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Induction of potent and long-lasting CD4 and CD8 T-cell responses against hepatitis C virus by immunization with viral antigens plus poly(I:C) and anti-CD40

Aintzane Zabaleta [a], Laura Arribillaga [a], Diana Llopiz [a], Javier Dotor [a], Juan J. Lasarte [a], Jesús Prieto [a], Francisco Borrás-Cuesta [a], Juan I. Esteban [b], Josep Quer [b], Francesc Vayreda [c], Pablo Sarobe [a,*]

[a] *University of Navarra, Center for Applied Medical Research (CIMA), Division of Hepatology and Gene Therapy, 31008 Pamplona, Spain*
[b] *Liver Unit, Internal Medicine, Hospital Vall d'Hebron, 08035 Barcelona, Spain*
[c] *R&D Biokit S.A., 08186 Lliçà d'Amunt, Barcelona, Spain*

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Abstract

Development of vaccination strategies against hepatitis C virus (HCV) is of paramount importance. With this aim, we tested the ability of dendritic cell-activating reagents polyinosinic-polycytidylic acid (poly(I:C)) and anti-CD40, as adjuvants to induce T-cell responses against HCV. Immunization of mice with these adjuvants induced dendritic cell maturation in vivo. Also, joint administration of poly(I:C) and anti-CD40 plus HCV antigens had a synergistic effect on the induction of anti-HCV T-cell responses. CD4 responses displayed a Th1 cytokine profile, and CD8 responses could be induced by immunization with a minimal CD8 epitope. Addition of a low amount of NS3 protein (as a source of Th epitopes) to the immunization mixture enhanced CD8 responses, whereas immunization with higher doses of NS3 induced both CD4 and CD8 responses. Surprisingly, immunization with NS3 protein but not with CD8 epitopes was able to induce CD8 responses and able to recognize cells expressing HCV antigens endogenously. Moreover, immunization with these adjuvants activated NK cells, which in turn helped to induce Th1 responses. Finally, this combined immunization protocol afforded long-lasting T-cell responses, suggesting that this strategy may prove to be useful in vaccination and/or treatment of HCV infection.

Keywords: Hepatitis C; Vaccination; Adjuvant; Dendritic cell; T-cell

1. Introduction

Hepatitis C virus (HCV) infection is characterized by its high tendency to chronicity, which may evolve to cirrhosis and liver cancer (Dienstag, 1983). Although antiviral antibodies are elicited after infection, chronic state is characterized by the lack of cellular CD4 and CD8 T-cell immune responses (Cerny et al., 1995; Hoffmann et al., 1995; Lasarte et al., 1998; Rehermann et al., 1996; Sarobe et al., 1996). Thus, HCV may have developed strategies to specifically evade antiviral immune responses. Indeed, interaction of HCV proteins, such as core proteins, with T-cells, impairs T-cell effector functions (Kittlesen et al., 2000). Moreover, although there is some controversy, it has been postulated that dendritic cells (DC) expressing HCV proteins (Hiasa et al., 1998; Sarobe et al., 2002, 2003), as well as DC obtained from HCV patients (Bain et al., 2001; Kanto et al., 1999), have an impaired stimulatory ability, which may lead to a lack of response towards HCV antigens. To circumvent these problems, strategies need to be developed in order to induce potent and long-lasting responses against HCV for the prevention and treatment of HCV infection.

DC are a heterogeneous group of cells that constitute the most important antigen presenting cell (APC) population (Shortman and Liu, 2002). These cells capture antigens in the periphery, and after receiving an activation stimulus they migrate to lymphoid organs, where they present processed antigens on MHC

* Corresponding author at: University of Navarra, Center for Applied Medical Research (CIMA), Division of Hepatology and Gene Therapy, Pío XII 55, 31008 Pamplona, Spain. Tel.: +34 948 194700x3009; fax: +34 948 194717.
E-mail address: psarobe@unav.es (P. Sarobe).

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molecules, and produce chemokines and cytokines to attract and activate T-cells (Banchereau and Steinman, 1998). The activation process of DC, also known as maturation, is characterized by a higher expression of MHC molecules (signal 1), co-stimulatory molecules (signal 2) and polarizing cytokines such as IL-12 (signal 3). Maturation is induced by factors like pathogen components or host molecules common during inflammation or cell damage. These factors act on DC through receptors for microbial derived products, such as Toll like receptor (TLR) molecules (Janeway and Medzhitov, 2002; Kaisho and Akira, 2004), receptors for cytokines (TNF-α, IL-1, IFN-α) or receptors for cell associated ligands (e.g. CD40) (Caux et al., 1994). Since DC maturation is a requisite for efficient T-cell priming, immunization protocols have included different molecules able to activate DC. In the last years, the elucidation of the different molecular processes leading to DC maturation, and the characterization of the ligands able to bind to DC-activating receptors, have allowed the development of several immunization strategies combining antigens with different maturation stimuli. These studies have shown that antigens, when administered under these circumstances, are able to induce potent immune responses (Diehl et al., 1999; Liu et al., 1998; Schwarz et al., 2003; Thompson et al., 1998; Vabulas et al., 2000; Weiner et al., 1997). All these data prompted us to study the efficacy of inducing cellular immune responses against HCV antigens using HCV peptides and proteins in combination with two well known DC stimuli: polyinosinic-polycytidylic acid (poly(I:C)), a TLR3 ligand (Alexopoulou et al., 2001) similar to double stranded RNA generated during replication of viruses such as HCV, and anti-CD40, an antibody mimicking the effect of CD40L present on activated T-cells.

2. Materials and methods

2.1. Antigens and reagents

Peptides were synthesized manually in a multiple peptide synthesizer using Fmoc chemistry. Ninhydrin test of Kaiser was used to monitor every step. At the end of the synthesis they were cleaved and deprotected with trifluoroacetic acid and washed with diethyl ether. The purity and identity of peptides were checked by analytical HPLC and mass spectrometry. Purity of the peptides was always above 90%. HCV NS3 protein was obtained from different sources: NS3 produced in bacteria expressing the first 500 aa of the protein (genotype 1a) was obtained from Mikrogen (Martinsried, Germany), and was used to stimulate cells in some in vitro assays. NS3 used for immunization purposes was produced in our laboratory. A first batch of NS3 protein was produced in the yeast expression system *Pichia pastoris* by cloning a full-length NS3 sequence of genotype 1a (GenBank accession number DQ068198 B3 clone) (Quer et al., 2005). This protocol will be described in detail elsewhere (Quer et al., manuscript in preparation). A second batch was expressed in bacteria by cloning the full-length sequence of NS3 (genotype 1b, GenBank accession number D90208). It was obtained by digestion with Sal*I* and Not*I* of plasmid gWIZ expressing NS3 (kindly provided by Dr. G Inchauspe, Lyon, France) and cloned between the Bsr*GI* and Not*I* sites of plasmid pET-45b(+) (Novagen, Madison, WI). After obtaining the protein from inclusion bodies, it was purified by affinity chromatography (Histrap, Pharmacia) and ion exchange chromatography (Hitrap-DEAE sepharose FF, Pharmacia) using an FPLC platform (AKTA, Pharmacia). Endotoxin levels of NS3 proteins were tested using a quantitative chromogenic Limulus Amebocyte Lysate QCL 1000 assay (Cambrex, Walkersville, MD) and were <0.5 EU/μg protein. Poly(I:C) was obtained from Amersham (Barcelona, Spain) and anti-CD40 antibodies were purified from FGK-45 hybridoma (Rolink et al., 1996).

2.2. Mice

Six- to 8-week-old BALB/c and C57BL/6 mice were obtained from Harlan (Barcelona, Spain). HHD mice, transgenic for human HLA-A2.1 and beta 2 microglobulin molecules (Pascolo et al., 1997), were a kind gift from Dr. F. Lemonnier (Institut Pasteur, Paris, France). They were maintained in pathogen-free conditions and treated according to guidelines of our institution, after study approval by the review committee.

2.3. Cell lines

T2 cells were a kind gift from J.A. Berzofsky (NIH, Bethesda, MD) and were used as target cells in chromium release assays with CTL from HHD mice. The NK cell-sensitive YAC-1 cell line was obtained from ATCC (Manassas, VA). T1 cells transfected with the entire HCV polyprotein (T1/HCVcon) (Wolk et al., 2005) and non-transfected T1 cells were a kind gift from D. Moradpour (Lausanne, Switzerland). These lines were grown in complete medium [RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 50 μM 2-mercaptoethanol; CM (complete medium)]. Medium for T1/HCVcon cells contained also 2 mg/ml of G418. EL-4 cells transfected with NS3/4A (EL-4 NS3/4A) were obtained from Dr. M. Sallberg (Karolinska Institute, Sweden) and were grown in CM containing 800 μg/ml of G418 (Frelin et al., 2004).

