18). In the case of antigen-specific naive CD8$^+$ T cells, precursor frequencies have been reported to vary from 1 to 100 per 1 million CD8$^+$ T cells in humans. The immunodominant HLA-A2-restricted epitope HCV NS3-1073 is reported to be one of the epitopes with the highest precursor frequencies of up to 60 per million CD8$^+$ T cells (19, 20). Further, memory T cells generated by one pathogen can respond to another unrelated pathogen due to T cell cross-reactivity, which may influence the immune response toward the second infection (21). Cross-reactivity between NS3-1073 and one influenza A virus (IAV) epitope has been documented previously (22, 23). A cross-recognition of different peptides by a given T cell is determined by the respective cell's T cell receptor. Since the generation of the T cell receptor on a somatic level is a complex process influenced by random events, the T cell receptor repertoire and thus also the T cell repertoire (i.e., the whole entity of all T cell receptors and all T cells present in a given organism) are unique to each individual, even among genetically identical ones. The uniqueness of these repertoires, as well as the hierarchies of epitope recognition, has been termed "private specificity" (24, 25).

In this study, HCV NS3-1073 was used as a model epitope because it generates one of the most frequent responses within HCV-infected HLA-A2$^+$ individuals, was shown to be cross-reactive to other epitopes, and was used in a peptide vaccine trial (11). We found that preexisting NS3-1073-specific T cells are abundant in healthy HCV-seronegative donors and partially have a memory phenotype. Potential low-dosage exposure of HCV did not seem to be the cause for these prevalent preexisting NS3-1073-specific CD8$^+$ T cells in our cohort. We observed new cross-reactive responses between HCV NS3-1073 and other epitopes from common pathogens with surprisingly little amino acid homology, suggesting that they might originate from previous heterologous infections. Importantly, we found that preexisting NS3-1073-specific CD8$^+$ T cells may contribute to the magnitude and speed of T cell responses elicited by a T cell-inducing HCV peptide vaccine, revealing a possible clinical impact of these cells.

MATERIALS AND METHODS

Ethics statement. The present study was conducted in accordance with the guidelines of the Declaration of Helsinki. The study was approved by the local ethics committee of Hanover Medical School. All individuals gave written informed consent.

Patient cohort. Between 2008 and 2015, blood samples from 121 HLA-A2$^+$ HCV-SNs were collected and included in the study. Samples were negatively tested for the presence of HCV-RNA and anti-HCV antibodies. The characteristics of all individuals included are summarized in Table 1 and see also Table S1 in the supplemental material. The cohort of HCV-SNs vaccinated with the experimental HCV peptide vaccine was described previously (11, 26) and was considered to be risk-free to HCV exposure in the present study.

IC41 HCV peptide vaccine. Detailed vaccine component and vaccination strategy were described previously (11). Briefly, the IC41 HCV peptide vaccine generated by Intercell AG (Vienna, Austria) consists of peptide antigens and the adjuvant poly-L-arginine, both synthesized by chemical means. The vaccine harbors highly promiscuous T-helper epitopes and five HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitopes. The five synthetic peptides in this vaccine (Ipep83, Ipep84, Ipep87, Ipep89, and Ipep1426) are derived from HCV genotype 1 core 23-44 and 132-140, NS3 1073-1081, NS3 1248-1261, and NS4 1764-1786. The peptide vaccine was administered every 4 weeks (first clinical trial) or biweekly (second clinical trial) until week 20.

Isolation of PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated using a standard Ficoll Hypaque density centrifugation method (BioColl separating solution; Biochrom AG, Berlin, Germany). The cells were either used directly *ex vivo* for experiments or cryopreserved in freezing medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) in liquid nitrogen.

Monoclonal antibodies, synthetic peptides, and MHC-I multimeric complexes. The following fluorochrome-labeled mouse anti-human monoclonal antibodies were used for flow cytometric staining: anti-CD8-APC-H7, anti-CD45RA-FITC, anti-IFN-γ-FITC, anti-TNF-APC, anti-MIP-1β-PE, anti-CD14-PE-Cy7, anti-CD19-PE-Cy7, and anti-CD56-PE-Cy7 (BD Pharmingen, Heidelberg, Germany) and anti-CCR7-PerCP/Cy5.5 (BioLegend, San Diego, CA). Phycoerythrin-labeled HLA-A*0201-restricted MHC-I multimers were purchased from Immudex (Copenhagen, Denmark). Peptide sequences of the multimers were identical to the synthetic peptides used. Synthetic HLA-A*0201-restricted peptides originating from HCV NS3-1073 (CINGVCWTV), Epstein-Barr virus (EBV) BMLF1-259 (GLCTLVAML), EBV LMP2-329 (LLWTLVVLL), EBV LMP2-426 (CLGGLLTMV), IAV NA-231 (CVNGSCFTV), IAV M1-58 (GILGFVFTL), cytomegalovirus (CMV) pp65-495 (NLVPMVATV), and HIV Gag-77 (SLYNTVATL) were used, tyrosinase-368 (YMDGTMSQV) served as a negative control. Peptide variants of NS3-1073 according to different HCV genotypes were as follows: GT1b (CVNGVCWTV), GT2a (SISGVLWTV), GT3a (TIGGVMWTV), GT4a (AVNGVMWTV), and GT5a (CISGVCWTV). All peptides were purchased from ProImmune,

Ltd. (Oxford, United Kingdom) with a purity >98% and were dissolved in sterile endotoxin-free DMSO (Sigma-Aldrich). Final concentrations of peptides used for the cytokine assays depended on the sequence of the peptide and were determined previously by independent titration experiments to achieve optimal stimulation of cells for each of the different peptides. The concentrations were 0.2 μg/ml for IAV M1-58, 1 μg/ml for EBV BMLF1-259 and CMV pp65-495, and 10 μg/ml for HCV NS3-1073 and all other peptides, including the NS3-1073 variants.

Cell surface staining, multimer staining, and multimer-associated magnetic bead enrichment. PBMCs were stained with the respective antibodies for 10 to 15 min at 4°C. After a washing step, the cells were analyzed by flow cytometry (BD FACSCanto II; BD Biosciences). For detection of antigen-specific $CD8^+$ T cells, samples were stained with 50 μl of MHC-I multimers/ml for 20 min at room temperature, followed by cell surface staining. A dump channel containing anti-CD14, anti-CD19, and anti-CD56 was used to remove unspecific events. All samples were analyzed within 60 min by flow cytometry, and data were evaluated using FlowJo Software (Tree Star, Inc., Ashland, OR). For the analysis of the memory phenotype enrichment of HCV-$multimer^+$ $CD8^+$ T cells was performed by staining 20×10^6 to 30×10^6 PBMCs with 20 to 30 μl of NS3-1073 multimer, as described above. After washing and incubation with anti-phycoerythrin microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany), the cells were isolated according to the manufacturer's instructions. Multimer-enriched cells were stained for cell surface expression of CCR7 and CD45RA and analyzed by flow cytometry. Gating of memory cell populations was performed according to the staining of bulk $CD8^+$ T cells.

Antigen-specific *in vitro* long-term T cell lines. For the establishment of antigen-specific T cell line, two different protocols were used depending on the availability of cell samples. Preferentially, $CD8^+$ T cells were isolated from freshly processed PBMCs using a magnetic bead isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. Peptide-pulsed (1 μg/ml), irradiated (40 Gy), HLA-A*0201-transgenic TAP transporter-deficient T2 cells were used as antigen-presenting cells. A total of 5×10^5 isolated $CD8^+$ T cells/ml were stimulated with 10^5 T2 cells/ml. In the case of cryopreserved samples, PBMCs were thawed and plated at 2×10^6 cells/ml, together with peptides, for the first week. Every 7 days, the cells were in all cases harvested and again plated at 5×10^5 cells/ml with peptide-pulsed, irradiated T2 cells. The cells were kept in CTL medium (AIM-V; Invitrogen, Carlsbad, CA) containing 10% human AB serum (Lonza, Basel, Switzerland), 100 U of penicillin/ml, 0.1 mg of streptomycin/ml, 1% minimal essential medium/nonessential amino acids, 1% sodium pyruvate, and 2 mM L-glutamine (PAA Laboratories GmbH, Parching, Austria) and 5 U of recombinant human interleukin-2 (Invitrogen Life Technologies, Darmstadt, Germany)/ml. The growth and functionality of antigen-specific $CD8^+$ T cells was analyzed after 21 days of culture, and cell lines with frequencies of NS3-1073^+ $CD8^+$ T cells >0.5% were considered responders. Importantly, since the T2 cells used as antigen-presenting cells are immortalized by EBV transformation, background expansion of EBV-specific memory $CD8^+$ T cells can occur even in without a specific peptide being added.

For memory sorting of cells, fresh PBMCs were stained with anti-CD8 and dump channel antibodies (CD14-CD19-CD56) and for identification of the memory status with anti-CCR7 and anti-CD45RA. The cells were then sorted using fluorescence-activated cell sorting (FACS) into naive ($CCR7^+$ $CD45RA^+$) and memory ($CCR7^+$ $CD45RA^-$ T_{CM} and $CCR7^-$ $CD45RA^-$ T_{EM} and $CCR7^-$ $CD45RA^+$ T_{EMRA}) $CD8^+$ T cells. Next, 0.3×10^6 sorted T cells were stimulated for a total of 3 weeks *in vitro* in a 48-well plate, together with 0.06×10^6 peptide-pulsed and irradiated T2 cells, in CTL medium. The cells were fed every 3 to 4 days, harvested after 7 days, and again plated at 0.3×10^6 cells/well, together with fresh pulsed and irradiated T2 cells.

