Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and Core proteins

Aurelia A. Haller a, Georg M. Lauer b, Thomas H. King a, Charles Kemmler a,1, Valerie Fiolkoski a, Yingnian Lu a, Don Bellgrau a,c, Timothy C. Rodella a, David Apelian a, Alex Franzusoff a, Richard C. Duke a,c,∗

a GlobeImmune, Inc., 1450 Infinite Drive, Louisville, CO 80027, USA
b Partners AIDS Research Center and Infectious Disease Division, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA
c Departments of Immunology and Medicine, University of Colorado at Denver and Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA

Received 7 August 2006; received in revised form 13 October 2006; accepted 20 October 2006
Available online 10 November 2006

Abstract

Control of primary infection with hepatitis C virus (HCV) is associated with robust and broad T cell immunity. In contrast, chronic infection is characterized by weak T cell responses suggesting that an approach that boosts these responses could be a therapeutic advance. Saccharomyces cerevisiae is an effective inducer of innate and adaptive cellular immunity and we have generated recombinant yeast cells (GI-5005) that produce an HCV NS3-Core fusion protein. Preclinical studies in mice showed that GI-5005 induced potent antigen-specific proliferative and cytotoxic T cell responses that were associated with Th1-type cytokine secretion. In studies in which GI-5005 was administered up to 13 times, no detectable vector neutralization or induction of tolerance was observed. Prophylactic as well as therapeutic administration of GI-5005 in mice led to eradication of tumor cells expressing HCV NS3 protein. Immunotherapy with GI-5005 is being evaluated in chronic HCV infected individuals in a Phase 1 clinical trial.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Hepatitis C; Vaccine; Immunotherapy; Yeast; HCV; S. cerevisiae

1. Introduction

Hepatitis C virus (HCV) is a major causative agent of acute and chronic hepatitis worldwide. It is estimated that there are 200 million chronically HCV-infected individuals worldwide, 4 million of whom reside in the United States [1]. The inability to clear acute HCV infection leads to chronic hepatitis, with the potential sequelae of liver cirrhosis, liver failure and liver cancer [2]. There is at present no preventative vaccine and therapeutic options are limited to interferon/ribavirin therapy, which is often poorly tolerated, is contraindicated in many subjects and is very expensive. In addition, the efficacy of the current standard treatment with interferon and ribavirin is limited, especially in genotype 1, the most prevalent genotype in the US and most industrialized countries [3]. Thus, only a proportion of HCV-infected persons can be successfully treated at this time and alternative treatment modalities, including immunotherapies and prophylactic vaccines, are clearly needed.

Successful control of HCV is associated with strong and broadly directed HCV-specific CD4+ and CD8+ T cell responses, while chronic HCV infection is characterized by attenuated and functionally impaired T cells [4,5]. Therefore,
an immunotherapeutic approach that stimulates potent cellular immunity against one or more HCV antigens could be beneficial in chronic HCV disease. A number of experimental approaches to immunotherapy in general have been investigated, including the use of DNA-, recombinant viral-, and autologous dendritic cell-based strategies. DNA vectors are good at priming immune responses in humans but are poor at boosting whereas recombinant viruses, in particular adenoviruses, are good at boosting but suffer from the limitation of vector neutralization [6–8]. Finally, ex vivo manipulated autologous dendritic cell-based approaches are patient-specific and labor intensive. These considerations suggest that the ideal HCV immunotherapy might consist of a non-pathogenic vector that can deliver multiple HCV antigens into the MHC classes I and II antigen presentation pathways to stimulate potent CD4+ and CD8+ T cell responses. If possible, this vector would also be capable of repeated administration similar to what is done with therapeutic drugs.

Recently, the role of pattern recognition receptors, including Toll-like receptors (TLR), in the activation of innate and acquired immunity has been elucidated [9–14]. These receptors are found on antigen-presenting cells (APC) including dendritic cells and macrophages. Saccharomyces cerevisiae yeast cell wall components interact with more dendritic cell and macrophage receptors than perhaps any other microbe, and are especially effective at stimulating antigen presentation. These receptors include TLR-2, TLR-4, TLR-6, CD14, Dectin-1, Dectin-2, DEC-205 and the mannose receptor family [10,13]. Uptake of zymosan, a crude S. cerevisiae yeast cell wall preparation, results in up-regulation of a multitude of pro-inflammatory genes [14–17]. Our own data indicate that uptake of whole yeast by mouse and human dendritic cells and macrophages results in up-regulation of a variety of cell surface molecules including adhesion molecules (ICAM-1, CD54), co-stimulatory molecules (B7-1, B7-2, CD80, CD86) and classes I and II MHC molecules, as well as promoting the secretion of pro-inflammatory cytokines including IL-12 [18]. Yeast-associated proteins are efficiently presented via the secretion of pro-inflammatory cytokines including IL-12 and classes I and II MHC leading to protective antigen-specific helper and cytotoxic T lymphocyte (CTL)-mediated immunity to tumor cells [18–20].

In the present study, a recombinant yeast strain producing an HCV NS3-Core fusion protein, termed GI-5005, was tested in pre-clinical studies in mice for its ability to induce T cell-mediated immune responses and to eradicate HCV antigen-expressing cells in vivo.

