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**Immunization with mannosylated nanovaccines and inhibition of the immune-suppressing microenvironment sensitizes melanoma to immune checkpoint modulators**

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3 **Ibrutinib sensitizes melanoma to immune checkpoint modulators following**  
4 **immunization with nano-vaccines by relieving immune-suppressing**  
5 **microenvironment**

6 **One Sentence Summary:**

7 Combination of dendritic cell-targeted nano-vaccines with a myeloid-derived  
8 suppressor cell inhibitor and immune checkpoint modulators expands the host  
9 antitumor immune cells, restricts tumour growth and prolongs survival in an orthotopic  
10 melanoma model.

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2 nanomedicines.

3 **ABSTRACT**

4 Low response rate, acquired resistance, and severe side effects have limited the  
5 clinical outcomes of immune checkpoint therapy. Here we show that combining  
6 cancer nano-vaccines with anti-PD-1 for immunosuppression blockade, and anti-  
7 OX40 for effector T cell stimulation, expansion, and survival, can potentiate the  
8 efficacy of melanoma therapy. Prophylactic and therapeutic combination regimens of  
9 dendritic cell-targeted mannosylated nano-vaccines with anti-PD-1/anti-OX40 ( $\alpha$ PD-  
10 1/ $\alpha$ OX40) demonstrate synergism, stimulating T cell infiltration into tumours at early  
11 treatment stages. However, treatment at the therapeutic regimen does not result in  
12 enhanced inhibition of tumor growth compared to  $\alpha$ PD-1/ $\alpha$ OX40 alone and is  
13 accompanied by increased infiltration of myeloid-derived suppressor cells (MDSC) in  
14 tumours. Combining the double therapy with ibrutinib, an MDSC-inhibitor, leads to  
15 remarkable tumour remission and prolonged survival in treated melanoma-bearing  
16 mice. The synergy between the mannosylated nano-vaccines, ibrutinib and  $\alpha$ PD-  
17 1/ $\alpha$ OX40 provides essential insights to devise alternative regimens to improve the  
18 efficacy of immune checkpoint modulators in solid tumours by regulating the  
19 endogenous immune response.

20

## 1 INTRODUCTION

2  
3 Advances in immunotherapy have improved the treatment outcomes of  
4 melanoma, based on antitumor immune responses elicited by the patient's own  
5 immune system. Cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and  
6 programmed cell death protein 1 (PD-1) blockade have enhanced specific T cell-  
7 mediated antitumor immunity in clinical practice <sup>1,2</sup>. Since these findings, efforts are  
8 being made not only to inhibit negative immune checkpoints but also to stimulate  
9 activation signalling pathways of the immune system. For example, OX40 (CD134 or  
10 TNFSFR4) is a co-stimulatory receptor member of the tumour necrosis factor (TNF)  
11 family that is transiently expressed on activated T cells. Once activated, OX40  
12 induces expansion, trafficking, and survival of effector T cells, and increases pro-  
13 inflammatory cytokine secretion <sup>3,4</sup>. OX40-signaling can also block the inhibitory  
14 activity of tumour-infiltrating regulatory CD4<sup>+</sup> T cells, by hindering Foxp3, TGF- $\beta$ , and  
15 IL-10 signalling, known to suppress vaccine-induced immune responses <sup>5-7</sup>.

16 Despite the promising clinical results, immune checkpoint therapy has been  
17 associated with low percentages of effective and durable responses to single agent  
18 therapies, due to resistance or relapse <sup>8-10</sup>. The presence of tumour infiltrating  
19 lymphocytes (TIL) has been correlated with favourable outcomes of immune  
20 checkpoint modulation therapies <sup>11</sup>. Based on these observations, and in an effort to  
21 integrate a novel therapeutic approach, we hypothesized that cancer vaccines  
22 delivering tumour-associated antigens to dendritic cells (DC) and consequent T-cell  
23 priming and activation, could potentiate the antitumor responses and survival  
24 outcomes of the combination of PD-1 blockade and OX40 co-stimulation. Therefore,  
25 we set to test this hypothesis in two murine models of melanoma.

1 In order to modulate the generation and function of myeloid-derived  
2 suppressor cells (MDSC), we further administered ibrutinib, an irreversible inhibitor  
3 of Bruton's tyrosine kinase (BTK). Under cancer-associated inflammation, an  
4 aberrant myelopoiesis leads to the accumulation of MDSC, compromising the  
5 efficient maturation of DC, affecting the antigen presentation process and driving T  
6 cell transcriptional programs towards T cell anergy and exhaustion. The presence of  
7 MDSC has been also correlated to decreased efficacy of anti-CTLA-4, constituting an  
8 important target for enhancing the efficacy of immune checkpoint inhibitors <sup>12,13</sup>.

9 Here, we report the design, synthesis and characterization of biodegradable  
10 polymeric nano-sized particles made of mannose-grafted polylactic-co-glycolic  
11 acid/polylactic acid (PLGA/PLA) (man-NP) (**Fig. 1A**). Man-NP were designed to  
12 improve the nanoparticle interactions with DC by targeting the mannose receptor  
13 (MR/CD206) <sup>14</sup>. The mannosylated nano-vaccines contained the Toll-like receptor  
14 (TLR) ligands CpG and monophosphoryl Lipid A (MPLA), as immune potentiators,  
15 and the melanoma-associated melan-A/MART-1 peptides as antigens. The  
16 mannosylated nano-vaccines carried short (10 amino acids) and long (20 amino  
17 acids) melan-A/MART-1 peptides, aiming for the major histocompatibility complex  
18 (MHC) class I and class II antigen presentation pathways, respectively. Melan-  
19 A/MART-1 (26-35(A27L)) MHCI-restricted peptide (MHCI-ag) and melan-A/MART-1  
20 (51-73) MHCII-restricted peptide (MHCII-ag) were used. Activation of MHC class I  
21 and MHC class II potentiated these active vaccination strategies, by engagement of  
22 both CD4<sup>+</sup> and CD8<sup>+</sup> T cells <sup>15</sup>.

23 Our results demonstrate amplified antitumor immune response mediated by (i)  
24 targeting DC through vaccination as an active immunotherapy, (ii) inhibiting MDSC,  
25 and (iii) modulating OX40 and PD-1 on T cells. Here we show that this strategy led to

1 DC recruitment and differentiation to re-shape the tumour microenvironment (TME)  
2 cellular plasticity and induced a remarkable melanoma growth inhibition.

3

## 4 **RESULTS**

### 5 **Polymeric nanoparticles as antitumor nano-vaccines**

6 To deliver tumour-associated antigens to DC, we developed two different types  
7 of biodegradable nanoparticles: PLGA/PLA NP (NP) and mannose-PLGA/PLA NP  
8 (man-NP) (**Fig. 1A**). To prepare man-NP, mannose-grafted PLGA polymer was  
9 synthesized by amidation of the terminal carboxylic acid groups of PLGA with  
10 mannosamine <sup>16</sup>. The reaction was confirmed by <sup>1</sup>H NMR of mannose-PLGA  
11 (**Supplementary Fig. 1**) compared with the individual <sup>1</sup>H NMR spectra of  
12 mannosamine and PLGA. The multiplet signal between 3.2 and 3.8 ppm indicates  
13 the presence of mannose in the mannose-PLGA polymer, as previously reported <sup>17</sup>.  
14 NP and man-NP were prepared by the double emulsion solvent evaporation method  
15 <sup>18</sup>. The average hydrodynamic diameters of NP and man-NP were similar and ranged  
16 between  $166 \pm 5$  nm and  $181 \pm 8$  nm, depending on the entrapped molecules (**Table**  
17 **1**), with a low polydispersity index ( $0.13 \pm 0.04$  to  $0.18 \pm 0.04$ ). Electron microscopy  
18 and atomic force microscopy showed homogenous spherical particles, with slight  
19 roughness on the surface (**Fig. 1B, C, D**) and near-neutral surface charge (**Table 1**).  
20 Mannose residues on the surface of man-NP were detected by the Lectin Recognition  
21 Assay, using Concanavalin A (Conc A) (**Supplementary Fig. 2A**). Conc A binds to  
22 exposed mannose residues, forming aggregates <sup>15,19</sup>. After incubation of man-NP,  
23 Conc A induced particle aggregation, revealed by a 5-fold increase in average size  
24 (**Supplementary Fig. 2B**). Particle aggregation (in green) was also confirmed by  
25 fluorescence microscopy following incubation of fluorescein isothiocyanate (FITC)-

1 labelled Conc A with man-NP (**Supplementary Fig. 2C**). Non-mannosylated NP were  
2 the negative control.

3 NP and man-NP showed high levels of entrapment efficiency (EE) and loading  
4 capacity (LC) (**Table 2**). For the MHC I-ag, EE  $>97.5 \pm 0.2\%$  and LC  $>48.8 \pm 0.1$   
5  $\mu\text{g}/\text{mg}$ , while for the MHC II-ag EE  $>74.6 \pm 3.5\%$  and LC  $>37.3 \pm 1.7 \mu\text{g}/\text{mg}$ . The EE  
6 of CpG was above  $80.8 \pm 2.5\%$ , which corresponds to LC of  $8.1 \pm 0.3 \mu\text{g}/\text{mg}$ .

