Pre-clinical development of *Listeria*-based nanovaccines as immunotherapies for solid tumours: insights from melanoma

Hector Terán-Navarro, Ricardo Calderon-Gonzalez, David Salcines-Cuevas, Isabel García, Marco Marradi, Javier Freire, Erwan Salmon, Portillo-Gonzalez, Elisabet Frande-Cabanes, Almudena García-Castaño, Virginia Martinez-Callejo, Raquel Tobes, Fernando Rivera, Sonsoles Yañez-Diaz, and Carmen Álvarez-Domínguez

**ABSTRACT**

Gold glyconanoparticles loaded with the listeriolysin O peptide 91–99 (GNP-LLO<sub>91-99</sub>), a bacterial peptide with anti-metastatic properties, are vaccine delivery platforms facilitating immune cell targeting and increasing antigen loading. Here, we present proof of concept analyses for the consideration of GNP-LLO<sub>91-99</sub> nanovaccines as a novel immunotherapy for cutaneous melanoma. Studies using mouse models of subcutaneous melanoma indicated that GNP-LLO<sub>91-99</sub> nanovaccines recruit and modulate dendritic cell (DC) function within the tumour, alter tumour immunotolerance inducing melanoma-specific cytotoxic T cells, cause complete remission and improve survival. GNP-LLO<sub>91-99</sub> nanovaccines showed superior tumour regression and survival benefits, when combined with anti-PD-1 or anti-CTLA-4 checkpoint inhibitors, resulting in an improvement in the efficacy of these immunotherapies. Studies on monocyte-derived DCs from patients with stage IA, IB or IIIB melanoma confirmed the ability of GNP-LLO<sub>91-99</sub> nanovaccines to complement the action of checkpoint inhibitors, by not only reducing the expression of cell-death markers on DCs, but also potentiating DC antigen-presentation. We propose that GNP-LLO<sub>91-99</sub> nanovaccines function as immune stimulators and immune effectors and serve as safe cancer therapies, alone or in combination with other immunotherapies.

**Introduction**

Cutaneous melanoma is probably the most common solid tumour, which originates from melanocytes, exhibits multidrug resistance and has a low survival rate. The annual incidence of melanoma continues to grow in the European Union at the rate of 3–7% per year. Melanoma is also the best example of an immunological “hot” tumour as it is heavily infiltrated by immune cells and can be treated with immunological checkpoint inhibitors such as anti-CTLA-4 or anti-PD-1/PD-L1 antibodies that block T-cell-negative regulators. These new immunotherapies approved by the FDA for advanced melanoma, mainly stages IIIB or IV, are not effective in all melanoma cases due to a low number of responders and other effects that minimize their success, such as the high rate of immunological adverse events, the appearance of immunotherapy resistance or the lack of benefit on long-term survival.

Immunotherapies for solid tumours, which also attenuate bacterial mutants of the human pathogen *Listeria monocytogenes* (LM) lacking the C-terminal of the bacterial toxin listeriolysin O (LLO), have been widely used in prostate cancer, cervix carcinoma and even pancreatic cancer. However, cancer patients are immunocompromised individuals and caution is necessary when using attenuated *Listeria* mutants in cancer patients. The main virulence factor of this pathogen, LLO, appears to be responsible for many biological activities related to the ability of LM as anti-tumour therapy such as lower concentrations required in *vivo* to induce apoptosis than when...
acting as a bacterial cytolytic toxin, the recruitment of DCs, binding to membranes, the induction of cytotoxic T cell responses and tumour homing.\textsuperscript{10-12} These LLO properties explain the very low doses of pathogenic LM which disable the immune tolerance of tumours and cause regression of experimental melanoma, while mutants deficient in the gene coding LLO, failed to serve as anti-melanoma therapy.\textsuperscript{12} To avoid the use of pathogenic LM, but to focus on LLO-based therapies, we identified LLO peptides that can cause melanoma regression and studied the anti-neoplastic properties of the 91–99 peptide of LLO (LLO\textsubscript{91–99}) to prevent adhesion and dissemination of experimental melanoma-induced carcinomatous peritonitis as adjuvant therapy, either using DCs loaded with this peptide\textsuperscript{14} or gold nanoparticles (GNPs) loaded with LLO\textsubscript{91–99} peptide and β-D-glucose.\textsuperscript{15} GNPs can be loaded with multiple copies of the desired (bio)molecules (ligands) by means of thiol chemistry,\textsuperscript{16} and depending on the chosen ligands, can be used to intervene in pathological processes such as metastasis,\textsuperscript{17} cancer,\textsuperscript{18-20} bacterial infection,\textsuperscript{21-23} HIV infection\textsuperscript{24,25} and listeriosis.\textsuperscript{26-28} Thus, we hypothesized that GNPs could also be favourable alternatives to DC-LLO\textsubscript{91–99} vaccines and therapies against solid tumours.

In the present study, we evaluated the therapeutic activity of GNP-LLO\textsubscript{91–99} nanovaccines as safe immunotherapies for cutaneous melanoma using subcutaneous transplants of primary or metastatic murine melanoma. We also tested, as a proof of concept, GNP-LLO\textsubscript{91–99} nanovaccines in combination with immunological checkpoint inhibitors in mouse models and monocyte-derived DCs (MoDC) from melanoma patients.