2. Materials and methods

2.1. Reagents and tissue culture medium

Concanavalin A (Con A), lipopolysaccharide (LPS) (S. typhimurium), dextran sulfate (DS), phorbol myristic acid (PMA) and ionomycin were purchased from Sigma. Na2[51Cr]O4 (S.A. 250 mCi/mg Cr) and [3H]-TdR (S.A. 35 Ci/mmol) were purchased from MP Biomedical. A tissue culture medium (cRPMI-10) consisting of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (HyClone), 2 mM l-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 10 mM HEPES was used for general cell culture and for all immune assays employing mouse spleen cells.

2.2. Cells and viruses

C57BL/6 (H-2b)-derived EL4, BALB/c (H-2d)-derived A20 B and DBA/2 (H-2d)-derived P815 tumor cells were obtained from the ATCC. Transfectants of these cell lines that stably express the HCV NS3 protein (termed EL4-NS3, A20-NS3 and P815-NS3) were prepared as follows. A single region of the HCV genome (genotype 1a, H77 strain; cDNA was a gift of Dr. J. Bukh, NIH, Bethesda [21]) was amplified by PCR to generate a DNA sequence encoding an inactivated NS3 protein as a single polypeptide with the following sequence elements fused in frame from N- to C-terminus (HCV polyprotein numbering in parentheses): (1) the sequence MADEAP to impart resistance to proteasomal degradation and (2) amino acids 1–631 (1027–1657) of the HCV NS3 protease protein (the amino acid at HCV polypeptide residue 1165 was changed from a serine to an alanine in order to inactivate the proteolytic activity). This DNA was cloned into the pUP1 vector that also contains the NeoR drug resistance gene. The NS3/pUP plasmid was transfected into P815, A20 and EL4 tumor cells using the Effectene Transfection reagent (Qiagen). Expression of the NS3 protein was confirmed by immunoblot using a polyclonal antibody directed specifically against HCV NS3 (Virostat). Once a NS3-expressing clone was identified, the stable cell line was propagated in cRPMI-10 containing 1 mg/ml G4.18 sulfate. Viruses encoding HCV NS3 (rVV-NS3; subtype 1b) and HCV-Core (rVV-Core; subtype 1b) were a kind gift of Alfred Prince (Lindsey F. Kimball Research Institute, New York). Viruses encoding HIV-1 gag (rVV-gag; HXB2 strain) and β-galactosidase (rVV-lac) were a kind gift of Dr. Daniel Kuritzkes (MGH, Boston). Viruses were propagated in HeLa cells (ATCC) and were titered by plaque assay on BSC40 cells (ATCC). Cell-free supernatants of infected cell cultures were stored at −70°C.

2.3. Recombinant yeast

GI-5005 yeast were engineered to express an HCV NS3-Core fusion protein under the control of the copper-inducible CUP1 promoter. Two regions of the HCV genome (genotype 1a, H77 strain) were amplified by PCR in order to generate a yeast expression plasmid termed pGI-113. The NS3-Core fusion protein encoded by pGI-113 is a single polypeptide with the following sequence elements fused...
and grown for 1 h at 30 °C for 1 h. It is important to note that all HCV antigen production occurs during the period that copper is present in the yeast culture media. No further HCV antigen production occurs during the period that copper is present in GI-5005. GI-5003 cells were washed three times in PBS to remove residual copper, adjusted to 10 YU/ml in PBS and heat-inactivated at 56 °C for 1 h. It is important to note that all HCV antigen production occurs during the period that copper is present in the yeast culture media. No further HCV antigen production occurs once the yeast cells have been heat-killed or upon in vivo administration.

In frame from N- to C-terminus (Fig. 1; HCV polyprotein numbering in parentheses): (1) the sequence MADEAP to impart resistance to proteasomal degradation, (2) amino acids 89–350 (1115–1376) of the HCV NS3 protease protein, (3) a single threonine amino acid residue introduced in cloning, (4) amino acids 2–140 (2–140) of the HCV Core protein and (5) the sequence ED to increase the hydrophilicity of the Core variant. pGI-113 was transfected into competent W303a S. cerevisiae yeast (ATCC) resulting in GI-5005. Expression of the fusion protein was confirmed by Western blot analysis of lysates from copper-induced, heat-inactivated GI-5005 yeast using monoclonal antibodies specific for HCV NS3 (Virostat) or HCV Core protein (Anogen). The results revealed a single ~47 kDa protein that was detected with NS3- and Core-specific monoclonal antibodies thereby confirming that the fusion protein harbors both NS3 and Core antigenic epitopes (data not shown). Expression levels of the fusion protein ranged from 2000 to 6000 ng/yeast unit (YU) GI-5005. GI-5003 (expressing inactivated full-length HCV NS3 protein), GI-4014 (expressing mutant human K-Ras: [19]) and GI-1000 or GI-1001 (vector control [18]) yeast were used as controls in several experiments described below. These yeast strains were prepared and maintained in an identical manner to GI-5005 except for the heterologous antigen that is expressed.

