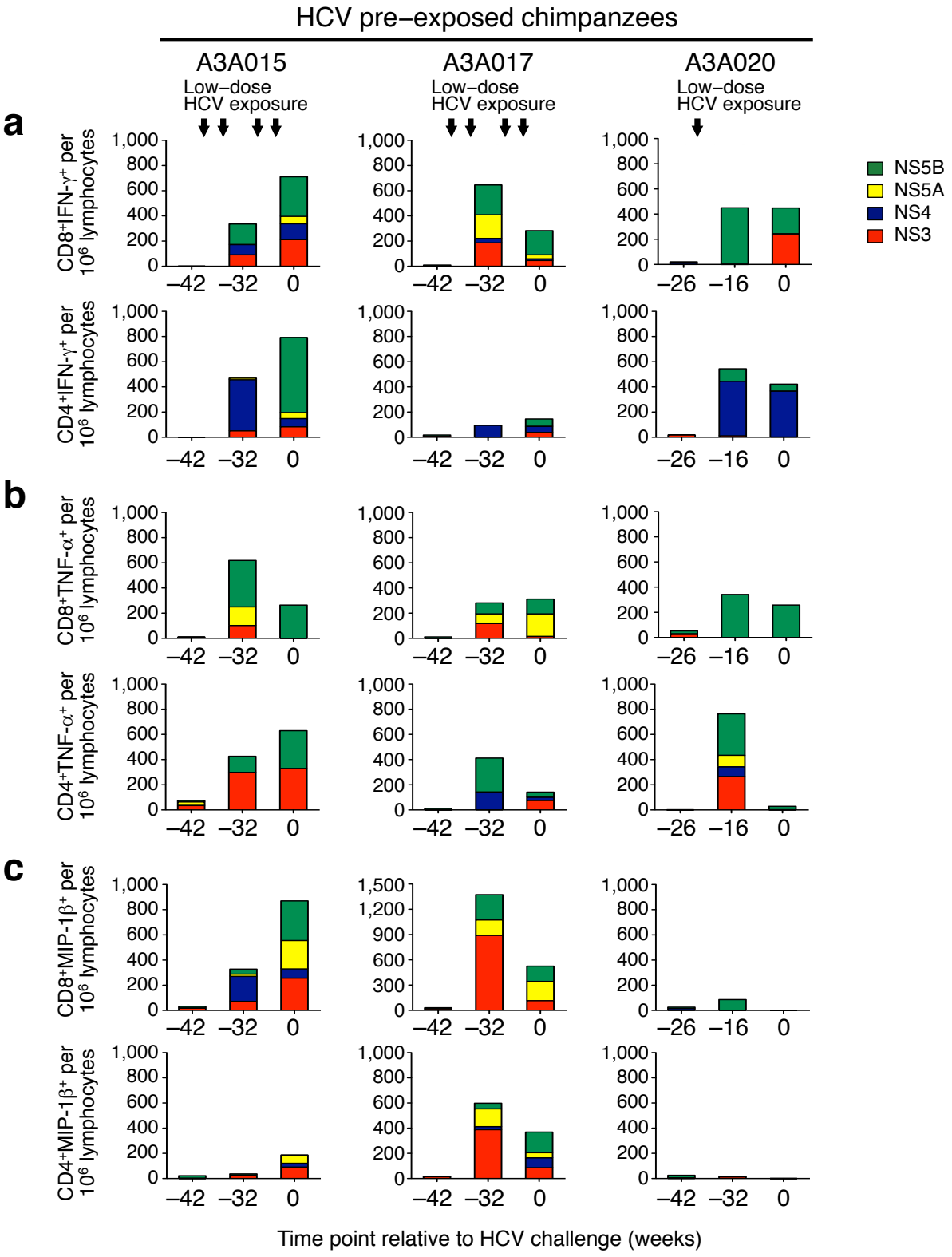
SUPPLEMENTARY INFORMATION

Subinfectious Hepatitis C Virus Exposures Suppress T Cell Responses Against Subsequent Acute Infection

