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Short communication

Engineered exosomes boost the HCV NS3-specific CD8^{+} T lymphocyte immunity in humans

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ABSTRACT

At the present, no anti-Hepatitis C virus (HCV) HCV vaccine is available, and many patients failed the treatment with new class of HCV inhibitors. In HCV infection, both experimental and clinic evidences indicate that a strong CTL-immune response could have significant therapeutic effects. We developed an innovative anti-HCV CD8^{+} T immunogen based on the uploading in engineered exosomes of full-length HCV-NS3 protein. HCV NS3 exosomes appeared immunogenic when injected in mice, as proven by the detection of a memory CD8^{+} T lymphocyte pool two weeks after the last of three immunizations. On the other hand, dendritic cells isolated from PBMCs of HCV infected patients activate autologous HCV NS3-specific CD8^{+} T lymphocytes upon challenge with HCV NS3 exosomes. These results provide the proof-of-principle that engineered exosomes can boost the CD8^{+} T cell immunity in HCV-infected patients, thus representing a suitable option for patients resisting the therapies with recently discovered HCV inhibitors.

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1. Introduction

Although an effective new class of HCV NS3/NS4A and NS5B inhibitors (i.e., boceprevir and sofosbuvir) is available [1], however drug resistance, suboptimal activity against diverse HCV genotypes, and their extremely high cost limit the expectancy of HCV eradication, while prompting the research for innovative vaccine strategies [2]. A both strong and broad CTL immune response targeting cells expressing HCV antigens is expected to be a key step towards viral clearance. In fact, the presence of multispecific IFN-γ producing, HCV-specific CD8^{+} T cells have been reproducibly detected in infected hosts recovering from acute HCV infection [3,4]. Consistently, in chronic HCV infection the functionality of HCV-specific CD8^{+} T cells is significantly altered [5].

Exosomes are vesicles of 50–100 nm released constitutively by all cell types [6]. They form intracellularly upon inward invagination of endosome membranes. Leading to the formation of intraluminal vesicles which thereby form multivesicular bodies. They can traffic either to lysosome vesicles or to the plasma membrane thereby releasing their vesicular contents in the extra-cellular milieu upon fusion with plasma membrane. Vesicles released by this mechanism are defined exosomes.

Exosomes are nanoparticles having a low intrinsic immunogenic profile. Their immunogenicity is basically related to the amounts and quality of antigens they incorporate. Exosomes have been tested in a number of clinical trials demonstrating both feasibility and good tolerance of exosomes as cell-free vaccines. However, the therapeutic efficacy appeared quite limited posing the need for new methods to increase their immunogenicity.

Engineering exosomes to upload heterologous proteins represents the last frontier in terms of nanoparticle-based technology. We optimized a method of protein incorporation in exosomes by exploiting the unique properties of a non-functional mutant of the HIV-1 Nef protein referred to as Nef^{mut}. It incorporates at high extents in exosomes meanwhile acting as carrier of protein antigens fused at its C-terminus [7]. In this way, the Nef^{mut}-based fusion products remain protected from external behavior. These features, together with the flexibility in terms of incorporation of foreign antigens and ease of production, make Nef^{mut}-based exosomes a convenient vehicle for immunogens.

Abbreviations: HCV, Human hepatitis C virus; CTLs, cytotoxic T lymphocytes; kDa, kilodaltons; VSV-G, vesicular stomatitis virus G protein; PBMCs, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorting; HPV, Human Papilloma Virus; FCS, fetal calf serum; APCs, antigen-presenting cells; CTL, cytotoxic T lymphocytes; TAA, tumor-associated antigens; iDCs, immature dendritic cells; AchE, acetylcholinesterase; PMA, phorbol 12 myristate 13-acetate; SD, standard deviation; SFU, spot-forming units; ALT, alanine transaminase.

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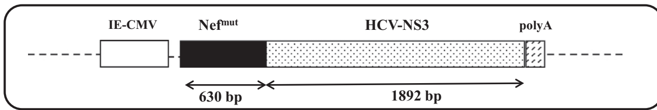

Fig. 1. Scheme of the pcDNA3-based vector expressing the Nef^{mut}/NS3 fusion protein. The positions of promoter (IE-CMV), Nef^{mut}/HCV-NS3 fused open reading frame, and poly A signal are signed. Base pair lengths of each moiety are also indicated.