To prepare the vaccine, yeast cell starter cultures were grown from frozen stocks in ULDM medium (6.7 g/l yeast nitrogen base with ammonium sulfate, 20 g/l glucose, 0.02 mg/ml adenine, 0.02 mg/ml histidine and 0.02 mg/ml tryptophan) to mid log phase (3 YU/ml; where 1 YU equals 10 0.02 mg/ml adenine, 0.02 mg/ml histidine and 0.02 mg/ml yeast nitrogen base with ammonium sulfate, 20 g/l glucose, grown from frozen stocks in ULDM medium (6.7 g/l yeast nitrogen base with ammonium sulfate, 20 g/l glucose, 0.02 mg/ml adenine, 0.02 mg/ml histidine and 0.02 mg/ml tryptophan) to mid log phase (3 YU/ml; where 1 YU equals 10.02 mg/ml adenine, 0.02 mg/ml histidine and 0.02 mg/ml yeast nitrogen base with ammonium sulfate, 20 g/l glucose, grown from frozen stocks in ULDM medium (6.7 g/l yeast nitrogen base with ammonium sulfate, 20 g/l glucose, 0.02 mg/ml adenine, 0.02 mg/ml histidine and 0.02 mg/ml tryptophan) to mid log phase (3 YU/ml; where 1 YU equals 10

BALB/cBy and C57BL/6J mice, aged 6–8 weeks (Jackson Labs, Bar Harbor), were maintained in the Center for Laboratory Animal Care of the University of Colorado Health Sciences Center (Aurora, Colorado). Mice were injected subcutaneously weekly for 3 weeks with 5 YU GI-5005 unless stated otherwise. Mice were sacrificed 7 days after the last immunization and spleen cell suspensions were prepared by gently macerating the spleens through 70 μM cell strainers (Falcon).

Ten million spleen cells were placed in individual wells of 24-well tissue culture plates (Corning) and stimulated in vitro (IVS) with nothing or with the indicated number of yeast or plaque forming units (pfu) of infectious vaccinia virus expressing HCV or irrelevant antigens in a total of 2 ml cRPMI-10. Unless otherwise indicated, the CTL assay was performed on day 5 after initiation of the IVS period. On the day of the CTL assay, 1 × 10⁶ P815-NS3, A20-NS3 or EL-4-NS3 or P815, A20, EL-4 cells that had been infected overnight with recombinant vaccinia viruses at a multiplicity of infection of 10, were labeled in their cytoplasm with 100 μCi Na₂⁵¹CrO₄ and resuspended in cRPMI-10 at 1 × 10⁵ target cells/ml. While the target cells were labeling, the spleen cells were resuspended, an aliquot was counted and 200 μl of the suspension were placed undiluted in triplicate in individual wells of 96-well V-bottomed tissue culture plates (Corning). Three to five doubling dilutions of the undiluted spleen cells were then performed such that a final volume of 100 μl of spleen cells was present in each well. Additional wells consisting in 100 μl of cRPMI-10 and 2% Triton X-100 in triplicate, and without added effector spleen cells were set up as “spontaneous” and “maximum” release conditions, respectively. Hundred microliters of the ⁵¹Cr-labeled target cells were then added and the plates were centrifuged at 1000 rpm × 10 min to establish effector-target cell contact. The plates were incubated for 6 h at 37 °C after which 100 μl of cell-free supernatant was removed and radioactivity present as ⁵¹Cr was determined by liquid scintillation counting using a MicroBeta TriLux instrument (PerkinElmer). The average cpm for the spontaneous and maximum release conditions was determined and the % specific release for each triplicate and doubling dilution of effector cells (termed “experimental”) was calculated using the following formula:

\[
\% \text{Specific lysis} = \frac{\text{CPM}_{\text{expt}} - \text{CPM}_{\text{spont (avg)}}}{\text{CPM}_{\text{max}} - \text{CPM}_{\text{spont (avg)}}} \times 100
\]

Data are presented as mean percent specific lysis ± S.D.
2.6. Lymphocyte proliferation assays

Spleen cells from immunized mice were diluted to 4 × 10^8/ml in cRPMI-10 and 100 µl (4 × 10^5 cells) were transferred to individual wells of 96-well U-bottomed plates containing 100 µl of various stimuli (yeast, infectious vaccinia viruses, mitogens) at the indicated cell number, pfu or concentration and in triplicate. The plate was centrifuged at 500 rpm for 5 min and placed at 37 °C. After 3–6 days, the plate was “pulsed” by adding 1 µCi of ^3[H]-thymidine to each well (in 25 µl cRPMI-10) and after an additional 18 h the cells were “harvested” onto filter mats using a TomTek cell harvester. The data for each well is recorded as the number of counts of incorporated ^3[H]-thymidine per minute (cpm) and is presented as mean CPM ± S.D.

2.7. Cytokine studies

Spleen cells from immunized mice were stimulated in vitro by placing them in 24-well tissue culture plates at a concentration of 1 × 10^6 spleen cells/well and either nothing, GI-5005 at a concentration of 1 × 10^6 yeast cells/well, Con A (5 µg/ml), LPS (50 µg/ml) plus DS (40 µg/ml), infectious rVV-NS3 (1 × 10^6 pfu/well) or PMA (15 ng/ml) plus ionomycin (750 ng/ml), in a total of 2 ml cRPMI-10 and incubated at 37 °C. On days 2 and 5 after initiation of the IVS culture period, 200 µl of cell-free supernatants were collected from each well of the 24-well plate and stored frozen at −70 °C. The frozen samples were thawed and assayed using a Luminex Mouse Cytokine Ten-Plex kit (BioCompare). Cytokine concentrations were determined by comparison to standard curves generated with control reagents and are presented as pg/ml. Initial analysis of these assays showed maximal cytokine production in response to yeast and non-specific mitogens (e.g., Con A, LPS + DS and PMA + ionomycin) in samples from day 2 after initiation of the IVS culture period. In contrast, very little cytokine production was observed in response to recombinant vaccinia virus-encoded antigens on day 2, but rather maximal production was observed on day 5. Hence, cytokine study results are presented from day 2 samples when yeast and non-specific mitogens are used as stimuli and from day 5 samples when recombinant vaccinia viruses are used as stimuli.