2.4. Immunization

Groups of three mice were immunized with poly(I:C) (50 μg/mouse), anti-CD40 (50 μg/mouse) or both, combined with different antigens: peptide E1-121 (50 μg/mouse), peptide 1073 (50 μg/mouse), peptide 1073 (50 μg/mouse) + NS3 protein (5 μg/mouse), or NS3 protein alone (5–500 μg/mouse). When using peptides or peptides plus NS3, adjuvants and antigens were injected simultaneously i.p. resuspended in 0.5 ml of PBS, whereas when using a higher amount of NS3 (>25 μg/mouse), anti-CD40 was injected intraperitoneally (i.p.) (500 μl), and 6 h later NS3 was administered i.p (500 μl) and poly(I:C) i.v. (200 μl). In NK cell depletion experiments mice were injected i.p. with rabbit anti-AsGM1 (40 μl/mouse) (Wako Chemicals, Neuss, Germany) or an equivalent amount of rabbit IgG (Sigma, Madrid, Spain) as a control, on days 2 and 0, being 0 the day of immunization. Six days after immunization (or 60 days, in the

case of memory responses), animals were sacrificed and their spleens were removed, homogenized and pooled for immunological analysis.

2.5. Stimulation of spleen cells to measure cytokine production in supernatants

Spleen cells were resuspended in CM and plated at 8×10^5 cells/well in 0.2 ml in U-bottomed 96-well plates in the absence or in the presence of recombinant HCV NS3 protein or peptides. In antibody blocking experiments, cells were incubated with 10 μg/ml of anti-CD4 or anti-CD8 antibodies, obtained from GK1.5 and H35.17.2 hybridomas, respectively. When measuring responses against EL-4 NS3/4A cells, 3×10^5 splenocytes were stimulated with 3×10^4 mitomycin C-treated EL-4 NS3/4A or EL-4 non-transfected cells. In all cases, 48-h supernatants were harvested and IFN-γ and IL-4 were measured by ELISA (BD-Biosciences, San Diego, CA), according to the Manufacturer's instructions.

2.6. ELISPOT

ELISPOT assays for IFN-γ were carried out using a kit from Mabtech (Sweden) according to the Manufacturer instructions. Briefly, plates (Multiscreen HTS; Millipore, Bedford, MA) were coated with purified anti-IFN-γ AN18 antibody (15 μg/ml). After overnight incubation, plates were washed with PBS and blocked for 3 h with DMEM containing 10% FCS. Then, 10^5 immune splenocytes were cultured in triplicate in the absence or in the presence of CTL peptides (10 μM) or rNS3 (1 μg/ml). One day later, plates were washed with PBS and incubated with biotinlylated anti-IFN-γ R4-6A2 antibody (1 μg/ml). After 4 h, plates were washed and incubated with a 1/500 dilution of streptavidin-peroxidase. One hour later, plates were washed and developed with freshly prepared DAB solution. The reaction was stopped with distilled water and spots were counted using an automated ELISPOT reader (CTL, Aalen, Germany).

2.7. Measurement of CTL and NK cell lytic activity

To measure CTL responses against peptides, splenocytes from immunized animals were incubated with peptides (10 or 0.1 μM) for 2 h at 37 °C, washed twice and cultured in 24 well plates at 7.5×10^6 cells/well. To measure responses against T1/HCVcon cells, 7.5×10^6 immune splenocytes were stimulated with 7.5×10^5 mitomycin C-treated T1/HCVcon cells. In both cases, 2 days later, IL-2 (Boehringer-Mannhein GmbH, Germany) (2.5 U/ml) was added to the wells, and on days 5–7, effector cells were harvested for chromium release assays. Lytic activity was measured by incubating for 4 h different numbers of effector cells with 3000 $Na_2{}^{51}CrO_4$-labeled target cells: T2 cells with or without peptide, or T1 and T1/HCVcon cells. In the case of NK cell lytic activity, freshly obtained splenocytes were incubated with 3000 chromium labelled YAC-1 cells and supernatants were harvested 4 h later. Percentage of specific lysis was calculated according to the formula: (cpm experimental − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100, where spontaneous lysis corresponds to target cells incubated in the absence of effector cells and maximum lysis is obtained by incubating target cells with 5% Triton X-100.

2.8. Flow cytometric analysis of DC

Analysis of the expression of DC surface molecules was done 15 h after treatment with different stimuli. Spleen cells were doubled-stained at 4 °C in PBS containing 2% FCS, with the following FITC-labelled antibodies: anti-CD80, anti-CD86, anti-I-A^d, anti-H-2 K^d and isotype control, in combination with anti-CD11c-PE antibodies (all from BD-Biosciences). After 30 min, cells were washed and surface expression of the different markers was analyzed on the $CD11c^+$ population by using a FACSCalibur™ flow cytometer (Becton Dickinson).

2.9. Statistical analysis

Statistical analyses were performed using two-way ANOVA test with the SPSS 9.0 program for Windows.

3. Results

3.1. Poly(I:C) and anti-CD40 administration induces in vivo maturation of dendritic cells

Administration of different DC activating agents together with antigens has proved to be very useful for the induction of potent immune responses. In the case of HCV infection, lack of cellular anti-viral responses has been associated with disease chronicity. Since developing strategies for the induction of potent anti-HCV immune responses is a subject of great interest, we decided to study the effect on the activation of DC of the combination of an anti-CD40 activating antibody with poly(I:C), a TLR3 ligand which mimics the dsRNA generated in a viral infection.

In a first set of experiments, BALB/c mice were injected i.p. with PBS, poly(I:C), anti-CD40 or a combination of both stimuli. Fifteen hours later, activation of splenic DC was analyzed by flow cytometry. As shown in Fig. 1A and B, analysis of $CD11c^+$ cells from spleen showed that treatment with anti-CD40 was only able to slightly up-regulate the expression of I-A^d class II molecules, compared to PBS treated mice, without any effect on the other markers. Poly(I:C) treatment increased the expression of all four analyzed markers, CD86 being the most up-regulated. Finally, combination of poly(I:C) and anti-CD40 also increased the expression of activation markers, reaching levels slightly higher than those obtained with poly(I:C) alone. Thus, administration of poly(I:C) and anti-CD40 is able to induce maturation of DC in vivo.

3.2. Poly(I:C) and anti-CD40 administration synergizes in the induction of anti-HCV T-cell responses

Since we had seen that injection of poly(I:C), and to a minor extent anti-CD40, was able to induce in vivo activation of DC, the efficacy of these DC activating stimuli to induce

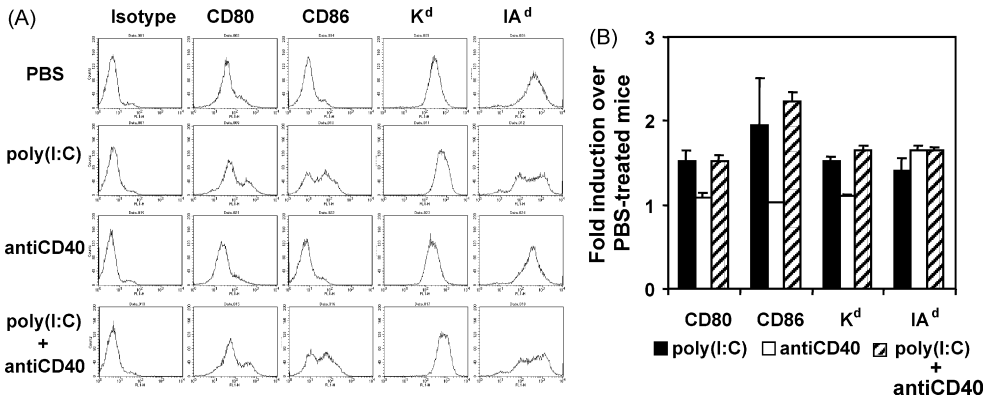

Fig. 1. Administration of anti-CD40 and poly(I:C) induces in vivo maturation of dendritic cells. BALB/c mice (three per group) were injected i.p. with PBS, 50 μg of poly(I:C), 50 μg of anti-CD40 or poly(I:C) plus anti-CD40. Fifteen hours later, animals were sacrificed and splenocytes were doubled-stained with PE-labeled anti-CD11c antibodies in combination with FITC-labeled isotype control, anti-CD80, CD86, K^d or IA^d antibodies. Expression of DC activation markers was analyzed for each mouse on the $CD11c^+$ population. (A) FACS results obtained in a representative mouse from each treatment group are shown. (B) Results (mean ± S.D. of three mice) are expressed as fold-induction of activation markers relative to PBS-treated control mice, and are representative of two different experiments.