HIV-1 Nef is a 27 kDa protein lacking enzymatic activities, however acting as a scaffold/adaptor element [8]. Nef^{mut} lacks basically all Nef functions [9], whereas incorporating in exosomes up to 100-fold most efficiently than the wild-type isoform. Recently, we showed that a viral TAA uploaded in Nef^{mut}-based engineered exosomes is cross-presented in targeted APCs, thereby eliciting a strong CTL immunity in mice which associates with clearing of syngeneic tumor cells implanted after immunization [10]. In the perspective to apply our previous findings to the treatment of HCV infection, engineered exosomes uploading HCV NS3 were pro-

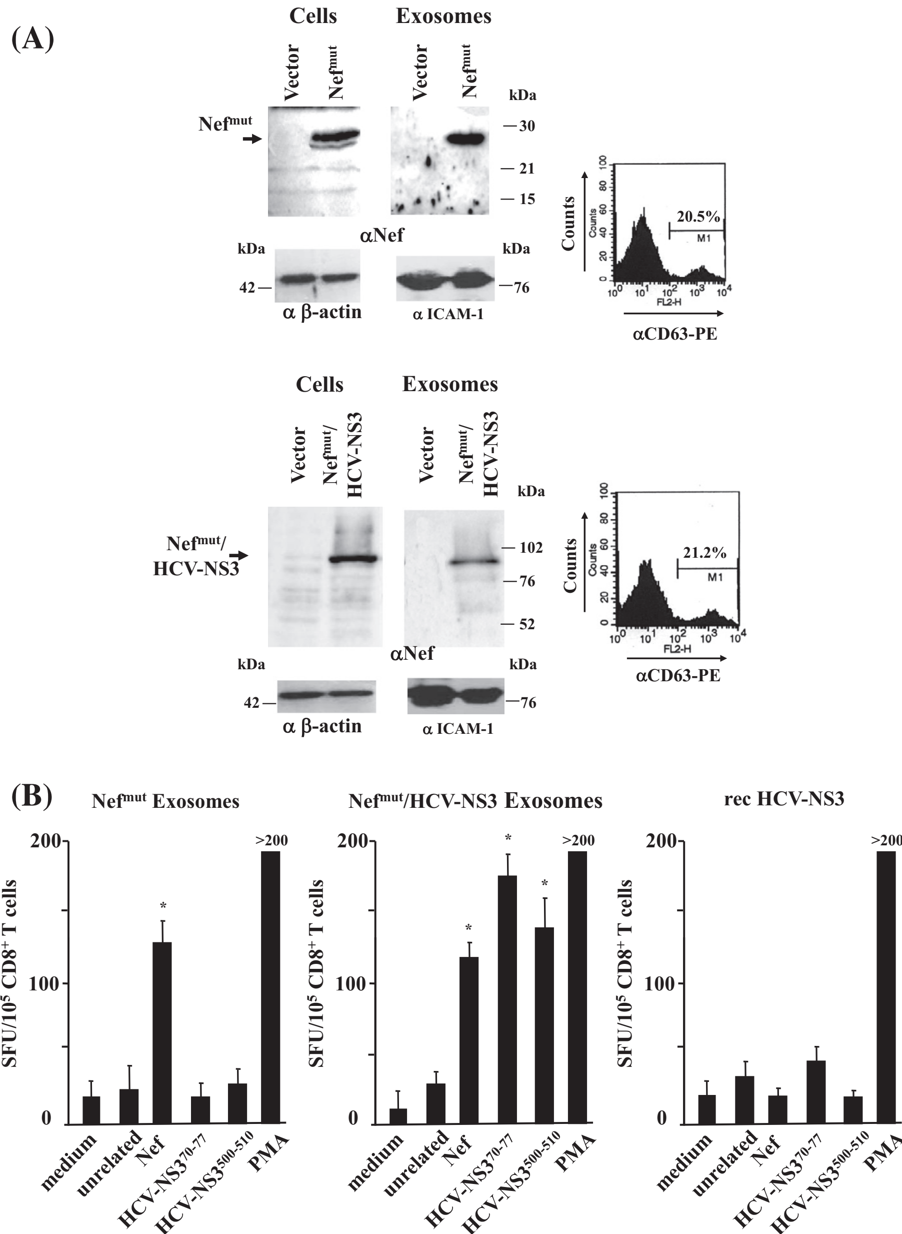

Fig. 2. HCV NS3-specific immunity induced in mice by inoculation of Nef^{mut}/NS3 exosomes. (A) Molecular characterization of exosome preparations uploading either Nef^{mut} (upper panels) or Nef^{mut}/NS3 (lower panels). A total of 30 μg (for cell lysates) and 200 μU equivalent of AchE activity of exosomes were assayed in western blot assays probed with anti-Nef, anti-β-actin (for cell lysates only), and anti-ICAM-1 (for exosomes only) antibodies. Arrows sign the relevant protein products. Molecular markers are given in kDa. In addition, both exosome preparations were tested for CD63 contents (right panels). Quadrants were set on the basis of the fluorescence of beads alone incubated with anti-CD63 mAb. Percentages of positive events are indicated. Results are representative of the assays performed on three different exosome preparations. (B) CD8$^+$ T cell immune response in mice inoculated with either Nef^{mut}-, Nef^{mut}/NS3 exosomes, or recombinant NS3 protein. C57Bl/6 mice (5 per group) were inoculated three times and, two weeks after the last inoculation, splenocytes were isolated and incubated 5 days in the presence or not of 5 μg/ml of either unrelated, Nef-, or NS3-specific peptides. Afterwards, cell activation extents were evaluated by IFN-γ Elispot assay carried out in triplicate with 10^5 cells/well. As a control, untreated cells were also incubated with 5 ng/ml of PMA and 500 ng/ml of ionomycin. Shown are the mean + SD number of SFU/10^5 cells. $^*p < 0.05$. The paired Student's *t*-test was used and confirmed using the non-parametric Wilcoxon rank sum test.