7 NP and man-NP did not affect DC viability ( $>90\%$ ) in the concentration range  
8 tested (**Supplementary Fig. 3A**), supporting their physiological biocompatibility. This  
9 was also assessed *ex vivo* by red blood cell (RBC) lysis determination. NP and man-  
10 NP did not disrupt RBC membranes at concentrations up to 20 mg/ml  
11 (**Supplementary Fig. 3B**).

12

### 13 **Mannosylated nano-vaccines are highly internalized by dendritic cells**

14 To determine internalization, Cy5.5-labeled NP and man-NP were incubated  
15 with murine immature dendritic cells (JAW SII). FACS measurements showed higher  
16 uptake with increasing time of contact. Man-NP exhibited much higher internalization  
17 levels ( $P = 0.001$ ) than non-mannosylated NP (**Fig. 1E**). Particle internalization was  
18 also confirmed by confocal microscopy, corroborating the FACS results (**Fig. 1F**).  
19 Cy5.5-labeled NP and man-NP prepared with PVA instead of TPGS were used as  
20 control. We observed a dramatic increase in NP uptake while using TPGS compared  
21 with PVA, including those NP functionalized with mannose (**Supplementary Fig. 4**).  
22 The higher uptake obtained for mannosylated NP (TPGS) was reduced in the  
23 presence of soluble mannose, which confirms that the higher internalization obtained  
24 for mannose-decorated NP was mediated by the interaction of this targeting moiety  
25 with CD206 receptor at the DC surface.

1 We show that our man-NP (TPGS) was preferentially taken-up *in vivo* by  
2 circulating DC (gated as CD11b<sup>+</sup>CD11c<sup>+</sup>), compared to macrophages  
3 (CD11b<sup>+</sup>CD11c<sup>-</sup>) and resident DC (CD11b<sup>-</sup>CD11c<sup>+</sup>) (**Fig. 1G, Supplementary Fig.**  
4 **5**). In addition, our nano-vaccine was able to significantly increase the expression of  
5 activation/maturation markers at the surface of these APC (**Fig. 1H**).

6

### 7 **Mannosylated nano-vaccines induce splenocyte activation and cytotoxicity** 8 **against melanoma cells**

9 After subcutaneous (s.c.) injection of Cy5.5-labeled NP or man-NP (20 mg/ml),  
10 50  $\mu$ l in the right hind and 50  $\mu$ l in the left hind, particles remained at the site of  
11 immunization 48 hours following injection ( $N=4$ ). Fluorescence signal measurements  
12 revealed the preferential accumulation of particles in the lymph nodes (LN). Residual  
13 accumulation was detected in the liver and the kidney, due to the excretion of  
14 metabolic particle derivatives (**Fig. 2A-B**).

15 To determine the ability of NP and man-NP to induce specific immune  
16 responses, we performed a 3-time immunization, seven days apart, to C57BL/6J  
17 mice. Ten days after the last immunization, splenocytes were harvested and re-  
18 activated in cell culture for 6 days with anti-CD28 and MHCI-ag alone, or in  
19 combination with MHCII-ag (**Fig. 2C**). The groups treated with NP and man-NP  
20 showed an increased secretion of inflammatory cytokines, such as IFN- $\gamma$ , a Th1  
21 cytokine, and GM-CSF. The highest levels of IFN- $\gamma$  and GM-CSF were induced by  
22 man-NP. Elevated levels of CCL1/TCA-3 and TARC/CCL17 also suggested that our  
23 particles played a major role in priming antigen-specific T cells (**Fig. 2D**). There was  
24 no evidence of body weight change in all groups, attesting to vaccine tolerability and  
25 safety (**Supplementary Fig. 6**).

1           Analysis of Th2 cytokines revealed that the administration of NP MHCI-ag/NP  
2 MHCII-ag and man-NP MHCI-ag/man-NP MHCII-ag decreased MIP-1 $\beta$ /CCL4  
3 secretion, whereas both man-NP MHCI-ag and the combination man-NP MHCI-  
4 ag/man-NP MHCII-ag equally reduced the MCP-5/CCL12 levels. These results  
5 indicate the role of nanoparticulate vaccines in the modulation of Th2 chemokine  
6 secretion profile.

7

8           The major goal of a successful antitumor immunotherapy is to trigger antigen-  
9 specific cytotoxic CD8<sup>+</sup> T cells (CTL) to recognize and destroy target cells.  
10 Splenocytes collected from mice immunized with man-NP MHCI-ag/man-NP MHCII-  
11 ag exhibited the highest *ex vivo* cytotoxicity when co-cultured with mCherry-labelled  
12 murine Ret melanoma cells (RMS) (**Fig. 2E**). These data suggest that the  
13 immunization with man-NP MHCI-ag or man-NP MHCI-ag/man-NP MHCII-ag  
14 efficiently triggered CTL responses. Man-NP MHCI-ag/man-NP MHCII-ag elicited the  
15 highest CTL activity. These were superior to man-NP MHCI-ag ( $P = 0.014$ ), to non-  
16 mannosylated NP co-entrapping both antigens ( $P = 0.02$ ), and dramatically stronger  
17 than NP MHCI-ag/MHCII-ag ( $P = 9.6 \times 10^{-6}$ ). MHCI-ag/MHCII-ag/CpG/MPLA (free  
18 antigen/immune potentiators) or empty particles induced a CTL activity similar to PBS  
19 (**Fig. 2E**).

20           The CTL activity induced by man-NP MHCI-ag/man-NP MHCII-ag is clearly  
21 shown by the increased fluorescent staining related to caspase 3/7 activation (in  
22 green), a marker of apoptosis (**Fig. 2F**).

23

1 **Prophylactic mannosylated nano-vaccines synergize with anti-PD-1/anti-OX40**  
2 **( $\alpha$ PD-1/ $\alpha$ OX40) treatment, restricting melanoma growth and prolonging**  
3 **survival**

4         Given the *in vitro* and *ex vivo* findings, we next asked whether the immune  
5 response elicited by the mannosylated nano-vaccines would synergize with the PD-  
6 1 blockade and OX40 activation for melanoma therapy. To test this hypothesis, mice  
7 were treated according to the schedule in **Fig. 3A**. When man-NP MHCII-ag/man-NP  
8 MHCII-ag was combined with  $\alpha$ PD-1/ $\alpha$ OX40, a remarkable tumor growth inhibition  
9 was obtained (**Fig. 3B-E, Supplementary Fig. 8**), with minimal systemic toxicity  
10 (**Supplementary Fig. 7**).

11         At day 17 after tumour cell inoculation, the tumour volume in animals treated  
12 with  $\alpha$ PD-1/ $\alpha$ OX40, or with the combination of non-mannosylated (NP MHCII-ag/NP  
13 MHCII-ag) or mannosylated nano-vaccines (man-NP MHCII-ag/man-NP MHCII-ag)  
14 with  $\alpha$ PD-1/ $\alpha$ OX40 was much lower compared to animals treated with PBS ( $P <$   
15  $0.0016$ ) (**Fig. 3D**). At day 27, the tumour volume intra-group variability increased for  
16 all the groups, except for the one treated with the combination of man-NP MHCII-  
17 ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40, in which the tumours remained small (**Fig.**  
18 **3E, Supplementary Fig. 8**). Empty NP, with and without mannose were used as  
19 controls. Tumour volumes of animals vaccinated with empty NP or empty man-NP  
20 were similar to those treated with PBS (**Supplementary Fig. 8**).

21         Of note, the combination of mannosylated nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40  
22 induced a prolonged overall survival. The combination man-NP MHCII-ag/man-NP  
23 MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 resulted in 100% survival 42 days following tumour  
24 inoculation, with a survival rate of 50% two months following tumour inoculation. On

1 the other hand, treatment with  $\alpha$ PD-1/ $\alpha$ OX40 resulted in only 20% of survival (**Fig.**  
2 **3C**).

3 Immunohistochemistry staining revealed high levels of caspase-3 and tumour-  
4 infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in all groups immunized with nano-vaccines  
5 (**Supplementary Fig. 9**). The highest level of CD8<sup>+</sup> T cell infiltration was observed in  
6 groups treated with mannosylated nano-vaccines, alone or in combination with  $\alpha$ PD-  
7 1/ $\alpha$ OX40, and non-mannosylated nano-vaccines alone, suggesting that indeed the  
8 nano-vaccines are capable of reactivating cell-mediated cytotoxicity in the TME  
9 (**Supplementary Fig. 9**).

10

### 11 **Combination of therapeutic nano-vaccines with $\alpha$ PD-1/ $\alpha$ OX40 immune** 12 **checkpoint therapy at an intervention dosing schedule**

13 Motivated by the synergism observed with the combination of prophylactic  
14 nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40, we hypothesized that the same antitumor  
15 immune-mediated effect could be achieved by treating melanoma-bearing mice at a  
16 therapeutic regimen. Therefore, man-NP MHCI-ag/man-NP MHCII-ag were  
17 combined with  $\alpha$ PD-1/ $\alpha$ OX40 according to the dosing schedule described in **Fig. 4A**  
18 and tested in Ret melanoma cells and mCherry-labelled Ret melanoma (RMS) cells.  
19 mCherry-labelling of Ret cells did not alter tumour cell proliferation, neither *in vitro*  
20 (data not shown) nor *in vivo*. Ret cells and RMS induced similar tumour growth  
21 profiles in C57BL/6J mice, and the response to our triple combination therapies, man-  
22 NP MHCI-ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib, was also similar  
23 (**Supplementary Fig. 10**).