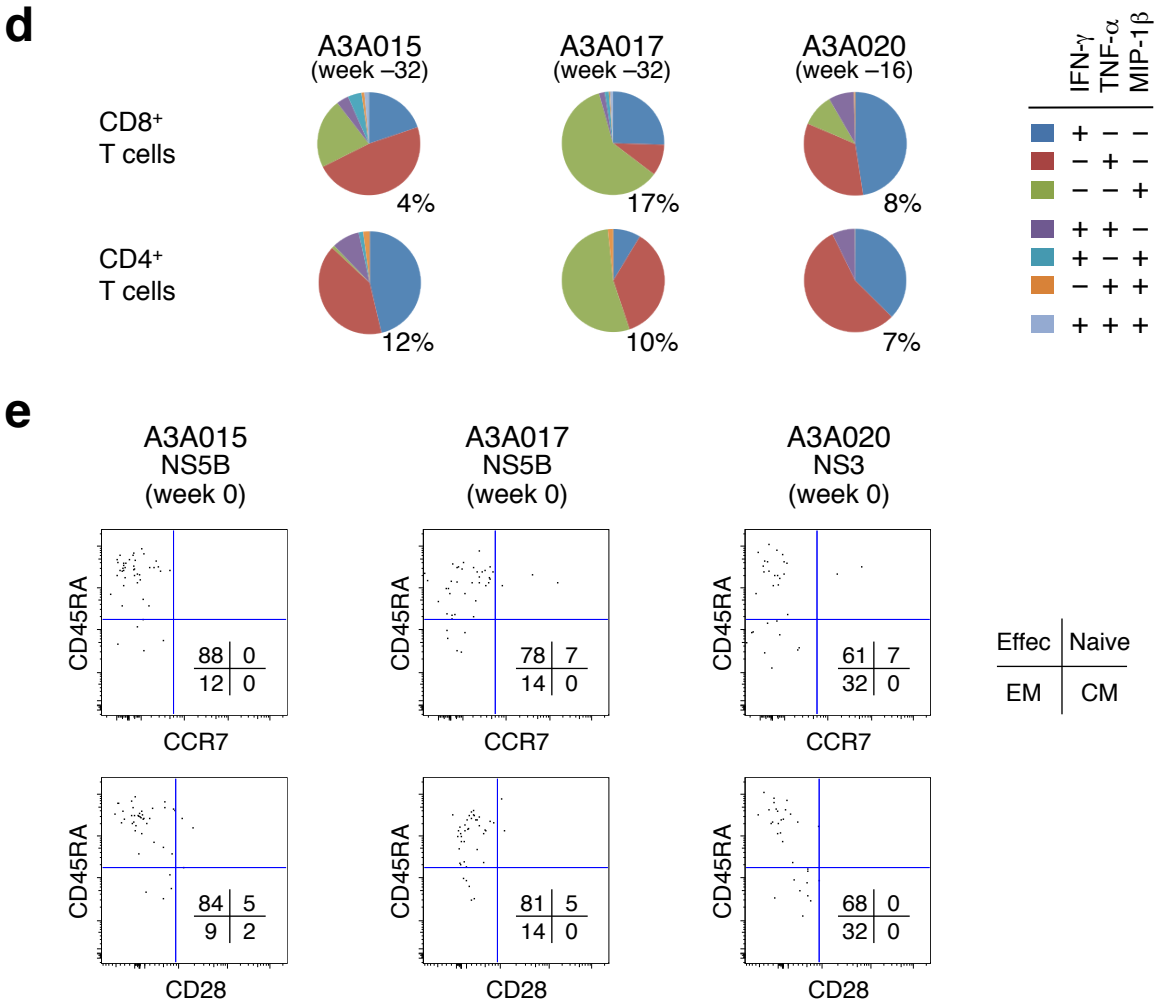
Su–Hyung Park, Naga Suresh Veerapu, Eui–Cheol Shin, Angélique Biancotto, J. Philip McCoy, Stefania Capone, Antonella Folgori and Barbara Rehermann

Table of Contents:

Supplementary Figure 1

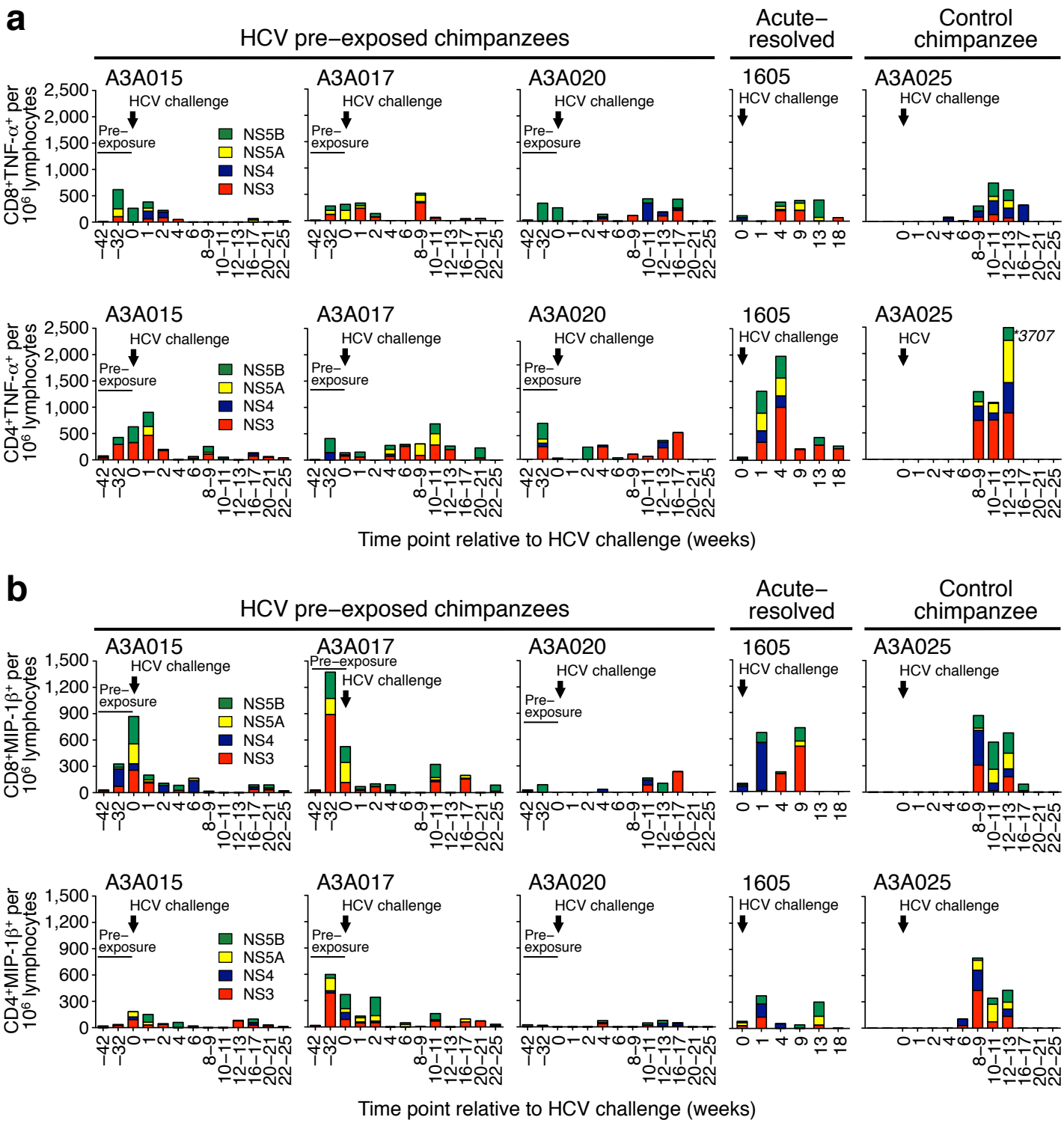


Supplementary Figure 1 (continued)

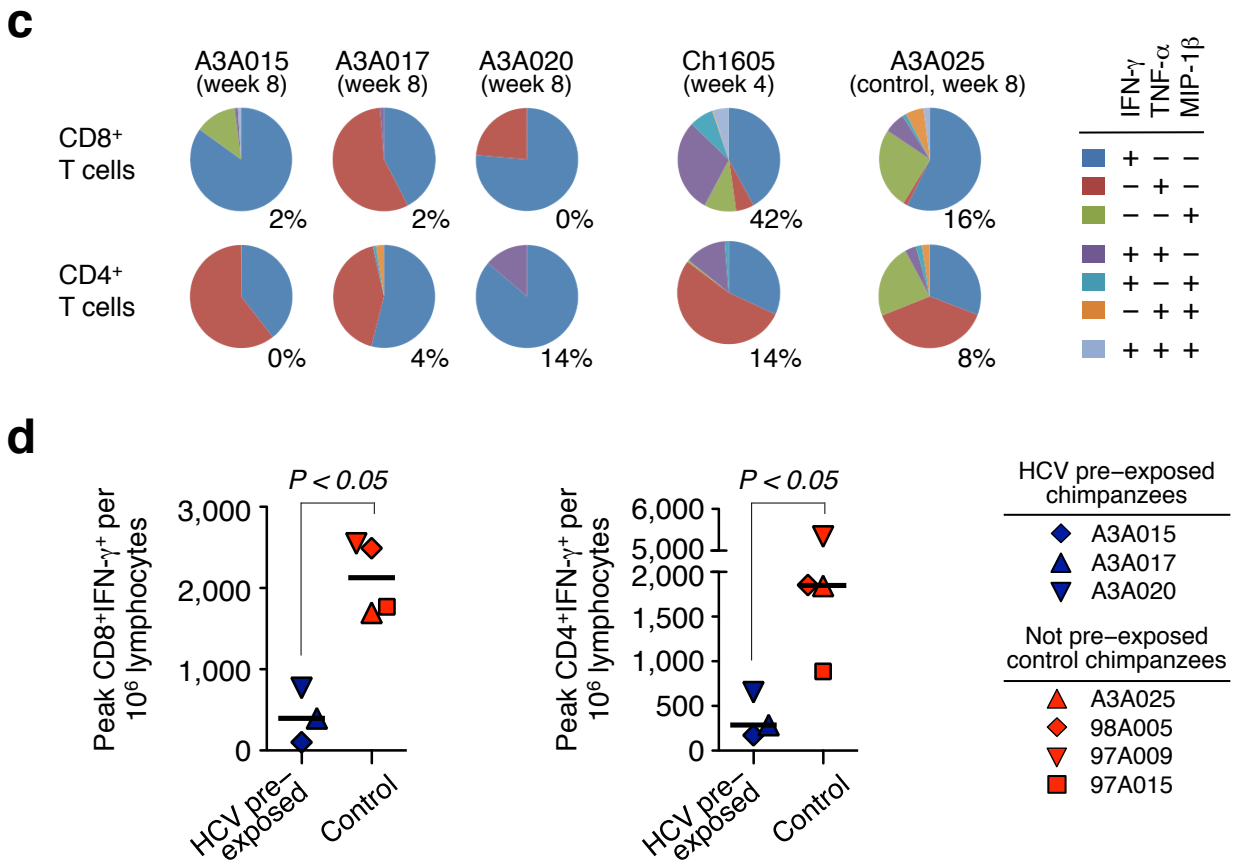


Supplementary Figure 1. Characterization of HCV–specific T cell responses that are induced by repeated exposure to blood samples from anti–HCV positive patients with trace amounts of HCV. (**a–c**) As indicated by vertical arrows chimpanzees A3A015, A3A017 were intravenously infused at weeks -42, -33, -24 and -15, and chimpanzee A3A020 was intravenously infused at week -26 with plasma or PBMC from anti–HCV–positive patients with trace amounts of HCV below the detection limit of the qualitative COBAS Amplicor HCV Test 2.0 (Roche) **(Supplementary Table 1)**[13]. PBMC were stimulated with 6 pools of overlapping HCV peptides, and the frequency of IFN-γ$^+$ (**a**) TNF-α$^+$ (**b**) and MIP-1β$^+$ (**c**) CD8$^+$ and CD4$^+$ T was determined by flow cytometry. (**d**) The relative frequencies of HCV–specific T cells that expressed different combinations of cytokines after stimulation with pools of overlapping HCV peptides at the indicated study time points are shown in the pie chart. The numbers to the bottom right of each pie chart indicate the percentage of HCV–specific T cells that produce 2 or more cytokines. (**e**) Memory and differentiation phenotype of HCV–specific IFN-γ–producing CD8$^+$ T cells at week 0 after stimulation with HCV–NS5B and HCV–NS3–specific peptide pools. Effec, effector cells; naïve, naïve cells, EM, effector memory cells; CM, central memory cells.