duced and tested for both their immunogenicity in mice and antigenicity in PBMCs from HCV infected patients.

2. Materials and methods

2.1. Exosome production, purification, quantification, and characterization

The recovery of the pcDNA3-based vector expressing the fusion protein Nef^{mut}/HCV-NS3, genotype 1b (Fig. 1) has been already described [11]. Engineered exosomes were produced by transfecting 293T cells as reported [7], and purified through differential centrifugations [12]. Their amounts were measured in terms of the activity of AchE, i.e., a classical exosome marker [13] through the Amplex Red kit (Molecular Probes). Exosome preparations were characterized by both western blot and FACS analysis as reported [7]. For semi-quantitative western blot, scaled amounts of recombinant Nef produced and quantified as described [14] were included as reference samples.

2.2. Mice immunization and detection of IFN-γ producing CD8$^+$ T lymphocytes

All studies with animals here described have been approved by the Ethical Committee of the Istituto Superiore di Sanità, Rome, Italy (protocol n. 555/SA/2012) according to Legislative Decree 116/92 which has implemented in Italy the European Directive 86/609/EEC on laboratory animal protection. Animals used in our research have been housed and treated according to the guidelines inserted in here above mentioned Legislative Decree. C57Bl/6 mice (Charles River Laboratories) were inoculated subcutaneously (s.c.) 3 times at 2-week intervals with either exosomes carrying Nef^{mut} and Nef^{mut}/NS3, or recombinant NS3 genotype 1b (ProSpec). Two weeks after the last inoculation, mice were sacrificed, and splenocytes cultured with the following H-2^b peptides: Nef$_{48-56}$ TAATNADCA [15], NS3$_{370-377}$ ITQMYTNV, and NS3$_{500-510}$ WYELTPAETSV [16]. After 5 days of incubation, IFN-γ Elispot assay was performed in triplicate conditions using reagents from Mabtech AB. SFU were counted 16 h later using an Elispot reader.