24 The groups  $\alpha$ PD-1/ $\alpha$ OX40 and man-NP MHCI-ag/man-NP MHCII-ag +  $\alpha$ PD-  
25 1/ $\alpha$ OX40 showed similar average tumour volume at day 18, which was 6-fold

1 significantly smaller compared to the PBS-treated ( $P < 0.0012$ ) (**Fig. 4B**), with  
2 negligible systemic toxicity observed (**Supplementary Fig. 11**).

3 On day 18 after tumour inoculation, the combination of mannosylated nano-  
4 vaccines with  $\alpha$ PD-1/ $\alpha$ OX40 did not result in further tumour growth restriction  
5 compared to  $\alpha$ PD-1/ $\alpha$ OX40 alone; however, the animals treated with the combination  
6 had the highest levels of CD8<sup>+</sup> T cells in tumour infiltrates ( $P < 0.001$ ) (**Fig. 4D**,  
7 **Supplementary Fig. 12**). These data support the synergism we previously observed  
8 in the prophylactic regimen.

9 The treatment with  $\alpha$ PD-1/ $\alpha$ OX40 induced the lowest levels of CD4<sup>+</sup> T-cell  
10 infiltrates (**Fig. 4E**, **Supplementary Fig. 12**) displaying reduced infiltration of  
11 regulatory T (Treg) cells (**Fig. 4F**, **Supplementary Fig. 13**). Animals treated with the  
12 mannosylated nano-vaccines, alone or in combination, had higher levels of Treg cells  
13 compared to  $\alpha$ PD-1/ $\alpha$ OX40-treated group. Therefore, the highest CD8:Treg ratio was  
14 observed for  $\alpha$ PD-1/ $\alpha$ OX40 treatment, followed by the combination of mannosylated  
15 nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40 immune checkpoint therapy (**Fig. 4G**).

16 At the final endpoint, the animals treated with  $\alpha$ PD-1/ $\alpha$ OX40 remained with the  
17 highest CD8:Treg ratio. This was 7-fold higher compared to the groups treated with  
18 mannosylated nano-vaccines, alone or in combination with  $\alpha$ PD-1/ $\alpha$ OX40 ( $P < 0.01$ ).

19 Interestingly, from day 18 to the final endpoint, the infiltration of CD11b<sup>+</sup>Gr-1<sup>+</sup>  
20 MDSC increased significantly in the two groups treated with mannosylated nano-  
21 vaccines, alone or in combination (**Fig. 4H**, **Supplementary Fig. 14**). At the final  
22 endpoint, the levels of MDSC in those groups were approximately 4-fold higher than  
23 those obtained for the  $\alpha$ PD-1/ $\alpha$ OX40-treated group. These extremely elevated levels  
24 of MDSC seem to correlate with the marked decrease of CD8<sup>+</sup> T cell infiltration (**Fig.**  
25 **4D**, **Supplementary Fig. 12**) and with the high percentage of Treg cells (**Fig. 4F**,

1 **Supplementary Fig. 13)** at the final endpoint in the two groups treated with the  
2 mannosylated nano-vaccine. This contributes to lower CD8:Treg ratios at the final

3

4 **Trivalent combination of mannosylated nano-vaccines with ibrutinib and  $\alpha$ PD-**  
5 **1/ $\alpha$ OX40 strongly restricts melanoma growth and prolongs overall survival**

6 Considering the high levels of MSDC observed in the tumour infiltrates, we  
7 hypothesized that combined targeted inhibition of these cells with our mannosylated  
8 nano-vaccines and immune checkpoint modulators would lead to enhanced  
9 therapeutic efficacy. To inhibit MDSC, we selected ibrutinib, an irreversible inhibitor  
10 of the Bruton's tyrosine kinase (BTK) and interleukin-2-inducible T-cell kinase (ITK),  
11 which can modulate the generation and function of MDSC <sup>20,21</sup>.

12 Melanoma-bearing mice were treated according to the schedule in **Fig. 5A**. On  
13 day 20 after tumour inoculation, we observed that the average tumour volume of  
14 groups treated with ibrutinib or with mannosylated nano-vaccines + ibrutinib were  
15 identical to the PBS-treated group. In the groups treated with mannosylated nano-  
16 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 or  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, however, the tumour volume  
17 was significantly reduced by more than 5-folds ( $P = 0.038$  and  $P = 0.0060$ ,  
18 respectively) (**Fig. 5B**). In addition, the mannosylated nano-vaccines (man-NP) were  
19 significantly superior to the non-mannosylated nano-vaccines (NP) from day 19  
20 onwards ( $P = 0.005$ ), demonstrating the crucial role of the mannose targeting  
21 moieties for achieving an effective immune-mediated control of tumour growth  
22 (**Supplementary Fig. 15A-B**). In fact, on day 23, the man-NP-treated group had an  
23 average tumour volume of 54 mm<sup>3</sup>, in contrast to the NP-treated group, which had an  
24 average tumour volume of 638 mm<sup>3</sup>.

25 The trivalent therapy, comprising of the combination of mannosylated nano-

1 vaccines induced the most potent specific anti-tumour immune response ( $P = 0.001$ )  
2 (**Fig. 5C-D, Supplementary Fig. 15C-D**). The superior antigen-dependent immune  
3 response was irrespective of the antigen used (gp100 or melan-A/MART-1) as shown  
4 by a tetramer staining assay (**Fig. 5C, Supplementary Fig. 15C-D**). Melan-A/MART-  
5 1 specificity was further confirmed by the IFN- $\gamma$  ELISpot assay (**Supplementary Fig.**  
6 **15E**). The highest levels of antigen-specific intracellular IFN- $\gamma^+$  stained in tumour-  
7 infiltrating cytotoxic CD8 $^+$  T lymphocytes of RMS-bearing mice measured by FACS,  
8 at the 1<sup>st</sup> endpoint, were obtained for animals treated with the mannosylated nano-  
9 vaccines (**Supplementary Fig. 15F**).

10 On day 33, animals treated with the man-NP MHCI-ag/man-NP MHCII-ag +  
11  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib presented a 3-fold higher survival percentage ( $P = 0.0049$ )  
12 compared to animals treated with NP MHCI-ag/NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 +  
13 ibrutinib, among which 8 out of 13 already reached a tumour volume of at least 1000  
14 mm $^3$  (**Supplementary Fig. 15G**).

15

16 Fourteen out of 15 animals of the group mannosylated nano-vaccine +  $\alpha$ PD-  
17 1/ $\alpha$ OX-40 + ibrutinib remained alive at day 40, in comparison to 7 out of 15 animals  
18 of the group  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib (**Fig. 5E**). These animals treated with  $\alpha$ PD-  
19 1/ $\alpha$ OX-40 + ibrutinib presented higher variability in terms of tumour size (**Fig. 5F**).  
20 Moreover, 7% of the animals treated with mannosylated nano-vaccine +  $\alpha$ PD-1/ $\alpha$ OX-  
21 40 and 23% of the animals treated with  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib remained alive after  
22 65 days, while 67% of animals treated with the trivalent combination mannosylated  
23 nano-vaccine +  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib survived during that period. The survival  
24 curves of the triple regimen are statistically different from those obtained for PBS ( $P$   
25 = 0.0001), man-NP +  $\alpha$ PD-1/ $\alpha$ OX-40 ( $P = 0.0001$ ) and  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib ( $P$

1 = 0.0256) treatments. Furthermore, we detected only slight body weight changes  
2 relative to the initial body weight, throughout the study, reflecting negligible systemic  
3 toxicity (**Supplementary Fig. 11**).

4 We determined two endpoints to analyse immune cell infiltrates within the  
5 TME. The first endpoint, day 20 after tumour inoculation, corresponds to the day of  
6 the first death in the PBS-treated group, whereas the second endpoint was tumour  
7 size-matched, occurring on day 27 for PBS, ibrutinib only and mannosylated nano-  
8 vaccines + ibrutinib, and on day 35 for  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib and mannosylated  
9 nano-vaccines +  $\alpha$ PD-1/ $\alpha$ OX40. During this period, the tumours of animals treated  
10 with mannosylated nano-vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib were very small and  
11 therefore, those were kept for the immunohistochemistry (IHC) analysis.

12 Higher infiltration of T lymphocytes, as expected, was associated with stronger  
13 tumour growth inhibition (**Fig. 6A, Supplementary Fig. 12**). The number of CD8<sup>+</sup> T  
14 lymphocytes was more than 20-fold higher for mice treated with  $\alpha$ PD-1/ $\alpha$ OX40 +  
15 ibrutinib or with mannosylated nano-vaccines +  $\alpha$ PD-1/ $\alpha$ OX40, compared to the PBS-  
16 treated group (**Fig. 6B, Supplementary Fig. 12**).

17 The highest level of CD8<sup>+</sup> TIL, at the second endpoint, was induced by  $\alpha$ PD-  
18 1/ $\alpha$ OX40 + ibrutinib. At this time point, low levels of Treg cells were observed within  
19 the tumours of animals treated with  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib or mannosylated nano-  
20 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 (**Fig. 6D, Supplementary Fig. 13**). Both treatments also  
21 resulted in high CD8/Treg ratios, which correlated with the enhanced therapeutic  
22 efficacy (**Fig. 6E**).