Supplementary Figure 2

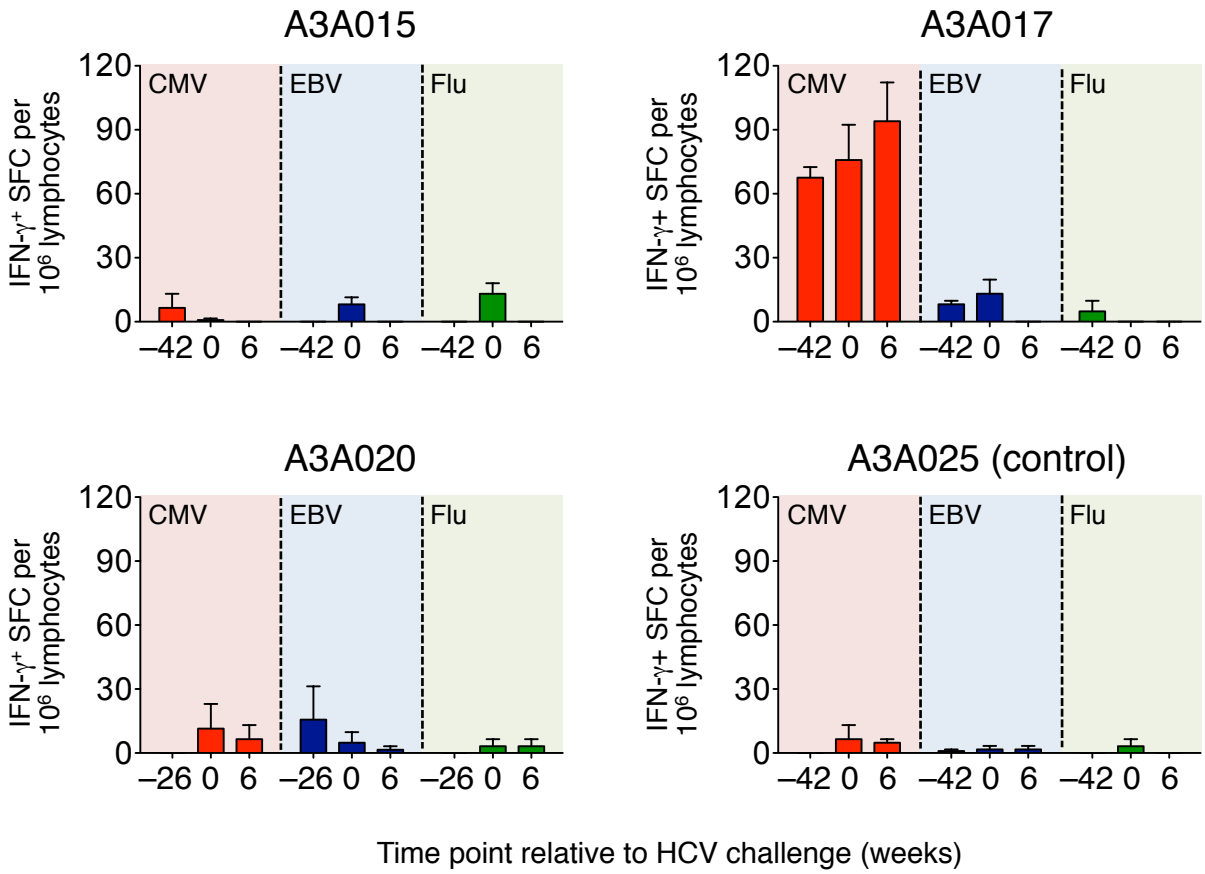


Supplementary Figure 2 (continued)



Supplementary Figure 2. Repeated exposure to blood samples with trace amounts of HCV suppresses HCV-specific CD8⁺ and CD4⁺ T cells upon HCV challenge.
(**a–b**) Chimpanzees A3A015, A3A017 and A3A020 were infused with human plasma or PBMC from anti–HCV–positive patients with trace amounts of HCV RNA, and chimpanzee A3A025 was infused with plasma and PBMC from anti–HCV–negative, HCV RNA–negative blood donors **(Supplementary Table 1)** prior to challenge with 100 CID_{50} HCV genotype 1a at week 0. Chimpanzee 1605 had spontaneously resolved a past acute HCV infection with systemic viremia prior to challenge with 100 CID_{50} HCV genotype 1a at week 0 **(Supplementary Table 1)**[14]. PBMC were stimulated with 6 pools of overlapping HCV peptides, and the number of TNF-α⁺ (**a**) and MIP-1β⁺ (**b**) $CD8^+$ and $CD4^+$ T cells per 10^6 lymphocytes was determined by flow cytometry. (**c**) The relative frequency of HCV–specific T cells that expressed different combination of cytokines after stimulation with pools of overlapping HCV peptides at the indicated study time points are shown in the pie chart. The numbers to the bottom right of each pie chart indicate the percentage of HCV–specific T cells that produce 2 or more cytokines. (**d**) The peak frequency of IFN-γ⁺$CD8^+$ and IFN-γ⁺$CD4^+$ T cells after challenge with 100 CID_{50} HCV genotype 1a is compared between HCV–pre-exposed chimpanzees and control chimpanzees. Statistical analysis: Mann–Whitney U test.

