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Generation of cellular immune responses to HCV NS5 protein through *in vivo* activation of dendritic cells

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SUMMARY

Chronic hepatitis C (HCV) infection is a substantial medical problem that leads to progressive liver disease, cirrhosis, and hepatocellular carcinoma (HCC). The aim of this study was to achieve sustained cellular immune responses *in vivo* to a HCV nonstructural protein using dendritic cell (DC)-based immunization approach. We targeted the HCV NS5 protein to DCs *in vivo* by injecting microparticles loaded with this antigen. The DC population was expanded in BALB/C mice (H-2^d) by hydrodynamic injection of a plasmid pUMVC3-hFLex expressing the secreted portion of the human Fms-like tyrosine kinase receptor-3 ligand (hFlt3). Mice were subsequently injected with microparticles coated with HCV NS5 protein via the tail vein. Cellular immune responses were determined with respect to secretion of INF γ and IL2 by CD4⁺ cells and cytotoxic T-lymphocyte (CTL) assays *in vitro*; inhibition of tumour cell growth was employed for the assessment of CD8⁺ generated activity *in vivo*. We found that Flt3L treatment expanded the DC population in the spleen to 43%, and such cells displayed a striking upregulation of CD86 as well as CD80 and CD40 co-stimulating molecules. Viral antigen-specific T_H1 cytokine secretion by splenocytes was generated, and CTL activity against syngeneic NS5 expressing myeloma target cells was observed. In addition, these cells inhibited tumour growth indicating that NS5-specific robust CTL activity was operative *in vivo*. Thus, the capability of activating DCs *in vivo* using the methods described is valuable as a therapeutic vaccine strategy for chronic HCV infection.

Keywords

cellular immune response; cytotoxicity; dendritic cells; hepatitis C virus

INTRODUCTION

Hepatitis C virus (HCV) infection is caused worldwide by a positive strand RNA virus [1]. Exposure to HCV results in acute infection in approximately 15% of persons; however, in the remaining 85%, there is the establishment of persistent viral infection. Most individuals with chronic HCV have mild to no clinical symptoms; however, there is often the development of progressive liver disease, cirrhosis, and hepatocellular carcinoma (HCC). Therefore, chronic HCV infection is a substantial medical problem on a global basis. The

mechanism(s) responsible for persistent viral infection are unclear, but may involve alterations in the host cellular immune response to HCV.

In this regard, dendritic cells (DCs) are highly specialized antigen-presenting cells (APC). DCs are characterized by their ability to take up, process, and present peptides in the context of MHC Class I and Class II molecules to effector T-cells. Thus, DCs act at the interface between innate and adaptive immune responses [2]. Upregulation of co-stimulatory molecules is essential and influenced by several factors such as activation of Toll-like receptors or binding of CD40 by the CD40 ligand. Interactions of DCs with T-cells allows for highly efficient antigen presentation and the generation of viral-specific cellular immunity [3].

There is increasing evidence that DC-based vaccination represents an attractive approach to elicit sustained anti-viral responses to HCV structural and nonstructural proteins [4–6]. To develop and employ a successful immunological approach against HCV, it is important to understand the essential process(es) in the host that leads to clearance of viral infection. Current studies suggest that HCV eradication from infected persons requires the early appearance of strong CD8⁺ cytotoxic T-cell (CTL) activity in the context of a vigorous and sustained CD4⁺ T-cell proliferation culminating in the secretion of T_H1-type cytokines in response to stimulation by multiple structural and nonstructural viral proteins [7–9]. Because DCs interact with both CD8⁺ and CD4⁺ T-cells, they play a pivotal role in the transition from innate to adaptive immunity. Interestingly, there is emerging data that suggests chronic HCV infection is associated with impaired DC activity [10,11]. Defective DC function has been observed in recent studies where chronic alcohol consumption has been found to alter the properties of DCs and resulted in impaired cellular immune responses against the HCV NS5 protein [12]. Taken together, these studies emphasize the importance of robust DC function for successful eradication of chronic HCV infection.