23 IHC staining of RMS tumour sections for CD4 and CD8 confirmed the flow  
24 cytometry results, showing a prominent infiltration of CD8<sup>+</sup> T cells at day 20 after  
25 tumour inoculation, in groups that received the mannosylated nano-vaccines with

1  $\alpha$ PD-1/ $\alpha$ OX40. The mannosylated nano-vaccines also induced higher expression of  
2 the OX40 receptor as shown in the groups treated with man-NP MHCII-ag/man-NP  
3 MHCII-ag + ibrutinib and man-NP MHCII-ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40  
4 (**Supplementary Fig. 16**). It should be noted that the expression of PD-1/PD-L1 was  
5 the lowest for the tumours of animals treated with man-NP MHCII-ag/man-NP MHCII-  
6 ag +  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, even compared to those immunized with the non-  
7 mannosylated NP (**Supplementary Fig. 17**).

8 High levels of caspase-3 were observed in the tumours of the groups that  
9 received man-NP MHCII-ag/man-NP MHCII-ag + ibrutinib, man-NP MHCII-ag/man-NP  
10 MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 and  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, although only man-NP  
11 MHCII-ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 and  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib strongly  
12 inhibited tumour growth and significantly prolonged survival of the mice (**Fig. 5B, Fig.**  
13 **5E, Supplementary Fig. 16**).

14 In groups treated with  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, and mannosylated nano-  
15 vaccines + ibrutinib, we observed a decrease in MDSC infiltrates from the first to the  
16 second endpoint. This suggests that ibrutinib was restricting the migration of MDSC  
17 into the TME (**Fig. 6G, Supplementary Fig. 14, Supplementary Fig. 17**).

18  
19 The trivalent combination showed superior antitumor efficacy also in two  
20 independent studies ( $N = 6-9$ /group in each experiment) using a second mouse  
21 model bearing orthotopic s.c. B16-F10 melanoma (**Supplementary Fig. 18**). In this  
22 case, the dosing regimen led to 79% (first study **Supplementary Fig. 18A**) and 81%  
23 (second study **Supplementary Fig. 18B**) tumour growth inhibition at day 19 for the  
24 combination of mannosylated nano-vaccines with ibrutinib and  $\alpha$ PD-1/ $\alpha$ OX40, *versus*  
25 67% (**Supplementary Fig. 18A**) and 68% (**Supplementary Fig. 18B**) for the nano-

1 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 combination *versus* 70% (**Supplementary Fig. 18A**) and  
2 57% (**Supplementary Fig. 18B**) for the  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib combination. The  
3 proposed mechanism was confirmed in this second melanoma model by the  
4 reduction of infiltrating MDSC and the increase of infiltrating T cells in the TME  
5 (**Supplementary Fig. 19**). We selected Day 19 for comparison as this is the last day  
6 of the studies at which all the animals in all the groups were still present.

7

## 8 **DISCUSSION**

9 The recent clinical validation of different immune checkpoint modulators has  
10 revolutionized cancer therapy. However, low response rate, adverse effects and  
11 resistance led to the clinical need for alternative combination strategies to overcome  
12 these limitations <sup>22</sup>.

13 Our results report the synergism of mannosylated antitumor nano-vaccines  
14 with ibrutinib and PD-1 blockade/OX40. Our trivalent treatment strategy re-  
15 established the dynamic equilibrium between the host effector antitumor immunity  
16 and the tumour-mediated evasion mechanisms, based on the modulation of immune  
17 cell activity within the TME, rather than the direct effect on tumour cells. This resulted  
18 in a remarkable restriction of melanoma growth and long-term survival (**Fig. 7**).

19

20 These nano-vaccines, as an active immunotherapeutic strategy, have the  
21 unique ability to expand host antitumor specific T effector phenotype, improving  
22 sensitivity and long-term tumour recognition. The synergistic effect between  $\alpha$ PD-  
23 1/ $\alpha$ OX40, already reported in an ovarian cancer model <sup>23</sup>, could be relevant for  
24 melanoma <sup>24</sup> and be potentiated by cancer vaccines delivering tumour-associated  
25 antigens to DC.

1 We designed and synthesized non-mannosylated (NP) and mannosylated  
2 nano-vaccines (man-NP) for the *in vivo* co-delivery of melanoma-associated antigens  
3 (MAA) and TLR agonists to DC, via phagocytosis-dependent ("passive") and ligand-  
4 mediated ("active" through the mannose receptor (MR/CD206)) targeting,  
5 respectively. The two different particles were biocompatible and had similar  
6 morphology and physico-chemical characteristics in terms of size, Pdl, surface  
7 charge, EE, and LC. Intravital imaging revealed that NP and man-NP remained near  
8 the site of injection, after s.c. hock immunization, promoting contact with DC at the  
9 periphery (inguinal lymph node), as well as proximity to the popliteal and axillary  
10 lymph nodes. These data explain the preferential accumulation of the nano-vaccines  
11 in these tissues, which is extremely important for the induction of antigen-specific  
12 adaptive immune responses and vaccine efficacy <sup>25</sup>. The mean average diameter of  
13 these nano-vaccines, below 200 nm, may also be suitable for their trafficking through  
14 the lymphatic drainage directly to the lymphoid organs <sup>26</sup>. Despite the similar  
15 biodistribution profiles, mannosylated man-NP were much more efficiently  
16 internalized by DC compared to non-mannosylated NP ( $P = 0.00067$ ). The mannose  
17 residues on the surface of man-NP, demonstrated by particle aggregation induced by  
18 Conc A, increased particle affinity to MR/CD206 of DC, improving particle  
19 internalization <sup>14</sup>. The added value of mannose grafted onto the surface of our nano-  
20 vaccine was further confirmed *in vitro*, as the soluble mannose in the medium  
21 significantly reduced the mannose-mediated NP internalization by DC.

22 The most important goal of antitumor vaccination is the induction of cytotoxic  
23 activity against tumour cells. The presence of mannose on the surface and the  
24 particulate nature of our nano-vaccines carrying MAA and TLR agonists, played a  
25 pivotal role in this process, by activating splenocytes in healthy animals. While the

1 same MAA and TLR agonists, administered freely, only induced a negligible immune  
2 response. The co-delivery of tumour-associated antigens and TLR ligands by the  
3 same nanoparticulate carrier enhances antigen internalization, processing, and  
4 subsequent presentation. This is a key step to overcome host tolerance to tumour  
5 cells by improving effective T cell priming and lymphocyte expansion <sup>15</sup>.

6 Recently, the TLR synergy has been reported <sup>27</sup> showing that their activation  
7 on DC is crucial to induce tumour-specific T cell responses <sup>28</sup>. The role of TLR4  
8 agonists (MPLA) on Treg cells is underexplored, but it was shown to be essential for  
9 the immune-mediated destruction of cancer cells <sup>29</sup>. CpG, a TLR9-agonist, on the  
10 other hand, was shown to suppress the activation of those Treg cells, increase CD4<sup>+</sup>  
11 T cell survival, and promote the infiltration of CD8<sup>+</sup> T cells into the tumour <sup>30</sup>. Our  
12 nanoparticles allowed the multi-targeting TLR co-stimulatory effect, by entrapping  
13 both CpG and MPLA within the same polymeric matrix, in contrast to their free  
14 administration. The co-delivery of these TLR agonists to a single DC potentiates the  
15 efficient maturation of these cells towards balanced Th1- and Th2-type immune  
16 responses, which are crucial to control homeostasis within the tumour site <sup>31,32</sup>. It was  
17 previously reported that the intra-tumour administration of CpG, in combination with  
18  $\alpha$ OX40, induced a superior antitumor effect in melanoma compared to each of these  
19 agonists alone <sup>33</sup>. Our nano-platforms constitute an alternative for the targeted  
20 delivery of CpG oligonucleotide, as the *ex vivo* and *in vivo* studies presented here  
21 support the successful protection of CpG, once entrapped within our nano-vaccines  
22 polymeric matrix.

23 Mannosylated and non-mannosylated nano-vaccines triggered the secretion  
24 of pro-inflammatory cytokine profiles. We observed a significant increase in the  
25 secretion of IFN- $\gamma$  and GM-CSF, after re-stimulating the splenocytes collected from

1 mice treated with nano-vaccines. IFN- $\gamma$  and GM-CSF are associated with enhanced  
2 antigen priming and subsequent presentation by antigen-presenting cells (APC) <sup>34,35</sup>,  
3 as well as cytotoxic T cell activity. IFN- $\gamma$  is extensively produced by CD8<sup>+</sup> T cells and  
4 it is a predictor of antigen-specific cytotoxic T cell-mediated responses <sup>36</sup>. The  
5 increased activation of CD8<sup>+</sup> T cells in mice treated with nano-vaccines is also  
6 supported by the increased secretion of CCL1/TCA-3 and TARC/CCL17, which are  
7 chemokines associated with activated T cells <sup>37,38</sup>.

8 An inclusive assessment of the triad, IFN- $\gamma$ , IL-2 and TNF- $\alpha$ , predicted an  
9 improved CTL activity for man-NP MHCI-ag/man-NP MHCII-ag. We also observed  
10 that our nano-vaccines increased the secretion of IL-6, which is involved in the  
11 recruitment and differentiation of T lymphocytes, as well as in the suppression of Treg  
12 cells <sup>39,40</sup>. The suppression of these cells is important for eliciting CTL cell activity  
13 triggered by our nano-vaccines. Furthermore, the decreased secretion of MIP-  
14 1 $\beta$ /CCL4 and CCL12, as detected in the splenocytes from animals treated with our  
15 nano-vaccines, has also been associated with the inhibition of Treg cells, reduction  
16 of tumour angiogenesis and enhanced antitumor efficacy <sup>41,42</sup>.