Analysis of functionality of antigen-specific T cells. The cells were stimulated with respective peptides for 6 h. Brefeldin A (2 μg/ml; Sigma-Aldrich) was added after 1 h. Cells were washed and stained for CD8 and exclusion markers. Cell fixation and permeabilization was performed using a CytoFix/CytoPerm kit (BD Biosciences) according to the manufacturer's protocol. The intracellular staining of gamma interferon (IFN-γ), tumor necrosis factor (TNF), and macrophage inflammatory protein 1β (MIP-1β) was analyzed by flow cytometry. Responses to the peptide candidates were to be considered cross-reactive if the cytokine production by NS3-1073-expanded cell lines upon stimulation with the peptide candidate was 2-fold higher than the respective background level determined from the medium control cell lines that were likewise restimulated.

***In silico* structure-based cross-reactivity prediction.** Six three-dimensional structures of pMHC complexes presenting selected peptides were recovered from the Protein Data Bank, and another six were recovered from the CrossTope Data Bank (27) (see Table S2 in the supplemental material). The electrostatic potential distribution over the pMHC surface was computed with the GRASP2 program, and images of the T cell receptor (TCR)-interacting surfaces were used as input to perform a structure-based hierarchical cluster analysis (HCA). The HCA was performed with SPSS software (PASW Statistics 18; IBM, Chicago, IL) using values extracted from seven selected regions, as previously described (28). Complementary bootstrap calculations were performed with R (http://www.r-project.org/).

Cell sorting and RNA extraction. After 21 days, $multimer^+$ $CD8^+$ T cells were isolated by high-resolution flow cytometric sorting at the Central Sorting Facility of Hanover Medical School using a BD FACSAria. Purity of sorted $multimer^+$ $CD8^+$ T cells was >95%. mRNA was then isolated using the Oligotex Direct mRNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Isolated mRNA was stored at −80°C until further use.

Analysis of TCR using template-switch anchored reverse transcription-PCR and sequencing. TCR analysis was performed by using an adapted SMART-RACE method as described by Quigley et al. (29) using a SMARTer RACE cDNA amplification kit (Clontech Laboratories, Inc.). Touchdown PCR was performed with an Advantage 2 PCR kit (Clontech) with custom-synthesized primers specific for the constant region of the TCR β chain as described in reference 29. Gel-purified SMART-RACE products were cloned into the pCR4-TOPO vector using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The plasmids were transformed into TOP10 chemically competent *Escherichia coli* and propagated overnight on ampicillin Luria-Bertani agar plates at 37°C. Single bacterial colonies were selected; for each sample, 50 clones were sequenced and analyzed. Alignment and identification of TCR gene family usage and of CDR3β regions was done according to IMGT standard sequences (http://www.imgt.org) using the Sequencher software (GeneCodes, Ann Arbor, MI). Clones with identical nucleic acid sequences were regarded as one clone. The frequency of individual clonotype distributions within the sequenced clones was analyzed. The frequencies of TCR β variable-chain (TRBV) families used for targeting the NS3-1073 epitope were analyzed among all 16 samples subjected to sequencing. Based on the median frequencies, the most common TRBV families were designated "preferentially used" to indicate similarities.

RESULTS

***In vitro*-expanded HCV NS3-1073-specific $CD8^+$ T cells from HCV-SNs show diverse proliferation capacity, functionality, and a private TCR repertoire.** *Ex vivo* frequencies of HCV-specific $CD8^+$ T cells in HCV-SNs were analyzed in 81 of 121 healthy HCV-SNs in total. However, the frequencies of specific $CD8^+$ T cells were generally low in most individuals (<0.05% of total $CD8^+$ T cells) and difficult to detect by multimer staining (Table 1 and see Table S1 in the supplemental material); therefore, we expanded NS3-1073-specific $CD8^+$ T cells *in vitro* from a total of 109 individuals. Of the HCV-SN individuals tested, 34.8% (38/109) showed NS3-1073-specific $CD8^+$ T cell proliferation after *in vitro*

stimulation for 3 weeks (with a threshold for a positive response 0.5% of the total CD8$^+$ T cells) (Fig. 1A, Table 1). The magnitude of the responses and the NS3-1073 multimer staining pattern varied widely between individuals. The frequencies of NS3-1073-specific CD8$^+$ T cells after *in vitro* stimulation ranged from null to 89%, with an average of 1.9% of the total CD8$^+$ T cells (see Table S1 in the supplemental material). No obvious differences in the frequencies or response rates of HCV-SN individuals were observed between cell lines established with isolated CD8$^+$ T cells or total PBMCs (data not shown; see also Table S1 in the supplemental material).

Intracellular cytokine staining of IFN-γ, TNF, and MIP-1β confirmed that *in vitro*-expanded NS3-1073-specific CD8$^+$ T cells are functional upon NS3-1073 peptide restimulation (Fig. 1A and B). This response was dose dependent, confirming its specificity (Fig. 1C). The strength and the quality of the response varied considerably between individuals. Upon comparing the ratio between frequencies of multimer$^+$ CD8$^+$ T cells and IFN-γ$^+$ CD8$^+$ T cells, we found that only a fraction of the NS3-1073 multimer$^+$ CD8$^+$ T cells produced IFN-γ in response to NS3-1073 in most cases, whereas the frequencies of MIP-1β$^+$ cells was consistently higher (Fig. 1b).

The individual differences in the proliferation and cytokine responses that we observed may be explained by the individual TCR repertoire. We performed unbiased sequencing of the TCR β variable chain (TRBV) from NS3-1073 multimer$^+$ CD8$^+$ T cells sorted using FACS from the 3-week cell lines from 16 HCV-SNs. The clonality of expanded NS3-1073 multimer$^+$ T cells showed great variability, with some individuals having a few clones dominating the response, while in others the response was rather polyclonal (Fig. 1A, bar graphs and pie charts). Among all of the individuals analyzed, no clearly prevalent CDR3 amino acid motif could be identified (data not shown). The only common pattern (public specificity) that we observed was a preferential usage of TRBV families TRBV4, TRBV6, and TRBV29 (Fig. 1A, pie charts, and Fig. 1D).

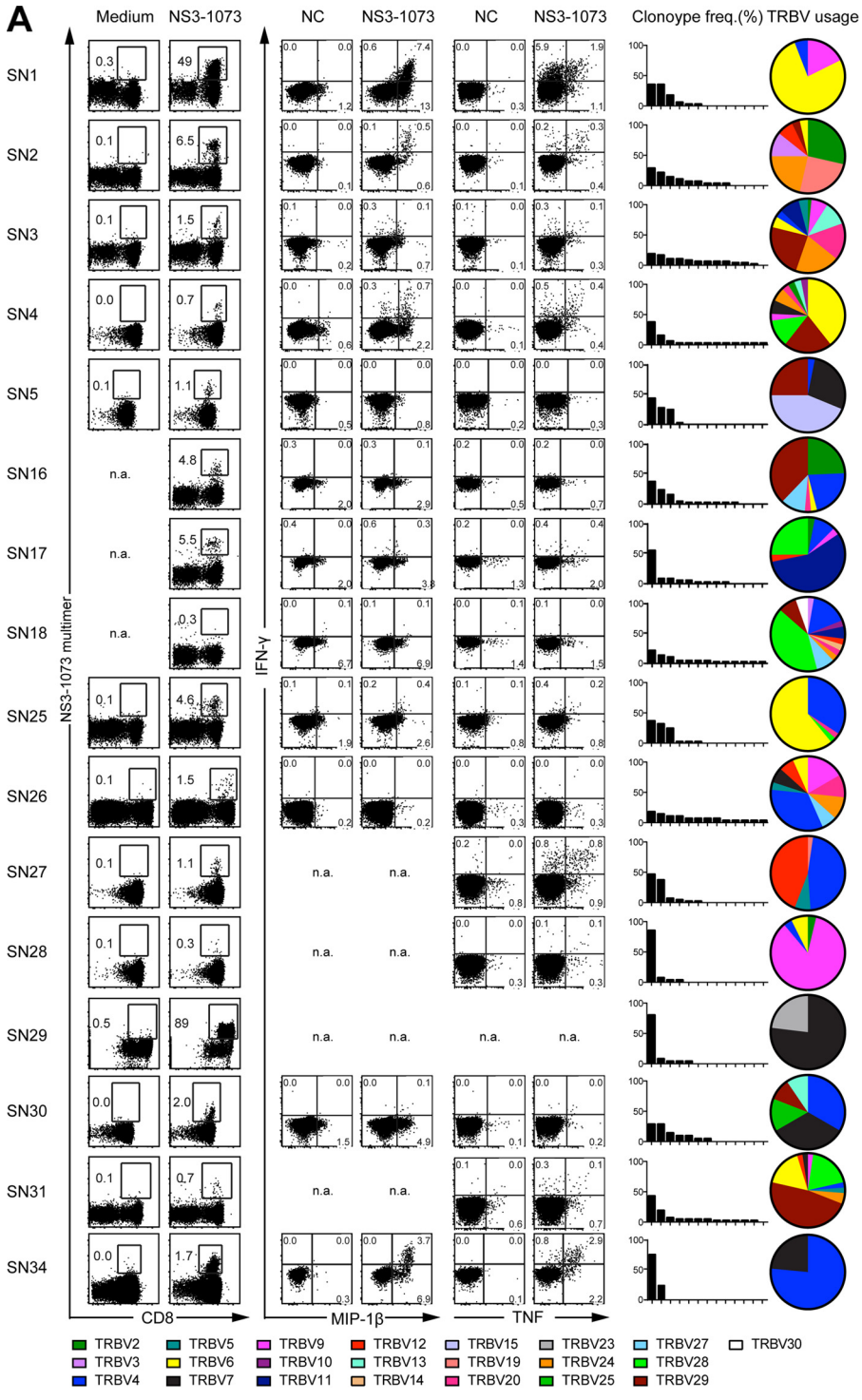
Preexisting HCV NS3-1073-specific CD8$^+$ T cells generate an *in vitro* response and demonstrate a partial memory phenotype. We next sought to confirm that NS3-1073-specific CD8$^+$ T cells expanded *in vitro* were indeed generated by *ex vivo*, preexisting NS3-1073-specific CD8$^+$ T cells. In order to determine the frequency of NS3-1073-specific CD8$^+$ T cells *ex vivo* in more depth, we further used a multimer-based magnetic-bead enrichment technique. HCV NS3-1073-specific CD8$^+$ T cells were enriched *ex vivo* from 20 PBMC samples (Fig. 2A). Our results revealed a precursor frequency ranging from 2 to 47 per 10^6 CD8$^+$ T cells, which is comparable to findings in previous publications (30). Further, we depleted NS3-1073-specific T cells *ex vivo* and cultured the remaining CD8$^+$ T cells *in vitro* with NS3-1073 peptide, as performed previously. Here, no expansion of NS3-1073-specific CD8$^+$ T cells was observed (Fig. 2B). We next correlated the preexisting T cell frequency with the magnitude of proliferation *in vitro*. Due to the limitation of cell numbers, both multimer enrichment and *in vitro* expansion were performed in eight HCV-SN individuals. We observed a clear positive correlation between the preexisting T cell frequency and the extent of NS3-1073-specific CD8$^+$ T cell proliferation *in vitro* (Fig. 2c). These results indicate that NS3-1073-specific CD8$^+$ T cells proliferating *in vitro* are generated by the specific cells detectable *ex vivo* and that the proliferation magnitude *in vitro* observed with our method may reflect the *ex vivo* preexisting T cell frequency.