The purpose of this study was to achieve sustained cellular immune responses to a HCV nonstructural protein *in vivo* using a DC-based immunization approach. In this study, we attempted to target the HCV NS5 protein to DCs *in vivo* by injecting microparticles coated with this antigen. Previous studies revealed that antigen-loaded beads represent an excellent vehicle for targeting DCs *in vitro* because they are very efficiently internalized by such cells [13]. The results suggest that DCs are activated *in vivo* and induced a viral-specific CD4⁺ and CD8⁺ cellular immune response following prior DC expansion with Flt3L administration and subsequent intravenous (i.v.) injection of NS5 protein-coated microparticles.

MATERIALS AND METHODS

Mice

Six- to eight-week-old female BALB/c mice (H-2^d) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and kept under specific pathogen-free conditions in the animal facility of Rhode Island Hospital. Experiments were conducted according to animal protocols reviewed and approved by the Lifespan Animal Care and Use Committee.

Culture conditions

Splenocytes derived from immunized and control mice were cultured in serum-free HEPES-buffered RPMI 1640 medium (Cambrex Bio Sciences, Wakersville, MD, USA) supplemented with 2 mM L-glutamine, 1% essential and nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 U/mL gentamycin (all from Sigma–Aldrich, St Louis, MO, USA).

Cultivation medium for splenocytes obtained from immunized animals was additionally supplemented with heat-inactivated 10% foetal bovine serum (FBS).

Coating of magnetic beads with HCV NS5

Immunomagnetic beads (Calbiochem, EMD Bioscience, San Diego, CA, USA) with a diameter of 1.3 μm were suspended in CDI buffer (Sigma-Aldrich, St Louis, MO, USA) and incubated for 10 min at 50 °C, pelleted and washed three times in borate buffer (pH 8–9) (Fluka/Sigma-Aldrich, St Louis, MO, USA). After the final wash, borate buffer was added with or without 100 μg of NS5 protein (RDI Fitzgerald, Concord, MA, USA), followed by an overnight incubation at room temperature under constant slow agitation. Then beads were pelleted through magnetic forces, and the supernatant was removed by pipetting; the beads were then washed and resuspended in 200 μL Hank's balanced salt solution (HBSS). To prevent bead aggregation, centrifugation was omitted in all steps.

In vivo enrichment of dendritic cells

Expansion of the DC population before immunization was achieved by using methods previously described [2,14]. Briefly, the plasmid pUMVC3-hFtLex expressing the secreted portion of human Fms-like tyrosine kinase receptor-3 ligand (hFt3: Vector Core Laboratory, University of Michigan) was injected twice (day 0 and 6) into the tail vein of mice (hydrodynamic gene delivery); the first course of immunization was then performed on day 12 after the first hFt3L plasmid injection.

Immunization

Four groups of mice were immunized after *in vivo* expansion of DCs with hydrodynamic injection of an hFt3L expression plasmid. Mice were injected with 100 μg of beads or beads coated with HCV NS5 protein and resuspended in 200 μL HBSS intravenously into the tail vein. The immunization schedule is described in Table 1.

Intracellular cytokine staining (ICS) and flow cytometric analysis

ICS and flow cytometry were performed in accordance with methods previously described [2,14]. In brief, $1\text{--}5 \times 10^5$ cells were incubated with excess anti-mouse CD16/32 (clone 93, rat isotype) to block Fc-receptor, then stained with 1 μg PE-, FITC-, or PerCP-labelled antibody specific for the following mouse cell surface markers: CD11c (clone N418), CD4 (clone L3T4), CD8a (clone Lyt-2), CD40 (clone 1C10); the recommended isotypes controls were included. ICS was performed with anti-mouse IFN- γ (clone XMG1.2) or anti-mouse IL-2 (clone JES6-5H4), and the Cytfix/Cytoperm Kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. All antibodies were purchased from eBiosciences (San Diego, CA, USA) if not otherwise indicated.