**Results and discussion**

Since Coley’s treatment of cancer with bacterial vaccines to boost the immune system against host tumours, and the approved Bacillus Calmette-Guerin (BCG) vaccine for bladder cancer, the immunotherapy field has grown enormously. In this regard, immunological checkpoint inhibitors or LM-based immunotherapies using attenuated LM are two examples of cancer therapies. Several studies have suggested that melanoma might be a good target for LM-based immunotherapies, using either low doses of pathogenic LM, or attenuated LM vaccines expected to lack virulence and cytolytin ability.\textsuperscript{12,13,29,30} However, the development of severe systemic listeriosis due to the use of one of these attenuated LM vaccines in a cancer trial,\textsuperscript{9} and significant increases in the annual incidence of listeriosis in several European countries, particularly Spain,\textsuperscript{31,32} strongly suggest the need to engineer safer LLO-based cancer immune therapies.

We present pre-clinical and proof of concept studies of a novel LM-based nanotherapy for cutaneous melanoma using gold nanoparticles (GNPs) coupled to both β-D-glucose and the 91–99 peptide of LLO, i.e. GNP-LLO\textsubscript{91–99} nanovaccines. We studied GNP-LLO\textsubscript{91–99} nanovaccines as either monotherapy or combination therapy with immunological checkpoint inhibitors, as a viable alternative to DC cancer vaccines against cutaneous melanoma.

**Preparation of GNP-LLO\textsubscript{91–99} nanovaccines and cell targeting characterization**

GNP-LLO\textsubscript{91–99} nanovaccines are synthetic particles with a nanoscale gold core, which maximises antigen loading and provides homogeneous structure and size (chemical structure in Figure S1, panel A and detailed procedure in Supplemental file). This immunotherapy is non-toxic, both in vivo using C57BL/6 mice and in vitro using human monocyte derived DCs (MoDC) (Figure S1, panel B). Similar to pathogenic LM, GNP-LLO\textsubscript{91–99} nanovaccines have tumour and DC targeting abilities, as shown by co-localization of GNP-LLO\textsubscript{91–99} and MHC-I molecules within intracellular compartments (yellow fluorescent images in Figure S1, panel C). The perinuclear localization of MHC-I and GNP-LLO\textsubscript{91–99} containing compartments, appears to correspond with antigen cross-presentation organelles (Figure S1, panel C and single staining shown in Figure S2). We also observed another GNP-LLO\textsubscript{91–99} and MHC-I co-localization pattern in cell surface spots of melanoma (left images in Figure S1, panel C), showing that GNP-LLO\textsubscript{91–99} nanovaccines induce different signals in tumours and DCs.

**Tumour effects in vivo after treatment with GNP-LLO\textsubscript{91–99} nanovaccines**

On confirmation of the melanoma targeting ability of these nanovaccines (Figure S1, panel C), we investigated the anti-melanoma efficacy of GNP-LLO\textsubscript{91–99} Nanovaccines. The therapeutic effect on primary B16OVA\textsuperscript{37} or metastatic B16.F10 melanoma started when the subcutaneous (s.c) melanoma size reached 400 mm\textsuperscript{3} on day 7 (Figure S3, panel A) and tumours had a consistent and visible structure (Figure S3, panel B). B16.F10 melanoma resulted in a lower number of surviving mice and larger tumours which appeared earlier than B16OVA melanoma, as expected in metastatic tumours (Figure S3, panels A-C). Treatment was administered intravenously (i.v) as a single dose of GNP-LLO\textsubscript{91–99} nanovaccines (50 μg/mouse, n = 10 mice/group). One week later, we assessed tumour size, lung metastases and immune responses in tumour infiltrated lymphocytes (TILs) and spleens (protocol in Figure 1(a)). GNP-LLO\textsubscript{91–99} nanovaccines caused regression of melanoma auto- and allo-transplants and reduced the tumour volumes of B16.F10 and B16OVA melanoma in C57BL/6 congenic and CD-1 allogenic mice by 97–98% (Figure 1(b) and Table S1). GNP-LLO\textsubscript{91–99} nanovaccines also inhibited the formation of lung metastasis (Figure 1(c)) and induced lasting responses, as the mice demonstrated 100% survival rates (SR) (grey squares in Figure 1(d)) and remained healthy up to 30 days or longer (Table S1). Only 2–3% of non-treated (NT) animals survived up to day 30 (plot with black circles in Figure 1(d)) and showed 10-fold higher tumour volumes than the average initial size (day 30 column labelled TV in Table S1).

GNP-LLO\textsubscript{91–99} nanovaccines showed higher overall therapeutic efficacy than DC-LLO\textsubscript{91–99} vaccines, which resulted in a maximum 72% reduction in tumour volume (Figure 1(b) and Figure S3, panel D), 80% inhibition of metastases (Figure 1(c)) and 60% SR (black triangles in Figure 1(d)). Moreover, GNP-LLO\textsubscript{91–99} anti-tumour effects were LLO 91–99 peptide specific, as empty GNPs, soluble LLO 91–99 or 189–201 peptides or other LM-based nanovaccines effective in listeriosis prophylaxis.\textsuperscript{27,28}
provided no perceived benefit on tumour growth, metastases, or mouse health (Figure 1(b-d)).