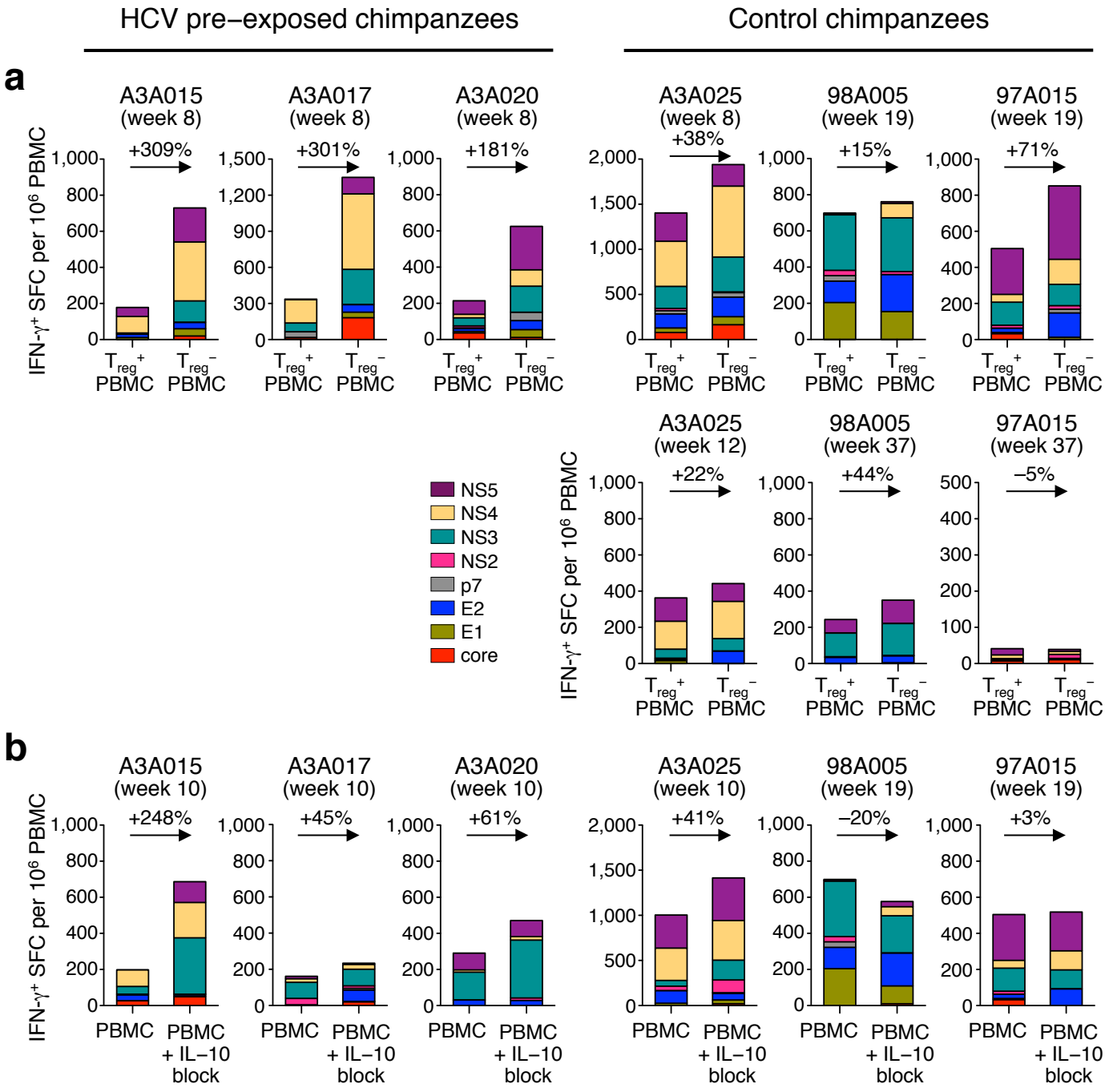
Supplementary Figure 3



Supplementary Figure 3. T cell responses to CMV are not affected by low–dose HCV exposure and 100 CID_{50} HCV–challenge.