Proliferation assay

Cells (4×10^6) were labelled with 1.0 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) in HBSS by incubation for 15 min at 37 °C. Then, the cells were centrifuged, resuspended in prewarmed medium, and incubated for another 30 min to ensure complete modification of the probe. The labelled cells were incubated for 5 days in 6-well plates containing 5 mL culture medium supplemented with 10% FBS and 1 $\mu\text{g}/\text{mL}$ HCV NS5. The cells were then washed, stained for CD4 or CD8, and evaluated for proliferation by flow cytometry.

Measurement of cytokine secretion

To quantify cytokine production, splenocytes derived from immunized and nonimmunized animals were cultured at 5×10^5 cells/200 μ L/well in 96-well flat-bottom plates at 37 °C. Cells were stimulated with 0.1–1 μ g/mL recombinant HCV NS5 protein. After 24 h, the supernates were collected, and the levels of IFN- γ and IL-2 were quantified using commercial ELISA kits purchased from eBiosciences according to manufacturer's instructions.

In vitro cytotoxicity assay

The CTL activities expressed by splenocytes derived from immunized and control mice were assessed following *in vitro* stimulation in which 1×10^8 cells in 30 mL of culture medium were incubated with recombinant murine IL-2 (5 U/mL; eBiosciences, San Diego, CA, USA) and recombinant HCV NS5 protein (0.3 μ g/mL; RDI Fitzgerald, Concord, MA, USA). After three days culture, the cells were harvested, and a standard 4-h 51 Cr release assay was performed. The 51 Cr labelled parental syngeneic murine myeloma cells (SP2/0); (ATCC, Manassas, VA, USA) and stably expressing clones of HCV NS-5 (SP-2/21-NS-5) were used as the targets. All assays were conducted in quadruplicate. Antigen-specific lysis is calculated using the following formula: $[(E-M)/(T-M)] \times 100$; T = maximum release, M = spontaneous release, E = experimental release.

Tumour challenge model

CTL activity was assessed *in vivo* by tumour challenge in accordance with methods described previously [2,14]. Briefly, stably expressing HCV NS5 cells (SP-2/21-NS-5) were harvested and washed three times in serum-free medium. The backs of mice were shaved, and each animal was inoculated s.c. with 4×10^6 SP2/21-NS5 cells in the left flank and with parental SP2/0 cells in the right flank. Cells were resuspended in 100 μ L serum-free medium. On day 15, mice were euthanized; tumours were dissected, measured, and weighed.

Statistical analysis

Results were analysed using the SigmaStat 3.0 statistics program (Jandel Scientific, San Rafael, CA, USA). Individual means were compared using a nonpaired Student's *t*-test. When comparing more than two groups, a one-way ANOVA was performed followed by a Tukey test to determine which groups differed significantly ($P < 0.05$).

RESULTS

Expansion and maturation of dendritic cell populations in vivo

To expand DCs in BALB/c mice prior to immunization, we employed the technique of hydrodynamic delivery of the hFlt3-L expressing plasmid (pUMVL3hFlex) as described [15]; the total number of splenocytes was increased tenfold to approximately 4×10^8 cells/spleen of which 25% were positive for CD11c [13]. This represents approximately 100-fold expansion of the murine DC splenic population [6,13]. In this context, animals were immunized with NS5 or poly IC-coated beads. It was not possible to determine the number of amplified DCs in individual animals following immunization. Nevertheless, we characterized the DC population following i.v. injection of NS5-coated magnetic microparticles in animals with or without hydrodynamic injection ($\times 2$) of the Flt3L expression plasmid. Splenocytes were isolated and bead-containing phagocytic cells were separated using MACS® columns. Isolated cells were then stained with antisera to CD11c and CD11b, and double-positive cells were designated as DCs. Without Flt3L treatment, DCs represented 6.9% of the total cell population. However, following Flt3L injection, 43%

of the cells in the spleen were characterized as DCs as shown in Fig. 1. In addition, all such cells were found to contain NS5-coated microbeads by direct microscopic examination, and thus the HCV antigen-coated microparticles reached the spleen by IV injection (data not shown). Furthermore, injection of beads coated with poly I:C, a ligand to the Toll-like receptor 3 expressed on DCs, led to a striking upregulation of CD86 (Fig. 2a), as well as CD 80 and CD 40 (Fig. 2b,c). Again, it appears that the coated beads reached their intended target cells in the spleen and matured the DC population.