anti-HCV immune responses was tested by immunization with different HCV antigens. We first used peptide E1-121 (HVSGHRMAWDMMMNW, spanning residues 121–135 from HCV E1 protein) previously characterized as immunodominant in the context of E1 after immunization of BALB/c mice (Bruna-Romero et al., 1997; Lopez-Diaz de Cerio et al., 1999). To test the effect of poly(I:C) and anti-CD40 on the induction of responses against E1-121, BALB/c mice were immunized i.p. with the peptide in PBS in combination with these stimuli, and responses were measured 6 days later. Fig. 2A shows that neither E1-121 nor E1-121 plus anti-CD40 were able to induce IFN-γ production. Combination of peptide with poly(I:C) induced around 0.5 ng/ml of IFN-γ, similar to that induced when immunizing with this peptide in IFA (Lopez-Diaz de Cerio et al., 1999). Finally, immunization of E1-121 in combination with poly(I:C) and anti-CD40 showed strong synergy, inducing a response six to eight-fold higher than that induced by peptide and poly(I:C) ($p < 0.001$; anti-CD40 plus poly(I:C) versus all the other groups, considered all comparisons separately). Responses induced against E1-121 when using these adjuvants were mediated by $CD4^+$ cells, since they were completely inhibited when splenocytes were cultured in the presence of anti-CD4 antibodies, whereas anti-CD8 antibodies had no effect (Fig. 2B). IL-4 production was not detected in any of the groups studied, suggesting that Th1 responses are induced by this immunization protocol (data not shown). $CD8^+$ responses are also important for anti-HCV immunity; therefore, we decided to use peptide 1073 (CINGVCWTV, spanning residues 1073–1081 from HCV NS3 protein), which has been described as an HLA-A2-restricted CTL epitope recognized by HCV patients (Cerny et al., 1995). In order to induce responses against this peptide, we used HHD transgenic mice, which carry a chimeric human HLA-A2 molecule, and measured lytic activity against peptide-loaded MHC class II negative T2 cells. Experiments, equivalent to those carried out with peptide E1-121, showed that, when measuring lytic responses, immunization with 1073, or 1073 plus anti-CD40, did not induce any measurable response, whereas 1073 plus poly(I:C), induced a response able to recognize peptide-loaded target cells. Finally, combination of 1073

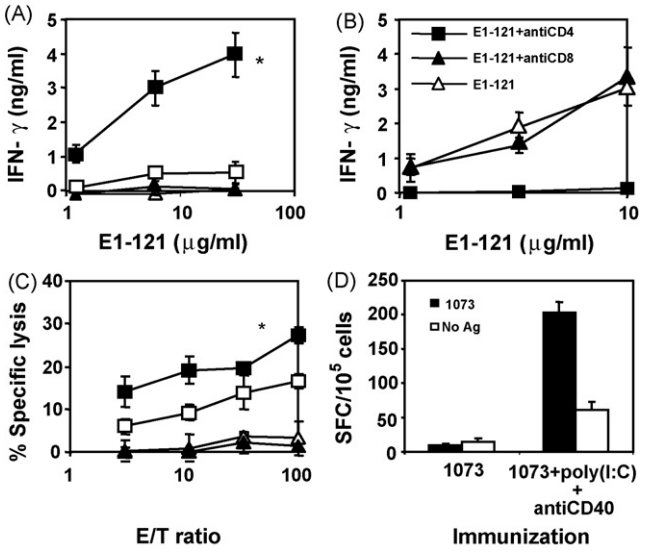

Fig. 2. Synergy between anti-CD40 and poly(I:C) on the induction of anti-HCV CD4 and CD8 responses. (A) BALB/c mice (three per group) were immunized i.p. with 50 μg/mouse of peptide E1-121 alone (open triangles), E1-121 plus anti-CD40 (closed triangles), E1-121 plus poly(I:C) (open squares) or E1-121 plus anti-CD40 and poly(I:C) (closed squares). Six days later their splenocytes were pooled and stimulated with different concentrations of peptide E1-121, and supernatants were harvested after 48 h of culture to measure IFN-γ production ($^*p < 0.001$; E1-121 plus anti-CD40 and poly(I:C) vs. all the other groups, considering all peptide concentrations and comparing with each group separately). (B) Pooled splenocytes from mice immunized with E1-121 plus anti-CD40 and poly(I:C) were cultured with E1-121 with or without anti-CD4 or anti-CD8 antibodies and IFN-γ was measured as in A. (C) HHD mice (three per group) were immunized i.p. with 50 μg/mouse of peptide 1073 alone (open triangles), 1073 plus anti-CD40 (closed triangles), 1073 plus poly(I:C) (open squares) or 1073 plus anti-CD40 and poly(I:C) (closed squares). Six days later their splenocytes were pooled and stimulated in vitro with peptide 1073 and IL-2 for 6–7 days. Then, lytic activity was measured against T2 target cells loaded with 10 μM of 1073. Responses against T2 cells incubated in the absence of 1073 were always below 3% ($^*p < 0.001$; 1073 plus anti-CD40 and poly(I:C) vs. all the other groups, considered all E/T ratios and comparing with each group separately). (D) HHD mice (three per group) were immunized i.p. with 50 μg/mouse of peptide 1073 alone or with 1073 plus anti-CD40 and poly(I:C). Six days later their splenocytes were pooled and responses against 1073 were measured as IFN-γ production in an ELISPOT assay. Results are expressed in all figures as mean ± S.D. of triplicate cultures.

with poly(I:C) and anti-CD40 induced the strongest responses (Fig. 2C) ($p < 0.001$; anti-CD40 plus poly(I:C) versus all the other groups, all comparisons considered separately). CD8 responses were also measured as IFN-γ production by ELISPOT (Fig. 2D), showing that they were not induced after 1073 immunization. In agreement with previous experiments, clear responses against peptide 1073 were induced after immunization with 1073 plus poly(I:C) and anti-CD40. Thus, combination of HCV antigens with poly(I:C) and anti-CD40 was able to synergistically induce CD4 and CD8 responses.

3.3. Low dose of NS3 protein helps induction of CTL responses induced by peptide plus poly(I:C) and anti-CD40, whereas high dose of NS3 directly induces T-helper and CTL responses

Previous experiments had shown that administration of a minimal CTL epitope plus poly(I:C) and anti-CD40 was able to induce CTL responses, in spite of the absence of antigens recognized by $CD4^+$ T-cells which could provide CD4 T-cell help for CTL activation. Although the role of CTL is very important in viral clearance through the lysis of infected cells and cytokine production, $CD4^+$ T-cells help in CTL induction and may be crucial to stimulate efficient memory CTL. Thus, we decided to test whether the combination of a minimal CTL epitope with an antigen bearing Th epitopes, would increase the efficacy of this immunization protocol. As a source of Th epitopes we chose HCV NS3 recombinant protein (rNS3), which was included in the immunization mixture. First, we tested the ability of poly(I:C) and anti-CD40 to induce T helper responses against NS3 using a dose of 5 μg/mouse. As shown in Fig. 3A, strong IFN-γ production was induced after immunization with rNS3 and these adjuvants; it was mediated by $CD4^+$ cells. No IL-4 production was found in this case (data not shown), confirming the Th1 profile, as for immunization with peptide E1-121. Regarding CD8 responses, immunization with this amount of rNS3 did not induce any response against 1073, measured either as IFN-γ production or as lytic activity (data not shown). Then, we immunized with 1073 + rNS3, which induced stronger responses against 1073 than immunization with 1073 alone, as measured in ELISPOT assays (Fig. 3B). Clear responses against NS3 were also detected in the group immunized with 1073 + rNS3. When measuring lytic activity against target cells loaded with 1073 in these groups, it was found that although not as clear as when using ELISPOT assays, 1073 + rNS3 immunization induced higher responses than immunization with 1073 (Fig. 3C) ($p < 0.001$). Since Th cells may also help CTL induction through the activation of high avidity CTL (Kumaraguru et al., 2004), we analyzed CTL responses elicited after stimulation with a low peptide concentration (0.1 μM), instead of the high concentration (10 μM) used in Fig. 3B and C. As shown in Fig. 3D, stronger CTL responses of high avidity were induced after immunization with 1073 + rNS3 than after immunization with 1073 alone ($p < 0.001$). These results indicate that, although immunization with 1073 plus anti-CD40 and poly(I:C) is able to induce CTL responses independently of Th cells, inclusion of Th epitopes in the immunization mixture enhances the efficacy of high avidity CTL induction.