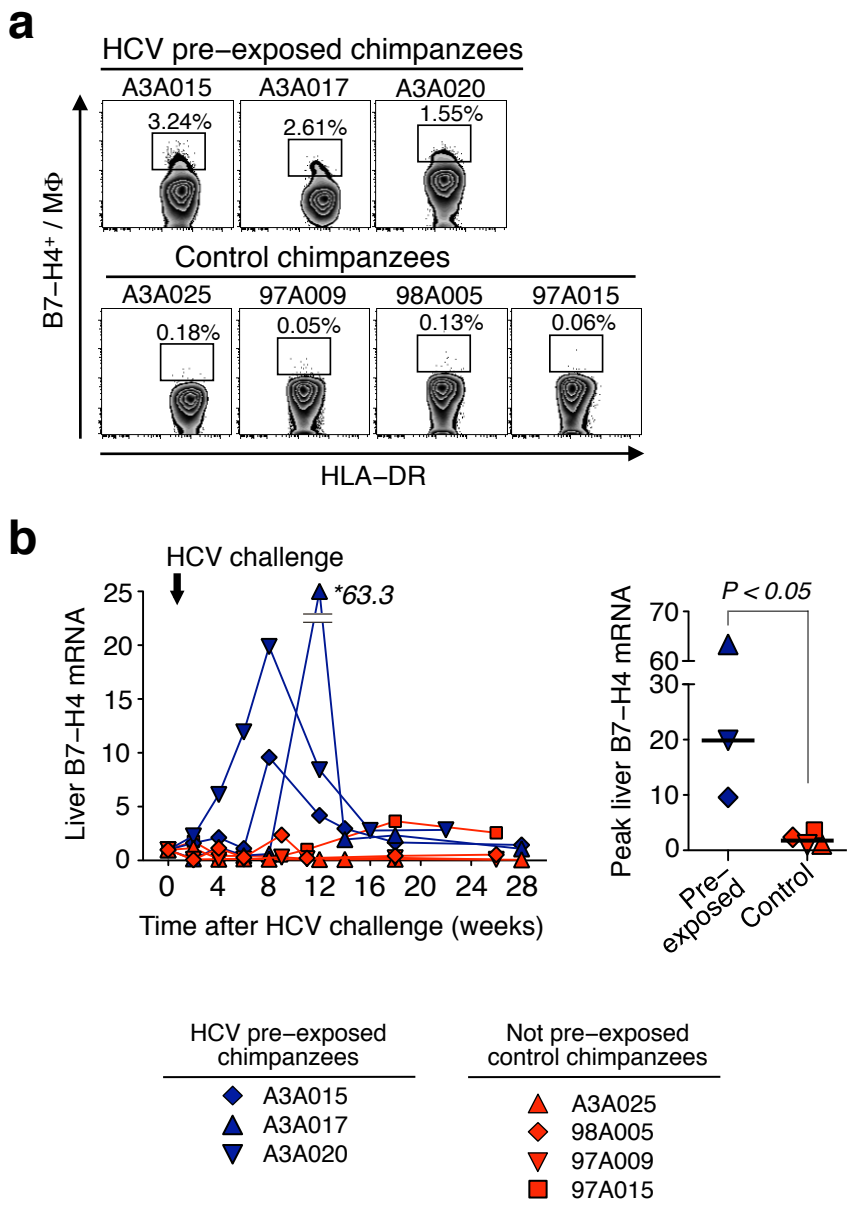
PBMC were obtained at the indicated study time points prior to low–dose HCV exposure (week -42 or week -26), after low–dose HCV exposure and prior to HCV challenge (week 0), and after HCV challenge (week 6) and stimulated in IFN-γ ELISpot assays with pools of influenza virus, CMV and EBV T cell epitopes.

Supplementary Figure 4



Supplementary Figure 4. ***In vitro*** **depletion of T_{reg} cells restores the suppressed HCV–specific T cell response after 100 CID_{50} HCV challenge of previously HCV–exposed chimpanzees.**
(**a**) PBMC were depleted of $CD4^+CD25^+CD127^{dim/–}$ T_{reg} cells (right bars) or depleted of $CD4^+CD25^+CD127^{dim/–}$ T_{reg} cells and subsequently reconstituted with the same cells (left bars) prior to stimulation with 18 pools of overlapping HCV peptides in IFN-γ ELISpot assays. (**b**) IFN-γ ELISpot assay with PBMC incubated with (right bars) or without (left bars) an IL–10 neutralizing antibody and an IL–10 receptor blocking antibody prior to stimulation with 18 pools of overlapping HCV. The weeks refer to the bleed date after challenge with 100 CID_{50} HCV.

