HCV Persistence and Immune Evasion in the Absence of Memory T Cell Help.

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Materials and Methods.

HCV infections and CD4+ T cell depletion.

Chimpanzees (Pan troglodytes) were maintained under standard conditions for humane care and in compliance with NIH guidelines at the New Iberia Research Center, Lafeyette, LA (CB0627) and the Southwest Foundation for Biomedical Research (4X287). They were infected intravenously with 100 CID of HCV-1/910 stock for the first time in 1994 (CB0627) or 2001 (4X287). CBO627 was re-challenged in 2001 with the same virus. Six months following clearance of the 2001 infections, three doses of the anti-CD4 antibody cM-T412 (S1, S2) were administered intravenously 14, 10, and 7 days prior to a new HCV challenge with 100 CID of HCV-1/910. An initial dose of 5 mg of cM-T412 per kg body weight was followed by two reinforcing doses of 2.5 mg/kg for a total of 500mg of antibody per animal.

HCV RNA.

HCV RNA levels in frozen plasma collected in EDTA were analyzed by HCV 3.0 RNA branched DNA (bDNA) (sensitivity 3,500 GE/ml) and HCV RNA TMA QL (sensitivity 50 GE/ml) assays by Bayer Diagnostics, Berkeley, CA.

Isolation of lymphocytes from blood and liver.

Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient. To recover intrahepatic lymphocytes (IHL), liver biopsies were gently homogenized in PBS containing 1% FCS. CD8+ T cells were enriched using anti-human
CD8\(^+\) dynabeads (Dynal, Oslo, Norway) and expanded in bulk using anti-human CD3 antibodies as previously described (S3, S4). Briefly, CD8\(^+\) T cells were seeded in one well of a 24 well plate in T cell clone media [RPMI medium (Invitrogen, Carlsbad, CA), 10% heat inactivated FCS and 40 U/ml recombinant human IL-2 (a gift from Chiron Corporation, Emeryville, CA)]. Cells were expanded using anti-human CD3 monoclonal antibody (clone X35) (Immunotech, Marseille, France) at 0.05 \(\mu\)g/ml in the presence of 2 \(\times\) 10\(^6\) irradiated (3000 rads) human PBMCs per well as feeder cells. Cultures were fed every 3-4 days by replacing half of the culture media. Expanded cells were tested in IFN-\(\gamma\) elispot assays.

A similar technique was used to isolate intrahepatic CD4\(^+\) T cells. However, attempts to expand these cells failed, indicating depletion of CD4\(^+\) T cells from liver.

**Elispot assay.**

Nine pools containing 30-40 peptides each were prepared using a set of 301 overlapping peptides (Mimotopes Pty, San Diego, CA) encompassing the entire HCV-1 polyprotein according to the published sequence by Choo et al. (S5). Peptides were 20 amino acids long overlapping by 10 residues. Human IFN-\(\gamma\) elispot kits were purchased from U-cytech (Utrecht, The Netherlands). Peripheral blood and liver elispot assays were performed as previously described (S6).

**Flow cytometry.**

Depletion of T cell subsets in the peripheral blood after cM-T412 treatment was monitored by 4 color flow cytometry using anti-CD3 and subset-specific antibodies
against CD4 (Leu 3a) (BD-Pharmingen, San Diego, CA) and CD8 (DK25) (Dako, Denmark). The anti-CD4 antibody was previously shown to bind the CD4 protein in the presence of cM-T412 (S2).

For intracellular cytokine staining, $1 \times 10^5$ autologous EBV transformed B cell lines (BLCLs) were pulsed with either the specific peptide or the ovalbumin derived peptide (SIINFEKL) as a negative control (1 µg/ml) for 1 hour at 37 ºC. Cells were washed then cocultured with $1 \times 10^5$ CD8$^+$ T cells for 18 hrs at 37ºC. Brefeldin A (Sigma, St. Louis, MO) (10 µg/ml) was added after the first hour. The reaction was stopped by re-suspension in FACS buffer (PBS, 2% FCS, 0.1% NaN$_3$). Cells were then stained with anti-CD8 antibody for 20 min at 4°C, permeabilized using cytofix-cytoperm solution (BD-Pharmingen, San Diego, CA) and finally stained with anti-IFN-γ–FITC for 30 min. at 4°C. Cells were re-suspended in 4% formaldehyde solution and analyzed on a FACS Calibur instrument using CELLQuest software. 10,000 events were acquired in the CD8$^+$ T lymphocyte gate.

**Generation of CTL lines.**

Chimpanzee CBO627 was treated with cMT-412 and 168 days after infection CD8$^+$ T cell lines specific for the NS5A-2251 epitope were derived from liver tissue as described (S3, S4). Briefly, CD8$^+$ T cells enriched using anti-human CD8 dynabeads (Dynal, Oslo, Norway) as described above were cloned at a limiting dilution of 10 or 50 cells/well in 96 well plates. CD8$^+$ T cells were seeded in T cell clone media and expanded using anti-human CD3 monoclonal antibody (clone X35) (Immunotech, Marseille, France) at 0.05 µg/ml in the presence of $5 \times 10^4$ irradiated (3000 rads) human PBMCs per well as feeder
cells. Cultures were fed every 3-4 days by replacing half of the culture media. After 2 weeks, growing cell lines were transferred to 24 well plates and subjected to another round of anti-CD3 antibody stimulation in the presence of $2 \times 10^6$ irradiated human PBMCs. Cultures were fed every 3-4 days as described above. 250 independently derived CD8$^+$ T cell lines were tested in a CTL assay against autologous EBV transformed B cell lines (BLCLs) infected with recombinant vaccinia viruses expressing different regions of the HCV-1/910 polyprotein. 721.221 B cells transfected with individual Patr-A, -B or -C MHC class I molecules of CBO627 were used to determine the MHC class I restricting molecule. Epitope fine mapping was performed by intracellular staining for IFN-γ using peptide matrices spanning the antigen of interest. Peptides were 20 amino acids (aa) long overlapping by 10 residues. Truncations of the specific 20 aa peptide were synthesized to determine the minimal epitope required for recognition.