17 The pro-inflammatory cytokine secretion profile induced by our nano-vaccines  
18 clearly correlated with increased *ex vivo* CTL activity. Man-NP carrying MHCI-ag and  
19 MHCII-ag induced the most robust CTL activity against murine RMS cells *ex vivo*.  
20 The benefit of MHC class II peptides in cancer vaccination, however, is still unclear.  
21 In clinical trials, it has been associated with poor prognosis, due to increased activity  
22 of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells or apoptosis of activated CD8<sup>+</sup> T cells <sup>43,44</sup>. Our results, on  
23 the contrary, support that the engagement of synergistic MHCI and MHCII responses  
24 can lead to stronger CD8<sup>+</sup> cytotoxic T cell responses <sup>45</sup>. Man-NP with MHCI-ag and  
25 MHCII-ag not only induced the strongest *ex vivo* CTL activity, but also slightly

1 enhanced antitumor immunity *in vivo*, when compared to treatment with man-NP  
2 carrying only MHCI-ag.

3 Our prophylactic nano-vaccines alone, as expected, induced a modest tumour  
4 growth inhibition *in vivo*. This is in agreement with the poor to moderate efficacy  
5 reported for cancer vaccines, due to tumour aggressiveness and the  
6 immunosuppressive TME <sup>46</sup>. The combination of prophylactic man-NP MHCI-ag/man-  
7 NP MHCII-ag with  $\alpha$ PD-1/ $\alpha$ OX40, however, highlights the synergistic antitumor effect  
8 of these complementary approaches. This combination resulted in the most potent  
9 antitumor response, leading to a survival of 50% of the mice for more than 2 months  
10 following tumour inoculation. We observed only minimal and yet reversible systemic  
11 toxicity, reflected by slight body weight change relative to the initial body weight.

12 The use of MHCI-ag and MHCII-ag peptides was essential for the induction of  
13 a robust antitumor response. The nano-platform was also crucial, as the free  
14 administration of the same MAA induced much lower efficacy. The combination of  
15 MHCI-ag/MHCII-ag (free) +  $\alpha$ PD-1/ $\alpha$ OX40 induced 20% survival, similar to  $\alpha$ PD-  
16 1/ $\alpha$ OX40 alone. This survival rate is 30% lower compared to the combination of  
17 mannosylated nano-vaccines (man-NP MHCI-ag/man-NP MHCII-ag) +  $\alpha$ PD-  
18 1/ $\alpha$ OX40.

19 Antitumor activity was achieved solely in those groups that were treated with  
20 antigen-loaded NP, as opposed to those treated with empty NP. This is an indication  
21 that the immune-mediated effect obtained with NP entrapping antigens resulted from  
22 the increased immunogenicity of those epitopes conferred by the adjuvant effect of  
23 our nano-vaccine.

24 The tumours collected from mice treated with the combination prophylactic  
25 man-NP MHCI-ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 had extensive infiltration of

1 CD8<sup>+</sup> T cells, a good prognosis marker for immunotherapy. We also observed  
2 increased caspase-3 levels in these tumours, correlating with the superior CTL  
3 activity observed *ex vivo*.

4 The remarkable results achieved with the combination of prophylactic  
5 mannosylated nano-vaccines (man-NP MHC I-ag/man-NP MHC II-ag) +  $\alpha$ PD-  
6 1/ $\alpha$ OX40 prompted the design of a therapeutic intervention strategy. Therapeutic  
7 mannosylated nano-vaccines (man-NP MHC I-ag/man-NP MHC II-ag), rather than  
8 prophylactic, increase the applicability of the strategy and the chances for translation  
9 to the clinic. Clinical studies combining antitumor cellular vaccines with immune  
10 checkpoint therapy have had promising outcomes in pancreatic cancer <sup>47</sup>, colorectal  
11 cancer <sup>48</sup>, prostate cancer <sup>49</sup> and melanoma <sup>50</sup>. Our therapeutic strategy could offer a  
12 clinical alternative to those therapies with autologous DC, which are associated with  
13 complex, long and expensive procedures <sup>51</sup>.

14 In the beginning of the treatment, the combination of mannosylated nano-  
15 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 clearly increased the tumour infiltration of CD8<sup>+</sup> T cells,  
16 limiting PD-1-mediated immunosuppression, essential for a CTL activity against  
17 melanoma cells. The treatment using these mannosylated nano-vaccines  
18 undoubtedly increased the expression of the co-stimulatory OX40, which was then  
19 available at higher extent to be targeted by the immune checkpoint therapy.

20 Nevertheless, the levels of CD8<sup>+</sup> T cells within the tumour tend to decrease  
21 with time, while there was an increasing infiltration of Treg cells. As a consequence,  
22 the CD8:Treg ratio was low, indicating an immunosuppressive TME, which limits the  
23 expected antitumor immunity. In parallel, the levels of tumour infiltrated MDSC  
24 increased significantly in the TME, hampering infiltration of CD8<sup>+</sup> T cells and CTL  
25 activity <sup>52,53</sup>. Our mannosylated therapeutic nano-vaccines seem to induce infiltration

1 of MDSC into the TME over time. The infiltration of MDSC hindered the early effect  
2 of CD8<sup>+</sup> T cell stimulation and proliferation, inhibiting T cell infiltration and CTL activity  
3 in the TME. This imbalance promoted an immunosuppressive TME, and the  
4 combination of mannosylated nano-vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 failed to show benefit  
5 in comparison with  $\alpha$ PD-1/ $\alpha$ OX40 alone.

6         Considering the high levels of MDSC, we hypothesized that the inhibition of  
7 these suppressor cells with ibrutinib, could remarkably improve the clinical outcomes  
8 of our strategy. Ibrutinib was shown to limit the generation and migration of MDSC,  
9 and was proposed as a strategy to enhance cancer immunotherapeutic strategies  
10 <sup>20,54</sup>.

11         Our findings demonstrate that the mannosylated nano-vaccines +  $\alpha$ PD-  
12 1/ $\alpha$ OX40 + ibrutinib induced remarkable tumour remission and/or tumour growth  
13 inhibition in two tumor-bearing mouse models, with long-term survival. The  $\alpha$ PD-  
14 1/ $\alpha$ OX40 + ibrutinib contributed to a homeostasis in the TME ideal for antitumor  
15 vaccination. By depleting MDSC, ibrutinib revealed the full potential of our  
16 mannosylated nano-vaccines in enhancing T cell responses at the TME, rendering  
17 them to be available for immune checkpoint therapy with  $\alpha$ PD-1 and  $\alpha$ OX40. The  
18 addition of ibrutinib also improved DC maturation and CD4<sup>+</sup> T cell proliferation,  
19 increasing the secretion of cytokines essential for the expansion of CD8<sup>+</sup> T cells, while  
20 trafficking into the TME.

21         Of note, this strong therapeutic synergism of our nano-vaccines with ibrutinib  
22 and  $\alpha$ PD-1/ $\alpha$ OX40 immune checkpoint therapy was only obtained when the  
23 mannose moieties were grafted on the surface of the nano-vaccines. These data  
24 prove the superior impact of mannosylation of our NP in the DC-mediated antigen

1 presentation and the activation of tumour-antigen specific T cells in the melanoma  
2 therapeutic setting.

3 Taken together, although each of these components had modest results as  
4 monotherapy, their combination resulted in robust and widespread complementary  
5 outcomes. These results provide a strong basis to devise novel regimens and  
6 combination therapies for solid tumours, unravelling the full potential of particulate  
7 mannosylated cancer nano-vaccines.

8

9

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10

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22

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6

## 7 **AUTHOR CONTRIBUTIONS**

8

9 R.S.F. and H.F. conceived and designed all experiments. (ongoing)

10 XXX and XXX. performed the experiments: G. H. analyzed the data: I. J. contributed  
11 materials/analysis tools: A. B. and E. F. co-wrote the paper.

12

13 The authors do not have any conflict of interests to declare.

14

15

16

## 17 **DATA AVAILABILITY**

18 The authors declare that the data supporting the findings of this study are available

19 within the paper and its Supplementary Information. Additional data and source files

20 are available from the corresponding authors (RSF, HF) upon reasonable request.

21

## 22 **ADDITIONAL INFORMATION**

23 “Supplementary information is available in the online version of the paper. Reprints

24 and permission information is available online at [www.nature.com/reprints](http://www.nature.com/reprints).

25 Correspondence and requests for materials should be addressed to RSF and HF.”

26

1 **FIGURE CAPTIONS**

2 **Fig. 1. NP and Man-NP are potential delivery systems for vaccination. A.**

3 Schematic representation of mannose-PLGA/PLA nanoparticles (man-NP). **B.** TEM

4 image of spherical man-NP. **C.** SEM image of spherical man-NP. **D.** AFM images of

5 spherical man-NP, showing narrow size polydispersity. **E.** Particle internalization by

6 DC determined by FACS. Non-treated cells and non-labeled NP were used as

7 negative controls. Data are presented as mean  $\pm$  SD,  $N = 4-6$ , from three independent

8 experiments performed in triplicate. **F.** Confocal images of DC after 3 hours of

9 incubation with NP (left) and man-NP (right). Z-stacks (top) and projections (bottom).