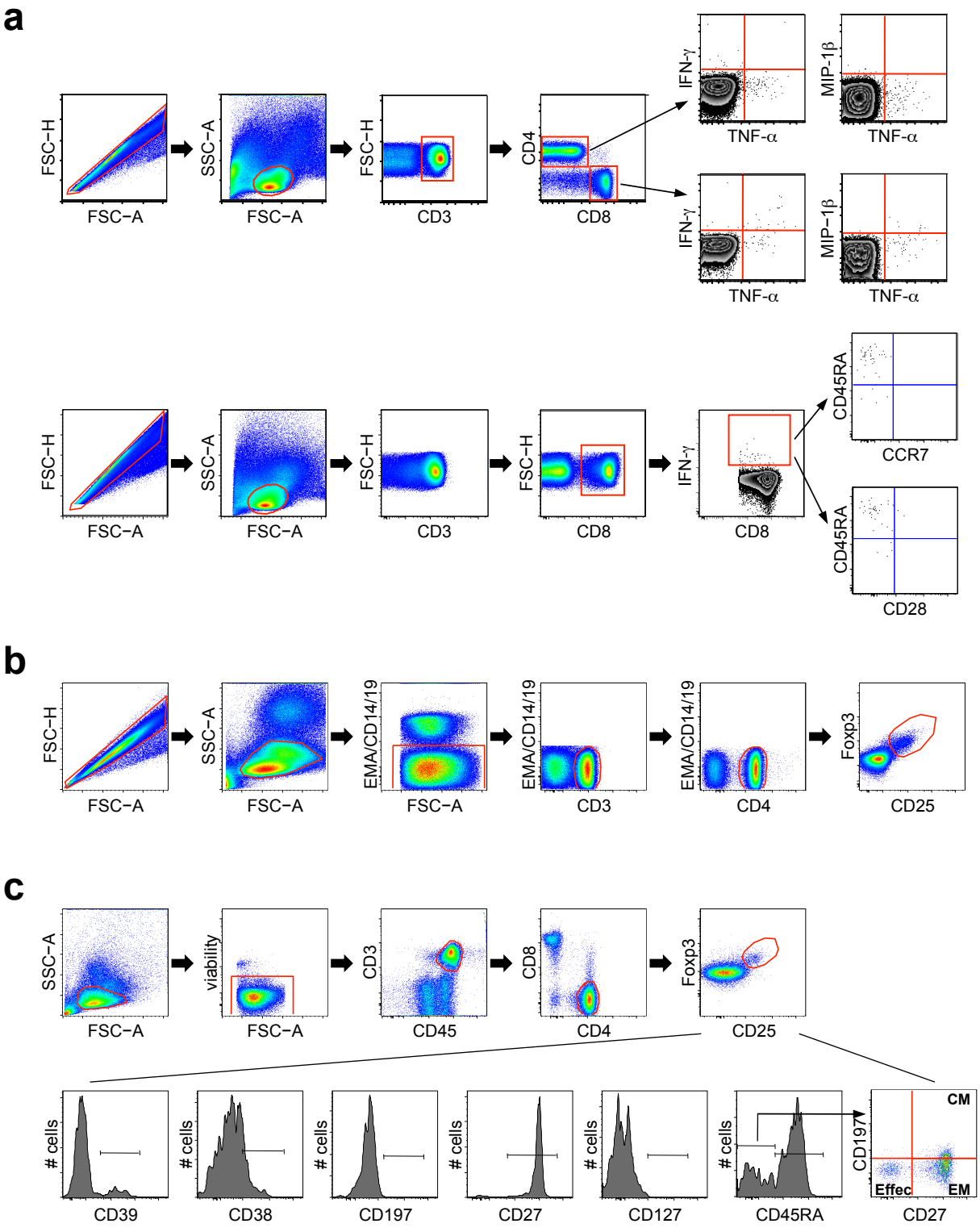
Supplementary Figure 5



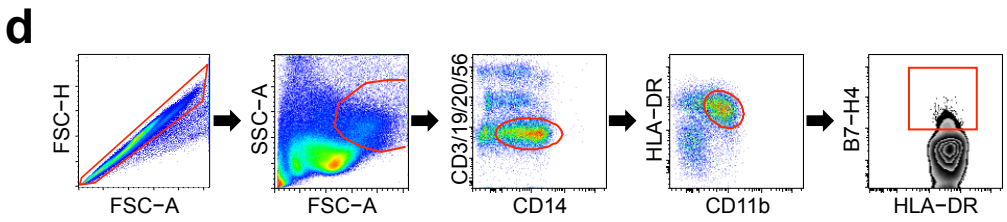
Supplementary Figure 5: Repeated exposure to blood samples with trace amounts of HCV predisposes to an increased frequency of circulating B7–H4⁺ macrophages and an increased intrahepatic B7–H4 mRNA levels upon subsequent HCV challenge.

(**a**) Frequency of B7–H4⁺ macrophages in the blood at 10 weeks after HCV challenge. (**b**) Intrahepatic B7–H4 mRNA levels were determined by real–time PCR, normalized to endogenous references (GAPDH and β7) and expressed as relative increase over mRNA levels at the time point of HCV challenge (week 0). Statistical analysis: Mann–Whitney U test. The timing of the intrahepatic B7-H4 mRNA peak coincided with the absence of HCV-specific T cell responses 8-12 weeks after HCV challenge in the HCV-exposed chimpanzees while the control chimpanzees mounted strong T cell responses at that time (**Fig. 2b,c**).

Supplementary Figure 6



Supplementary Figure 6 (continued)



Supplementary Figure 6. Flow cytometry gating strategy. (**a**) Gating strategy to determine the percentage of cytokine–producing $CD4^+$ and $CD8^+$ T cells by flow cytometry. $CD4^+$ and $CD8^+$ T cells were analyzed for IFN-γ, TNF-α and MIP-1β expression after sequential gating on single events, $CD3^+$ lymphocytes and $CD4^+$ and $CD8^+$ T cells (upper panels). $CD8^+$ IFN-γ^+ T cells were analyzed for CD45RA, CCR7 and CD28 expression after gating on single events and $CD3^+$ lymphocytes (lower panels). (**b**) Gating strategy to determine the percentage of $Foxp3^+CD25^+$ cells in the peripheral blood $CD4^+$ T cell population. The percentage of $Foxp3^+CD25^+$ T cells within in the $CD4^+$ T cell population was determined after gating on single events, exclusion of EMA^+, $CD14^+$ and $CD19^+$ cells and gating on $CD3^+$ lymphocytes. (**c**) Gating strategy to analyze subsets of $Foxp3^+CD25^+CD4^+$ T cells. Effec, effector cells; CM, central memory cells; EM, effector memory cells. (**d**) Gating strategy to determine the frequency of B7–$H4^+$ macrophages in the blood. The $CD14^+$HLA–DR^+CD11b^+ macrophage population was analyzed for B7–H4 expression after gating on single events in forward scatter–area versus forward scatter–height plots, and exclusion of $CD3^+$, $CD19^+$ $CD20^+$ and $CD56^+$ cells.

Supplementary Table 1. Infection schedule

Chimpanzee			Exposure 1		Exposure 2		Exposure 3		Exposure 4		HCV Challenge		
Number	**Sex**	**Age[a]**	**Week**	**Geno-type**	**Week**	**Geno-type**	**Week**	**Geno-type**	**Week**	**Geno-type**	**Week**	**Dose**	**Geno-type**
HCV pre-exposed chimpanzees [b]													
A3A015	M	6	-42	2	-33	1	-24	1b	-15	2b	0	100 CID_{50}	1a
A3A017	F	6	-42	3	-33	1	-24	2b	-15	1	0	100 CID_{50}	1a
A3A020	M	8	-26	3							0	100 CID_{50}	1a
Control chimpanzees [c]													
A3A025	F	6	-42	n.a. [d]	-33	n.a.	-24	n.a.	-15	n.a.	0	100 CID_{50}	1a
98A005	F	7									0	100 CID_{50}	1a
97A009	M	7									0	100 CID_{50}	1a
97A015	F	7									0	100 CID_{50}	1a
Chimpanzee with immunological memory after resolved acute HCV infection [e]													
1605	F										-93	3.2 CID_{50}	1a
		5									0	100 CID_{50}	1a

[a] Age at time of challenge with 100 CID_{50} HCV genotype 1a.