2.3. Cross-presentation assay in human PBMCs

Selected HCV chronically infected subjects had an average age of 51 years, were in good general health conditions, and did not receive anti-HCV treatment in the last 3 years, to avoid confounding results arising from the immunosuppressive effect of drugs. The patients had average levels of ALT of 103 ± 32 U/L, and serum HCV RNA mean titers of 2.7 × 10^6 IU/mL. PBMCs isolated from 3 chronically HCV infected patients and, as a control, 3 healthy donors were cultured in RPMI, 10% AB human serum for 5 days in the presence of PeptTivator NS3 (Miltenyi) (i.e., a pool of HCV NS3 genotype 1b peptides consisting in 15-mers with 11 aa overlap covering the whole NS3 sequence) following the manufacturer's recommendations. As a control, a mix of HIV-1 Gag peptides including most frequent potential T epitopes (PTE) [17] was used. In parallel, monocytes were separated from PBMCs using anti-CD14 microbeads (Miltenyi), and differentiated to iDCs with 500 Units/mL of IL-4 (R&D Systems) and 30 ng/ml of GM-CSF (Serotec Ltd). Their phenotype was routinely checked by FACS analysis for the expression of CD11c and the absence of CD14 markers. CD8$^+$ T cells were separated from PBMCs using anti-CD8 microbeads (Miltenyi). iDCs were challenged with the exosomes and, after 6 h of incubation, washed and co-cultured in triplicate conditions at 1:2 ratio with the CD8$^+$ T lymphocytes isolated from PBMC in an IFN-γ Elispot microwell plate. After 16 h incubation, IFN-γ Elispot assay was performed, and SFU were counted.

2.4. Statistical analysis

When appropriate, data are presented as mean + SD. In some instances, the paired Student's *t*-test was used and confirmed using the non-parametric Wilcoxon rank sum test. $p < 0.05$ was considered significant.

3. Results

3.1. HCV NS3 engineered exosomes elicit CD8$^+$ T lymphocyte adaptive immunity when injected in mice

Engineered exosomes uploading Nef^{mut}/NS3 were characterized compared to exosomes incorporating Nef^{mut} alone in terms of the contents of both Nef^{mut}-related products and CD63, the latter being a cellular marker typically associated to exosomes (Fig. 2A). We noticed that the Nef^{mut}/NS3 fusion product readily associated with exosomes, even if apparently at slightly lower levels than Nef^{mut} alone. This decrease can be at least in part consequence of the nearly 4-fold higher molecular weight of the fusion product compared to Nef^{mut}, which would lead to a steric hindrance in the process of exosome incorporation.

The immunogenicity of the Nef^{mut}/NS3 exosomes was tested in C57Bl/6 mice by s.c. injections of amounts of exosomes incorporating 100 ng of the Nef^{mut}/NS3 fusion protein, as assessed by semi-quantitative western blot (not shown). Immunizations were repeated three times at two week intervals. As controls, mice were inoculated with exosomes uploading either 100 ng of Nef^{mut} alone, or the same amount of recombinant NS3. Two weeks after the last

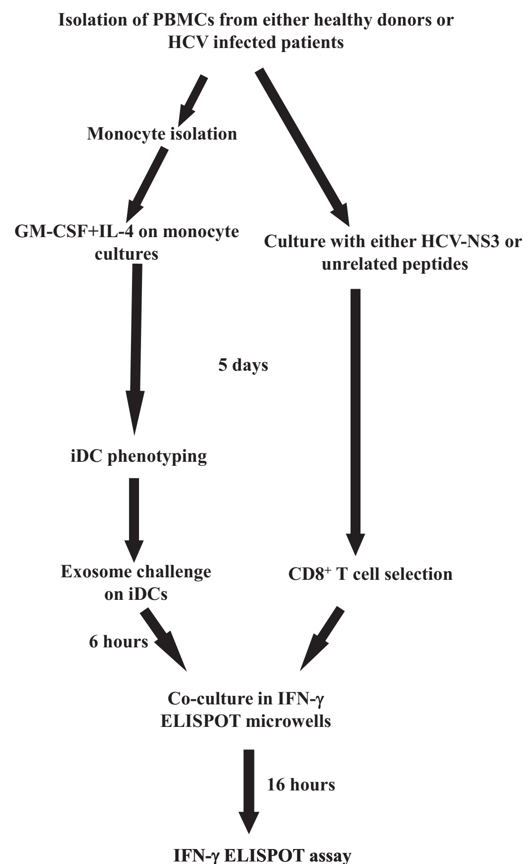

Fig. 3. Flow of the experimental design leading to cross-presentation assay on PBMCs from both healthy donors and HCV patients.