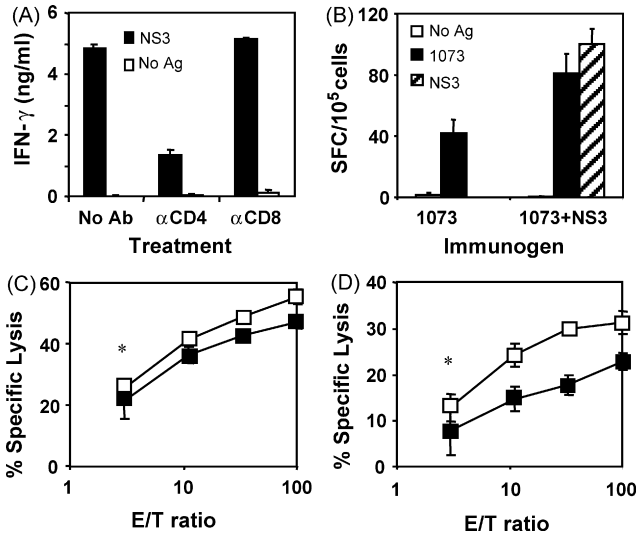

Fig. 3. Immunization with low dose of rNS3 protein plus poly(I:C) and anti-CD40 induces CD4 responses and helps activation of peptide-induced CD8 responses. (A) HHD mice (three per group) were immunized i.p. with rNS3 (5 μg/mouse) plus poly(I:C) and anti-CD40. Six days later their splenocytes were pooled and stimulated in vitro with rNS3 (1 μg/ml) in the presence or in the absence of blocking antibodies, and IFN-γ production in supernatants was measured 2 days later. (B) HHD mice (three per group) were immunized with poly(I:C) and anti-CD40 plus 1073 or plus 1073 + rNS3 (5 μg/mouse) and their splenocytes were pooled and stimulated in vitro with peptide 1073 (10 μM), rNS3 (1 μg/ml) or left unstimulated. Responses were measured as IFN-γ production in an ELISPOT assay. (C and D) HHD mice (three per group) were immunized with poly(I:C) and anti-CD40 plus 1073 (closed symbols) or plus 1073 + rNS3 (5 μg/mouse) (open symbols). Six days later their splenocytes were pooled and stimulated in vitro with peptide 1073 at 10 μM (C) or 0.1 μM (D). Five days later, CTL activity of stimulated cells was tested against T2 cells loaded with the same peptide concentration used for stimulation. Results are expressed in all cases as mean ± S.D. of triplicate cultures ($p < 0.001$; 1073 + rNS3 vs. 1073, considered all E/T ratios).

Since experiments with model proteins such as OVA (Ahonen et al., 2004) had shown that immunization with a high amount of protein was able to induce CD8 responses, we decided to test whether higher amounts of rNS3 were able to induce CD8 responses. To this purpose, we immunized HHD mice with 500 μg of rNS3 plus poly(I:C) and antiCD40. In this case, due to the denaturing character of the buffer used in the rNS3 solution, we tried to avoid mixing rNS3 with anti-CD40 and poly(I:C), so the antibody was administered i.p. 6 h before the antigen and poly(I:C). In order to measure CD8 responses, we used again peptide 1073, as well as peptides 1038 and 1406 from NS3 (GLLGCIITSL, spanning residues 1038–1047, and KLVGLGINAV, residues 1406–1415) also described as HLA-A2-restricted CTL epitopes (Cerny et al., 1995; Martin et al., 2004). As shown in Fig. 4A, splenocytes cultured in vitro for several days with these peptides were able to lyse target cells pulsed with the corresponding CTL epitopes. Moreover, clear IFN-γ production against these peptides was detected in the culture supernatants of splenocytes, 1073 being the most immunogenic (Fig. 4B), as in lytic assays. In order to measure CD4 responses (Fig. 4C) immune spleen cells were stimulated

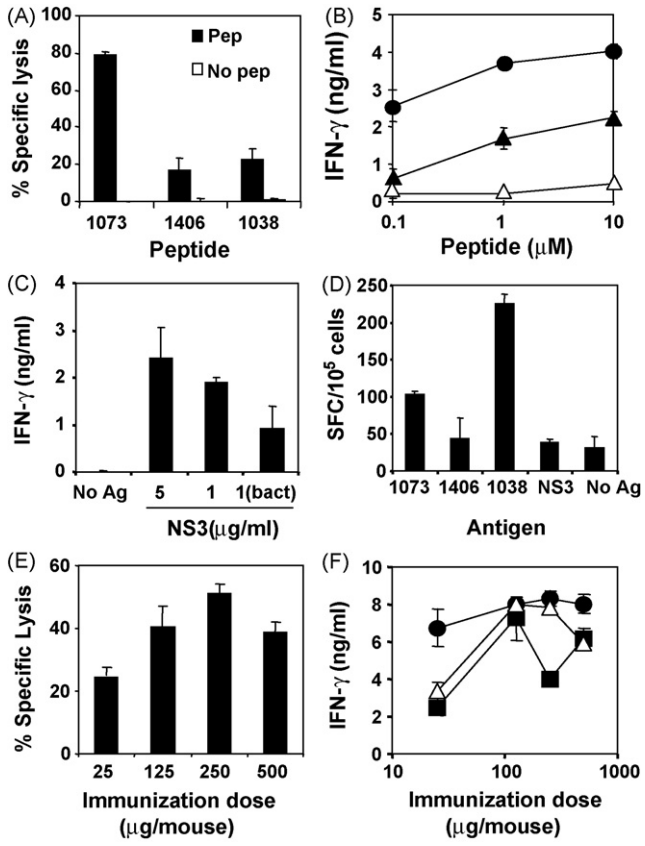

Fig. 4. Immunization with high dose of rNS3 protein plus poly(I:C) and anti-CD40 induces CD4 and CD8 responses. (A) HHD mice (three per group) were immunized with poly(I:C) and anti-CD40 plus rNS3 (500 μg/mouse). Six days later, their splenocytes were pooled and stimulated with CTL peptides 1073, 1406 or 1038 (10 μM). After 5 days of culture, lytic activity of effector cells (E/T ratio of 100) was tested against T2 cells loaded with the corresponding peptide. (B) Splenocytes from mice shown in A were cultured in the presence of CTL epitopes 1073 (closed circles), 1406 (open triangles) or 1038 (closed triangles), and after 48 h IFN-γ production was measured in the supernatants. (C) Splenocytes from mice shown in A were also stimulated with rNS3 protein used for immunization (5 or 1 μg/ml) or with a commercial NS3 (1 μg/ml) to measure IFN-γ production in the supernatants. (D) Splenocytes from mice immunized as in A were pooled and stimulated with CTL epitopes (10 μM) or rNS3 (1 μg/ml) and IFN-γ production was measured in ELISPOT assays. (E) HHD mice (three per group) were immunized with different doses of rNS3. Six days after immunization, their splenocytes were pooled and stimulated for 1 week with peptide 1073 (10 μM). Then, lytic activity was measured against peptide-pulsed T2 target cells (E/T ratio of 100). Lytic activity against T2 cells incubated in the absence of peptide was in all experiments below 3%. (F) Splenocytes from mice shown in (E) were also stimulated with CTL peptides 1073 (closed circles) and 1038 (closed squares) at 10 μM or with rNS3 (open triangles) at 1 μg/ml and IFN-γ production was measured in the supernatants 2 days later. Results are expressed in all figures as mean ± S.D. of triplicate cultures.

with rNS3, and they induced high amounts of IFN-γ. Moreover, these cells not only recognized our yeast-produced rNS3 used for immunization, but also commercial rNS3 expressed in bacteria, confirming the specificity of the response. Finally, CD8 and CD4 responses were also measured as IFN-γ production in ELISPOT assays (Fig. 4D). This experiment confirmed previous results against immunodominant epitopes 1073 and 1038, with strong responses, and against subdominant epitope 1406, with a very low response. However, a weak response was detected against rNS3 protein.