2.8. Tumor challenge and therapy experiments

BALB/c or C57BL/6 mice (5 per group), were injected subcutaneously weekly for 3 weeks with either nothing (Naïve), GI-5003, GI-5005 or with GI-4014 at the doses that are indicated in the text. Mice were challenged via subcutaneous implantation in the flank 7 days after the final immunization with the indicated numbers of A20-NS3 cells or EL4-NS3 cells. For tumor therapy, the indicated number of A20-NS3 cells were implanted into the flank of BALB/c mice and immunization with GI-5005 was initiated 7 days after tumor implantation. In both challenge and therapy experiments, tumors were measured using digital calipers on the indicated day after implantation. Tumor burden is reported as the average tumor volume (in mm^3) ± S.D.

3. Results

3.1. GI-5005 induces cytotoxic effector cells that kill cells expressing HCV NS3 including virally infected cells

In an initial attempt to determine whether GI-5005 yeast could induce HCV antigen-specific cytotoxic effector cells, C57BL/6 mice were immunized subcutaneously weekly for 3 weeks with 5 YU (5 × 10^7 yeast cells) GI-5005. The mice were sacrificed 7 days thereafter and their spleen cells were stimulated in vitro with GI-5005 yeast (one yeast cell per three spleen cells), for 5 days prior to testing on 51Cr-labeled EL4 lymphoma cells or on EL4 cells stably expressing HCV-NS3 (EL4-NS3). The results presented in Fig. 2A show that the stimulated spleen cells killed EL4-NS3 targets in a dose-dependent manner. In contrast, significantly less killing was observed on non-transfected EL4 cells. The results presented in Fig. 2B show that in vitro stimulation of spleen cells from immunized mice with GI-5005 is also antigen specific; that is, IVS with GI-5005 yeast reveals cytotoxic effector cells capable of enhanced cytotoxic activity against EL4-NS3, as compared to IVS with nothing (nil) or with vector control yeast (GI-1000). The requirement for exposure to some form of HCV NS3 antigen to activate cytotoxic effector cell activity during the IVS period was further investigated using stimulation with recombinant vaccinia virus encoding NS3 (rVV-NS3; Fig. 2C). These data show that NS3-specific cytotoxic effector cells present in the spleen of immunized mice are re-stimulated by IVS with rVV-NS3 but not with rVV-lac encoding the irrelevant antigen, β-galactosidase.

The results presented above demonstrated that immunization with GI-5005 leads to induction of cytotoxic effector cells that can kill syngeneic tumor cells expressing NS3. However, GI-5005 also expresses the HCV Core antigen. Attempts to derive stably transfected tumor cell lines expressing HCV Core protein were unsuccessful. To overcome the lack of a Core-expressing target cell, the studies presented in Fig. 2D and E were performed. H-2d-bearing P815 leukemia cell targets were infected overnight with rVV-NS3 or rVV-Core prior to their use in a cytotoxicity assay testing spleen effector cells (IVS with GI-5005) from BALB/c mice that had been immunized with either 5 or 0.1 YU GI-5005, 5 or 0.1 YU GI-5003 (a Tarmogen expressing full-length HCV-NS3 but not Core), or 5 YU GI-4014 (a Tarmogen expressing mutant human K-Ras as an irrelevant vector control). The data presented in Fig. 2D and E show that GI-5005 induces cytotoxic cells that can kill tumor cells infected with either rVV-NS3 or rVV-Core whereas killing induced by GI-5003 is restricted to NS3 and no HCV-specific killing is induced with GI-4014.
Fig. 2. GI-5005 induces cytotoxic effector cells that kill cells expressing HCV NS3 including virally infected cells. (A–C) Spleen cells (30 × 10^6/flask or 6 × 10^6/well) from C57BL/6 mice that were injected weekly for 3 weeks with 5 YU GI-5005 were stimulated in vitro (IVS) with either: 1 YU GI-5005/flask (A), with nothing (nil), 1 YU/flask GI-1001 or 1 YU/flask GI-5005 (B) or with 6 × 10^6 pfu/well rVV-lac or rVV-NS3 (C). At the end of the IVS period, doubling dilutions of the spleen cell cultures were tested on 51Cr-labeled EL4 or EL4-NS3 cells as indicated. (D and E) Spleen cells from BALB/c mice that were injected weekly for 3 weeks with the indicated YU of GI-5005, GI-5003 or GI-4014 were stimulated in vitro with GI-5005 for 5 days. At the end of the IVS culture period, doubling dilutions of the spleen cell cultures were tested on 51Cr-labeled P815 leukemia cells that had been infected overnight with either rVV-NS3 (D) or rVV-Core (E). E:T ratio refers to the effector:target cell ratio based on spleen effector cell concentrations at the start of the IVS culture period. Results are expressed as the average percent specific lysis ± S.D. for triplicate samples.