10 ( $N = 3$ ;  $n = 6$ ). Scale bars = 50  $\mu\text{m}$ . **G.** Percentage of NP internalization 17 hours after

11 immunization with empty NP or antigen-loaded NP. man-NP (TPGS) were

12 preferentially internalized by circulating DC in immunized C57BL/6J mice and

13 increased the expression of the activation and maturation markers of these APC. **H.**

14 Median fluorescence intensity (MFI) of activated DC that internalized NP, present in

15 the lymph nodes (LN), 17 hours after immunization. Mean  $\pm$  SD;  $N = 3$ ,  $n = 3$ , where

16  $N$  denotes the number of independent experiments and  $n$  denotes the number of

17 measurements per experiment. Statistics: Two-way ANOVA followed by Tukey Post-

18 Hoc test (**G.**) or Bonferroni test (**H.**)  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$ .

19

20 **Fig. 2. NP and man-NP vaccines induce splenocyte activation and *ex vivo***

21 **cytotoxicity against melanoma cells. A.** Non-invasive intravital fluorescence

22 imaging of C57BL/6J mouse 3 hours and 48 hours following hock immunization with

23 NP (left) and man-NP (right). **B.** Organ biodistribution according to fluorescence

24 signal ( $N = 4$ ) with NP and man-NP. Data represent mean  $\pm$  SD. **C.** Immunization

25 scheme of C57BL/6J mice and *ex vivo* splenocyte cytotoxic activity timeline. **D.**

1 Secretion of IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , IL-2, IL-6, CCL1/TCA-3, MIP-1 $\beta$ , MCP-5/CCL12  
2 and TARC/CCL17 upon re-stimulation of splenocytes in culture. **E.** Cytotoxic activity  
3 of splenocytes harvested from immunized C57BL/6J mice, after incubation with  
4 Melan-A/MART-1 and CD28 in solution for 6 days. Data are presented as mean  $\pm$   
5 SEM ( $N = 6$ ). \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ . **F.** Images of RMS cells co-cultured with  
6 reactivated splenocytes from the group immunized with NP MHCI-ag and MHCII-ag  
7 (top) and the group immunized with man-NP MHCI-ag/man-NP MHCII-ag (bottom).  
8 Cell death was detected with an apoptosis reagent that couples to activated caspase-  
9 3/7 recognition motif and quantifies apoptosis (in green).

10

11 **Fig. 3. Prophylactic nano-vaccines have synergistic effect with PD-1 blockade**  
12 **and OX40 activation, restricting melanoma growth and prolonging survival. A.**  
13 Timeline of immunization, tumor inoculation and immune checkpoint therapy. **B.**  
14 Tumor growth curve.  $P$  values correspond to tumor volume at day 17. Data are  
15 presented as mean  $\pm$  SEM ( $N=4-5$ ). **C.** Kaplan-Meier overall survival over time graph,  
16 for mice inoculated with  $4.5 \times 10^5$  RMS cells ( $N = 4-5$ ). **D.** Individual tumor volume at  
17 day 17 and **E.** Day 27 following tumor inoculation.

18

19 **Fig. 4. Low CD8<sup>+</sup>/Treg ratio and high infiltration of Myeloid-derived Suppressor**  
20 **Cells (CD11b<sup>+</sup>Gr-1<sup>+</sup>MDSC) compromise the therapeutic efficacy of the**  
21 **combination of mannosylated nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40. A.** Timeline of  
22 tumor inoculation and treatments. **B.** Tumor growth curve. Data are presented as  
23 mean  $\pm$  SEM ( $N = 7$ ).  $P$  values correspond to tumor volume at day 18 after tumor  
24 inoculation. **C.-H.** Tumor-infiltrating immune cell populations. Tumors were isolated  
25 on day 18 after tumor cell inoculation ( $N \geq 3$ ) and when the tumor volume for final

1 endpoint was reached. Quantification was performed by flow cytometry. Data are  
2 presented as mean  $\pm$  SD of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P <$   
3 0.001.

4

5 **Fig. 5. Trivalent combination of mannosylated nano-vaccines with ibrutinib and**  
6  **$\alpha$ PD-1/ $\alpha$ OX40 strongly restricts melanoma growth, leading to long-term**

7 **survival. A.** Timeline of tumor inoculation and treatments. **B.** Tumor growth curve.

8 Data are presented as mean  $\pm$  SEM ( $N = 7$ ).  $P$  values correspond to tumor volume at  
9 day 20 after tumor inoculation. **C.** Percent of EGSRNQDWL (gp100)-specific CD8<sup>+</sup> T

10 cells in the lymph nodes (LN). **D.** ELISpot representative images of IFN- $\gamma$  spot forming

11 cells among splenocytes after *ex vivo* re-stimulation with melan-A/MART-1 peptides

12 on day 22. **E.** Kaplan-Meier overall survival over time graph, for mice inoculated with

13  $3 \times 10^5$  RMS ( $N = 13-15$ ). **F.** Individual tumor volume at days 27, 36 and 43 after tumor

14 inoculation, with mean  $\pm$  SEM represented ( $N=13-15$ ). All data are presented as

15 mean of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

16

17 **Fig. 6. High CD8<sup>+</sup>/Treg ratio and inhibition of MDSC are associated with**  
18 **improved therapeutic efficacy. A.-H.** Tumor-infiltrating immune cell populations.

19 Tumors were isolated on the first endpoint, day 20 after tumor cell inoculation ( $N \geq$   
20 3), and at the tumor size-matched second endpoint: day 27 for PBS, ibrutinib only

21 and nano-vaccines + ibrutinib; day 35 for  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib and nano-  
22 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40. Quantification was performed by flow cytometry. Data are

23 presented as mean  $\pm$  SD of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P <$

24 0.001.

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**METHODS 3019 words**

**MATERIALS AND METHODS**

**Materials**

Poly(L-lactic acid) (PLA, 2000 Da) with an average molecular weight (Mw) of 2000 was purchased from PolySciences, Inc., UK. Tumour-associated peptides MHCII-restricted Melan-A:26-35(L27), ELAGIGILTV, (MHCII-ag), and MHCII-restricted Melan-A:51-73(RR-23), RNGYRALMDKSLHVGTVQCALTRR (MHCII-ag) were purchased to GeneCust Europe. Alternatively, the synthesis and purification of these Melan-A peptides was performed by the Blavatnik Center for Drug Discovery at the Tel Aviv University. The synthesis and purification of the gp100 peptides (MHCII-restricted gp100:25-33 (EGSRNQDWL) and MHCII-restricted gp100:44-59 (WNRQLYPEWTEAQRDL) was performed by the Blavatnik Center for Drug Discovery at Tel Aviv University. H-2D<sup>b</sup>-restricted EGSRNQDWL PE-labelled tetramer was supplied by Quimigen S.L. (Madrid, Spain). CpG ODN 1826 (TCCATGACGTTCCCTGACGTT) was purchased from InvivoGen (San Diego, CA, USA). MEM  $\alpha$ , nucleosides, ascorbic acid was purchased from Invitrogen. RPMI 1640, heat inactivated foetal bovine serum (FBS), trypsin EDTA 0.05%, penicillin (10,000 Unit/ml) and streptomycin (10,000  $\mu$ g/ml), sodium pyruvate (100 mM), GM-CSF recombinant mouse protein (5 ng/ml), HEPES buffer (1 M), ACK lysing buffer, paraformaldehyde (PFA) 4% (v/v), Wheat Germ Agglutinin (WGA), Alexa Fluor<sup>®</sup> 633 Conjugate, Hoechst<sup>®</sup> 33342 and AlamarBlue<sup>®</sup> reagent were purchased from Thermo Fisher Scientific. Proteome Profiler Mouse XL Cytokine Array Kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Matrigel Matrix (Cat. no. 356231) was

1 purchased from BD Biosciences - Discovery Labware, Erembodegem, Europe.  
2 IncuCyte® Caspase-3/7 Apoptosis Assay Reagent was acquired from Biological  
3 Industries Israel Beit-Haemek Ltd. PLGA (Resomer 503H; Mw 24 000 – 38 000),  
4 mannosamine·HCl, dimethylformamide, 4-dimethylaminopyridine, *N,N*-  
5 Dicyclohexylcarbodiimide, methanol, anhydrous sodium sulphate, Concanavalin A,  
6 FITC-labelled Concanavalin A from *Canavalia ensiformis* (Jack bean) Type IV  
7 lyophilized powder, Lipid A monophosphoryl from *Salmonella enterica* serotype  
8 minnesota Re 595 (Re mutant) (MPLA), bovine serum albumin, d- $\alpha$ -tocopherol  
9 polyethylene glycol 1000 succinate (TPGS), Poly(vinyl alcohol) (PVA) Mw 13,000 to  
10 23,000 Da, 99% hydrolysed, dichloromethane (DCM) and chloroform-d were  
11 purchased from Sigma Aldrich. PD-1 and OX40 monoclonal antibodies were acquired  
12 from Bio X cell. Rabbit monoclonal anti-caspase 3 was purchased from Epitomics  
13 (CA, USA). Antibodies anti-mouse CD4 (clone: CK1.5), CD8 $\alpha$  (clone: 53-6.7) and  
14 DAPI were acquired from BioLegend (San Diego, CA, USA).  
15 Streptavidin–horseradish peroxidase conjugate was purchased from Histostain, Life  
16 Technologies (CA, USA). Fluorochrome labelled antibodies for flow cytometry were  
17 purchased from Miltenyi Biotec, unless otherwise specified. ELISpot kit was  
18 purchased by R&D Systems Inc. (Minneapolis, USA).