To further characterize the preexisting HCV-specific T cells from HCV-SNs, we analyzed the memory phenotype of NS3-1073 multimer$^+$ T cells after enrichment. Adequate amounts of NS3-1073-specific CD8$^+$ T cells were enriched in 12 of 20 individuals to enable a reliable phenotyping. Costaining with memory markers CD45RA and CCR7 revealed that part of the preexisting NS3-1073-specific CD8$^+$ T cells had a naive phenotype since they expressed both markers. However, a considerable fraction of the NS3-1073-specific CD8$^+$ T cells were of a memory phenotype (T_{CM}, T_{EM}, and T_{EMRA}) (Fig. 2D). Within an individual, a mixed population of cells with different memory phenotypes could often be observed. The frequencies of memory cells varied between 1.9 and 90%. No correlation was found between the memory status of the T cells *ex vivo* and the *in vitro* proliferation magnitude.

Frequency of *in vitro*-generated HCV NS3-1073-specific CD8$^+$ T cells in HCV-SN is not associated with the risk for HCV exposure. Low-level exposure to HCV without an establishment of viremia or seroconversion has been discussed as one reason for the presence of memory NS3-1073-specific CD8$^+$ T cells in HCV-SNs. Grouping and stratifying our cohort according to the risk of contact to HCV revealed no significant differences between the groups in terms of NS3-1073-specific proliferation *in vitro* (Fig. 2E and Table 1). The frequency of individuals responding was not associated with the risk group (RF, 9/23 individuals, 39%; HBD, 17/54 individuals, 31%; PE, 12/32 individuals, 38%). Similarly, the magnitude of responses was comparable, with median frequencies of NS3-1073-specific CD8$^+$ T cells as follows: RF, 0.3%; HBD, 0.2%; and PE, 0.3%. Moreover, the functionality or avidity of the NS3-1073-specific CD8$^+$ T cells did not differ between the different risk groups (data not shown).

HCV NS3-1073-specific CD8$^+$ T cells can respond to multiple peptide variants derived from HCV but also unrelated pathogens. Another possible explanation for the origin of NS3-1073-specific CD8$^+$ T cells is T cell cross-reactivity. As a first step to investigating the cross-reactivity of NS3-1073-expanded cell lines, we tested peptide variants of NS3-1073 derived from different HCV genotypes that have comparatively high sequence homology. Stimulating NS3-1073 GT1a-expanded T cell lines with five different peptide variants in the cytokine assay showed that two of the variants were recognized efficiently (GT1b and GT4a), whereas cross-recognition of the other three peptides was low (GT5a) or absent (GT2 and GT3a; Fig. 3A). These findings were comparable between all 13 individuals analyzed and thus reveal a considerable degree of flexibility of NS3-1073-specific CD8$^+$ T cells to respond to peptide variants.

We next extended our analyses of cross-reactivity to peptides derived from unrelated viruses. An *in silico* structure-based approach was adopted to facilitate the selection of potential cross-reactive peptide candidates. Known HLA-A2-restricted epitopes IAV NA-231 and M1-58, EBV BMLF1-259, LMP2-329, LMP2-426, K12-17, BARF0-356, and LMP1-125, HIV Gag-77, and CMV pp65-495 were included in this screening based on a previous study (Fig. 3B) (28). Previously suggested cross-reactive peptide IAV NA-231 and HCV NS3-1073 GT1b were used as positive controls (22, 23). Twelve structures of pMHC complexes presenting selected peptides, as well as NS3-1073 GT1a (Table 2), were recovered from the Protein Data Bank or the CrossTope Structural Data Bank (27). Electrostatic potential distribution over the pMHC