To find the minimal amount of rNS3 able to induce CTL responses, we carried out titration experiments by immunizing mice with 500, 250, 125 or 25 μg/mouse of rNS3. Splenocytes from these immunized animals were stimulated with peptide 1073 and after 1 week, lytic responses against peptide-loaded target cells were measured. As shown in Fig. 4E, a dose as low as 25 μg/mouse was able to induce CTL responses recognizing peptide 1073. This ability to activate CD8 cells was confirmed when responses were measured as IFN-γ production in supernatants against 1073 and 1038 epitopes (Fig. 4F). As expected, all immunization doses could activate CD4 responses against rNS3 (Fig. 4F). Thus, immunization with low amounts of rNS3 plus poly(I:C) and anti-CD40 induced only CD4 responses, whereas immunization with amounts above 25 μg/mouse were able to induce both CD4 and CD8 responses.

3.4. Immunization with poly(I:C) plus anti-CD40 and rNS3 induces T-cell responses in different mouse strains

Induction of T-cell responses by immunizing with a protein, instead of defined peptide epitopes, would have the advantage of using a panel of several epitopes that might be presented by different MHC molecules. To test this property in our immunization model we immunized C57BL/6 mice with rNS3 (100 μg/mouse) and measured CD8 and CD4 T-cell responses. A second group of mice receiving a booster immunization was also included. These experiments showed that immunization with rNS3 plus poly(I:C) and antiCD40 was able to induce CD8 responses against the D^b-restricted CTL epitope 1629 (GAVQNEVTL, residues 1629–1637 from NS3) (Frelin et al., 2003), measured as IFN-γ production (Fig. 5A). Also, it induced Th responses against rNS3 (Fig. 5B). In this mouse strain, two immunizations were required to detect clear T-cell responses.

3.5. Immunization with HCV antigens plus anti-CD40 and poly(I:C) induces strong memory responses

Immunization with recombinant viruses is able to induce long-term memory responses against the antigen of interest,

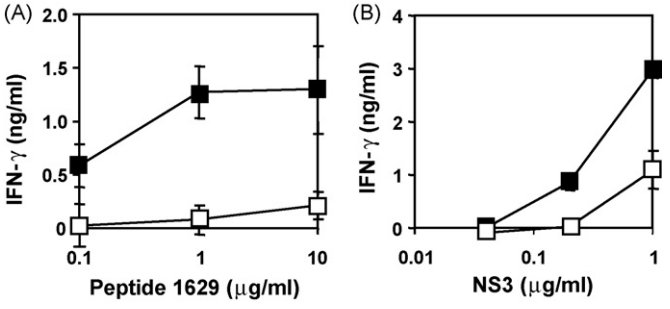

Fig. 5. rNS3 can induce CD4 and CD8 T-cell responses in other mouse strain. C57BL/6 mice (three per group) were immunized once (open symbols) or twice (closed symbols) with rNS3 (100 μg/mouse) plus poly(I:C) and anti-CD40. Six days after the last immunization their splenocytes were pooled and stimulated with the H-2 D^b-restricted CTL epitope 1629 (A) or with rNS3 (B) and IFN-γ production was measured 48 h later. Results are expressed as mean ± S.D. of triplicate cultures.

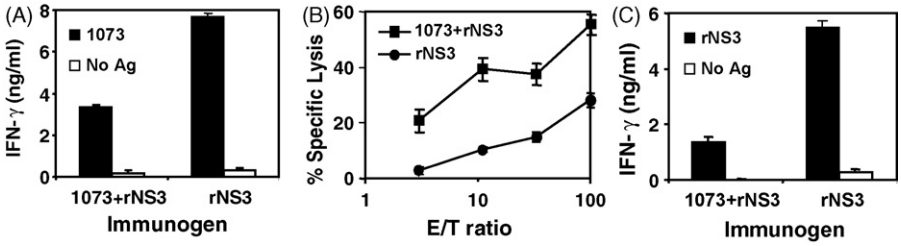

Fig. 6. Immunization with HCV antigens plus poly(I:C) and anti-CD40 induces strong CTL and T-helper memory responses. HHD mice (three per group) received two immunizations with rNS3 (100 μg/mouse) or with 1073 (50 μg/mouse) and rNS3 (5 μg/mouse) plus poly(I:C) and anti-CD40. Sixty days after the last immunization animals were sacrificed to measure CTL and T-helper responses. (A) Splenocytes were pooled and stimulated in 96-well plates with or without peptide 1073 (10 μM), and IFN-γ production in the supernatants was measured 2 days later. (B) Splenocytes from mice shown in A were also stimulated in 24-well plates with peptide 1073 at 10 μM. Five days later, their CTL activity was tested against T2 cells loaded with peptide 1073. Responses against T2 cells incubated in the absence of 1073 were always below 3%. (C) T-helper responses against NS3 induced in the same groups of animals were measured as IFN-γ production in culture supernatants. Results are expressed in all cases as mean ± S.D. of triplicate cultures.

whereas peptide immunization does not frequently lead to the induction of these long-term responses (Casares et al., 2001). Since our immunization mixture contains poly(I:C), a component that mimics viral dsRNA, we analyzed if this immunization protocol was able to induce long-lasting memory responses. For these experiments we used again as model antigens high dose rNS3 (100 μg/mouse) or peptide 1073 in combination with low dose rNS3 (5 μg/mouse), immunization protocols which induce both CD4 and CD8 responses. Animals received two immunizations and 60 days after the last immunization CTL and T-helper responses were analyzed. Fig. 6A shows that 60 days after immunization splenocytes from these animals were able to specifically produce IFN-γ in response to peptide 1073. When lytic activity against 1073 was measured, strong responses were still detected (Fig. 6B), but in this case 1073 + rNS3 induced more potent responses. Finally, when T-helper responses against NS3 were measured, it was found that cells from both groups of animals still displayed a high memory response (Fig. 6C). These results show that HCV peptides and proteins in combination with poly(I:C) and anti-CD40 are able to induce strong memory CD8 and CD4 responses.

3.6. Immunization with poly(I:C) plus anti-CD40 induces NK cell activity which helps the induction of Th1 responses

It has been reported that NK cells express TLR3, and in vitro stimulation with poly(I:C) leads to NK cell activation (Sivori et al., 2004). Therefore, we decided to study whether our immunization protocol was able to activate these cells. Experiments carried out with the NK cell sensitive line YAC-1 showed that 6 days after administration of poly(I:C) and anti-CD40, a clear NK cell lytic activity was detected, whereas very low levels were observed in animals treated with PBS (Fig. 7A). We thus studied the role played by these activated NK cells on the induction of T-cell responses after immunization with poly(I:C) and anti-CD40. A group of mice was treated with anti-AsGM1 to deplete NK cells, whereas another group was administered control rabbit IgG before immunization with 1073 and low dose of rNS3 plus poly(I:C) and anti-CD40. In both cases, T-cell responses were measured 6 days later. As shown in Fig. 7B, mice depleted of NK cells had lower IFN-γ production against NS3. Moreover, after depletion of NK cells, detectable levels of IL-4 against NS3 were measured, confirming the role of NK cells on the induction of Th1 responses (Fig. 7C). A decrease in CTL activity against peptide 1073 was observed in anti-AsGM1-treated mice (data not shown). However, since anti-AsGM1 administration directly decreases CTL activity (Stitz et al., 1986), this experiment cannot unequivocally attribute an effect of NK cells on the induction of CTL responses when using poly(I:C) and anti-CD40. Thus, NK cell activity induced by poly(I:C) and anti-CD40 administration contributes to the induction of CD4 Th1 responses.