[b] Exposures 1, 2 and 3 consisted of intravenous infusion of 15-31 ml of plasma and exposure 4 consisted of intravenous infusion of 3.5×10^7 PBMC from patients with trace amounts of HCV below the detection limit of the qualitative COBAS Amplicor HCV Test 2.0 (Roche).

[c] Exposures 1, 2 and 3 consisted of intravenous infusion of 22-31 ml of plasma and exposure 4 consisted of intravenous infusion of 3.5×10^7 PBMC from healthy HCV-RNA-negative blood donors without a history of HCV infection infection.

[d] n.a., not applicable.

[e] Chimpanzee 1605 had been infected with 3.2 CID50 HCV genotype 1a and developed acute hepatitis with systemic viremia, which was spontaneously cleared [14].

Supplementary Table 2. Virological and clinical course of high–dose HCV challenge

	HCV viremia			Peak ALT level[b] [U/L]	Infection outcome
	Initial titer (week 2) [RNA copies/ml]	Maximum titer [RNA copies/ml]	Duration [weeks]		
Control chimpanzees					
A3A025	1.05×10^5	0.69×10^6	> 28	129	chronic
98A005	1.03×10^5	6.1×10^6	17	486	cleared
97A009	0.11×10^5	1.9×10^6	13	81	cleared
97A015	0.10×10^5	0.73×10^6	> 28	726	chronic
Mean ± s.e.m.	**0.57×10^5** $\pm 0.27 \times 10^5$	**2.34×10^6** $\pm 1.28 \times 10^6$		**356** ± 153	
HCV pre-exposed chimpanzees					
A3A015	1.24×10^5	1.55×10^6	>28	201	chronic
A3A017	1.33×10^5	0.32×10^6	>28	126	chronic
A3A020	1.74×10^5	4.62×10^6	16	91	cleared
Mean ± s.e.m.	**1.43×10^5** $\pm 0.15 \times 10^4$	**2.16×10^6** $\pm 1.28 \times 10^6$		**140** ± 33	
Chimpanzee 1605 with immunological memory after resolved acute HCV infection					
Primary infection	2.5×10^3	1×10^6	12	176	cleared
Rechallenge	n.t.[a]	6.2×10^4	4	70	cleared

[a] n.t., not tested at this time point.
[b] the timing of the ALT peak coincided with the decrease in viremia. There was no significant difference in peak ALT levels in control chimpanzees and pre-exposed chimpanzees (p=0.629, Mann–Whitney U test).

SUPPLEMENTARY METHODS

Influenza A virus, cytomegalovirus (CMV) and Epstein Barr virus (EBV) peptides. The influenza virus epitope pool contained the nucleoprotein peptides CTELKLSDY, ILRGSVAHK, SRYWAIRTR, KTGGPIYKR, RVLSFIKGTK, LPFDKTTVM and ELRSRYWAI; the matrix peptides ASCMGLIY, GILGFVFTLT, SIIPSGPLK; and the polymerase peptide FMYSDFHFI. The CMV epitope pool contained the UL–83 peptide LEGVWVPCPLPKRRRYRQ; the IE1 peptide SDEEEAIVAYTL and the pp65 peptides IPSINVHHY, EFFWDANDIY, TPRVTGGGAM and NLVPMVATV. The EBV peptide pool contained the LMP–2A peptide CLGGLLTMV; the BMLF–1 peptide GLCTLVAML; the BZLF–1 peptide RAKFKQLL; the EBNA–2 peptide RLRAEAQVK; the EBNA3A peptides FLRGRAYGL, RPPIFIRRL, QAKWRLQTL, YPLHEQHGM; the EBNA3C peptides RRIYDLIEL and EENLLDFVRF;the EBNA4 peptides IVTDFSVIK and AVFDRKSDAK; and the BRLF–1 peptides DYCNVLNKEF, RVRAYTYSK and ATIGTAMYK.

Detection of HCV E2–specific antibodies. HCV E2–specific antibodies were detected as previously described[1] using recombinant HCV E2 genotype 1a protein (ImmunoDiagnostics).

Analysis of B7H4 expression on macrophages. PBMC were stained with antibodies to $CD3_{PacBlue}$ (clone UCHT1, 1:50), $HLA–DR_{APC\ Cy7}$ (clone L243, 1:20) from BD Biosciences, $CD19_{PacBlue}$ (clone HIB19, 1:100), $CD56_{PacBlue}$ (clone MEM-188, 1:20) from BioLegend, $CD20_{PacBlue}$ (clone 2H7, 1:33), $CD11b_{AlexaFluor700}$ (clone CBRM1/5, 1:20), $B7–H4_{PE}$ (clone H74, 1:20) from eBioscience and $CD14_{PE–Cy5}$ (clone 61D3, 1:100, Serotec) (Supplementary Fig. 6d). All stained cells were analyzed on a LSRII flow cytometry (BD Biosciences) using FACS Diva version 6.1.2 (BD Biosciences) and FlowJo version 8.8.6 (Tree Star) software.

REFERENCE

1. Raghuraman, S., *et al*. Spontaneous clearance of chronic hepatitis C virus infection is associated with appearance of neutralizing antibodies and reversal of T cell exhaustion. *J Infect Dis* 205, 763-771 (2012).