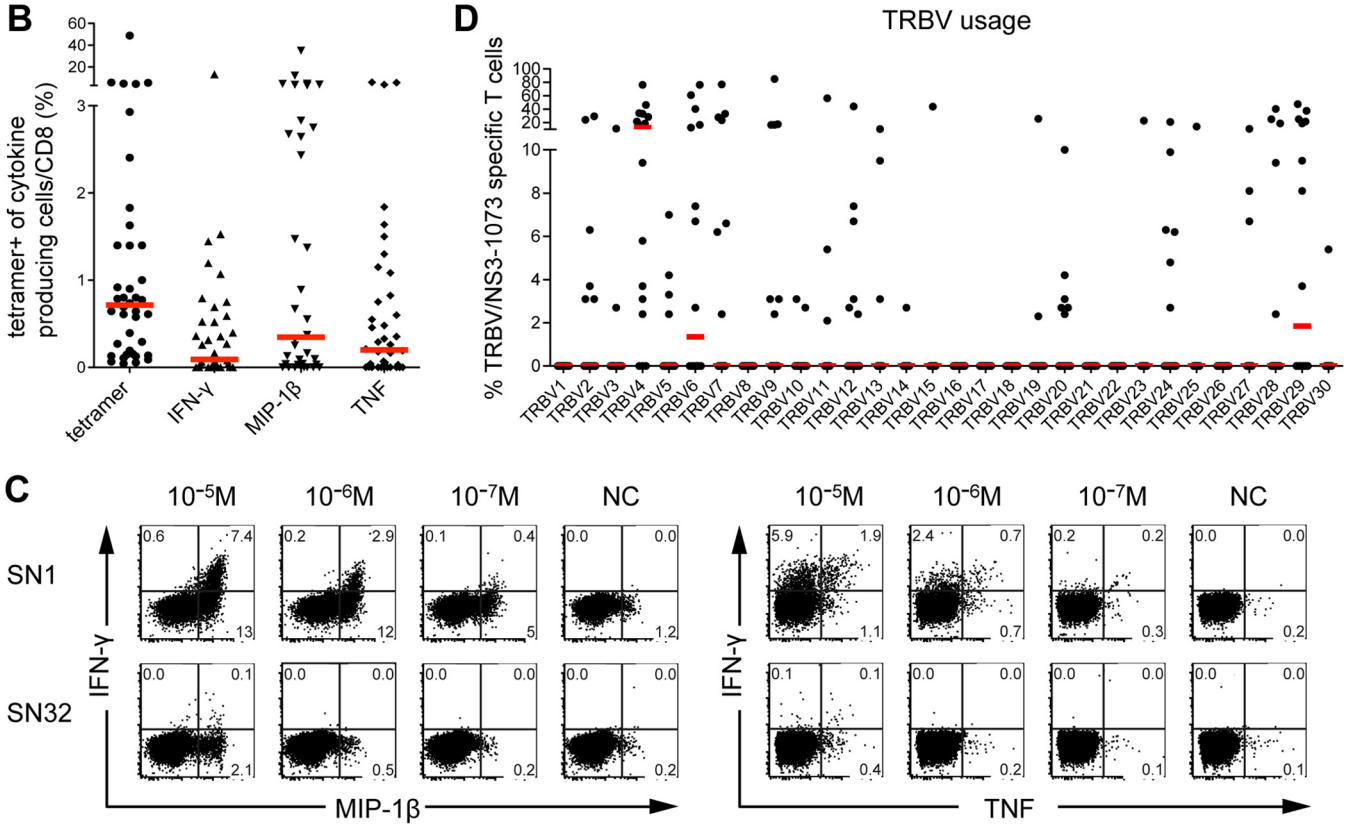


FIG 1 Expansion and functionality of HCV NS3-1073-specific CD8$^+$ T cells from HCV-SN individuals. (A) Expansion of HCV NS3-1073-specific CD8$^+$ T cells upon *in vitro* peptide stimulation varied between HCV-SN individuals.Results for 16 individuals for whom all readouts of multimer staining, cytokine production, and TRBV analysis could be performed are shown. FACS plots of multimer staining (left) and cytokine production upon peptide restimulation (right) from respective medium controls (left columns) and peptide stimulated cell lines (right columns) are shown, and the numbers refer to the frequencies of NS3-1073-specific cells gated on the total CD8$^+$ T cells and IFN-γ$^+$ MIP-1β$^+$ cells gated on the CD8$^+$ T cells after exclusion of dump channel. Bar graphs depicting data from sequencing of the T cell receptor of NS3-1073-specific CD8$^+$ T cells from 16 HCV-SN individuals show an individually variable repertoire, and each bar represents the frequency of an individual T cell clone. TRBV usage of NS3-1073-specific CD8$^+$ T cell responses of all individuals analyzed are shown as pie charts, and the frequencies of different TRBV families are summarized for each individual, respectively. (B) The frequencies of NS3-1073-specific CD8$^+$ T cells after *in vitro* expansion (●) are indicated for each sample tested ($n = 40$). In addition, the production levels of IFN-γ (▲), MIP-1β (▼), and TNF (■) are summarized. All frequencies refer to total CD8$^+$ T cells. Horizontal red lines indicate median frequencies. (C) Dose-dependent cytokine response of NS3-1073-specific T cell lines. The production of IFN-γ and MIP-1β, as well as IFN-γ and TNF, by NS3-1073-specific CD8$^+$ T cell lines upon *in vitro* stimulation with decreasing amounts of NS3-1073 peptide was analyzed by intracellular cytokine staining. FACS plots of cells stimulated with negative-control peptide (left) or with the NS3-1073 peptide are shown from a representative individual, and the cells are gated on the total CD8$^+$ T cells. (D) Summary of individual percentages of TRBV family usages by NS3-1073-specific CD8$^+$ T cells for all individuals analyzed ($n = 16$). Red horizontal bars indicate the median frequencies.

surface was used as input to perform a structure-based HCA. In agreement with an HCA published previously, EBV LMP2-329, HIV Gag-77, and IAV M1-58 fall into the same cluster as HCV GT1b. This newly performed HCA indicated an even bigger cluster of possible cross-reactive targets for NS3-1073, including CMV pp65-495 and EBV LMP2-426. This cluster (Fig. 3B, node 4) was also confirmed by bootstrap calculations ($P < 0.05$). The other complexes presented highly divergent electrostatic potential distribution (Fig. 3B), falling in an unrelated branch (node 7) and being therefore excluded from further analysis.

We first screened our NS3-1073-expanded cell lines for the recognition of the IAV NA-231 peptide. Indeed, cross-reactivity toward IAV NA-231 was detectable, since the NS3-1073-expanded cell lines responded with cytokine production upon NA-231 rechallenge (Fig. 3C; see also Table S1 in the supplemental material). However, only 3 of 30 cell lines displayed cross-reactivity to NA-231. We therefore screened more NS3-1073-expanded cell lines for the cross-recognition of the newly selected peptide candidates with the same approach. Overall, EBV LMP2-426, IAV M1-58, CMV pp65-495, and HIV Gag-77 showed cross-reactivity in 13 of 43 NS3-1073-expanded cell lines, whereas EBV LMP2-329 only very weakly stimulated cytokine responses by NS3-1073-specific CD8$^+$ T cells, which was mostly below the set threshold. However, no dominant or common cross-reactive response could be identified, and the recognition of the different peptide candidates showed a highly private pattern (Fig. 3C). Interestingly, in some cases, we also observed NS3-1073-expanded cell lines that did not respond to the NS3-1073 peptide itself but to a peptide candidate instead, which might indicate a higher affinity for the peptide candidate than for NS3-1073.

HCV NS3-1073 builds a network of cross-reactivities with multiple directionalities. The previous data showed that different peptide candidates can be recognized by NS3-1073-specific CD8$^+$ T cells, as evaluated by cytokine production. However, it is not clear whether these responses are the "cause" for the NS3-1073-specific CD8$^+$ T cells found in HCV-SN individuals. To further

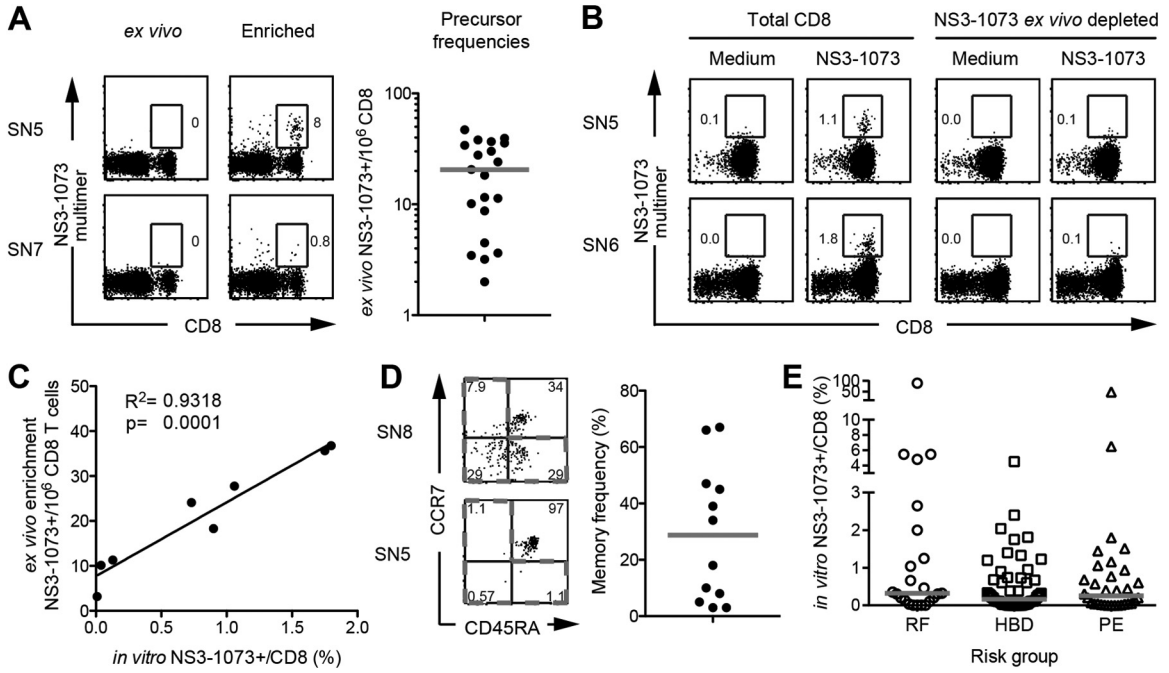


FIG 2 *Ex vivo* detection and phenotype of NS3-1073-specific CD8$^+$ T cells. (A) *Ex vivo* detection of antigen-specific CD8$^+$ T cells by NS3-1073 multimer staining and multimer-associated enrichment from HCV-SN. FACS plots show populations of NS3-1073-specific CD8$^+$ T cells before (*ex vivo*, left) and after enrichment (right) for two representative individuals, and the numbers refer to the frequencies of NS3-1073-specific CD8$^+$ T cells gated on the total CD8$^+$ T cells. From enriched populations, the precursor frequencies were calculated, and these are summarized (scatter plot). A horizontal red line indicates the mean frequency. (B) *In vitro* stimulation of total CD8$^+$ T cells (left) and NS3-1073-depleted cells with NS3-1073 peptide ("negative enrichment fraction," right); the latter resulted in no expansion of NS3-1073-specific T cells. FACS plots gated on the total CD8$^+$ T cells for two individuals analyzed are shown. (C) The association of the *ex vivo* frequency of NS3-1073-specific CD8$^+$ T cells from HCV-SN individuals (referring to the absolute number of total CD8$^+$ T cells) and the respective frequency of NS3-1073 CD8$^+$ T cells after *in vitro* expansion revealed a positive correlation ($n = 8$). (D) Analysis of the memory phenotype of NS3-1073-specific CD8$^+$ T cells by *ex vivo* multimer-associated enrichment and costaining for CCR7 and CD45RA showed a mixed population consisting of both naive and memory cells. FACS plots are gated on NS3-1073 multimer$^+$ CD8$^+$ T cells; the results for two representative individuals are shown. The scatter plot (right) summarizes the frequencies of memory CD8$^+$ T cells (100% − [frequencies of naive CCR7$^+$ CD45RA$^+$ cells]) among the NS3-1073-specific cells for all samples analyzed. (E) Scatter plot summarizing the frequencies of NS3-1073-specific CD8$^+$ T cells after *in vitro* stimulation of all HCV-SNs analyzed ($n = 109$). Samples are grouped according to risk factors for HCV exposure (RF, risk free; HBD, healthy blood donor; PE, potentially exposed). Horizontal lines indicate the median frequencies of responses.

elaborate on this, we studied the directionality of the cross-reactivity using the approach of Kasprowicz et al. (31) by analyzing the expansion of CD8$^+$ T cells specific for peptide candidates by stimulation with NS3-1073 ("reverse" cross-reactivity) or the expansion of NS3-1073-specific CD8$^+$ T cells by stimulation with peptide candidates ("forward" cross-reactivity).