3.2. GI-5005 induces cells that secrete Th1-type, pro-inflammatory cytokines

Fig. 3 shows the cytokines that are secreted when spleen cells from either naïve or immunized mice are placed in tissue culture with GI-5005 yeast. Cell-free supernatants were collected 48 h after initiation of culture and cytokine concentrations were determined using the flow cytometer-based Luminex™ assay. Fig. 3A shows the cytokine response of naïve BALB/c spleen cells to yeast and indicates that the innate response to yeast leads to secretion of IL-6, IL-12 and TNF-α, presumably derived from monocytes and dendritic cells in the population. The data presented in Fig. 3B shows the cytokine response of spleen cells from GI-5005-immunized mice and demonstrate that GI-5005 administration elicits cells that secrete IL-2 and IL-5, the pro-inflammatory Th1-type cytokines GM-CSF and IFN-γ, and enhanced amounts of IL-6 and TNF-α. Notably, IL-4 and IL-10 do not appear to be produced, even though the spleen cells are capable of producing these Th2-type cytokines in response to polyclonal stimulation with PMA plus ionomycin (Fig. 3C). Whereas several cytokines are secreted by spleen cells from immunized mice in response to yeast antigens, the HCV antigen-specific response appears to be somewhat more limited. As shown in Fig. 3D, GM-CSF, IFN-γ and TNF-α, are secreted by spleen cells from immunized mice in response to rVV-NS3, whereas secretion of IL-2, IL-5 and IL-6 are not observed; however, it is important to note, and as discussed in Section 2.7 above, that this response was measured after 120 h in culture, as opposed to after 48 h in the presence of intact yeast during IVS.

3.3. Effect of yeast cell number and antigen content on immune responses induced with GI-5005

Further experiments were undertaken to compare the induction of cytotoxic effector cells and cytokine-secreting cells, respectively, by GI-5005 yeast that express different amounts of antigen. In brief, GI-5005 yeast were produced that express approximately 1400, 500 and <50 ng/YU of HCV NS3-Core fusion protein. This was accomplished by varying the amount of copper present during the induction period (Fig. 4A). BALB/c mice were immunized weekly with the three different GI-5005 Tarmogens (designated H, M and L) at three different doses, 0.1, 1 and 10 YU. Mice were sacrificed 7 days after the third injection and their spleen cells were stimulated in vitro with GI-5005-H and tested for cytotoxicity on P815-NS3 targets. The data show clear dose responses
Fig. 3. GI-5005 induces cells that secrete Th1-type, pro-inflammatory cytokines. Spleen cells (10 × 10^6/well) from naïve C57BL/6 mice (A and C) or from C57BL/6 mice that received three weekly injections of 5 YU GI-5005 (B and D) were stimulated with either: GI-5005 (0.1 YU/well) (A and B), PMA (15 ng/ml) plus ionomycin (750 ng/ml) (C) or rVV-NS3 (100 × 10^6 pfu/well) (D). Cell-free supernatants were collected at 48 h (A–C) or 120 h (D) after initiation of culture and cytokines (presented in pg/ml) were quantified using the flow-cytometer-based Luminex™ assay.

based on both parameters; that is, the amount of HCV antigen being produced by the yeast and the number of yeast that were used for immunization (Fig. 4B–D). A similar effect was also noted for IVS using spleen cells from a single group of immunized mice placed in culture with different numbers of the various yeast (Fig. 4E and data not shown).

Fig. 4F and G show the levels of IFN-γ secreted in response to intact yeast versus rVV-NS3. In brief, these data indicate that the induction of yeast-specific cytokine-secreting T cells is dependent on the number of yeast that are used for immunization but is independent of the amount of HCV antigen being expressed in the yeast. In contrast, the induction of HCV antigen-specific T cells secreting IFN-γ is dependent on both criteria. Based on the data presented in Fig. 4, a minimum of 500 ng fusion protein/YU or 0.008 ng fusion protein/ng total yeast protein (0.8% of the total yeast protein) is required for inducing antigen-specific T cell responses in mice as measured by in vitro assays.