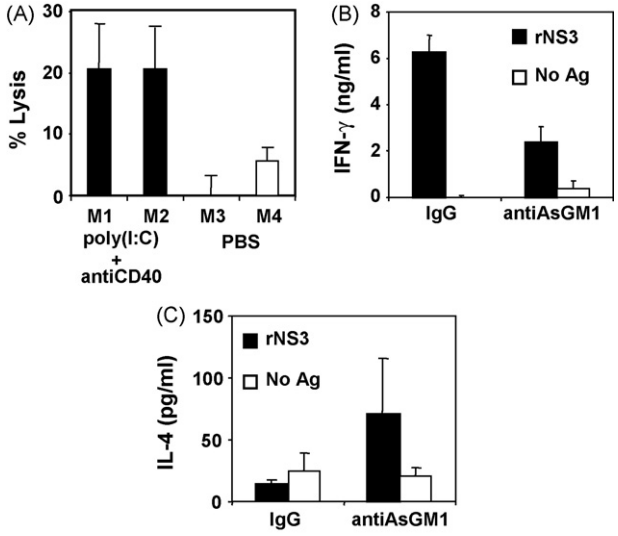

Fig. 7. Immunization with poly(I:C) plus anti-CD40 induces NK cell activity which helps to induce Th1 responses. (A) HHD mice (two per group) were injected with poly(I:C) and anti-CD40 (M1 and M2) or with PBS (M3 and M4). Six days later, NK cell lytic activity of their spleen cells was measured against the NK-sensitive cell line YAC-1 at an E/T ratio of 100. (B and C) HHD mice (three per group) were treated with 40 μl of anti-asialo GM1 or with rabbit IgG (control group) on days 2 and 0 and immunized with poly(I:C), anti-CD40, peptide 1073 (50 μg/mouse) and NS3 protein (5 μg/mouse). Six days later, their splenocytes were pooled and stimulated with NS3 (1 μg/ml) or with culture medium. Production of IFN-γ (B) and IL-4 (C) was measured in 48-h supernatants. Results are expressed in all cases as mean ± S.D. of triplicate cultures.

3.7. Immunization with rNS3 but not with NS3-derived CTL epitopes induces CTL responses that recognize endogenously expressed HCV antigens

The principal goal of immunization protocols aimed at inducing CTL responses is to activate CD8 lymphocytes able to recognize infected cells. We decided thus to study the ability of the different HCV antigens to induce CTL that would recognize T1/HCVcon cells, which endogenously express the HCV polyprotein. We immunized mice with 1073 or with rNS3 and we studied the responses against T1/HCVcon cells. None of these immunization protocols induced responses able to specifically recognize T1/HCVcon cells, although both induced strong responses recognizing peptide 1073 (Fig. 8A and B). The sequence of region 1073–1081 present in peptide 1073 and in rNS3, as well as the sequence of other CTL epitopes present in rNS3, have several changes compared to the sequence present in T1/HCVcon cells. We therefore decided to use a new peptide from region 1073 to 1081 similar to that present in T1/HCVcon cells (CVNGVCWTV, from now on 1073 CVN). CTL induced after immunization with rNS3 recognized very poorly peptide 1073 CVN (Fig. 8C), which might explain the lack of recognition of T1/HCVcon cells. Thus, in order to enhance the possibility of inducing CTL responses recognizing T1/HCVcon cells, a new recombinant NS3 protein (rNS3c) containing the 1073 CVN sequence as well as more similar sequences in other CTL epitopes (Kato et al., 1990), was produced in bacteria and used for immunization purposes. Immunization with 1073 CVN or with rNS3c induced CTL responses recognizing peptide 1073 CVN. However, only rNS3c was able to induce responses recognizing T1/HCVcon cells (Fig. 8D and E). These results suggested that immunization with protein NS3 plus poly(I:C) and anti-CD40 was more suitable than immunization with peptide to induce responses recognizing endogenously expressed antigens. To confirm these results, we immunized C57BL/6 mice with CTL epitope 1629 or with rNS3 and measured the response. Peptide immunization did not induce any measurable response against peptide-loaded cells (data not shown); so we did not carry out further experiments of recognition of NS3/4A-transfected EL4 cells. However, immunization with rNS3 activated a response able to recognize NS3/4A-transfected EL4 cells (Fig. 8F).

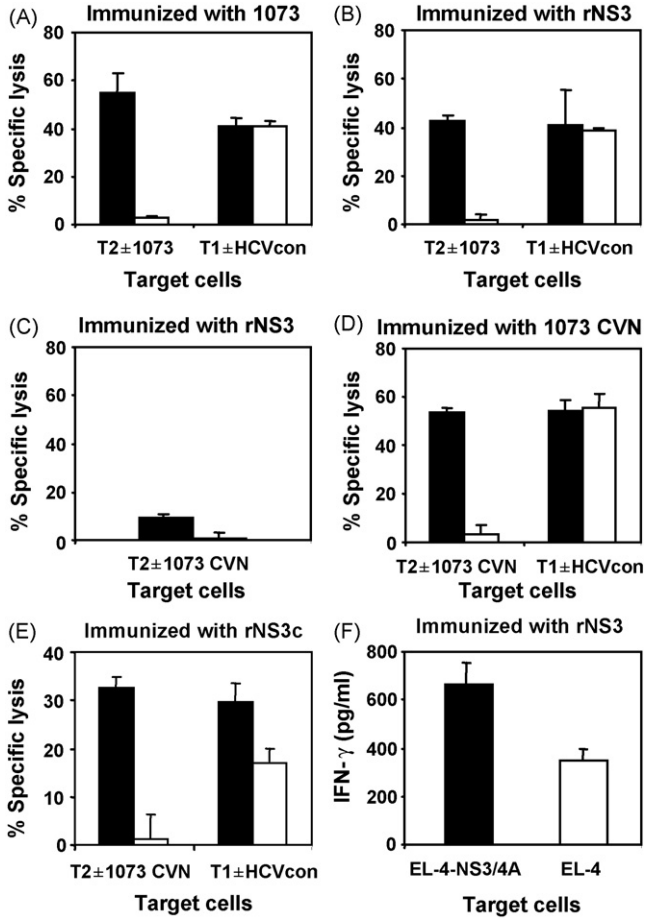

Fig. 8. Immunization with rNS3 protein but not with NS3-derived CTL epitopes induces CTL responses that recognize endogenously expressed HCV antigens. HHD mice (three per group) were immunized with peptide 1073 (A) or with rNS3 (100 μg/mouse) expressed in yeast (B). In both cases, immune splenocytes were pooled and stimulated with peptide 1073 (10 μM) or with mitomycin C-treated T1/HCVcon cells. Five days later, lytic activity was measured against target cells with antigen: T2 cells pulsed with peptide 1073 or T1 cells transfected with HCV genes (black bars). As control, cells without antigen were used: unpulsed T2 cells or non-transfected T1 cells (open bars). (C) HHD mice (three per group) were immunized with rNS3 (100 μg/mouse) and CTL responses against T2 cells pulsed with peptide 1073 CVN (black bar) or without peptide (open bar) were measured. (D and E) HHD mice were immunized with peptide 1073 CVN (D) or with rNS3c (100 μg/mouse) (E). As in (A) and (B), immune splenocytes were pooled and stimulated with peptide 1073 (10 μM) or with mitomycin C-treated T1/HCVcon cells. Lytic activity was measured against target cells with antigen: T2 cells pulsed with peptide 1073 CVN or T1 cells transfected with HCV genes (black bars). As control, cells without antigen were used: unpulsed T2 cells or non-transfected T1 cells (open bars). (F) C57BL/6 mice (three per group) were immunized with rNS3 (100 μg/mouse) and 6 days later their splenocytes were pooled and stimulated with NS3/4A-transfected EL-4 cells or with untransfected EL-4 cells to measure IFN-γ production. Results are expressed in all cases as mean ± S.D. of triplicate cultures.

4. Discussion

In the present study we showed that combination of poly(I:C), a ligand of TLR3, with anti-CD40, is a potent adjuvant mixture for the induction of cellular immune responses against HCV. With the aim of inducing efficient responses, we chose poly(I:C) as DC-activating reagent, because it has been reported that poly(I:C) induces DC maturation through TLR3, a receptor for double stranded RNA (Alexopoulou et al., 2001), which is commonly found in the replication of single-stranded RNA viruses such as HCV. Combination of this reagent with agonistic anti-CD40 antibodies has shown an important synergistic effect on the induction of T-cell responses against HCV antigens. These results confirm previous findings using anti-CD40 and different TLR ligands in the expansion of CD8 T-cells (Ahonen et al., 2004). However, in the present study we have found that for poly(I:C), this synergy is also able to enhance CD4 T-cell responses. Although induction of CD8 T-cell responses using this adjuvant does not require help provided by CD4 T-cells, we have found that the inclusion of antigens recognized by CD4 T-cells enhances the induction of CTL responses. This effect is clearer when studying the induction of high avidity CTL, as recently reported in other immunization systems (Kumaraguru

et al., 2004). In the case of HCV infection, chronically infected patients have much lower CD4 T-cell responses than patients that are able to eliminate infection (Hoffmann et al., 1995; Lasarte et al., 1998). However, this difference is not as clear in the case of CTL responses. Indeed, CTL responses are found in infected patients, although lower than in cured patients (He et al., 1999; Rehermann et al., 1996). Thus, CTL may behave as an important effector population in the clearance of HCV infection, but CD4 T-cells play a central role in the control of the disease. Moreover, it has been described that antiviral memory cells depend on CD4 cells for their protective activity (Grakoui et al., 2003).