We first evaluated the "reverse" cross-reactivity by multimer staining of peptide candidate-specific T cells in NS3-1073-expanded cell lines. Indeed, we could observe a proliferation of several different other antigen-specific CD8$^+$ T cells that was induced by NS3-1073 peptide stimulation. This included the expansion of EBV LMP2-329- and LMP2-426-, CMV pp65-495-, and IAV M1-58-specific CD8$^+$ T cells (Fig. 4A and E). Importantly, the expansion of CMV pp65-specific CD8$^+$ T cells induced by NS3-1073 occurred only in individuals who showed detectable CMV pp65-495-specific CD8$^+$ T cells *ex vivo* (9 of 14 individuals; Fig. 4B and E). These data clearly show that, using our settings, memory T cells are required to induce such a reverse cross-reactive response to NS3-1073 in HCV-SN. To investigate the role of CMV-specific memory T cells in the cross-reactive expansion of CD8$^+$ T cells, we performed experiments sorting CD8$^+$ T cells according to their memory phenotype by staining for CCR7 and CD45RA. We stimulated the naive (CCR7$^+$ CD45RA$^+$) and memory cells (T_{CM}, T_{EM}, and T_{EMRA}) with either CMV pp65 or NS3-1073 peptide and analyzed the proliferation of CMV pp65-specific CD8$^+$ T cells. As expected, when using the original CMV peptide, we found an expansion of CMV-specific CD8$^+$ T cells only in the memory cell compartment and not in the naive cell fraction showing a successful sorting process (Fig. 4C). Importantly, the cross-reactive expansion of CMV pp65-specific CD8$^+$ T cells induced by stimulation with the HCV NS3-1073 peptide as described before was also only visible in the memory cell compartment and not in the naive fraction (Fig. 4C). In agreement with this observation, we could not expand cross-reactive NS3-1073-specific T cells *in vitro* from five HLA-A2-positive cord blood samples when only few memory T cells were present (data not shown).

Since CMV is a common and ubiquitous virus in humans and we could observe a cross-reactive response between CMV pp65 and HCV NS3-1073, we sought to further follow-up on the impact of CMV infection on the presence of NS3-1073-specific CD8$^+$ T cells in HCV-SNs. After stratifying 44 HCV-SNs according to the *ex vivo* detectability of CMV pp65-specific CD8$^+$ T cells into positive and negative individuals and analyzing the expansion of NS3-1073-specific CD8$^+$ T cells *in vitro*, we found that no differences were visible between the two groups (median frequencies for NS3-1073: 0.3% in CMV$^+$ subjects versus 0.3% in CMV$^-$ subjects [$P >$

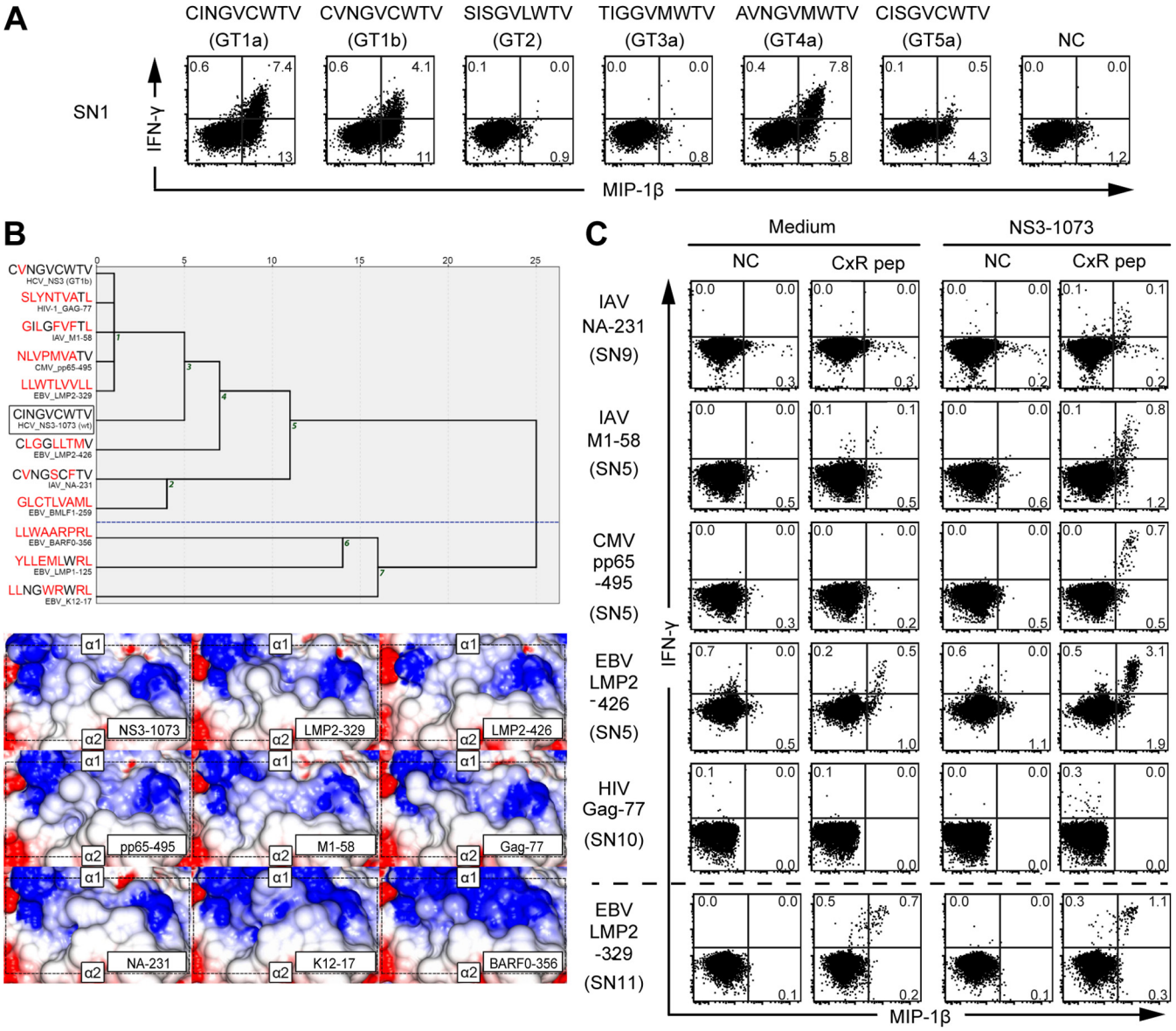


FIG 3 HCV NS3-1073-specific CD8$^+$ T cells recognize different cross-reactive peptides *in vitro*. (A) Restimulation of NS3-1073-expanded cell lines with NS3-1073 peptide variants revealed a selective cross-recognition of specific peptides. FACS plots showing IFN-γ and MIP-1β staining are shown for a representative individual, and the cells are gated on the total CD8$^+$ T cells. (B) *In silico* structure-based prediction revealed peptide candidates potentially cross-reactive to NS3-1073. Hierarchical cluster analysis (HCA) was performed based on charge distribution over the TCR-interacting surface of pMHC complexes. Green numbers indicate each node of the dendrogram, in increasing order of distance (*x* axis). Epitope information is presented on the left, and amino acid exchanges in relation to NS3-1073 are indicated in red. Only the complexes in the upper branch of the dendrogram were selected for testing (blue line). Selected pMHC surfaces are depicted (lower panel). The α1 and α2 domains of the MHC are indicated, delimiting the region occupied by the peptide. Charged areas over the pMHC surface are represented as red (negative) and blue (positive charges) spots ranging from −5 to +5 kT. (C) *In vitro* restimulation of NS3-1073-specific CD8$^+$ T cell lines from HCV-SN with different cross-reactive peptide candidates showed recognition of various peptides. FACS plots of IFN-γ and MIP-1β responses from representative individuals are depicted for medium control cell lines (left) and NS3-1073-expanded cell lines (right), and the plots show results for cells gated on the total CD8$^+$ T cells. Peptides used for restimulation during the cytokine assay are indicated.

0.05]). This shows that there is no evidence of an exclusive impact of CMV infection on the HCV-specific T cell responses in HCV-SN individuals. However, for the expansion of CMV pp65-specific CD8$^+$ T cells induced by NS3-1073 peptide stimulation, the requirement of CMV pp65-specific CD8$^+$ T cells *ex vivo* was crucial and necessary to induce such cross-reactive responses (median CMV pp65-specific CD8$^+$ T cells induced by NS3-1073 *in vitro* stimulation: 6.2% in CMV$^+$ subjects versus 0% in CMV$^-$ subjects [$P < 0.01$]).

We further also investigated the occurrence of "forward" cross-reactivity by stimulating cells with peptide candidates *in vitro* and analyzing the expansion of NS3-1073-specific CD8$^+$ T cells. However, we could only observe an expansion in NS3-1073-specific CD8$^+$ T cells using the EBV LMP2-329 peptide and only in few cases (2 of 14 individuals) tested (Fig. 4D). Other peptide candidates did not induce NS3-1073 responses (data not shown). Thus, "forward" cross-reactivity seems to be less frequent in the case of NS3-1073. In summary, we propose a network of cross-

TABLE 2 Characteristics of peptide candidates for *in silico* structure-based approach[a]

Source virus(es)	Source protein	Epitope positions	Sequence	Epitope ID (IEDB)	Structure source	Structure type	CrossTope ID
HCV (WT)	NS3	1073-1081	CINGVCWTV	6435	PDB	Crystal (3MRG)	
HCV (GT1b)	NS3	1073-1081	CVNGVCWTV	7292	CrossTope	Model (D1-EM-D2)	A0201_0031
IAV	NA	231-239	CVNGSCFTV	7291	CrossTope	Model (D1-EM-D2)	A0201_0109
	M1	58-66	GILGFVFTL	20354	PDB	Crystal (2VLL)	A0201_0097
HIV-1	GAG	77-85	SLYNTVATL	59613	PDB	Crystal (2V2W)	A0201_0095
EBV/HHV4	LMP2	329-337	LLWTLVVLL	37960	CrossTope	Model (D1-EM-D2)	A0201_0073
	BMLF1	259-267	GLCTLVAML	20788	PDB	Crystal (3MRE)	
	Putative BARF0 protein	356-364	LLWAARPRL	37938	CrossTope	Model (D1-EM-D2)	A0201_0072
	LMP1	125-133	YLLEMLWRL	74774	CrossTope	Model (D1-EM-D2)	A0201_0080
	K12	17-25	LLNGWRWRL	37607	CrossTope	Model (D1-EM-D2)	A0201_0110
	LMP2	426-434	CLGGLLTMV	6568	PDB	Crystal (3REW)	
CMV/HHV5	pp65	485-493	NLVPMVATV	44920	PDB	Crystal (3GSO)	

[a] The table indicates the CrossTope ID and the epitope ID identifying the epitope in the Immune Epitope Database (IEDB). The structure type used for *in silico* modeling approaches and the virus and proteins from which the peptides originate, as well as the positions, lengths, and amino acid sequences of the epitopes, are given. Different HLA-A*0201-restricted peptides were recovered from the CrossTope Data Bank and used for *in silico* structure-based approaches to identify peptides potentially cross-reactive to the HCV NS3-1073 peptide. HHV4, human herpesvirus 4; HHV5, human herpesvirus 5.

reactive responses to NS3-1073 with different directionalities, which we were able to discern by analyzing the 48 samples tested for cross-reactive expansion and cytokine production (Fig. 4E).