In vitro cytotoxicity assay

A standard ^{51}Cr -release assay was employed to measure CTL present in the animals immunized with NS5-coated microparticles when compared to beads alone. The target SP2/21-NS5 cells were a derived cell line that constitutively expresses NS5-related peptides following stable transfection of an NS5 expressing plasmid [6]. The control cells were derived from the parental SP2/0 myeloma cell line. Highly significant CTL activity was observed in Flt3L-pretreated mice immunized with NS5-coated beads at various effectors to target cell ratio(s). In contrast, splenocytes derived from animals immunized with beads alone had little, if any, NS5-specific cytotoxicity (Fig. 3). There was no CTL activity directed against the control SP2/0 cells derived from mice immunized with NS5-coated, or noncoated beads (data not shown). These results suggest that i.v. immunization of NS5-coated microparticles in Flt3L-expanded DC populations are capable of activating HCV-specific CTL activity. It then became of interest to determine if such CTL activity was operative *in vivo*.

Tumour challenge model for assessment of in vivo CTL activity

Generation of CD8⁺ CTL viral-specific immune response is a key to the eradication of HCV infection in the liver. We performed a tumour challenge to determine whether antigen-specific CTL activity was present *in vivo*. In this experimental paradigm, animals were inoculated with tumour cells that expressed NS5 protein (SP2/21-NS5), or the nonexpressing parental SP20 cell line (control). DCs were expanded by hydrodynamic injection of the Flt3L expressing plasmids on day(s) 0 and 6, respectively. On day 12, animals were immunized with HCV NS5-coated beads i.v. via the tail vein; an immunization boost was performed 2 weeks later. Control animals received no immunization, immunization with beads alone, or immunization with soluble recombinant NS5 protein as an additional control. After the second immunization, mice were inoculated with 4×10^6 tumour cells. To provide intra-animal comparisons, mice received NS5 expressing cells in the left flank and the SP2/0 parental cells in the right flank simultaneously. Two weeks later, tumours were dissected, removed, and weighed.

As shown in Fig. 4, there was no difference in growth rate between the NS5 expressing tumour cells and the SP2/0 nonexpressing parental cells in immunized mice that received no beads, uncoated beads, or NS5 soluble protein as shown in Fig. 4a,c,d. In contrast, animals immunized twice with NS5-coated beads showed a dramatic reduction in the growth rate of NS5 expressing tumour cells when compared to the control SP2/0 parental tumour cell line (Fig. 4b). These results suggest that the Flt3L-expanded DC population that ingested the NS5-coated microparticles generated robust CTL activity *in vivo* as measured by reduction in tumour growth.

HCV viral antigen-specific cytokine production

Natural clearance of HCV infection is strongly associated with a sustained T_H1 immune response. Therefore, we assessed the ability of splenocytes derived from immunized mice to secrete T_H1-type cytokines such as INF γ , and IL-2. We compared cytokine secretion in animals pretreated with or without Flt3L. As shown in Fig. 5a,b, both INF γ and IL-2

secretion were increased in an NS5 dose-dependent manner in animals that received both NS5-coated beads and Flt3L prestimulation when compared to mice who received NS5-coated beads without Flt3L expansion of DCs. The specificity of the CD4⁺ immune response to NS5 is indicated by cytokine production following stimulation with an increasing concentration of NS5 protein. There was no INF γ and IL-2 production by splenocytes following immunization with beads alone or immunization with NS5 protein (data not shown).