3.4. Effect of repeated administration on immune responses induced with GI-5005

To explore the effect of repeated immunization on the ability of GI-5005 to induce immune responses, experiments comparing no, one or three weekly immunizations with GI-5005 were conducted in both C57BL/6 and BALB/c mice. Fig. 5A and B show dose-dependent killing activity in BALB/c-derived spleen effector cells on syngeneic class I MHC-bearing P815 target cells infected with either rVV-NS3 or rVV-Core which increases in proportion to the number of immunizations. Similar results were obtained with spleen cells derived from GI-5005-immunized C57BL/6 mice and tested on EL4 cells infected with either rVV-NS3 or rVV-Core (data not shown). The results presented in Fig. 5C show the cytokine secretion profiles of spleen cells derived from BALB/c mice that received one, two or three immunizations with GI-5005 in response to IVS with GI-5005. These results demonstrate that more than one immunization is required to see the full spectrum of response. Similar results were obtained with spleen cells from C57BL/6 mice (data not shown). Finally, Fig. 5D shows the results of a lymphocyte proliferation assay performed with spleen cells from mice that received one, two or three weekly immunizations with GI-5005. The response of HCV NS3 and Core-specific lymphocytes increased in proportion with the number of immunizations and the calculated stimulation indices improved from 3.5 to 4.2 against rVV-NS3 and from 6.5 to 11.5 against rVV-Core with one versus three immunizations. No stimulation was observed against rVV-rastafar (encoding mutant human K-ras), confirming the antigen-specificity related to the antigen encoded by the recombinant vaccinia employed for IVS.
The data presented above indicated that immune responses induced by GI-5005 were enhanced by repeated weekly administrations. To explore durability and boosting of immune responses with GI-5005, the following experiment was undertaken. In brief, female BALB/c mice received five weekly injections of GI-5005 followed by no boosting (to examine durability) or by boosting at weekly, bi-weekly, monthly or bi-monthly intervals. Mice were sacrificed 16 days after the last boosting (day 100 of the experiment). The results importantly show that repeated weekly immunization does not result in induction of neutralization and/or tolerance in that even after 12 weekly injections a subsequent administration resulted in boosting as measured by lymphocyte proliferation and cell-mediated cytotoxicity assays (Fig. 6A and B). Fig. 6C shows that the magnitude of cytokine secretion can also be boosted and that there is no evidence for tolerance against either endogenous yeast proteins or the HCV NS3 antigen. Finally, this data also shows the durability of the immune responses induced by GI-5005 in that the mice that were injected weekly for 5 weeks (designated as Weekly × 5, no boost (durability)) and then rested for 72 days still showed potent yeast- and HCV NS3-specific immune responses.

In order to further evaluate the robustness of the cellular immune responses induced upon immunization with GI-5005, C57BL/6 and BALB/c mice that received three weekly doses of GI-5005 were sacrificed 1 and 2 months post-dosing and the durability of the response assessed by lymphoprolif-
Fig. 5. Effect of repeated administration on immune responses induced with GI-5005. (A and B) Spleen cells from BALB/c mice that received one, or three weekly injections with 5 YU GI-5005 were stimulated in vitro with GI-5005 for 5 days prior to assay on 51Cr-labeled P815 cells that had been infected overnight with either rVV-NS3 (A) or rVV-Core (B). (C) Spleen cells from BALB/c mice that received 1, 2 or 3 weekly injections with 5 YU GI-5005 were stimulated in vitro with GI-5005. Supernatants were collected at 48 h and cytokines detected using the Luminex™ assay. (D) Spleen cells were placed in individual wells of 96-well U-bottom tissue culture plates (400,000 cells/well) and stimulated in vitro with either nothing (Bkgd), rVV-NS3, rVV-Core or rVV-rastafar (100,000 pfu/well). 3HtdR was added on day 5 and the plates were harvested 18 hours thereafter. Results are expressed as the average CPM ± S.D. for triplicate samples.

3.5. GI-5005 induces protective immunity against NS3-expressing tumor cells

Because an in vivo animal model of protection or therapy against HCV is not available, we have used protection and therapy against HCV antigen-bearing tumors to demonstrate in vivo activity of GI-5005. The data presented in Fig. 7A show that mice that were immunized with GI-5003 or GI-5005 yeast that express HCV NS3 were protected from challenge with A20-NS3 tumor cells, whereas mice immunized with nothing or with GI-4014 yeast that express mutant human K-Ras were not. These results demonstrate that yeast cells producing HCV NS3 induce dose- and antigen-dependent immune responses that protect immunized mice from tumor cells expressing the relevant antigen. This experiment was repeated in C57BL/6 mice that were immunized with GI-5005 and challenged 7 days thereafter with EL4-NS3 lymphoma cells injected subcutaneously. The results presented in Fig. 7B show that three of five immunized mice were protected from challenge with EL4-NS3 whereas naïve mice were not. Injection of GI-5005 did not protect mice from challenge with EL4 alone indicating that protective immunity was antigen-specific (data not shown).

To determine whether the tumors that had grown in the immunized mice were still expressing HCV NS3, the tumors were excised from the two GI-5005-immunized mice that showed evidence of tumor growth, as well as from the five naïve mouse controls, and placed in tissue culture medium containing the antibiotic G4.18. Whereas EL4-NS3 tumor cells excised from naïve mice grew out in the presence of G4.18, tumors from the GI-5005-immunized mice did not, suggesting that they had lost expression of the HCV-NS3 protein. In contrast, loss of HCV NS3 expression was not observed...
Fig. 6. Effect of repeated administration on immune responses induced with GI-5005. Female BALB/c mice received either PBS or five weekly immunizations with GI-5005 followed by no boosting (No boost, durability) or by boosting at weekly (8×), bi-weekly (4×), monthly (2×) or at 2 months. Spleen cells were isolated 16 days after the final boost (i.e., at week 15). (A) Spleen cells were placed in individual wells of 96-well plates (4×10^5 cells/well) and stimulated with either nothing (Bkgd), GI-5005 (320,000 or 20,000 yeast), Concanavalin A (ConA) or lipopolysaccharide + dextran sulfate (LPS + DS). 3HTdR was added on day 3 and the plates were harvested 18 h thereafter. Results are expressed as the average CPM ± S.D. for triplicate samples. (B and C) Spleen cells were placed in 24-well tissue culture plates (10×10^6 cells/well) and stimulated with: GI-5005 for 5 days prior to assay on 51Cr-labeled P815 cells (B), or with either GI-5005-H (0.2 YU/well) or rVV-NS3 (100×10^6 pfu/well) (C). Cell-free supernatants were collected at 48 h (IVS w/GI-5005) or 120 h (IVS w/rVV-NS3) after initiation of culture. Cytokines were quantified using the Luminex™ assay.

with A20-NS3 tumor cells as excised tumors that grew in the BALB/c mice described in Fig. 7A were found to be G4.18 resistant and to still express HCV NS3 protein (data not shown).