Preexisting HCV-specific CD8$^+$ T cells in HCV-SNs influence responses to vaccination. The consequence of preexisting NS3-1073-specific CD8$^+$ T cells was first studied in a small cohort of healthy HCV-SN individuals who were vaccinated with an HCV peptide vaccine in a clinical trial (11). From 35 vaccinated individuals, we analyzed the *ex vivo* frequency of CD8$^+$ T cells specific for three of the five HLA-A2-restricted HCV epitopes included in the vaccine. In most of the individuals, the NS3-1073 CD8$^+$ T cell response was predominant after vaccination. There was a large variation in the frequency of NS3-1073 multimer$^+$ cells, but the mean frequency of NS3-1073-specific CD8$^+$ T cells was significantly higher than with core 132 or NS4B-1764 (Fig. 5A).

Next, we sought to determine whether preexisting NS3-1073-specific CD8$^+$ T cells might impact the response to vaccination. PBMCs from three of the vaccinated donors before receiving the vaccine (baseline) were available and stimulated *in vitro* with NS3-1073 as done previously. Two of the three individuals showed a significant NS3-1073-specific CD8$^+$ T cell proliferation *in vitro* and were thus regarded as "baseline responders" (SN16 and SN17; Fig. 5b). *Ex vivo* multimer staining of samples at the end of the vaccination schedule showed that the two baseline responders achieved a stronger *in vivo* NS3-1073-specific CD8$^+$ T cell response toward the vaccine (Fig. 5B). These data imply that the frequency of preexisting T cells may contribute to the magnitude of immune response to a T cell-inducing vaccine.

This association between preexisting NS3-1073-specific T cells at baseline and responses to the vaccine was further analyzed in a second cohort of six individuals derived from a second clinical trial using the same experimental HCV peptide vaccine (26). Here, three of the six vaccinated individuals were "baseline responders" since they showed a proliferation of NS3-1073-specific CD8$^+$ T cells *in vitro* above our threshold (0.5% of CD8$^+$ T cells) already before the first administration of the vaccine (SN19, SN20, and SN21; Fig. 5C). For these individuals, the increase in NS3-1073-specific CD8$^+$ T cell proliferation *in vitro* occurred earlier (during vaccination) versus the other three individuals with no or a low response to NS3-1073-specific CD8$^+$ T cell responses *in vitro* before vaccination (SN22, SN23, and SN24). Also in this cohort, the *ex vivo* frequencies of NS3-1073 CD8$^+$ T cells during vaccination were stronger for "baseline responders." The "baseline nonresponders" also mounted NS3-1073-specific CD8$^+$ T cells detectable *ex vivo* in response to the vaccine, but this response was often weaker and emerging later (Fig. 5C, right panel). These results indicate that the frequency of preexisting T cells may contribute not only to the magnitude but also to the promptness of an immune response to vaccination.

DISCUSSION

We show here that HCV-specific CD8$^+$ T cells can be frequently detected in HCV-seronegative (HCV-SN) individuals and that these cells may impact the response to vaccination. Virus-specific T cell responses in unexposed individuals have been reported before in several settings (15, 16, 19, 32), and the composition of this preexisting T cell repertoire may influence the response toward vaccines, as suggested by reports for other infections (14, 33). However, thus far, no study has systematically studied the frequency and repertoire of HCV-specific CD8$^+$ T cell responses in a large number of HCV-SN individuals and investigated how preexisting HCV-specific CD8$^+$ T cells may influence vaccine responses. Our study, which included a total of 121 HCV-SNs, revealed a high prevalence of HCV NS3-1073-specific CD8$^+$ T cell responses both *ex vivo*, as well as *in vitro* in about one-third of individuals tested, whereas the NS3-1073-specific T cell responses were low or absent in the remaining two-thirds. However, the magnitude of the responses, as well as the avidity toward the NS3-1073 peptide, varied greatly between individuals, as did the TCR repertoire.

The frequency and the pattern of multimer$^+$ CD8$^+$ T cells were

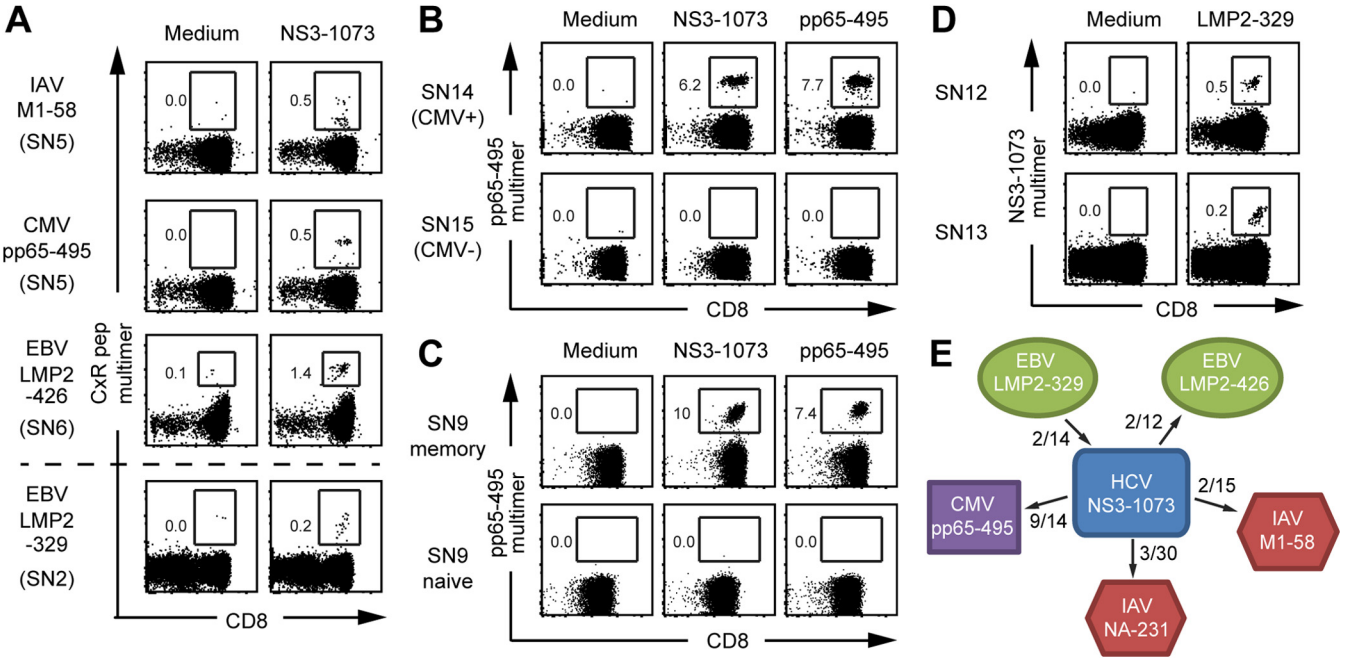


FIG 4 Proliferation of antigen-specific CD8$^+$ T cells upon peptide stimulation. (A) *In vitro* stimulation of CD8$^+$ T cells with the NS3-1073 peptide induced expansion of CD8$^+$ T cells specific for other viruses. FACS plots for cross-reactive peptide multimer staining of medium control and NS3-1073 expanded cell lines are shown for representative individuals. The respective epitope specificities are indicated on the left, and the cells were gated on the total CD8$^+$ T cells. (B) Expansion of CMV pp65-495-specific CD8$^+$ T cells by NS3-1073 peptide stimulation *in vitro* only occurred in individuals with *ex vivo* detectable CMV pp65-specific CD8$^+$ T cells (upper panel) and not in those without CMV pp65-specific CD8$^+$ T cells (lower panel). (C) Memory sorting of PBMC from HCV-SN individuals according to CCR7 and CD45RA expression revealed an expansion of CMV pp65-specific CD8$^+$ T cells in the memory T cell fraction (T_{CM}, T_{EM}, and T_{EMRA}) only. Of note, cross-reactive expansion of CMV pp65-specific CD8$^+$ T cells induced by NS3-1073-specific peptide stimulation also only occurred in the memory and not in the naive T cell compartment. FACS plots of multimer staining from one representative individual are shown, and the cells were gated on the total CD8$^+$ T cells. (D) *In vitro* stimulation of CD8$^+$ T cells using EBV LMP2-329 peptide induced an expansion of NS3-1073-specific CD8$^+$ T cells. FACS plots for NS3-1073 multimer staining of medium control and LMP2-329 expanded cell lines are shown for the two responding individuals. (E) Schematic matrix of peptides identified as cross-reactive to the HCV NS3-1073 epitope. The frequencies of individuals showing a cross-recognition of the respective peptides in our experiments (cross-reactive expansion and cytokine production) are shown, and the "directionalities" of the responses are indicated by arrows (total, $n = 48$).