**GNP-LLO91-99 nanovaccines induce cell death due to immune activities**

The blocking of tumour growth by GNP-LLO91-99 nanovaccines also suggested the induction of cell death. In this regard, histological analysis of B16OVA and B16.F10 melanoma from mice treated with GNP-LLO91-99 nanovaccines showed high numbers of necrotic foci following haematoxylin-eosin (HE) staining (Figure 2(a-b)). Using FACS analysis, early apoptosis was characterized as single annexin-V+ positive cells and late apoptosis as double 7-AAD+ and annexin-V+ positive cells. Melanoma from transplanted mice indicated that GNP-LLO91-99 nanovaccines increased significantly late apoptosis of primary and metastatic melanoma in vivo, from 45–50% to 73–76%, respectively (Figure 2(c)), an asterisk indicates a significant
induction with nanovaccines, compared to NT). Also, nanovaccines enhanced early apoptosis of metastatic B16.F10 melanoma in vivo, from 9% to 17% (Figure 2(c)), marked with an asterisk in the NT groups; while caused no significant effect in primary B16OVA melanoma. If nanovaccines are used directly in melanoma (B16OVA, B16.F10, A-375) or other tumours (ovary CHO cells or macrophage-derived cells as IC-21) in vitro, they induced very low percentages of early apoptosis, less than 10% (AnnV+ cells) (black bars in lower plot of Figure 2(c)). However, if nanovaccines are previously pre-incubated with mouse bone-marrow derived DC for 16 hours, and, next supernatants incubated with melanoma (B16OVA, B16.F10, A-375) or other tumours (ovary CHO or macrophage-derived IC-21 cells), they induce high percentages of early apoptosis in vitro, from 50 to 55% (grey bars in Figure 2(c)). Therefore, it seems that nanovaccines induce cell death due to immune activities.

GNP-LLO91-99 nanovaccines also caused significant increases in classic surface markers involved in immune activation and antigen presentation such as CD40 and CD80 (Figure 2(d)), suggesting that this treatment activated the immune system in primary and metastatic melanoma. The following findings support that GNP-LLO91-99 nanovaccines activate the immune response in melanoma and disable immune tolerance: First, the high numbers of

Figure 2. GNP-LLO91-99 nanotherapy affects melanoma programmed cell-death and antigen-presentation markers. (A, B) B16OVA melanoma auto-transplants were established s.c. in Figure 1 (n = 10/group of mice) and vaccinated i.v. with control GNP (50 µg/mouse), GNP-LLO91-99 (50 µg/mouse) or non-treated (NT). After 7 days, histochromy and immunohistochemistry were performed on melanoma embedded in paraffin to calculate the percentage of necrosis (A) or stained (B) with haematoxylin-eosin (HE). Asterisks over NT bars indicate that all groups of mice were compared to NT. The results are expressed as the mean number of necrotic foci ± SD (P ≤ 0.05). In (B), we show representative images of each group of mice stained with HE and mark with an asterisk, the NT group to which we compared all samples. (C) Removed melanoma were analysed for apoptosis by FACS analysis. We measured levels of early apoptosis using single staining with the apoptotic marker annexin-V (Annexin V) or late apoptosis using double staining with the DNA marker 7-AAD and the apoptotic marker annexin-V. Upper plots labelled as in vivo correspond to a representative experiment and the percentages reflect the cells localized in each quadrant from a total of 50,000 cells passed by the flow cytometer. GNP-LLO91-99 treated samples are compared to NT and significant differences are marked with an asterisk (*). The experiment was performed three times and results of each group of 10 mice are the following for late apoptosis (7-AAD+AnnV+ double positive cells): B16OVA/GNP-LLO91-99, 73 % ± 0.5; B16OVA/NT*, 50 % ± 1.5; B16.F10/GNP-LLO91-99, 76 % ± 1.5; B16.F10/NT*, 45 % ± 1.0. Results for early apoptosis (7-AAD−AnnV+) are: B16OVA/GNP-LLO91-99, 7 % ± 0.5; B16OVA/NT, 13% ± 1.0; B16.F10/GNP-LLO91-99, 17 % ± 1.5; B16.F10/NT, 9 % ± 0.5 (P ≤ 0.05). We also performed in vitro experiments of different tumour cells (lower bar plot), melanoma (B16OVA, B16.F10, A-375), ovary CHO or macrophage IC-21 cell lines treated for 16 h with GNP-LLO91-99 (50 µg/ml) (black bars) or supernatants of DC cells pre-treated with GNP-LLO91-99 (50 µg/ml) (grey bars) and, next, analysed for early apoptosis by FACS. Results correspond to the mean of five different experiments and are expressed as the mean percentage of positive cells ± SD (P ≤ 0.05). (D) Analysis of antigen-presentation and cell-death markers on the cell surface of different melanoma cell lines, either murine B16OVA or human A375 as primary melanoma or murine B16.F10 or human MeWo as metastatic melanoma, were treated in vitro with GNP-LLO91-99 nanovaccines (50 µg/mL, 16 h, 37°C) (+ GNP bars). Cells were stained with different fluorescent-labelled antibodies and examined by FACS analysis. The results are expressed as the mean percentage of positive cells ± SD (P ≤ 0.05).
Figure 3. GNP-LLO\textsubscript{91-99} nanotherapy as an immune effector: analysis of TILs, spleens and cytokines in sera. (a) B16.F10 and B16OVA melanoma auto-transplants were established s.c as in Figure 1-2 (n = 10/group of mice) and then i.v inoculated or not (NT) with a single dose of GNP-LLO\textsubscript{91-99} (50 µg/mouse). After 7 days, melanomas were removed and TILs isolated. TILs were analysed by FACS and tumour infiltrated (Ti)-DCs with CD11c\textsuperscript{+}CD8α\textsuperscript{+} phenotypes examined for different markers with specific monoclonal antibodies. Data in the plots correspond to B16.F10 melanoma and images correspond to melanoma embedded in paraffin and stained with anti-CD123 to stain DCs. We also quantified the amount of CD123\textsuperscript{+} cells in recovered melanoma by FACS, marked with an asterisk, and detected the following percentages: untreated B16.F10, 10% ± 0.9; GNP-LLO\textsubscript{91-99} treated B16.F10, 87% ± 1.3; untreated B16OVA, 9% ± 0.6; GNP-LLO\textsubscript{91-99} treated B16OVA, 84% ± 1.2. The results are expressed as percentages of positive cells ± SD (P ≤ 0.05). (b) T\textsubscript{reg} cell populations in spleens and melanoma were analysed by FACS. GNP-LLO\textsubscript{91-99} treated samples are compared to NT samples and marked with an asterisk (*) to indicate significant decreases. These experiments have been performed five times. The summarizing data of percentage so F C D 4\textsuperscript{+}FoxP3\textsuperscript{+} positive cells (T\textsuperscript{reg}) are the following in the spleens: NT group: 16% ± 2.0 and GNP-LLO\textsubscript{91-99} group: 14.4% ± 1.6. While T\textsuperscript{reg} percentages in melanoma are: NT group: 18% ± 1.5 and GNP-LLO\textsubscript{91-99} group: 3.6% ± 0.4 (P ≤ 0.05). (c) CTL activities specific of each peptide were examined with the frequencies of LLO\textsubscript{91-99} or OVA\textsubscript{257-264} peptide-specific CD8\textsuperscript{+} cells and IFN-γ producers in TILs. We examined these specific CTL activities using recombinant soluble dimeric mouse H-2b: Ig fusion protein that bind to each peptide, as described in the Methods and materials section. The results are expressed as percentages of positive cells ± SD.
Table 1. Cytokine pattern in mice auto-transplanted with melanoma and treated with GNP-LLO91-99 nanovaccines.