Regarding the induction of T-cell memory responses, immunization with peptides in conventional adjuvants such as IFA, is not as efficient as immunization with recombinant viruses to induce these memory responses (Casares et al., 2001). Thus, the strong memory responses found after immunization with HCV antigens combined with poly(I:C) and anti-CD40, suggest that this protocol is able to overcome the poor ability of peptides to induce memory responses. These results might be explained by the enhanced survival of CD4 T-cells after poly(I:C) stimulation (Gelman et al., 2004). Poly(I:C) induces T-cell proliferation and survival through the production of type I IFN (Kolumam et al., 2005; Tough et al., 1996), which in turn activates IL-15 production, an important cytokine in the homeostatic proliferation of memory T-cells (Lodolce et al., 1998; Zhang et al., 1998). Moreover, stimulation of CD8 T-cells through CD40 has been demonstrated to be very important for the induction of memory responses (Bourgeois et al., 2002; Koschella et al., 2004; Lefrancois et al., 2000). Our results on the induction of potent memory responses using poly(I:C) and anti-CD40 are similar to those recently reported using a synthetic oligonucleotide which behaves as a TLR9 ligand (Toka et al., 2005). In both cases, administration of anti-CD40 enhances the signal provided by the corresponding TLR ligand. It is important to note that in our case, not only CD8 but also memory CD4 responses are induced using this immunization protocol, probably due to the action of poly(I:C) and anti-CD40 in CD4 T-cells (Gelman et al., 2004).

Due to the activating effect of poly(I:C) and anti-CD40 on DC through TLR3 and CD40, respectively, these stimuli should lead to maturation and concomitantly to enhancement of the stimulatory capacity of DC. However, when studying maturation of these cells in vivo, we have not seen any synergy when measuring surface expression of activation markers. Indeed, maturation induced by poly(I:C) and anti-CD40 is very similar to that induced by poly(I:C) alone. These results suggest that in vivo administration of poly(I:C) and anti-CD40 may have synergistic effects on other DC functions, such as cytokine production or stimulatory ability, as demonstrated in vitro with CD40L in combination with the TLR ligands LPS and poly(I:C) (Lapointe et al., 2000) or with other microbial stimuli (Schulz et al., 2000). Alternatively, poly(I:C) and anti-CD40 may have effects on other cell populations, and all these effects synergize to induce T-cell responses. Indeed, it has been reported that NK cells express several TLR molecules, and after in vitro stimulation with CpGs or poly(I:C) they are activated and produce IFN-γ (Sivori et al., 2004). In agreement with this, we have seen that in vivo administration of poly(I:C) and anti-CD40 induces NK cell activity, and this activity helps to induce T-cell responses. Our results are also in agreement with those recently reporting that NK cells, recruited in lymph nodes by DC matured under the effect of some stimuli, provide IFN-γ useful for Th1 priming (Martin-Fontecha et al., 2004). Together with NK cells and DC, T-cells may also directly benefit from the effect of these agonist molecules. Activated CD4 T-cells express TLR3, and stimulation through this pathway enhances their survival (Gelman et al., 2004). Also, CD40L induces an increased activation of CD4 and CD8 T-cells after suboptimal TCR stimulation (Fanslow et al., 1994). Taken together, these results suggest that, at the time of immunization, the reagents are able to stimulate different populations, including DC, NK cells and T-cells, and the coordinate activity of all these cells finally synergizes on the induction of T-cell responses.

In the present work we have also compared the efficacy of different antigens to induce anti-HCV CD8 T-cell responses. We have found that both peptide epitopes and rNS3 induce potent peptide-specific responses. However, only protein is able to induce CTL recognizing endogenously expressed antigens. We do not have a clear explanation for it, but direct presentation of minimal CTL epitopes by non-professional APC when immunizing with peptide, as opposed to presentation by professional APC when immunizing with protein, might explain these differences. Experiments are currently in progress to elucidate these results. In addition, we have found that for rNS3, sequence homology between the antigen used for immunization and the antigen presented by target cells is important. Indeed, sequence homology between rNS3 expressed in yeast and NS3 present in T1/HCVcon cells is 92%, whereas the homology between the protein expressed in bacteria (rNS3c) and that of T1/HCVcon cells is 96%. Regarding the sequence of epitopes such as 1073 and 1406, they are more similar in this last case, which would explain the differences found in recognition.

5. Conclusions

Immunization with HCV antigens in combination with poly(I:C) and anti-CD40 induces potent and long-lasting T-cell immune responses against HCV. These responses are probably mediated by different cell populations, and their coordinate action might help inducing effector mechanisms which could be beneficial for an efficient anti-HCV immunization.

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References

Ahonen, C.L., Doxsee, C.L., McGurran, S.M., Riter, T.R., Wade, W.F., Barth, R.J., Vasilakos, J.P., Noelle, R.J., Kedl, R.M., 2004. Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. J. Exp. Med. 199, 775–784.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413, 732–738.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J.P., Trépo, C., Inchauspe, G., 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 120, 512–524.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245–252.

Bourgeois, C., Rocha, B., Tanchot, C., 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science 297, 2060–2063.

Bruna-Romero, O., Lasarte, J.J., Wilkinson, G., Grace, K., Clarke, B., Borras-Cuesta, F., Prieto, J., 1997. Induction of cytotoxic T-cell response against hepatitis C virus structural antigens using a defective recombinant adenovirus. Hepatology 25, 470–477.

Casares, N., Lasarte, J.J., de Cerio, A.L., Sarobe, P., Ruiz, M., Melero, I., Prieto, J., Borras-Cuesta, F., 2001. Immunization with a tumor-associated CTL epitope plus a tumor-related or unrelated Th1 helper peptide elicits protective CTL immunity. Eur. J. Immunol. 31, 1780–1789.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. J. Exp. Med. 180, 1263–1272.

Cerny, A., McHutchison, J.G., Pasquinelli, C., Brown, M.E., Brothers, M.A., Grabscheid, B., Fowler, P., Houghton, M., Chisari, F.V., 1995. Cytotoxic T lymphocyte response to hepatitis C virus-derived peptides containing the HLA A2.1 binding motif. J. Clin. Invest. 95, 521–530.

Diehl, L., den Boer, A.T., Schoenberger, S.P., van der Voort, E.I., Schumacher, T.N., Melief, C.J., Offringa, R., Toes, R.E., 1999. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. Nat. Med. 5, 774–779.

Dienstag, J.L., 1983. Non-A, non-B hepatitis. I. Recognition, epidemiology, and clinical features. Gastroenterology 85, 439–462.

Fanslow, W.C., Clifford, K.N., Seaman, M., Alderson, M.R., Spriggs, M.K., Armitage, R.J., Ramsdell, F., 1994. Recombinant CD40 ligand exerts potent biologic effects on T cells. J. Immunol. 152, 4262–4269.

Frelin, L., Alheim, M., Chen, A., Soderholm, J., Rozell, B., Barnfield, C., Liljestrom, P., Sallberg, M., 2003. Low dose and gene gun immunization with a hepatitis C virus non-structural (NS) 3 DNA-based vaccine containing NS4A inhibit NS3/4A-expressing tumors in vivo. Gene Ther. 10, 686–699.

Frelin, L., Ahlen, G., Alheim, M., Weiland, O., Barnfield, C., Liljestrom, P., Sallberg, M., 2004. Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus non-structural 3/4A gene. Gene Ther. 11, 522–533.

Gelman, A.E., Zhang, J., Choi, Y., Turka, L.A., 2004. Toll-like receptor ligands directly promote activated CD4+ T cell survival. J. Immunol. 172, 6065–6073.

Grakoui, A., Shoukry, N.H., Woollard, D.J., Han, J.H., Hanson, H.L., Ghrayeb, J., Murthy, K.K., Rice, C.M., Walker, C.M., 2003. HCV persistence and immune evasion in the absence of memory T cell help. Science 302, 659–662.