variable between individuals and, in some cases, the staining intensity was low and the multimer$^+$ population did not separate clearly from the bulk CD8$^+$ T cells. This may be due to the low affinity of the TCR toward this noncognate peptide. We are inclined to disregard a possibility of unspecific staining, since we first applied a stringent exclusion and dump channel strategy and, second, a peptide-specific and dose-dependent cytokine response *in vitro* toward the NS3-1073 peptide could be observed. The cytokine response of NS3-1073-specific T cells toward this peptide also varied between individuals. However, their frequencies were often lower than that of NS3-1073 multimer$^+$ CD8$^+$ T cells itself, and the production of TNF and, especially, MIP-1β was stronger compared to that of IFN-γ. The cytokine production profile further implies a low avidity of the T cells toward NS3-1073, since it has been reported that a higher TCR avidity is required to induce IFN-γ production, whereas a lower avidity is sufficient for the induction of TNF and MIP-1β responses, and an even lower one may induce T cell proliferation without cytokine production (34, 35). In a report by Neuveu et al. (15), a high avidity of HCV-specific CD8$^+$ T cells was associated with a clearance of infection. The HCV-specific CD8$^+$ T cells that they found in HCV-SN individuals showed a broad range of avidities, but this was lower overall than in recovered patients, which agrees with our data obtained in HCV-SNs.

The relative abundance of HCV NS3-1073-specific CD8$^+$ T cell responses in HCV-SN individuals led us to further explore possible mechanisms generating these responses. Responses toward NS3-1073 could also be found *ex vivo* by multimer enrichment, and the *ex vivo* memory phenotype of these cells revealed that they were a mixed population consisting of both memory and naive CD8$^+$ T cells. Similar findings of a mixed phenotype of HCV-specific CD8$^+$ T cells in seronegative persons have been described for another HCV-derived epitope (19) and also recently for HIV-specific T cells detected in uninfected persons (13). One conceivable explanation for the high number of naive precursor T cells specific for NS3-1073 might be that the NS3-1073 epitope is rather promiscuous and thus able to recruit many different T cell clones (36). However, we were not able to generate NS3-1073-specific CD8$^+$ T cells from cord blood samples and the expansion of naive T cells seems difficult using our cell culture system. Thus, we wanted to emphasize that both naive and memory NS3-1073-specific CD8$^+$ T cells are likely to contribute to this response. The origin and reason for the existence and abundance of HCV-specific T cells with a memory phenotype in HCV-seronegative persons is not well defined. Cytokines evoked by preceding infections or else homeostatic proliferation of T cells could be one reason for the cells acquiring a memory phenotype (14). Further, low-level exposure to HCV without seroconversion may induce NS3-1073-specific T cell responses and memory cells. However, in our results, the NS3-1073-specific CD8$^+$ T cell proliferation after *in*

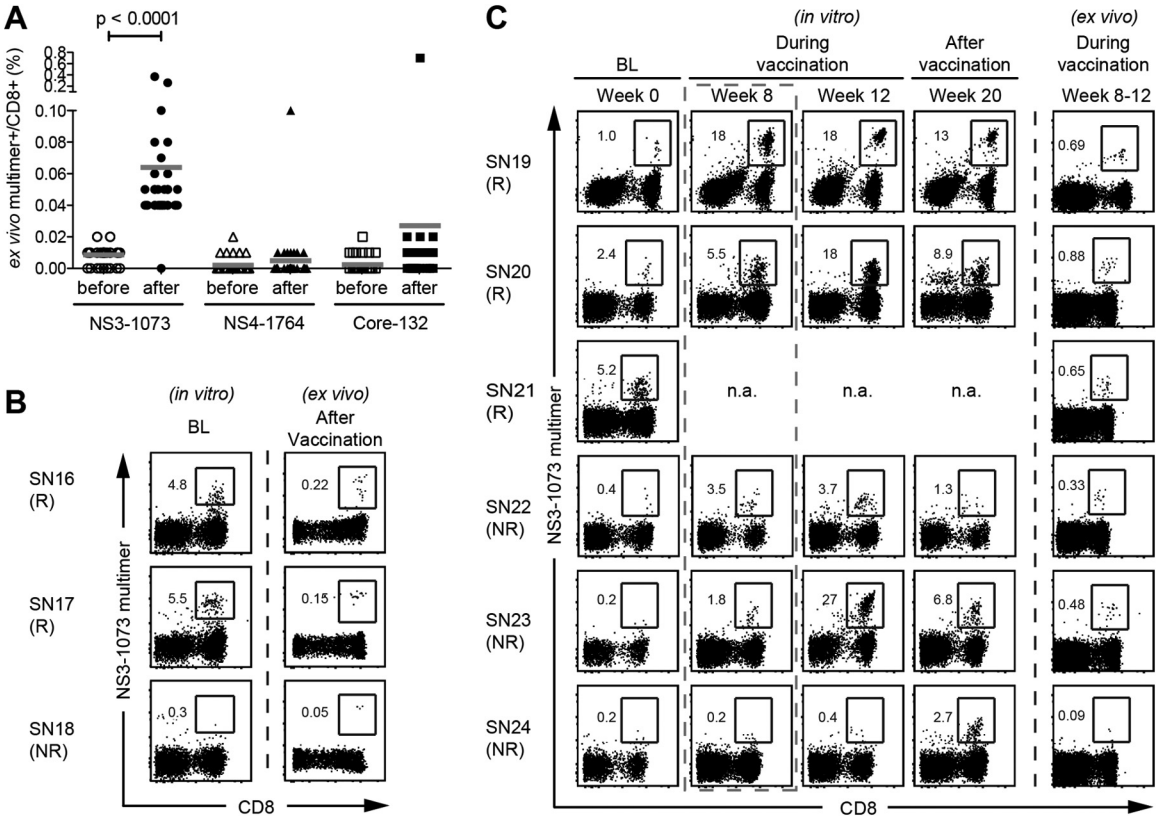


FIG 5 T cell responses to HCV peptide vaccination are influenced by preexisting NS3-1073-specific CD8$^+$ T cells. (A) *Ex vivo* frequency of HCV-specific CD8$^+$ T cells in vaccine receivers before and after HCV peptide vaccination showed that responses toward the NS3-1073 epitope were predominant. Horizontal red lines indicate median frequencies. (B) Three healthy individuals vaccinated with the HCV peptide vaccine were analyzed for the expansion of NS3-1073-specific CD8$^+$ T cells *in vitro* at baseline before vaccination (left). "Baseline responders" SN16 and SN17 showed higher *ex vivo* NS3-1073 multimer$^+$ T cell frequencies to the vaccination (right); respective FACS plots are shown, and the numbers indicate the frequencies of NS3-1073-specific CD8$^+$ T cells. (C) *In vitro* expansion of NS3-1073-specific CD8$^+$ T cells in six additional healthy individuals vaccinated within a second clinical trial were analyzed for the expansion of NS3-1073-specific CD8$^+$ T cells before (week 0), during (weeks 8 and 12), and after HCV peptide vaccination (week 20). According to baseline *in vitro* proliferation magnitude, SN19, SN20, and SN21 are "baseline responders" (R), and SN22, SN23, and SN24 are "baseline nonresponders" (NR). A response to NS3-1073 before vaccination corresponded to the strength of the *in vitro* expansion of NS3-1073-specific CD8$^+$ T cells at an early time point in the vaccination (week 8, indicated by dotted box). The numbers on the FACS plots indicate the frequencies of NS3-1073-specific CD8$^+$ T cells gated on the total CD8$^+$ T cells. The *in vivo* responsiveness to HCV peptide vaccination was stronger early during vaccination in "baseline responders" (right). n.a., not analyzed.

vitro stimulation showed no differences between risk-free, potentially exposed individuals and healthy blood donors of unknown origin and risk factors. In addition, it is unlikely that all of the responding individuals were exposed to HCV previously because HCV prevalence is low in northern Europe.

The presence of NS3-1073-specific CD8$^+$ T cells in seronegative individuals displaying a memory phenotype can also be explained by the cross-reactivity of memory cells specific to unrelated pathogens. Importantly, memory cells have an activation advantage over naive T cells (37) and thus may be easily activated. Several recent reports show that a T cell is able to recognize and respond to several different peptides with a certain degree of similarity in structure. This phenomenon, called T cell cross-reactivity, is based on the flexibility and degeneracy of the TCR in binding to pMHC complexes (21, 38, 39). T cell cross-reactivity might occur between peptide variants derived from different viral genotypes but also between peptides from different pathogens (21, 40). Indeed, NS3-1073-specific CD8$^+$ T cell lines from HCV-SNs were not only able to respond to peptide variants derived from different HCV genotypes but also, importantly, recognize epitopes originating from heterologous and often ubiquitous viruses with a large degree of sequence dissimilarity. The cross-recognition pattern of other peptides was different between individuals, and we could find no dominant peptide recognized by most NS3-1073-expanded cell lines. The private specificity of this cross-reactivity may be explained by the individually diverse NS3-1073-specific CD8$^+$ T cell receptor repertoire that we observed. As reported previously for a cross-reactive vaccinia virus epitope (41), we propose a matrix of cross-reactivities between the HCV NS3-1073 epitope and other peptides.