| CONDITION                   | IL-6  | IL-10 | TNF-α | IFN-γ | IL-17A | IL-12p40 |
|-----------------------------|-------|-------|-------|-------|--------|----------|
| B16F10-NT                   | 59.5 ± 0.5 | 8.36 ± 0.5 | 11.39 ± 0.5 | 9.2 ± 0.6 | 4.85 ± 0.6 | 1.95 ± 0.3 |
| B16OVA-NT                   | 45.6 ± 0.3 | 6.3 ± 0.2 | 12.01 ± 0.3 | 4.07 ± 0.2 | 3.9 ± 0.2 | 0.8 ± 0.1 |
| B16F10/GNP-LLO91-99         | 4.5 ± 0.6 | 0.7 ± 0.5 | 34.5 ± 0.5 | 95.5 ± 0.5 | 47.7 ± 0.6 | 12.08 ± 0.4 |
| B16OVA/GNP-LLO91-99         | 3.11 ± 0.2 | 0.3 ± 0.1 | 37.6 ± 0.2 | 105.1 ± 0.6 | 51.7 ± 0.4 | 11.1 ± 0.1 |
| CONTROL                     | 3.8 ± 0.5 | 5.41 ± 0.3 | 10.1 ± 0.5 | 1.79 ± 0.5 | 1.7 ± 0.5 | 0.5 ± 0.1 |

*aMice (C57BL/6 strain) were s.c transplanted with B16.F10 or B16OVA melanoma cells and then inoculated i.v. with GNP-LLO91-99 nanovaccines or non-treated (NT).

*bCytokine levels (ng/mL) in sera of mice. Results are expressed as the mean ± SD. (P < 0.5).

infiltrating DCs observed in transplanted melanoma surrounding the necrotic foci (CD123 brown staining in images of Figure 3(a)). Second, the high percentages of activated DCs (84–87%) detected in the tumour infiltrated DC (Ti-DC) with the following phenotype, CD11c+CD8α+CD40+CD83+CD11blow (plot in Figure 3(a)). These activated CD11c+CD8α+ DCs were reported as the cellular targets of IFN-γ, responsible for tumour regression and drivers of cytotoxic T cell immunity in tumours.38–40 Third, GNP-LLO91-99 nanovaccines shifted the Th2 cytokine profile to the Th1 cytokine profile in vivo after transplantation with primary B16OVA or metastatic B16.F10 melanoma, increasing the serum levels of Th1 cytokines with anti-tumour potential such as IFN-γ, TNF-α and IL-12, and decreasing Th2 cytokines such as IL-6 and IL-10 (Table 1). A classic Th2 profile was detected in mice transplanted with melanoma and in patients with primary or metastatic melanoma (Table S2).45,46 Fourth, GNP-LLO91-99 nanovaccines caused a 5-fold reduction in T regulatory cells (Treg, CD4+CD25+FoxP3+) (Figure 3(b)) and a 2-fold reduction in the percentage of myeloid derived suppressor cells (MDSC, CD11b+Gr1+) (Figure S3, panel E) in TILs; otherwise present at a high proportion in TILs of mice transplanted with melanoma and not receiving any therapy (NT plots in TILs of Figure 3(b)) and B16F10 and B16OVA bars in Figure S3, panel E). Fifth, GNP-LLO91-99 nanovaccines also resulted in a 3 to 5-fold increase in the frequency of cytotoxic CD8+ T cells specific for the melanoma epitope OVA257-264 and for the antigen LLO91-99 epitope (B16OVA melanoma) (black and grey bars in plots of Figure 3(c)) and a 4-fold increase in the total percentages of CD8+ melanoma specific and IFN-γ producers (B16.F10 melanoma in Figure S3, panel F), with no significant changes in the percentages of CD4+ melanoma specific and IFN-γ producers. We conclude that GNP-LLO91-99 nanovaccines induced a robust LLO and melanoma-specific cytotoxic T cell response, had a concerted action on DCs and CD8+ T cells, disabled the immune tolerance of tumours by increasing the levels of tumoricidal cytokines, and reduced the percentages of negative immune regulators such as Treg or MDSC to cause melanoma cell apoptosis.42