DISCUSSION

Similar to most phagocytic cells, DCs are equipped with receptors which include members of the C-type lectin family that mediate antigen uptake. This phagocytic process is initiated through the calcium-dependent binding of C-type lectins to carbohydrate-bearing pathogen-derived antigens through highly conserved carbohydrate recognition domains (CRD). Some of these receptors appear to be ubiquitously expressed on phagocytic cells such as macrophages, monocytes, B-cells, neutrophils, and DCs [16]. Two different types of transmembrane C-type lectins are expressed on DCs distinguished by their molecular characteristics. The prototype C-type-1 lectin is the macrophage mannose receptor (MMR; CD206). This molecule contains 8 CRD, a N-terminal cysteine-rich domain, and a region involved in antigen transport [16]. A homologue to the MMR is DEC-205 (CD205), which is a second member of the type I lectins. Unlike the MMR, it is expressed specifically and uniquely on DCs and thymic epithelial cells. Investigations reveal that antibodies binding to DEC-205 are presented with a 100-fold higher efficiency to effector T-cells than nonrelevant antibodies [17]. In addition, when antigen is conjugated to an anti-DEC-205 antibody and specifically taken up by DCs residing in lymphoid tissue, evidence suggest that peptide epitopes are presented with high efficiency to CD4⁺ and CD8⁺ T-cells [18].

To explore the role of DCs in generating anti-viral immune responses to HCV, it was necessary to highly enrich the DC population *in vivo* via the injection of FMS-like tyrosine kinase 3 ligand (Flt3L). Recombinant human (rh) Flt3L exhibits a substantial, time dependent, and reversible increase in the number of functionally mature DCs derived from spleen, bone marrow, liver, lymph nodes, and gut-associated lymphoid tissue [19]. Furthermore, an increase in the weight of the liver of Flt3L-treated mice has been observed, which correlates with a striking proliferation of DEC205⁺ and CD86⁺ DCs within the nonparenchymal cell (NPC) population [15,19,20]. In contrast, other procedures used to amplify DCs from bone marrow-derived cells *in vitro* involve the addition of GM-CSF to cells in culture; however, the Flt3L stimulation approach leads to a uniform enrichment of all DC subtypes, which is in contrast to GM-CSF stimulation [21,22].

To amplify DC populations, hydrodynamic-based administration of a plasmid expressing Flt3L effectively transfects hepatocytes *in vivo* [6]; rapid injection of a large volume (2 mL in 5 s) that exceeds the cardiac output of the mouse. The resulting high hydrostatic pressure in the inferior vena cava causes an inversion of the blood flow containing the expression plasmid, and transfects organs such as spleen, liver, heart, and kidney with high efficiency [15]. Two hydrodynamic injections of the Flt3L expression plasmid, the splenocyte population was expanded up to tenfold ($5 \times 10^7 - 5 \times 10^8$). The proportion of functionally mature DCs in the splenic cell population increased 30% compared to 1% found in normal spleen [6].

There is increased interest in the use of DCs in therapeutic vaccination of viral diseases such as HCV [6]. They have high level of antigen-presenting capability and are a key component in the transition from the innate to an adaptive immune response. Thus, numerous studies reveal the importance of using DC-processed antigens in immunization strategies

[4,6,13,18,23–29]. The most common procedure employs DCs as vaccine vectors through prior *ex vivo* loading of these cells with the antigen of interest and subsequent adoptive transfer into vaccinated animals [4–6,13]. Indeed, this approach has reached clinical application [24,30,33].

Another novel approach targets the antigen to DCs *in vivo* instead of loading such cells with the antigen *ex vivo* prior to immunization. Bonifaz *et al.* presented an attractive model, i.e. antigen, coupled to the DC-specific DEC 205 antibody, initiates uptake into the cell by a receptor-mediated process followed by DC antigen processing [18]. Immunization studies induced high-level stimulation of antigen-specific CD4⁺ and CD8⁺ T-cell responses. Thus, it is possible to target antigens to DCs *in vivo*, and subsequently stimulate a robust cellular immune response. Another successful approach employs expression plasmids targeted to DCs following encapsulation into microspheres; encapsulated antigens are processed in the context of MHC class molecules and presented to T-cells to stimulate a cellular immune response [31–33]. Therefore, additional studies demonstrate that DCs are a promising vector to produce antigen-specific immunological responses [34,35].