3.6. GI-5005 stimulates cytotoxic effector cell activity in vitro in spleen cells isolated from non-immunized, tumor-bearing mice

When cytotoxic effector cell responses were assessed in spleen cell populations derived from GI-5005-immunized C57BL/6 mice that had subsequently rejected EL4-NS3 tumor cells (“protected”), it was apparent that enhanced activity had been induced (Fig. 7C). This suggested that exposure of GI-5005-immunized mice to a secondary source of HCV antigen, namely tumor cells expressing HCV NS3, resulted in enhanced immune responses. In order to determine if GI-5005 yeast could stimulate T cell activity from non-immunized, antigen-bearing mice, thus mimicking the situation in chronic HCV-infection, spleen cells from non-immunized, EL4-NS3 tumor-bearing C57BL/6 mice were stimulated in vitro with GI-5005 yeast. The results presented in Fig. 7C show that GI-5005 can stimulate cytotoxic effector cells derived from mice bearing tumors expressing HCV-NS3.

3.7. GI-5005 induces therapeutic activity against NS3-expressing tumor cells

The results presented in Fig. 7C indicated that GI-5005 can re-stimulate NS3-specific cytotoxic effector activity from spleen cells of mice bearing NS3-expressing tumors. This suggested that a therapeutic effect might also be attainable. To assess this possibility, BALB/c mice were injected subcutaneously with A20-NS3 tumor cells. Beginning 7 days after tumor implantation, the mice were immunized once a
Fig. 7. GI-5005 induces protective immunity against NS3-expressing tumor cells. (A) BALB/c mice received three weekly immunizations with nothing (Naïve) or with GI-4014, GI-5005 or GI-5003. Mice were challenged subcutaneously 7 days later with $5 \times 10^5$ A20-NS3. Tumors were measured on day 21 after challenge. Numbers (e.g., 5/5) refer to the number of animals with measurable tumors. Results are expressed as mean tumor volume ± S.D. (B) C57BL/6 mice received three weekly immunizations with nothing (Naive) or with 5 YU GI-5005. Mice were challenged subcutaneously 7 days after the final immunization with $5 \times 10^4$ EL-4-NS3. Tumors were measured on the indicated day after challenge. *Tumors excised from immunized mice were found to no longer express NS3. (C) Spleen cells from the five naïve, tumor-bearing (non-immunized) and from the two tumor-free immunized (protected) mice described in (B) were stimulated in vitro for 5 days with GI-5005 and tested for cytotoxicity on 51Cr-labeled EL4-NS3 targets. (D) BALB/c mice were challenged with the indicated number of A20-NS3. Mice were immunized by subcutaneous injection at skin sites distal to the tumor on days 7, 14 and 21 after tumor implantation with either PBS or with 10 YU GI-5005. Tumor volume was measured on the indicated day after initiation of therapy.

week for 3 weeks with either PBS or with GI-5005. Tumor growth was monitored and the mice were sacrificed 28 days after tumor implantation when the tumors in the PBS group reached 2500 mm³. In brief, whereas all five tumor-bearing mice that were treated with PBS showed tumor growth, only three out five that were treated with GI-5005 exhibited tumor growth and the tumors that arose in the treated animals appeared to be growing much more slowly (mean tumor volume in tumor-bearing mice in the PBS treated group was $2488 \pm 636$ mm³ versus $1264 \pm 548$ mm³ in the GI-5005 treated group). The immunotherapeutic property of GI-5005 was further confirmed in a second study as shown in Fig. 7D in which the number of implanted tumor cells was varied. These data show that GI-5005 is able to induce a therapeutic effect in mice with established NS3-bearing tumors.

4. Discussion

Conventional vaccine approaches to neutralize HCV by inducing antibodies that target the surface glycoproteins E1 and E2 have so far shown only limited success. In chimpanzees, immunization with recombinant E1 and E2 glycoproteins was able to prevent experimental infection after challenge with homologous virus but not heterologous virus [22]. A study evaluating a recombinant E1 protein as a therapeutic HCV vaccine clearly showed that this strategy was not sufficient to achieve virus clearance. A small Phase I study of truncated recombinant HCV E1 protein intramuscularly showed good tolerability in chronic hepatitis C patients, a limited increase in E1-specific CD4+ T cell responses and E1 antibody levels, but failed to decrease viral RNA titers [23]. Overall, approaches using recombinant viral vectors delivering HCV surface glycoproteins seem to be of limited promise, especially as immunotherapy for chronic disease. In the setting of chronic infection, where HCV-harboring cells need to be eliminated, immunotherapeutic approaches inducing a cellular immune response are a logical approach to improved control of HCV. In addition, if sterilizing antibody-mediated immunity cannot be achieved for HCV, as is indicated by many studies, agents inducing CD4+ and CD8+ T cell responses specific for HCV might also be the best option for prophylactic vaccines, as they might help to prevent the establishment of chronic infection.