Proof of concept for GNP-LLO91-99 nanovaccines as immunotherapy for melanoma

The actions of GNP-LLO91-99 nanovaccines (Figure 1–3) strongly suggest that they are valid immunotherapies for cutaneous melanoma. To determine the proof of concept, we compared the effects of nanovaccines in vivo with anti-CTLA-4 and anti-PD-1 antibodies,5,43 using the same doses, adjusted for mouse weight, and route of administration used for metastatic melanoma patients.5,6 Mice s.c transplanted with B16OVA melanoma were i.v inoculated with a single dose of anti-CTLA-4 antibody, anti-PD-1 antibody (100 µg/mouse) or GNP-LLO91-99 nanovaccines (50 µg/mouse) and the animals were analyzed 14, 23 or 30 days after treatment (14D, 23D, 30D bars in Figure 4(a)). GNP-LLO91-99 treatment was found to be the most efficient monotherapy with rapid and long lasting effects on tumour burden, showing 4-fold and 8-fold reductions, respectively (Figure 4(a)), and the only monotherapy to improve SR, as all mice survived and remained healthy up to 30 days (black circles in Figure 4(b)). Anti-CTLA-4 treatment reduced tumour size 4-fold in a short time, and did not have a lasting effect (grey and white bars in Figure 4(a)). Anti-PD-1 antibody had the opposite effect on tumour size, had a long lasting effect with an 8-fold reduction in tumour size and a less prominent reduction within a short time (white bars in Figure 4(a)). Anti-CTLA-4 treatment had no benefit on SR or mouse health as most of the mice did not survive after 23 days († labelled white bars in Figure 4(a) and black triangles in Figure 4(b)). However, anti-PD-1 therapy improved SR but deteriorated after 14 days (white squares in Figure 4(b)). In combination immunotherapy, GNP-LLO91-99 nanovaccines synergised anti-PD-1 treatment to achieve long lasting effects at 14 days and beyond, obtained complete tumour remission (asterisk bars in Figure 4(a)) and resulted in 100% SR with sustained lasting effects (similar black quadrangle and circle plots in Figure 4(b)). The combination of GNP-LLO91-99 nanovaccines with anti-CTLA-4 treatment also had some benefit on tumour regression (Figure 4(a)) with an 85% SR at 23 days and beyond (white triangles in Figure 4(b)). Therefore, GNP-LLO91-99 nanovaccines appear to be efficient immunotherapies for melanoma regression, with significant benefits on SR, and indicated the time-frame where each checkpoint inhibitor failed. In this regard, GNP-LLO91-99 nanovaccines in combination with anti-PD-1 antibodies improved the short-term effects and in combination with anti-CTLA-4 antibodies improved the long-term effects.

Checkpoint inhibitors can also modulate the antigen-presentation capacities of DCs within the melanoma environment and have been approved by the FDA for patients with stage IIIIB and IV melanoma.5,14 Therefore, we isolated TILs from mice transplanted s.c with B16OVA melanoma, treated or not with GNP-LLO91-99 nanovaccines or checkpoint inhibitors, and evaluated...
antigen-presentation markers in DCs such as MHC-I and MHC-II molecules, or the co-stimulatory molecules, CD40, CD80, CD83 and CD86 (Figure S3, panel G). As expected, anti-CTLA-4 and anti-PD-1 antibodies had no effect on antigen-presentation markers in Ti-DCs (CD8α+ cells), as their action is focussed on T cell proliferation.\(^5\),\(^6\),\(^45\) Anti-PD-1 antibodies had a negative effect on the percentages of MHC-II molecules and a small effect on CD80 and CD83, whereas they had no effect on the cell surface expression of CD40 and CD86 molecules (anti-PD-1 and anti-CTLA-4 bars in Figure S3, panel G). However, GNP-LLO\(_{91-99}\) nanovaccines clearly affected all antigen-presentation markers, causing a 2.5-fold increase in the percentages of co-stimulatory molecules,
CD40, CD80, CD83 and CD86, either as monotherapy or combination therapy with anti-CTLA-4 or anti-PD-1 antibodies (GNP-LLO91-99 bars in Figure S3, panel G).

We next confirmed the effect of GNP-LLO91-99 in combination with checkpoint inhibitors expanding CTIL activities, when we measured melanoma and antigen specific CD8+ frequencies in melanoma TILs. Anti-CTLA-4 or anti-PD-1 only caused a 2-fold increase in the frequency of cytotoxic CD8+ T cell specific for the melanoma epitope OVA257-264 and IFN-γ producers, and, no increased for the antigen LLO91-99 (green and blue bars in Figure 4(c)). While we detected a 5-fold increase in the frequency of cytotoxic CD8+ T cells specific for melanoma using GNP-LLO91-99 nanovaccines as monotherapies or in combination with anti-CTLA-4 or anti-PD-1.