Recently, a more straightforward and direct strategy for antigen loading of DCs has been proposed. It has been shown that magnetic microbeads may serve as carriers for antigen and are taken up efficiently by DCs *in vitro*. Furthermore, these studies suggest that DCs generated in mice through hydrodynamic tail vein injection of Flt3L expression plasmid will take up antigen-loaded beads with varying efficiency [18]. More important, it was reported that to induce a strong T-cell response, it was necessary to combine the process of antigen loading of DCs with a second maturation stimulus using antibodies to CD40 [4]. These observations support the concept that in the absence of a second stimulus, such as crosslinking of CD40 or ligand binding to TLR receptors as well as generating CD80 and CD86 interaction with T-cells may render DCs tolerogenic [28]. In the present study, we propose a novel preclinical vaccination strategy for HCV, which enabled us to combine currently established *in vitro* methods with an *in vivo* approach. Of central importance is the use of an Flt3L expressing plasmid for the expansion of the DC population *in vivo*. Indeed, the clinical use of this protein ligand has been reported [35]. By pretreating BALB/c mice prior to vaccination, the expanded lymphoid repertoire is slanted towards a high expansion of the DC population that is sufficiently immature to allow for antigen-coated microparticle uptake. Importantly, such DCs still possess the distinct property of T-cell priming. To our knowledge, this is the first study to reveal that enrichment of DCs through Flt3L pretreatment enables the establishment of a substantial viral antigen-specific immune response upon single injection of antigen-loaded microspheres. Although these antigen-coated beads are engulfed by other phagocytic cells, there is a preferential ingestion of the microparticles by the expanded DC population. Indeed, Flt3L exposure produces DCs with a maturation status that is still low enough to keep their phagocytic properties while being sufficiently mature to induce antigen-specific T-cell responses without requiring additional co-stimulation such as crosslinking of CD40 or toll-like receptors activation via poly I:C or LPS [4].

This approach functionally fulfils the requirements for an effective vaccine strategy against HCV in a preclinical animal model system, in that, there is induction of a strong CD8⁺ CTL response accompanied by sustained CD4⁺ T-cell activity [11]. In this context, it was possible to induce a significant CD4⁺ T-cell response resulting in an NS5-specific induction of IFN γ and IL-2 secretion by splenocytes following two immunizations. Cytokine production was NS5 specific because it was clearly dependent upon the protein concentration in the splenocyte restimulation assay. However, we were unable to detect intracellular cytokine staining by flow cytometric analysis. It was apparent from earlier studies that to reach detectable cytokine levels by intracellular cytokine staining requires

strong additional immunogenic stimuli such as LPS when *ex vivo* loaded DCs were used for immunization [10]. However, the CD8⁺ CTL biological activity was robust as shown by the suppression of tumour growth of syngeneic NS5 expressing myeloma cells in immunized mice. This finding confirms that the NS5-specific CTL response measured *in vitro* is operative *in vivo* and highly active. Our studies further revealed that it was essential to use microbeads as carriers to achieve efficient uptake of the immunogen by DCs because i.v. injection of soluble NS5 protein did not induce detectable T-cell responses as demonstrated in the tumour challenge experiments and T_H1-type cytokine secretion.

This preclinical model provides evidence that DCs can be activated directly *in vivo* to generate HCV-specific cellular immune responses. It will be of interest to employ this same approach using biodegradable microparticles because we have shown that such microbeads are easy to use as carriers for *in vivo* targeting of antigens to DCs. Because Flt3L ligand has been administered to humans in clinical vaccine studies [35], the combination of immunization with a biodegradable microparticle antigen carrier system appears to be a promising approach. Thus, the capability of activating DCs *in vivo* using the methods described herein have value as a therapeutic vaccine strategy for chronic HCV infection.

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Fig. 1.

Expansion of CD11b⁺ and CD11c⁺ cells following hydrodynamic delivery of an Flt3L expressing plasmid. Panel A represents before and Panel B represents after administration of Flt3L. Note that the percentage of double-positive cells increased from 6.5% to 43.3% of the splenic population.

Fig. 2.