We used an *in silico* approach using bioinformatics tools to help us to screen new cross-reactive peptide candidates. This approach has previously been used to successfully verify cross-reactivity among various genotype variants of the HCV NS3-1073 peptide (28). Our new structure-based HCA suggested greater structural similarity between HCV NS3-1073 and five unrelated targets. It is important to stress that all of them have little sequence similarity with the HCV NS3-1073 epitope studied here (CINGV CWTV). The real similarity may only arise when the peptides are presented by HLA-A*0201 as pMHC complexes. This is possible

because of the interaction with the amino acids in the MHC binding groove, due to which peptide side chains exposed at the TCR interaction surface may have an altered conformation, give a different structure to the TCR interaction surface and thus resemble the structure of a pMHC complexed with another peptide with little or no sequence similarity. Likewise, a study by Valkenburg et al. (42) showed that the pMHC structure was a key determinant of protective efficacy of CD8$^+$ T cells recognizing IAV mutant epitopes.

In the case of HCV NS3-1073 and IAV NA-231, cross-reactivity has been reported to be weak in recent studies (15, 31), which fits our results in HCV-SNs, where only a few cases of weak cross-reactivity were found. This may be explained by the structural differences; despite a sequence similarity of 66% between NA-231 and NS3-1073, the pMHC structure is more different than other predicted candidates with less sequence similarity. In a previous publication, Kasprowicz et al. also defined the "directionality" of cross-reactivity (31). According to their definitions, we analyzed our data on the proliferation of NS3-1073-specific CD8$^+$ T cells *in vitro* induced by cross-reactive peptide candidates or vice versa. We observed that NS3-1073 could induce an expansion of several antigen-specific CD8$^+$ T cells *in vitro*, including CMV pp65, IAV M1-58, and EBV LMP2-426. However, in contrast, these peptides did not induce the expansion of NS3-1073-specific CD8$^+$ T cells. The phenomenon of HCV peptide inducing memory cells from heterologous antigens was regarded as "reverse" cross-reactivity. Importantly, CMV-specific T cells could only be expanded in individuals with CMV-specific memory CD8$^+$ T cells *ex vivo*, supporting the idea that immunological memory is required for the cross-reactive expansion of HCV-specific T cells in HCV-SN. This is further supported by the observation that expansion of CMV pp65-specific CD8$^+$ T cells induced by NS3-1073 stimulation *in vitro* only occurred in the memory fraction of sorted cells and not in naive cells, demonstrating once again the crucial role of memory T cells for cross-reactive expansion, at least in the case of CMV pp65 and NS3-1073 cross-reactivity. In contrast, we only observed two cases of "forward" cross-reactivity for the EBV LMP2-329 peptide, which was able to induce only a low level of proliferation of NS3-1073-specific CD8$^+$ T cells *in vitro*. Since all individuals are most likely not exposed to HCV, this may help to explain why forward cross-reactivity is less frequently observed in HCV-SNs. Also, Kasprowicz et al. (31) indicated that "reverse" cross-reactivity seems to occur more often than "forward" cross-reactivity for HCV NS3-1073 and IAV NA-231.

The presence of a considerable frequency of HCV NS3-1073-specific CD8$^+$ T cells that we found in HCV-SNs may well impact the immune response toward HCV during an infection or a vaccination. To investigate this hypothesis, we used a cohort of HCV-SNs that were vaccinated with an HCV peptide vaccine within a clinical trial (11). Although only CD8$^+$ T cells specific for three of the five epitopes included in the vaccine were analyzed, we observed a strong dominance of NS3-1073 after vaccination. Within the vaccinated individuals, a broad variability of the frequency of NS3-1073-specific CD8$^+$ T cells after vaccination was discernible. One explanation for the dominance and diversity could be the preexisting HCV NS3-1073-specific T cell frequency and individually diverse TCR repertoire. In our vaccination trial data, baseline responders usually achieved a higher *ex vivo* NS3-1073 multimer$^+$ T cell frequency after vaccination, suggesting that the frequency of preexisting T cells may correlate with the magnitude of T cell responses toward a T cell-inducing vaccine. Our results from the second vaccination trial also imply that, in addition to impacting the strength of the response, the frequency of preexisting T cells also correlates with the promptness of the immune response. Notably, a response to NS3-1073 induced by the vaccine also appeared in the baseline nonresponder individuals and, in some cases, the response to vaccination was also strong, but these responses seemed to be delayed during the time course of vaccination and weaker during the early phases. Thus, the time point, together with the strength of the vaccine response, seemed to be influenced by the preexistence of NS3-1073-specific CD8$^+$ T cells. The reason behind these differences could be the vaccinated donors' individual innate or adaptive immune status during the vaccination period. Our vaccination trials only focused on the correlation between preexisting T cells and the magnitude of T cell response to a vaccine. However, the T cell response magnitude does not always correlate with the efficacy of a vaccine. Our study also has other limitations that need to be considered. For example, the vaccine cohort is rather small and due to the limited number of cells we could not investigate further details such as cross-reactivity in the vaccine cohort. Also, in our cohort of 121 HCV-SN individuals we could not investigate all aspects (TCR repertoire, cross-reactivity, avidity) in all individuals due to restricted cell numbers. In addition, only responses toward one model epitope NS3-1073 have been studied. Whether a similar broad cross-reactivity toward other epitopes exists as well, or whether the NS3-1073 epitope represents a highly promiscuous and possibly pathogenic epitope (36), is as yet unclear. Nevertheless, this is the largest cohort of seronegatives analyzed thus far for virus-specific CD8$^+$ T cell responses.

In the present study, we investigated the cross-reactivity of HCV NS3-1073-specific CD8$^+$ T cells, which we merely regard as a model system that could be extendable to many other epitopes as well. In general, T cell cross-reactivity has to be regarded as a quite common immunological phenomenon, and several recent publications also support this view (14, 38). Although we were able to detect CD8$^+$ T cell responses toward HCV NS3-1073 in about one-third of all individuals tested by using different readouts (*ex vivo* multimer staining, *ex vivo* enrichment, *in vitro* expansion), the differences in the frequencies, cytokine production, avidity, and TCR usage highlight once more the clear private specificity of these responses, with every individual showing a unique pattern of response. This private specificity is likely dependent on the individual's corresponding T cell receptor repertoire, since it determines the specificity and range of epitopes that are recognized. The T cell/T cell receptor repertoire is shaped by the individual's history of infections and altered upon subsequently occurring infections; specific T cell clones are deleted, and others are activated, turning into memory cells. Thus, the detection of cross-reactive responses is impeded by all of these factors, and responses may appear or vanish over time, possibly leading to an underestimation of the frequencies of such responses.

The majority of cross-reactive responses investigated here focus on peptides derived from common pathogens such as CMV and EBV. However, we want to emphasize that, in addition to these two viruses, multiple other epitopes derived from other pathogens that were not included in the present study are also likely to be cross-reactive to HCV NS3-1073. A recent publication investigating heritable and nonheritable factors influencing the diversity of the human immune system (43) revealed CMV to be

one major nonheritable factors to impact this diversity. Yet, stratifying our cohort according to the *ex vivo* status for CMV by staining for pp65-specific $CD8^+$ T cells revealed no major impact of CMV infection on the response toward NS3-1073 in these cases, showing that CMV infection is not the exclusive reason for NS3-1073 cross-reactivity. This was not surprising, since we expect multiple pathogens to be involved in the generation of the NS3-1073-specific $CD8^+$ T cell responses that we observed in HCV-SN individuals, as mentioned above. Likewise, it can be assumed that EBV also does not constitute a major influence because >95% of the population is infected with EBV, and yet NS3-1073-specific T cell responses were only found in ca. 30% of individuals analyzed. In conclusion, we suggest that HCV-specific $CD8^+$ T cells are abundant in HCV-seronegative individuals due to the involvement of both naive precursors and cross-reactive memory cells. The private specificity of the preexisting repertoire may influence the response to vaccines and probably also toward infection. The differences in response magnitudes, TCR repertoires, and recognition of peptide variants that we could observe in our cohort is also important for the development of effective vaccines against HCV. For instance, a narrowed T cell and TCR repertoire might favor viral escape mutations (40) and thus the loss of protection, as shown previously (44). Further, mutations resulting in HCV quasispecies and different genotypes are a major hurdle in the development of effective vaccines. A broad protection across different genotypes is crucial for vaccine-induced immune responses, but often existing circulating HCV strains do not induce such a broad recognition of viral variants (45). Even for HCV, with all of the new potent direct acting antivirals potentially available, the discussion about the need for a vaccine has not yet ended (46). Strategies to induce or boost T cell responses might therefore be personalized based on an individual's T cell repertoire that is present before the immune intervention.

ACKNOWLEDGMENTS

We thank Liisa K. Selin and Raymond M. Welsh (Department of Pathology, University of Massachusetts Medical School, Worcester, MA) for helpful discussions of data. Also, we are grateful to all patients and healthy volunteers who donated blood for this study.

This study was supported by grants of the German Research Foundation (DFG; CO310/2-1 [M.C.]) and the Collaborative Research Centre SFB900 (Chronic Infections: Microbial Persistence and its Control). Part of this study was funded by BMBF 01KI0788 (M.C.). S.Z. and R.K.B. were supported by the Hanover Biomedical Research School.

We acknowledge the assistance of the Cell Sorting Core Facility of the Hannover Medical School, supported in part by Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft.

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