19

## 20 **Synthesis and characterization of mannose-PLGA polymer**

21 Mannose-PLGA (man-PLGA) was synthesized from PLGA (Resomer 503H;  
22 Mw 24,000 – 38,000 Da). Carboxylic acid terminal groups of PLGA were modified  
23 with mannosamine under nitrogen atmosphere in mild conditions <sup>17</sup>. Briefly,  
24 Mannosamine·HCl (10.8 mg, 0.05 mmol, 3.5 eq.) and 4-dimethylaminopyridine (6.5

1 mg, 0.05 mmol, 4 eq.) were added to 4 ml of dimethylformamide. The mixture was  
2 stirred for 10 minutes at room temperature to achieve complete dissolution of  
3 mannosamine. PLGA (Resomer 503H; Mw 24 000 – 38 000) (400 mg, 0.0133 mmol,  
4 1 eq.) was added to the previous solution and stirred for 10 minutes at room  
5 temperature. *N,N*-Dicyclohexylcarbodiimide (5.5 mg, 0.027 mmol, 2 eq.) was added  
6 to induce the reaction between PLGA and mannosamine. The reaction was allowed  
7 to stir for 48 hours at room temperature under argon atmosphere. The polymer was  
8 precipitated with water and recovered by centrifugation. Man-PLGA was dissolved in  
9 DCM and dried over anhydrous sodium sulphate. The solution was filtered. DCM was  
10 evaporated through rotary evaporation. Methanol was added to precipitate the  
11 polymer and wash the reaction crude. Man-PLGA was dissolved in DCM and the  
12 precipitation was repeated. Finally, man-PLGA was dried under vacuum overnight  
13 and weighed after 24 hours (186.7 mg;  $\eta$  = 47%).

14

## 15 **Nuclear Magnetic resonance**

16 Details are described in the Supplementary Methods.

17

## 18 **Synthesis of NP and man-NP**

19 PLGA/PLA NP were formulated by the double emulsion-solvent evaporation  
20 (w/o/w) method <sup>18</sup>. PLGA/PLA (2:8) blend was dissolved in dichloromethane (DCM)  
21 at 50 mg/ml. MPLA (100  $\mu$ g) was added to DCM polymer solution. A 10% (m/v) PVA  
22 aqueous solution (100  $\mu$ l) containing CpG at 0.5 mg/ml and melan-A/MART-1 (26-  
23 35(A27L), melan-A/MART-1 (51-73), gp100 (25-33) or gp100 (44-59) at 5 mg/ml was  
24 added to DCM. For empty NP, 100  $\mu$ l of 10% PVA aqueous solution was added. The  
25 mixture was emulsified with a microprobe ultrasonic processor for 15 seconds at 20%

1 amplitude. TPGS aqueous solution 2.5% (m/v) (400  $\mu$ l) was added and the second  
2 emulsion was formed using the same conditions. The double emulsion was added  
3 dropwise into a 0.25% (m/v) PVA aqueous solution and stirred for 1 hour at room  
4 temperature. Particle suspension was collected by centrifugation at 20,000 g for 45  
5 minutes, 4°C (SORVALL® RC-5B PLUS Superspeed centrifuge). Particles were  
6 washed with ultrapure water, collected by centrifugation and finally resuspended in  
7 PBS or ultrapure water. Mannose-PLGA/PLA nanoparticles (man-NP) were prepared  
8 as previously described with man-PLGA/PLA (2:8) blend instead. Cy5.5-labeled NP  
9 and man-NP were synthesized by adding 0.5  $\mu$ g to the polymer blend.

10

#### 11 **Size Distribution and $\zeta$ -Potential Measurements**

12 Particle size was measured by Dynamic Light Scattering with Malvern Nano  
13 ZS (Malvern Instruments, UK). Z-average size was determined by cumulative  
14 analysis.  $\zeta$ -Potential of particles was measured by Laser Doppler Velocimetry in  
15 combination with Phase Analysis Light Scattering with the same equipment. Particles  
16 were diluted in ultrapure water and electrophoretic mobility was determined at 25°C  
17 with the Helmholtz-von Smoluchowski model.

18

#### 19 **Particle morphology**

20 Atomic Force Microscopy (AFM). Particles were diluted at 5 mg/ml in ultrapure  
21 water. A drop of sample was placed onto freshly cleaved mica for 20 minutes and  
22 dried with pure N<sub>2</sub>. Samples were analysed by AFM in tapping mode in air at room  
23 temperature, using a Nanoscope IIIa Multimode Atomic Force Microscope (Digital  
24 Instruments, Veeco), and etched silicon tips (ca. 300 kHz), at a scan rate of ca. 1.6  
25 Hz.

1 Scanning Electron Microscopy (SEM). Particles were diluted in trehalose 5%  
2 (m/v) and fast frozen at -80°C for 2 hours. Samples were dried under vacuum, first at  
3 -20°C for 14 hours and then at 20°C for 2 hours. Dried specimens were coated with  
4 gold on a Peltier-cold stage sputter-coater and examined using a FEI Quanta 200  
5 FEG ESEM Phillips 500 scanning electron microscope at 5 kV accelerating voltage.  
6 Transmission Electron Microscopy. Particles were diluted in PBS and placed on a  
7 carbon-coated copper grid and dried. Samples were analysed by Philips CM 120 Bio-  
8 Twin TEM.

9

## 10 **Entrapment efficiency and loading capacity of antigens and immune** 11 **potentiators**

12 Supernatants collected from centrifugations were used for indirect  
13 quantification of entrapped antigens and immune potentiators. Entrapment efficiency  
14 (EE % (m/m), Eq. (1)) and loading capacity (LC µg/mg, Eq. (2)) of melan-A/MART-1  
15 (26-35(A27L)) and melan-A/MART-1 (51-73) were determined with FAM-labelled  
16 melan-A/MART-1 (26-35(A27L)) and melan-A/MART-1 (51-73), respectively.  
17 Relative Fluorescence Units (RFU) were measured with SpectraMax M5e plate  
18 reader (Molecular Devices, CA, USA) at 498/518 nm, excitation/emission  
19 wavelengths. The amount of CpG in the supernatant was determined by the  
20 Oligreen® ssDNA quantitation kit <sup>15</sup>. RFU were measured using the fluorometer at  
21 485 nm excitation and 530 nm emission wavelengths.

22

$$23 \text{ Entrapment Efficiency (EE \%)} =$$
$$24 \frac{\text{initial amount of agent} - \text{amount of agent in the supernatant}}{\text{initial amount of agent}} \times 100 \text{ (1)}$$

25

1            *Loading Capacity (LC μg/mg) =*  
2            
$$\frac{\text{initial amount of agent} - \text{amount of agent in the supernatant}}{\text{total amount of polymer}} \quad (2)$$
  
3  
4

5    **Mannose detection on the particles' surface by the Lectin Recognition Assay**

6    Details are described in the Supplementary Methods.

7    **Cell lines**

8    Details are described in the Supplementary Methods.

9    ***In vitro* cell viability in the presence of NP or man-NP**

10   Details are described in the Supplementary Methods.

11   **Hemolysis assay**

12   Details are described in the Supplementary Methods.

13

14   ***In vitro* particle internalization by dendritic cells**

15            JAW SII DC ( $5 \times 10^4$  cells/well) were seeded in 96-well plates and incubated  
16   overnight. Cells were then incubated with rhodamine-grafted NP or man-NP (500  
17   μg/ml) for 4, 12 and 24 hours. Cells were then washed with DPBS and resuspended  
18   in flow cytometry buffer. Non-treated cells and non-labelled NP were used as negative  
19   controls. An excess of soluble mannose (5 mM) was added to the medium, as a  
20   control, in order to confirm its ability to compete with man-NP for CD206 at DC  
21   surface. The individual fluorescence JAW SII DC was collected for each sample using  
22   LSR Fortessa cytometer (BD Biosciences) by FACS Diva, and analysed with FlowJo  
23   software version 7.6.5 for Microsoft (TreeStar, San Carlos, CA).

24            The 8-well Ibidi® μ-Slide microscopy chambers were incubated with 300 μL  
25   fibronectin (10 μg/mL) per well during 30 min in a humidified incubator with 5% CO<sub>2</sub>,  
26   at 37°C. After discarding the volume of fibronectin, JAW SII cells ( $5 \times 10^4$  cells/well)

1 were seeded in 8-well Ibidi®  $\mu$ -Slide microscopy chambers for 6 hours in a humidified  
2 incubator with 5% CO<sub>2</sub>, at 37°C. Cells were incubated with rhodamine-grafted NP or  
3 man-NP (500  $\mu$ g/ml) for 3 hours. Non-treated cells and non-labelled NP were used  
4 as negative controls. Cells were washed and fixed at room temperature in 4%  
5 paraformaldehyde, containing Hoechst®332 at 2  $\mu$ g/mL and WGA Alexa Fluor 633 at  
6 5  $\mu$ g/mL to stain the nucleus and the cell membrane, respectively. Each well was  
7 washed three times with PBS and 100  $\mu$ L of fluoromount were added to each one.  
8 Particle internalization was observed by confocal microscopy using Zeiss LSM 710  
9 with 63x amplification in oil. Images were processed with ImageJ software. Three-  
10 dimensional (3D) projection images were obtained from 0.4  $\mu$ m Z-stacks and  
11 processed using the Leica Application Suite-Advanced Fluorescence (LAS-AF)  
12 software.