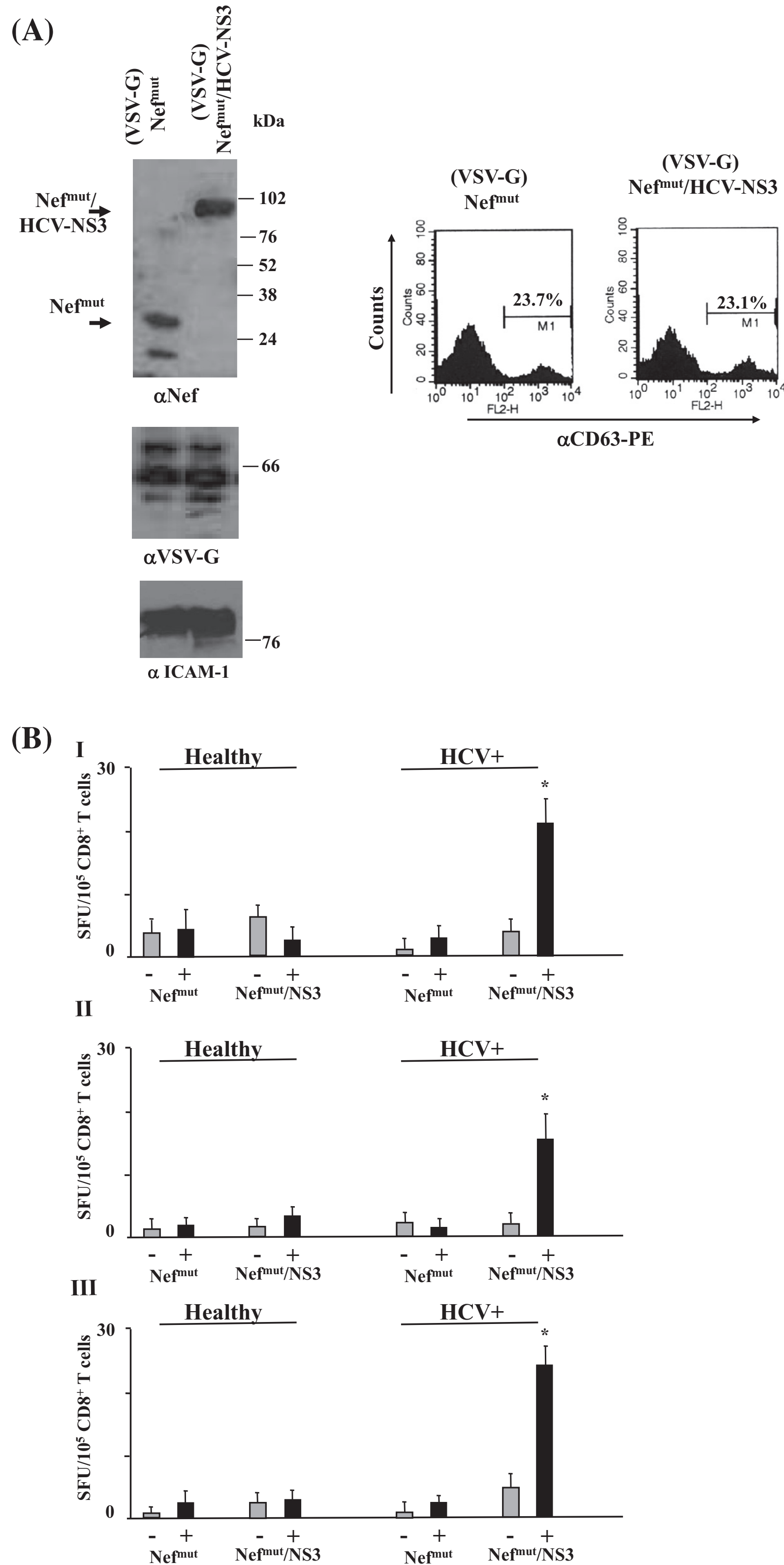

Fig. 4. Activation of HCV NS3-specific $CD8^+$ T lymphocytes after challenge of autologous iDCs with Nef^{mut}/NS3 engineered exosomes. A. Molecular characterization of exosome preparations uploading VSV-G together with either Nef^{mut} or Nef^{mut}/NS3. A total of 200 μU equivalent of AchE activity of exosomes was assayed in western blot assays probed with anti-Nef, anti-VSV-G, and anti-ICAM-1 antibodies. Arrows sign the relevant protein products. Molecular markers are given in kDa. In addition, both exosome preparations were tested for CD63 contents (right panels). Quadrants were set on the basis of the fluorescence of beads alone incubated with anti-CD63 mAb. Percentages of positive events are indicated. Results are representative of the assays performed on five different exosome preparations. B. Cross-presentation of NS3 delivered by exosomes in iDCs from three chronically HCV infected patients. Following the scheme depicted on Fig. 2, 10^5 iDCs from both healthy and HCV-infected patients were challenged with 100 μU equivalent of AchE activity of exosomes associating either Nef^{mut} or Nef^{mut}/NS3. After 6 h, the cells were put in co-culture overnight in IFN-γ Elispot microwells with $CD8^+$ T lymphocytes purified from PBMCs cultivated for 5 days in the presence of either unspecific (−) or NS3 (+) specific peptides. Shown are the mean + SD number of SFU/10^5 cells calculated from triplicate wells. $^*p < 0.05$. The paired Student's *t*-test was used and confirmed using the non-parametric Wilcoxon rank sum test.