We then assessed the effect of GNP-LLO91-99 nanovaccines as potential immunotherapy for patients with cutaneous melanoma, compared with the checkpoint inhibitors, ipilimumab (anti-CTLA-4 antibodies) and nivolumab (anti-PD-1 antibodies), examined the percentages of antigen-presentation and programmed cell-death markers in monocyte-derived DCs (HuMoDC) from a stage IIIB melanoma patient who had not received immunotherapy (Figure 4(d)). Nivolumab and ipilimumab had no effect on the percentages of CD86 molecules, induced a small increase in the percentage of MHC-I and CD80 molecules and reduced the percentages of programmed cell-death markers, PD-1, PD-L1 and annexin-V. In addition, nivolumab reduced the percentages of MHC-II molecules (nivolumab and ipilimumab bars in Figure 4(d)). GNP-LLO91-99 nanovaccines significantly increased the percentages of all antigen-presentation markers such as MHC-I, MHC-II, CD80 and CD86 and decreased the percentages of PD-1, PD-L1 and annexin-V, the programmed cell-death markers to basal levels (orange, maroon and black bars in Figure 4(d)). We also observed similar action of GNP-LLO91-99 nanovaccines in MoDC from patients with early stage melanoma (stages IA or IB) (Table 2). We also confirmed that the incubation of GNP-LLO91-99 with MoDC from a stage IIIB melanoma patient was able to induce cell death in different human melanoma, either primary (A-375 or MelJuSo) or metastatic melanoma cell lines (MeWo or SK-Mel24) due to immune activities, since GNP-LLO91-99 alone caused only basal level of early apoptosis (Figure 4(e)). All together, these results confirmed that GNP-LLO91-99 action is not restricted to the Ti-DC(CD8α+) of transplanted melanoma, but also increased immune activation and the antigen presentation abilities of MoDC in melanoma patients at primary or advanced stages (Figure 4(d)) to promote tumour cell death (Figure 4(e)). Moreover, this ability of GNP-LLO91-99 nanovaccines was also observed in combination with checkpoint inhibitors that lack these stimulatory capacities (+ GNP-LLO91-99 bars in Figure 4(d)), suggesting that this approach can also serve as a tool to confirm the putative action of immunotherapies.

**Conclusions**

We propose that GNP-LLO91-99 nanovaccines are safe immunotherapies, which act in the priming and efferent arms of the immune response, disable immune tolerance in melanoma (model in Figure 4(f)) and can benefit melanoma patients in the early and late stages of disease as follows: First, as an immune stimulator, GNP-LLO91-99 nanovaccines recruit DC-CD8α+ within the tumour environment, which produce Th1 cytokines with tumour killing potential such as IFN, TNF-α and IL-12, and improve DC antigen-presentation abilities. Second, GNP-LLO91-99 nanovaccines activate DCs and increase the intratumoural percentages of anti-Listeria and melanoma-specific cytotoxic T cells and IFN-γ producers, and, reduce Treg and MDSC suppressor cells. These two concerted actions of GNP-LLO91-99 nanovaccines induce melanoma cell apoptosis which restricts tumour growth and increases mouse survival. The ability of GNP-LLO91-99 nanovaccines to promote MoDC antigen-presentation abilities and synergise with ipilimumab and nivolumab in stage IA, IB and IIIB melanoma patients consolidates GNP-LLO91-99 nanovaccines as immunotherapies with extensive action. We propose that GNP-LLO91-99 nanovaccines are a promising nano-immunotherapy that can improve several immune parameters described in the proposed “cancer immunogram” such as: (1) induction of a strong immune status activating all DCs, (2) promotion of immune cell infiltration by cytotoxic T cells and CD8α+DCs, (3) decrease the expression of PD-1 and PD-L1 immune checkpoints, (4) reduce IL-6 and IL-10 soluble Th2 inhibitors and (5) improve tumour sensitivity to immune

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**Table 2.** GNP-LLO91-99 nanovaccines as human immunotherapies in comparison to check point inhibitors using MoDC.

| Patients code | Period 2012–15 | CONTROL | GNP-LLO91-99 | Nivolumab | Ipilimumab | Nivolumab + GNP-LLO91-99 | Ipilimumab + GNP-LLO91-99 |
|--------------|---------------|---------|-------------|----------|-----------|-------------------------|--------------------------|
| HUMV-2/MHC-II | 61.3 ± 0.5    | 93.32 ± 0.5 | 24.5 ± 0.3  | 60.3 ± 0.4 | 97.5 ± 0.5 | 62.59 ± 0.5             |                         |
| HUMV-2/CBD86 | 3.97 ± 0.2    | 76.8 ± 0.5 | 6.03 ± 0.2  | 6.01 ± 0.2 | 70.1 ± 0.3 | 65.56 ± 0.2             |                         |
| HUMV-2/PD-1  | 34.9 ± 0.3    | 12.9 ± 0.2 | 6.88 ± 0.4  | 9.02 ± 0.3 | 4.03 ± 0.1 | 10.01 ± 0.4             |                         |
| HUMV-3/MHC-II| 62.29 ± 0.5   | 92.41 ± 0.1| 23.9 ± 0.3  | 61.1 ± 0.5 | 92.0 ± 0.5 | 62.80 ± 0.5             |                         |
| HUMV-3/CBD86 | 4.12 ± 0.3    | 76.6 ± 0.4 | 6.07 ± 0.1  | 6.02 ± 0.12| 68.9 ± 0.4 | 67.1 ± 0.4              |                         |
| HUMV-3/PD-1  | 34.5 ± 0.4    | 12.9 ± 0.4 | 6.89 ± 0.2  | 9.1 ± 0.4  | 4.10 ± 0.3 | 10.13 ± 0.2             |                         |
| CONT/MHC-II  | 60.2 ± 0.5    | 64.1 ± 0.1 | 6.51 ± 0.3  | 61.1 ± 0.5 | 62.0 ± 0.5 | 62.80 ± 0.5             |                         |
| CONT/CBD86   | 3.12 ± 0.3    | 5.6 ± 0.4  | 5.07 ± 0.1  | 5.02 ± 0.1 | 5.9 ± 0.4  | 5.1 ± 0.4               |                         |
| CONT/PD-1    | 30.5 ± 0.4    | 30.9 ± 0.4 | 30.8 ± 0.2  | 30.1 ± 0.4 | 30.3 ± 0.3 | 30.2 ± 0.2              |                         |