He, X.S., Rehermann, B., Lopez-Labrador, F.X., Boisvert, J., Cheung, R., Mumm, J., Wedemeyer, H., Berenguer, M., Wright, T.L., Davis, M.M., Greenberg, H.B., 1999. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. Proc. Natl. Acad. Sci. U.S.A. 96, 5692–5697.

Hiasa, Y., Horiike, N., Akbar, S.M., Saito, I., Miyamura, T., Matsuura, Y., Onji, M., 1998. Low stimulatory capacity of lymphoid dendritic cells expressing hepatitis C virus genes. Biochem. Biophys. Res. Commun. 249, 90–95.

Hoffmann, R.M., Diepolder, H.M., Zachoval, R., Zwiebel, F.M., Jung, M.C., Scholz, S., Nitschko, H., Riethmuller, G., Pape, G.R., 1995. Mapping of immunodominant CD4+ T lymphocyte epitopes of hepatitis C virus antigens and their relevance during the course of chronic infection. Hepatology 21, 632–638.

Janeway Jr., C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197–216.

Kaisho, T., Akira, S., 2004. Pleiotropic function of Toll-like receptors. Microb. Infect. 6, 1388–1394.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., Hori, M., 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J. Immunol. 162, 5584–5591.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. 87, 9524–9528.

Kittlesen, D.J., Chianese-Bullock, K.A., Yao, Z.Q., Braciale, T.J., Hahn, Y.S., 2000. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. J. Clin. Invest. 106, 1239–1249.

Kolumam, G.A., Thomas, S., Thompson, L.J., Sprent, J., Murali-Krishna, K., 2005. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J. Exp. Med. 202, 637–650.

Koschella, M., Voehringer, D., Pircher, H., 2004. CD40 ligation in vivo induces bystander proliferation of memory phenotype CD8 T cells. J. Immunol. 172, 4804–4811.

Kumaraguru, U., Suvas, S., Biswas, P.S., Azkur, A.K., Rouse, B.T., 2004. Concomitant helper response rescues otherwise low avidity CD8+ memory CTLs to become efficient effectors in vivo. J. Immunol. 172, 3719–3724.

Lapointe, R., Toso, J.F., Butts, C., Young, H.A., Hwu, P., 2000. Human dendritic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes. Eur. J. Immunol. 30, 3291–3298.

Lasarte, J.J., Garcia-Granero, M., Lopez, A., Casares, N., Garcia, N., Civeira, M.P., Borras-Cuesta, F., Prieto, J., 1998. Cellular immunity to hepatitis C virus core protein and the response to interferon in patients with chronic hepatitis C. Hepatology 28, 815–822.

Lefrancois, L., Altman, J.D., Williams, K., Olson, S., 2000. Soluble antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. J. Immunol. 164, 725–732.

Liu, H.M., Newbrough, S.E., Bhatia, S.K., Dahle, C.E., Krieg, A.M., Weiner, G.J., 1998. Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. Blood 92, 3730–3736.

Lodolce, J.P., Boone, D.L., Chai, S., Swain, R.E., Dassopoulos, T., Trettin, S., Ma, A., 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 9, 669–676.

Lopez-Diaz de Cerio, A.L., Casares, N., Lasarte, J.J., Sarobe, P., Perez-Mediavilla, L.A., Ruiz, M., Prieto, J., Borras-Cuesta, F., 1999. T(h)1 but not T(h)0 cell help is efficient to induce cytotoxic T lymphocytes by immunization with short synthetic peptides. Int. Immunol. 11, 2025–2034.

Martin, P., Parroche, P., Chatel, L., Barretto, C., Beck, A., Trepo, C., Bain, C., Lone, Y.C., Inchauspe, G., Fournillier, A., 2004. Genetic immunization and comprehensive screening approaches in HLA-A2 transgenic mice lead to the identification of three novel epitopes in hepatitis C virus NS3 antigen. J. Med. Virol. 74, 397–405.

Martin-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F., 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat. Immunol. 5, 1260–1265.

Pascolo, S., Bervas, N., Ure, J.M., Smith, A.G., Lemonnier, F.A., Perarnau, B., 1997. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2. 1 monochain transgenic H-2Db beta2m double knockout mice. J. Exp. Med. 185, 2043–2051.

Quer, J., Esteban, J.I., Cos, J., Sauleda, S., Ocana, L., Martell, M., Otero, T., Cubero, M., Palou, E., Murillo, P., Esteban, R., Guardia, J., 2005. Effect of bottlenecking on evolution of the non-structural protein 3 gene of hepatitis C virus during sexually transmitted acute resolving infection. J. Virol. 79, 15131–15141.

Rehermann, B., Chang, K.M., McHutchison, J.G., Kokka, R., Houghton, M., Chisari, F.V., 1996. Quantitative analysis of the peripheral blood cytotoxic

T lymphocyte response in patients with chronic hepatitis C virus infection. J. Clin. Invest. 98, 1432–1440.

Rolink, A., Melchers, F., Andersson, J., 1996. The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching. Immunity 5, 319–330.

Sarobe, P., Jauregui, J.I., Lasarte, J.J., Garcia, N., Civeira, M.P., Borras-Cuesta, F., Prieto, J., 1996. Production of interleukin-2 in response to synthetic peptides from hepatitis C virus E1 protein in patients with chronic hepatitis C: relationship with the response to interferon treatment. J. Hepatol. 25, 1–9.

Sarobe, P., Lasarte, J.J., Casares, N., Lopez-Diaz de Cerio, A., Baixeras, E., Labarga, P., Garcia, N., Borras-Cuesta, F., Prieto, J., 2002. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. J. Virol. 76, 5062–5070.

Sarobe, P., Lasarte, J.J., Zabaleta, A., Arribillaga, L., Arina, A., Melero, I., Borras-Cuesta, F., Prieto, J., 2003. Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses. J. Virol. 77, 10862–10871.

Schulz, O., Edwards, A.D., Schito, M., Aliberti, J., Manickasingham, S., Sher, A., Reis e Sousa, C., 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity 13, 453–462.

Schwarz, K., Storni, T., Manolova, V., Didierlaurent, A., Sirard, J.C., Rothlisberger, P., Bachmann, M.F., 2003. Role of Toll-like receptors in costimulating cytotoxic T cell responses. Eur. J. Immunol. 33, 1465–1470.

Shortman, K., Liu, Y.J., 2002. Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. 2, 151–161.

Sivori, S., Falco, M., Della Chiesa, M., Carlomagno, S., Vitale, M., Moretta, L., Moretta, A., 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc. Natl. Acad. Sci. U.S.A. 101, 10116–10121.

Stitz, L., Baenziger, J., Pircher, H., Hengartner, H., Zinkernagel, R.M., 1986. Effect of rabbit anti-asialo GM1 treatment in vivo or with anti-asialo GM1 plus complement in vitro on cytotoxic T cell activities. J. Immunol. 136, 4674–4680.

Thompson, H.S., Davies, M.L., Watts, M.J., Mann, A.E., Holding, F.P., O'Neill, T., Beech, J.T., Thompson, S.J., Leesman, G.D., Ulrich, J.T., 1998. Enhanced immunogenicity of a recombinant genital warts vaccine adjuvanted with monophosphoryl lipid A. Vaccine 16, 1993–1999.

Toka, F.N., Gierynska, M., Suvas, S., Schoenberger, S.P., Rouse, B.T., 2005. Rescue of memory CD8+ T cell reactivity in peptide/TLR9 ligand immunization by codelivery of cytokines or CD40 ligation. Virology 331, 151–158.

Tough, D.F., Borrow, P., Sprent, J., 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. Science 272, 1947–1950.

Vabulas, R.M., Pircher, H., Lipford, G.B., Hacker, H., Wagner, H., 2000. CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. J. Immunol. 164, 2372–2378.

Weiner, G.J., Liu, H.M., Wooldridge, J.E., Dahle, C.E., Krieg, A.M., 1997. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc. Natl. Acad. Sci. U.S.A. 94, 10833–10837.

Wolk, B., Gremion, C., Ivashkina, N., Engler, O.B., Grabscheid, B., Bieck, E., Blum, H.E., Cerny, A., Moradpour, D., 2005. Stable human lymphoblastoid cell lines constitutively expressing hepatitis C virus proteins. J. Gen. Virol. 86, 1737–1746.

Zhang, X., Sun, S., Hwang, I., Tough, D.F., Sprent, J., 1998. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. Immunity 8, 591–599.