immunization, mice were sacrificed, and the splenocytes assayed for the presence of both Nef- and NS3-specific CD8$^+$ T lymphocytes by culturing them for 5 days in the presence of either Nef- or NS3-specific peptides. As controls, splenocytes were cultivated either in complete medium, or in the presence of an unrelated peptide. By IFN-γ Elispot assay, we detected Nef-specific CD8$^+$ T lymphocytes within splenocytes from mice inoculated with exosomes uploading either Nef^{mut} or Nef^{mut}/NS3. Most importantly, using either NS3-specific peptides, we also detected the presence of NS3-specific CD8$^+$ T lymphocytes from mice inoculated with Nef^{mut}/NS3 exosomes but not from those inoculated with Nef^{mut} alone, whereas neither Nef- nor NS3-specific immune responses were detected in splenocytes from mice inoculated with recombinant NS3 (Fig. 2B).

These results indicate that exosomes uploading HCV-NS3 are immunogenic.

3.2. Activation of HCV NS3 specific CD8$^+$ T lymphocytes by autologous iDCs challenged with HCV NS3 engineered exosomes

To investigate whether NS3 engineered exosomes are functional in humans, PBMCs from HCV chronically infected patients not under therapy and, as a control, healthy donors were isolated, and purified monocytes were differentiated to iDCs. Meanwhile, PBMCs were cultivated in the presence or not of a mix of peptides spanning the whole NS3 sequence. As a control, a mix of HIV-1 Gag peptides was used taking advantage from the fact that the selected HCV patients were HIV-1 seronegative. After 5 days, iDCs were challenged with the exosomes, and then put in co-culture with CD8$^+$ T lymphocytes isolated from the PBMC cultivated in the presence of peptides. Finally, iDCs/CD8$^+$ T lymphocytes co-cultures were carried out in IFN-γ Elispot microwells for 24 h. A scheme of this experimental design is reported in Fig. 3.