A T cell line specific for the Core-130 epitope was derived from anti-CD3 expanded CD8$^+$ intrahepatic lymphocytes (IHL) through 3 rounds of peptide stimulation. Briefly, CD8$^+$ IHL were expanded in bulk and tested in an IFN-γ liver elispot assay as described above and ref. S6. A response was detected against a peptide pool spanning the Core-E1 region. Fine specificity was mapped using peptide matrices spanning this pool by IFN-γ elispot. The specific 20 aa long peptide was used to pulse $2 \times 10^6$ autologous irradiated PBMCs at 10 µg/ml for 1 h at 37°C, cells were washed then used to stimulate $1 \times 10^6$ expanded IHL in one well of a 24 well plate in presence of T cell clone media. Proliferating cells were subjected to 2 more rounds of peptide stimulation at weekly
intervals. Truncations of the specific 20 aa peptide were synthesized to determine the minimal epitope by intracellular staining for IFN-γ as described above.

**Molecular Cloning and sequencing of HCV genomes.**

Plasma was collected in EDTA and frozen immediately at the indicated time points. RNA was extracted from 140 µl of thawed plasma using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. HCV RNA was reverse transcribed and PCR amplified by the sets of nested primers listed in Table S2. 5-30 µl of viral RNA were used for a single step reverse transcription followed by first round PCR with 20-100 pmoles of outer PCR primers and using the Access-RT PCR system (Promega, Madison, WI) or the SuperScript II One-Step RT-PCR for Long Templates (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, reverse transcription was performed at 48°C for 45 min, followed by heat inactivation at 94°C for 2 min. First round PCR was performed directly for 40 cycles (denaturing at 94°C for 30 sec, annealing at the temperatures indicated in Table 2 for 1 min, extension at 68°C for 2 min) and a final elongation cycle at 72°C for 7 min. First round PCR products were purified using the PCR Purification Kit (Qiagen) then 5 µl of the product was used in the second PCR using the nested set of primers listed in Table S2. Nested PCR was performed for 30 cycles (denaturing at 94°C for 30 sec, annealing at the temperatures indicated in Table 2, extension at 72°C for 1 min) followed by a final elongation step at 72°C for 15 min. PCR products were purified and cloned in pCR2.1-Topo (Invitrogen, Carlsbad, CA) using the Topo TA cloning kit (Invitrogen,
Calrsbad, CA) and sequenced. A minimum of 10 discrete molecular clones were sequenced for each sample.
Supporting online text.

cM-T412 is a mouse-human chimeric monoclonal antibody (S1). It was evaluated previously in chimpanzees to determine its safety for potential use in the treatment of human autoimmune diseases (S2). It was proven to be safe for use in chimpanzees, had no side effects and did not increase the frequency of opportunistic infections in the animals (S2). Efficiency of CD4⁺ T cell depletion was monitored by flow cytometry as described in Materials and Methods. CD4⁺CD3⁻CD11c⁺ cells representing the monocyte population, reached pretreatment levels within 7 days of administration of the last dose of antibody as previously described (S2).
### Table S1. HCV epitopes and MHC class I restriction in CBO627

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>Patr class I restriction</th>
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<tbody>
<tr>
<td>Core-130</td>
<td>FADLMGYIPL</td>
<td>B0401</td>
</tr>
<tr>
<td>NS5A-2251</td>
<td>LDSFDPLVA</td>
<td>B0401</td>
</tr>
<tr>
<td>NS5B-2509</td>
<td>CSLTPHSAK</td>
<td>A0303</td>
</tr>
<tr>
<td>NS5B-2541</td>
<td>SVWKDLLEDNVTPIDTTIMA</td>
<td>B0401</td>
</tr>
<tr>
<td>NS5B-2661</td>
<td>CDLDPQARVAI</td>
<td>B2401</td>
</tr>
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Table S2. Primer sequences used for amplification of genomic regions with selected CTL epitopes.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>PCR</th>
<th>Primer sequences (5’-3’)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core-130</td>
<td>First</td>
<td>Forward: CGC AGG GGC CCT AGA TTG GGT GTG</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GTA CAC AAT ACT CGA GTT AGG GCA ATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nested</td>
<td>Forward: GCG ACG AGA AAG ACT TCC GAG CGG</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGT GAC GTG GTA AAG CCC CGT GGA G</td>
<td></td>
</tr>
<tr>
<td>NS5A-2251</td>
<td>First</td>
<td>Forward: GAT CCC TCC CAT ATA ACA GCA GAG</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GAG GAC CAC CGT CCG CTT CTT CCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nested</td>
<td>Forward: GTG GCC AGC TCC TCG GCT AGC CAG</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTT TGG AGG TGG AAG CGG ACA GCC</td>
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</tr>
<tr>
<td>NS5B-2492-2686</td>
<td>First</td>
<td>Forward: CGT ACT CAA GGA GGT TAA AGC AGC GGC GTC AAA AG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GCA GCG GCG TCA AAA GTG AAG GCT AAC</td>
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</tr>
<tr>
<td></td>
<td>Nested</td>
<td>Forward: GTT CTC CCC CCT TGA ATT GGT AAG AG</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGT AAG AGG GCC CCC AAC ATA AAG C</td>
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References.