Expression of co-stimulatory molecules CD86 (Panel A), CD40 (Panel B), and CD80 (Panel C) on DC surface after Flt3L injection or/and immunization with beads coated with/without NS5, poly I:C. Mice were injected with different coated microbeads and sacrificed 12 h after injection for harvesting of splenocytes. Splenocytes were stained with anti-CD11c as a marker for DCs, and anti-CD80, anti-CD86, and anti-CD40 as indicators of maturation. CD11c⁺ cells were gated and analysed for earlier mentioned markers. DCs obtained from mice that were injected solely with Flt3L expression plasmid (Black dotted line/grey filling) were already CD86 and CD80 positive: 47.40% and 47.58%, respectively. Injection of NS5-coated microbeads (light pink line and filling) did not increase neither CD86 nor CD80 expression: 46.54% and 48.19%, respectively. Intravenous injection of microparticles coated with the TLR3-ligand poly I:C, a potent DC maturation stimulus upregulated all three markers regardless of NS5 (82.32%, 66.94%, 81.08%, CD86, CD40, and CD80 levels, respectively, for those received beads-poly IC and Flt3L represented by blue line; 80.29%, 52.18%, 71.98%, CD86, CD40, CD80 levels, respectively, for those received beads-NS5-poly I:C and Flt3L represented by orange line). This confirms that protein that is coated on microbeads reaches DCs where it is processed effectively. Percentile bars from top to bottom across all three panels are for the indicated line: blue (Bead-poly I:C-Flt3L), orange (Bead-NS5-poly I:C-Flt3L), black (Flt3L only), light pink (Bead-NS5-Flt3L).

Fig. 3.

In vitro CTL activity generated against NS5 expressing target cells. Splenocytes derived from immunized mice were restimulated with NS5 protein for 4 days. Restimulated cells were placed in co-culture with NS5 expressing or parental SP2/0 myeloma cell lines. Target cells were labelled with Cr⁵¹ chromium, and target cell lysis was measured by release of Cr⁵¹ chromium into the cell culture medium. Nonspecific SP2/0 cell lysis has been subtracted from the lysis of NS5 expressing cells (SP-2/21-NS5). Note that animals immunized with uncoated beads showed no CTL activity compared to beads coated with NS5 protein.

Fig. 4.

Generation of CD8⁺ CTL activity *in vivo* using a tumour challenge model. Mice were administered Flt3L expression plasmid via hydrodynamic delivery and then immunized $\times 2$ with microparticles that were either uncoated (a), coated with NS5protein (b), or following i.v. injection of NS5 protein alone (c), or no beads (d). Two weeks after the last immunization, mice were inoculated with hybridomas expressing NS5 protein (SP2/21-NS5) in the left flank and a hybridoma cell line negative for NS5 (SP2/0) in the right flank simultaneously. Animals were sacrificed two weeks later, and the tumours were excised and weight measured. Note the striking reduction in tumour growth rate in Panel B where animals are immunized with NS5-coated beads, and showed potent inhibition in growth rate of NS5 producing tumours. Animals immunized with uncoated beads, NS5 protein alone, or no beads showed no effect on tumour growth rate. The anti-tumour effect was antigen specific as shown in Panel B because there was no effect on the growth rate of the parental, nonexpressing NS5 cell line. * $P < 0.01$.

Fig. 5.

Demonstration of CD4⁺ activity against NS5. Splenocytes from immunized mice were restimulated for 18 h with NS5 protein at various concentrations. IFN γ (a) and IL-2 (b) were determined in cell culture supernate by ELISA. Note that animals immunized with NS5-coated beads and pretreated with Flt3L expression plasmids showed a striking dose-dependent increase in both IFN γ and IL2 expression when compared to animals immunized with NS5-coated beads without prior Flt3L ligand treatment. Animals immunized with beads alone had no effect on IFN γ or IL2 expression (data not shown).

Table 1**Immunization schedule**

Day 0	First hydrodynamic injection of Flt3L plasmid
Day 6	Second hydrodynamic injection of Flt3L plasmid
Day 12	First immunization (beads with or without NS5 protein)
Day 26	Second immunization
Day 40	Sacrifice animals for immunological studies Injection of tumour cells (4×10^6)
Day 47	Weigh tumours