As already described [7], to optimize the sensitivity of the cross-presentation assays, iDCs were challenged with exosomes associating VSV-G. The molecular characterization of the exosome preparations we used is shown in Fig. 4A. The co-expression of VSV-G in exosome-producing cells seemed to favor the incorporation of the fusion product. In fact, differently to that observed for exosomes produced in the absence of VSV-G (Fig. 2A), Nef^{mut} and Nef^{mut}/NS3 appeared associated with exosomes at similar extents. iDCs were challenged with either (VSV-G)Nef^{mut}/NS3 or, as a control, (VSV-G) Nef^{mut} engineered exosomes, and then co-cultivated with autologous, peptide-stimulated CD8$^+$ T lymphocytes in the presence of either NS3 or, as a control, HIV-1 Gag peptide mixtures. In view of the ascertained HIV-1 seronegativity of all donors, only CD8$^+$ T cells isolated from PBMCs from HCV infected patients and cultured with the HCV-NS3 specific peptides would be activated upon co-culture with the iDCs loaded with Nef^{mut}/NS3. Indeed, as shown in Fig. 4B, levels of activated lymphocytes significantly over background were detected only in co-cultures from HCV infected patients comprising iDCs challenged with Nef^{mut}/NS3 exosomes and CD8$^+$ T lymphocytes isolated from PBMCs cultured with NS3-specific peptides. The specificity of our assay was granted by the absence of activation in CD8$^+$ T lymphocytes isolated from PBMCs of: (i) HCV patients cultured with irrelevant peptides, and (ii) healthy donors stimulated with HCV-NS3 specific peptides.

These results indicate that exosomes engineered to upload HCV NS3 efficiently activated NS3-specific memory CD8$^+$ T lymphocytes generated in patients as part of the immune response mounted against HCV.

4. Discussion

We investigated whether the immunogenicity elicited by a viral TAA delivered by Nef^{mut}-based exosomes we previously described [8] also applied to HCV-related antigens engineered in exosomes. The results we obtained from the immunogenicity assays we carried out in mice proved that NS3-engineered exosomes can prime and expand a NS3-specific CD8$^+$ T cell immune response. Considering that exosomes have been produced in 293T human cells, it was expected that they associated molecules of human origin which would be represented at similar levels in both Nef^{mut} and Nef^{mut}/NS3 exosomes. However, the NS3 expression in exosome-producing cells would theoretically induce the uploading in exosomes of human products not represented in Nef^{mut} control exosomes, thus eliciting an immune response representing a potentially confounding factor. We believe that it was not the case since we observed that, upon stimulation with unrelated peptides, the activation extents of CD8$^+$ T lymphocytes from mice inoculated with Nef^{mut} did not significantly differ from that observed in the cells from mice injected with Nef^{mut}/NS3 exosomes. On this basis, evaluating the efficiency of the adaptive immune response elicited by NS3-exosomes in an animal model of HCV infection, e.g., EFT-Stat1$-/-$ mice [18], could be of interest. On the other hand, data we obtained with PBMCs from HCV patients for the first time demonstrate the CD8$^+$ T cell antigenicity in *ex vivo* human primary cells of the heterologous products uploaded in Nef^{mut}-based engineered exosomes. Considering that HCV NS3 is a quite conserved protein, and the immune response elicited by Nef^{mut}/NS3 exosomes is expected to be Class I MHC unrestricted in view of the incorporation of the full-length protein, Nef^{mut}/NS3 exosomes might be considered a candidate for innovative anti-HCV therapeutic strategies.

HCV-NS3 is considered an immunogen of election in several ongoing anti-HCV vaccine protocols [reviewed in [19]]. For instance, NS3 is expressed by both adenoviral- and MVA-based vectors already proven to induce both potent and long-lasting NS3-specific CD8$^+$ T cell immunity. HCV-NS3 is also part of inactivated *Saccharomyces cerevisiae*-based anti-HCV immunogens, and DNA vectors expressing HCV-NS3 have been tested for their immunogenicity upon injection by electroporation. In this scenario, the here described anti-HCV vaccine strategy represents a starting point for the development of both simple and innovative immunization protocols having the potentiality to compete with the most advanced anti-HCV therapeutic approaches.

Conflicts of interest

None.

Acknowledgments

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