In this study, we evaluated a novel yeast-based immunotherapeutic for its ability to induce HCV-antigen-specific T cell responses. These data showed that GI-5005, which expresses a HCV NS3-Core fusion protein, can induce high levels of antigen-specific proliferative as well as cytotoxic T cell activity in mice. The cytotoxic effector cells
derived from GI-5005-immunized mice were capable of killing target cells expressing HCV NS3 and Core antigens in a dose-dependent manner. The cellular immune responses increased in magnitude with increasing number of GI-5005 doses administered. Yeast vector neutralization was not observed in the mouse studies presented here (Fig. 6), or in rabbit toxicology studies, even after administration of as many as 13 weekly doses of GI-5005. The immune responses were long lasting suggesting the presence and activation of memory T cells. Of interest, the data presented in Fig. 6 failed to discriminate between the various boosting regimens following initial weekly immunizations. The effect of dosing schedule is currently being investigated more fully in mice in anticipation that such data will be valuable for designing prophylactic vaccine trials in humans.

The cytokines secreted by immune spleen cells in response to whole yeast consisted of TNF-α, IFN-γ, GM-CSF, IL-2, IL-6 and IL-12, suggesting the activation of a pro-inflammatory Th-1 type immune response. The cellular immune responses elicited upon GI-5005 administration achieved immune protection upon challenge of the mice with tumor cells that expressed the HCV NS3 protease constitutively. Immune protection in these mice prevented tumor formation, and the few GI-5005 immunized mice that did develop small tumors had lost expression of the HCV antigen due to immune selection pressures. Interestingly, the proliferative and cytotoxic T cell responses from immunized mice that rejected tumors were significantly higher than those observed using spleen cells from mice that were immunized with GI-5005 but not challenged with tumor cells. These results suggest that exposure of the animals to the same antigen, in this case NS3, multiple times (in a different context, i.e., NS3 present in yeast or NS3 expressed by mouse tumor cells) sensitizes the immune system and yields a highly boosted level of cytoxic effector cell activity.

As an immunotherapeutic, GI-5005 was shown to boost pre-existing HCV-specific cellular immune responses very efficiently in mice bearing syngeneic tumor cells expressing HCV NS3 allowing for complete eradication in a high proportion of animals. A similar augmentation of HCV-specific immune responses is expected to occur in chronic HCV patients upon receiving GI-5005 immunizations. GI-5005 targets two highly conserved HCV proteins, Core and NS3, both of which are essential for virus replication [5,24,25]. The use of large segments of conserved viral proteins in GI-5005 should render this immunotherapeutic effective even for the genetically diverse populations of HCV present in chronic infection. The inclusion of two HCV antigens in GI-5005 should further enhance the breadth of the cellular immune responses induced upon immunization. Repeat administration of GI-5005 should therefore induce long-lasting, broad-based immune responses targeting pre-existing as well as novel epitopes present in HCV proteins of such a magnitude that virus clearance will be achieved. However, it is important to be cautious in interpreting results from mice bearing HCV antigen-expressing tumors as compared to humans chronically infected with HCV. In the human situation, individuals have been infected for a prolonged period and multiple mechanisms including both active immunosuppression and immune tolerance may be preventing HCV clearance. These mechanisms may be difficult to overcome using an immunotherapeutic approach.

That GI-5005 has indeed the potential to elicit HCV-specific immune responses associated with control of HCV in humans is highlighted by preliminary studies using peripheral blood lymphocytes from HCV-exposed humans. In a subject with spontaneous eradication of HCV during acute infection, GI-5005 was able to induce recall responses similar to those that could be detected using overlapping peptides in an ELISpot assay and also to stimulate proliferative responses comparable to recombinant HCV proteins (unpublished observations).

In addition to being able to interact directly with dendritic cells, yeast have a variety of other characteristics that make them an ideal platform for immunotherapy. Multiple antigens may be engineered for expression within a single yeast strain [26–30], and these formulations share many advantages with DNA vaccines, including ease of construction and the ability to target multiple antigens. Unlike DNA vaccines, yeast-based immunotherapeutic formulations do not require extensive purification to remove potentially toxic contaminants. In addition, pre-clinical studies in mice and rabbits with recombinant yeast producing a variety of heterologous antigens have failed to show evidence for emergence of neutralizing antibodies directed against the yeast vector upon repeated administration.

In summary, we demonstrate that recombinant-yeast-based immunotherapy can induce potent HCV-specific cellular immunity with a Th1 profile, that this induction of cellular responses is dose-dependent and can be boosted with repeat injections, and that no neutralization or tolerance to the product was induced. We also show a biological effect of GI-5005 as both prophylactic and therapeutic vaccination was able to eradicate HCV-expressing tumors in mice. Together our data provide a strong rationale for evaluating GI-5005 as immunotherapy in chronic HCV infection.

Acknowledgements

We would like to thank Dr. Alfred Prince for the generous gift of rVV-NS3 and rVV-Core and Dr. Daniel Kuritzkes for providing rVV-Gag and rVV-lac.

References