*Selected cutaneous melanoma patients from the Dermatology Department during 2012–15, identified by internal codes and diagnosed with stage IA (HUMV-2) or stage IB (HUMV-3) melanoma as described in Table S2. Controls (CONT) were healthy donors. "Ex vivo differentiated MoDC from HUMV-2 and HUMV-3 melanoma patients or healthy donors (CONT) with CD45+CD11c+CD14- phenotype, were treated with GNP-LLO91-99 (50 µg/mL), nivolumab or ipilimumab (100 µg/mL) for 48 h and analysed by FACS using anti-DR-PE, anti-CD86-Bv21, and anti-PD-1-APC antibodies. Results are expressed as the mean ± SD (P < 0.5)."
effectors by increasing the antigen-presentation capacities of DCs and anti-melanoma cytotoxic cells.

GPN-LLO_{91-99} anti-melanoma abilities seem to be comparable to the STINGVAX vaccines prepared for melanoma, but with several advantages: (i) a synthetic nanostucture with a globular and gold core vs. irradiated allogenic tumour cells in the above-mentioned vaccines, which avoids systemic inflammatory reactions and immune adverse effects; (ii) the neo-epitopic and cytotoxic abilities of the LLO_{91-99} peptide enhance anti-melanoma specific cytotoxic T cell responses with no risk when including live bacteria as in the case of VACCIMEL, which includes BCG; (iii) improves DC antigen-presenting abilities, being a useful adjuvant therapy alone (43) or in combination with anti-PD-1 and anti-CTLA-4 immunotherapies and (v) a simple diagnostic tool to identify melanoma patients who may benefit from these treatments.

Materials and methods

**B16OVA transplants and GPN-LLO_{91-99} therapy, alone or in combination with anti-ctla-4 or anti-pd-1 antibodies**

B16OVA or SM1-OVA cells were transplanted into 8 to 12-week-old female C57BL/6 mice (autotransplants) and P4 newborn CD-1 mice were transplanted with B16OVA (allografts) by a single subcutaneous injection (10^6 cells) in a volume of 100 µL (n = 10/group). At 7 days, the melanoma transplanted mice received or not (NT) a single intravenous (i.v.) injection of GPN-LLO_{91-99} therapy (50 µg/mouse) alone (n = 10/group) or in combination with anti-CTLA-4 or anti-PD-1 antibodies (100 µg/mouse every two days), DC-LLO_{91-99} vaccines (10^6 cells), control GNP coated with glucose (GNP, 50 µg/mouse), anti-CTLA-4 or anti-PD-1 (clone RPMI-14) antibodies (100 µg/mouse every two days) or saline (NT, untreated mice). Allografts were established subcutaneously (s.c) in P4 mice only for 4 days (n = 5) receiving GPN-LLO_{91-99} in situ injection for 3 days. On day 7 after injection (auto- and allo-transplants) and before the mice were killed, serum was collected and processed within 50 min and stored at −80°C for cytokine analysis. Splens and melanomas were also collected. Melanoma size was measured using a calliper. Tumour volume (TV) was calculated using the following formula: (length x (width)^2)/2 as reported previously. The mean and SD of tumour volume per group were calculated.

Melanomas were minced, homogenized and passed through a 70-µm strainer and TILs isolated by centrifugation over a Ficoll gradient at 1,077 g/mL density (Histopaque-1,077, Sigma-Aldrich, St. Louis, MO, USA). We recovered TILs in the interphase gradient, while collecting melanoma cells in pellets.

**Patients**

Stage I and III cutaneous melanoma patients (HUMV-1/IIIB, HUMV-2/IA, HUMV-3/IB and HUMV-4/IA) diagnosed during the period 2012–2015 at the Dermatology Department were included in the study before receiving any interferon adjuvant treatment or having received interferon at least 5 years before initiation of this study. Stage IV cutaneous metastatic melanoma patients (ONCM-1, ONCM-2, ONCM-3) diagnosed during the same period at the Medical Oncology Department were included in the study before enrolling in any treatment. Patients participated in the study voluntarily and signed an informed consent at the physician consultation and received an Information Document on the research study. Patients were able to revoke the informed consent at any time. Blood samples were collected in EDTA-tubes at the Dermatology or Medical Oncology Departments of our institution (HUMV, Hospital Universitario Marqués de Valdecilla, Santander, Spain) on the day of patient consultation and processed in the IDIVAL laboratory within 2 h.

**Isolation of MoDC**

Peripheral blood mononuclear cells (PBMC) from cutaneous melanoma patients or healthy donors were isolated from a Ficoll gradient of whole blood cells (EDTA-containing). PBMC recovered from the interface were washed twice in Hank’s buffered solution and cultured. In other samples, PBMC were prepared in MACSTM buffer (PBS-0.5% BSA-2 mM EDTA) with microbeads conjugated to mouse IgG2a monoclonal human CD14 antibody (Miltenyi, Bergisch Gladbach, Germany) for monocyte (Mo) positive selection. CD14+ positive cells were selected using MACSTM columns (Miltenyi). FACS analysis following CD14+MACSTM selection indicated 99% of CD14+CD14+ cells were positive. MoCD14+ cells were differentiated to MoDC at 1 x 10^6 cells/mL in 6-well plates (FalconTM) over 7 days using GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) in RPMI-20% FCS medium. All differentiated cells were 98% CD45+CD11c+DRlowCD86+CD14+ positive cells using specific monoclonal antibodies (Miltenyi). MoDC were incubated with GNP-LLO_{91-99} nanovaccines (50 µg/mL), ipilimumab or nivolumab (100 µg/mL of each checkpoint inhibitor), prepared in RPMI-20% FCS medium for 48 h to determine the cell surface phenotype of activated MoDC by FACS analysis.

**FACS analysis and antibodies**

Spleen cell surface markers and recovered melanomas were analysed by FACS using the following antibodies for mice: anti-CD11c-PE, anti-MHCII-BV421, anti-CD8-APC, anti-CD4-PE, anti-CD11b-FITC, anti-F480-PE, anti-CD40-APC, anti-CD86-APC, anti-CD83-PE, anti-mouse IFN-γ-FITC and anti-CD25-FITC, and the following antibodies for humans: anti-CD11c-PE, anti-CD45-PerCP, anti-CD14-APC, anti-DR-PE (MHC-II), anti-CD80-PerCP, anti-CD84-FITC and anti-CD86-BV (Miltenyi Biotech Inc., Auburn, CA, USA). Intracellular FoxP3 was measured by FACS using FoxP3 Staining Buffer and anti-FoxP3-PE antibody (Miltenyi Biotech Inc.). The frequencies of LLO_{91-99} or OVA_{257-264} specific CD8 T cells producing IFN-γ were measured with recombinant soluble dimeric mouse H-2Kb: Ig fusion protein (DimerX1, BD Biosciences, Palo Alto, CA, USA) as reported previously. In brief, LLO_{91-99} or OVA_{257-264} peptides (40 µM) were pre-incubated with PE-conjugated H-2Kb: Ig (1 µM) in PBS, at 37°C for 16 h. TILs (2 x 10^6 cells/mL) were incubated with IFN-γ and CD8 antibodies and the staining cocktail mix described above for 10 min at 4°C. The percentages of
CD8⁺ gated cells were expressed as the mean ± SD of triplicate samples ($P < 0.05$). Data were analysed using FlowJo software. Mouse or human sera were used to quantify cytokines using the CBA Mouse Inflammation Kit or CBA Human Inflammation Kit, respectively (BD Biosciences).

**Immunohistochemistry**

Melanoma transplanted mice treated or not with GNP-LLO and were immersed in 4% formaldehyde for 24 h. Melanomas were embedded in paraffin, processed and sections stained with hematoxylin-eosin (HE) and immunohistochemically analysed for antibody staining (Dako, Carpintera, CA, USA). Necrotic foci were analysed and counted as described previously.²

**Statistical analysis**

For statistical analysis, the Student’s $t$ test was applied. $P \leq 0.05$ was considered significant.

**Ethics statement**

This study was approved by the Ethical Committee of Clinical Research of Cantabria at Instituto de Investigación Marqués de Valdecilla (Santander, Spain) reference number 30.2012. All participants signed the informed consent documents and these documents are in the custody of physicians in accordance with Spanish Law (Ministry of Health). The study was carried out in accordance with the Guide of Care and Use of Laboratory Animals of the Spanish Ministry of Science and Innovation. The Ethical Committee of Animal Experiments of the University of Cantabria approved the protocol (Permit Number: PI-01–17) that followed Spanish legislation (RD 1201/2005). Surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimise suffering. This study is protected by approved patent application PCT/ES2017/0170103.

**Author contributions**

R. C-G, H T-N, E F-C, D S-C, F R, S Y-D and C A-D designed the research, F J F and J G-R performed histochemical analyses and interpreted the results, A G-C, V M-C, S Y-D, F R, R C-G and C A-D designed the immunotherapies analysis, S Y-D and F R provided the melanoma patients, obtained and had custody of the informed consents and clinical history of the patients, I G and M M synthesized the GNP-LLO and GNP controls and performed the control and toxicity analysis, R T helped with statistics, elaboration of the paper and figures, and design of the immunological assays, R C-G, H T-N and C A-D wrote the paper. R C-G, E F-C, I G, S Y-D and C A-D participated as authors in PCT patent application and recent approval PCT/ES2017/0170103.

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**ORCID**

Ricardo Calderon-Gonzalez  http://orcid.org/0000-0003-0841-3814
David Salcines-Cuevas  http://orcid.org/0000-0003-2627-0597
Marco Marradi  http://orcid.org/0000-0003-0831-6942
Mar Portillo-Gonzalez  http://orcid.org/0000-0002-6346-4836
Elisabet Frande-Cabanes  http://orcid.org/0000-0003-1748-0584
Raquel Tobes  http://orcid.org/0000-0001-7032-8720

**Abbreviations**

ANOVA analysis of variance

CD cluster differentiation

DC dendritic cells

LLO listeriolysin O

MDSC myeloid-derived suppressor cells

MHC major histocompatibility complex

MoDC monocyte-derived dendritic cells

NK natural killer

Treg regulatory T cells

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