13

#### 14 **Animal studies**

15 All animal procedures were performed in compliance with Tel Aviv University,  
16 Sackler Faculty of Medicine guidelines and with the Portuguese competent authority  
17 for animal protection, Direcção Geral de Alimentação e Veterinária, Lisbon, Portugal.  
18 The protocols were approved by the institutional animal care and use committee  
19 (IACUC) and performed in accordance with NIH guidelines.

20 Male C57BL/6J mice (8 weeks old) were purchased from Envigo LTD  
21 (Jerusalem, Israel) or Charles River (Écully, France), and housed in the animal facility  
22 of Tel Aviv University or at the Faculty of Pharmacy, University of Lisbon. The number  
23 of animals in each group was determined according to previous studies (26, 58).

24 Mice body weight change was monitored 3 times per week. Mice were  
25 euthanized according to ethical protocol when showing signs of distress or with rapid

1 weight loss (above 10% within a few days or 20% from the initial weight). Tumour-  
2 bearing mice were euthanized in case the tumour size exceeded 2000 mm<sup>3</sup> or if the  
3 tumour was necrotic or ulcerative. Mice were perfused intracardially with PBS,  
4 immediately after euthanasia, tumours were dissected and incubated in 4% PFA and  
5 collected for histology.

6

### 7 ***In vivo* study of man-NP internalization by DC in draining lymph nodes**

8 Male C57BL/6, 8 weeks old mice (n = 3 / group) were injected into the right  
9 flank by the s.c. hock immunization with one of the Cy5.5 fluorescently-labelled plain  
10 and antigen-loaded NP formulations (2 mg of NP/mouse). Left non-injected flank  
11 served as negative control. All formulations contained MART-1 antigens and TLR  
12 ligands. Popliteal and inguinal lymph nodes were harvested 16 h post-immunization  
13 (p.i.). A single-cell suspension was stained with fluorescent-labelled anti-mouse  
14 antibodies against CD11c, MHCII (I-Ab), MHCI (H-2Kb), CD80 and CD86, for 20 min  
15 at 4°C. Samples were acquired with an LSR II Fortessa flow cytometer (BD  
16 Bioscience) and analysed with FlowJo software (Treestar).

17

### 18 **Immunization of animals with tumour-associated antigens**

19 For immunization studies, 8 weeks old C57BL/6J male mice were randomized  
20 into 8 groups (N = 6) (**Supplementary Table 1**).

21 Treatments (100 µl) were injected into each mouse by hock immunization, via  
22 s.c. injection proximal to popliteal LN. Half dose was injected into the right side and  
23 the other half into the left side. When the two peptide antigens melan-A/MART-1 (26-  
24 35(A27L)) and melan-A/MART-1 (51-73) were administered to the same mouse  
25 (groups 4, 6, and 8), 25 µl of each treatment was administered on each side. Each

1 dose contained 100 µg of antigen (50 µg of melan-A/MART-1 (26-35(A27L)) and 50  
2 µg of melan-A/MART-1 (51-73), when two antigens were used), plus 20 µg of CpG  
3 and 20 µg of MPLA, either free in solution or entrapped in 2 mg of particles (20 mg/ml).

4

#### 5 **Tumour antigen specific-proliferation of splenocytes from immunized mice**

6 Splenocytes from whole spleens of treated C57BL/6J mice (*N* = 6) were  
7 harvested and seeded in sterile 60 mm petri dishes, 10 days after the last  
8 immunization. Splenocytes were seeded for 6 days in complete RPMI medium, in the  
9 presence of melan-A/MART-1 (26-35(A27L)) or melan-A/MART-1 (26-35(A27L)) +  
10 melan-A/MART-1 (51-73) (0.1 mg/ml) (for groups 4, 6, and 8) and CD28 (2 µg/ml).

11

#### 12 **Splenocyte cytokine and chemokine secretion from immunized mice**

13 A membrane-based sandwich immunoassay was performed according to the  
14 manufacturer protocol of Proteome Profiler Mouse Cytokine Array Panel A Kit  
15 (catalogue number: R&D-ARY0068; R&D Systems Inc., Minneapolis, USA).  
16 Splenocyte's culture media from each group (**Supplementary Table 1**) were pooled  
17 (*n* = 6) and concentrated in concentration tubes (Amicon). Each sample was applied  
18 on a nitrocellulose membrane containing capture antibodies that bind to specific  
19 target protein. After overnight incubation at 4°C, streptavidin-HRP and  
20 chemiluminescent reagents were added, and incubation steps were performed  
21 according to the protocol provided by the analysis kit. Membranes were exposed to  
22 X-rays for 10 minutes. Results were detected by a transmission-mode scanner  
23 proportionally to the quantity of analyte and determined by protein array analyser  
24 software.

25

## 1 **Ex vivo immune cell killing assay**

2 Immune cell cytotoxic activity was assessed using IncuCyte® ZOOM live-cell  
3 instrument. RMS cells ( $6 \times 10^3$  cells/well) were seeded in 96-well plates in complete  
4 RPMI without phenol red, on the day before the addition of splenocytes. Pre-  
5 stimulated splenocytes were added to RMS cells in a 1:100 ratio. IncuCyte™  
6 Caspase-3/7 Apoptosis Assay Reagent was diluted in RPMI without phenol red and  
7 added to each well to final concentration of 5  $\mu$ M. Data was collected every 2 hours  
8 for 12 hours. Triton X-100 0.5% (v/v) and cell culture medium were used as positive  
9 and negative controls, respectively.

10

## 11 **Tumour inoculation, combination therapies, and tumour volume** 12 **measurements**

13 Male C57BL/6J mice (8 weeks old) were randomized into different groups ( $N$   
14 = 4-5) according to **Supplementary Table 2** for the prophylactic combination study.  
15 Different schedules were used for the prophylactic (**Fig. 3A**) and intervention  
16 combinatorial studies without or with the addition of ibrutinib (**Fig. 4A** and **Fig. 5A**,  
17 respectively). For the prophylactic study, on day 0, 50  $\mu$ l of cell suspension containing  
18  $4.5 \times 10^5$  murine RMS cells mixed with growth-factor reduced matrigel (1:1) were  
19 inoculated subdermally on the right dorsal region as reported before <sup>55</sup>. For the  
20 intervention studies, the amount of murine RMS and Ret cells that were inoculated  
21 on day 0 was  $3 \times 10^5$  instead. For the intervention study on the second melanoma  
22 model, 100  $\mu$ l of saline with  $1.5 \times 10^5$  B16-F10 cells in suspension were inoculated  
23 subdermally in the right flank. For both tumour models, mice were anesthetized with  
24 ketamine (100 mg/kg) and xylazine (12 mg/kg). The right dorsal area was treated with  
25 depilatory cream before the injection.  $\alpha$ PD-1 and  $\alpha$ OX40 were administered

1 intraperitoneally at 10 mg/kg. Ibrutinib was also administered intraperitoneally at 6  
2 mg/kg. Tumour size was measured every 3 days with a calliper. Tumour volume was  
3 determined by  $X^2 \cdot Y \cdot 0.5$  ( $X$  – small diameter;  $Y$  – large diameter) and body weight  
4 was monitored twice a week.

5

## 6 **Immunohistochemistry**

7 Details are described in the Supplementary Methods.

8

## 9 **Flow cytometric analysis of tumour-infiltrated immune populations**

10 RMS tumours were isolated from the animals directly after euthanasia. Tumour  
11 single-cell suspensions were obtained by mechanical disruption of the tissues and  
12 enzymatic digestion in RPMI medium with 0.5% BSA, 0.1% collagenase type II  
13 (LS004177, Worthington), 0.1% dispase (LS02109, Worthington) and DNase  
14 (LS002007, Worthington) for 1 hour at 37°C. After digestion, the suspension was  
15 filtered through a 70 µm filter (BD Biosciences) to remove the debris. For the RMS  
16 cells, the obtained single-cell suspension was then stained with fluorochrome labelled  
17 antibodies and analysed using an LSR Fortessa (BD Biosciences) and FlowJo  
18 software (Tree Star Inc.). Intracellular staining was performed with the Inside stain kit  
19 (Miltenyi Biotec, Cat.# 130-090-477), according to the manufacturer's protocol.  
20 Details of the antibodies used in each panel are in Supplementary information.

21

## 22 **Functional assessment of T-cells**

23 For the ELISpot assay, on day 0, 50 µl of cell suspension containing  $4.5 \times 10^5$   
24 murine RMS cells were inoculated subdermally. Starting on day 7, mice were treated  
25 with two weekly doses as reported in **Supplementary Table 3**.

1           On day 21, mice were euthanised and spleens harvested and splenocytes  
2 isolated. Splenocytes were seeded at  $2 \times 10^5$  cells per well in 96-well plates coated  
3 with IFN- $\gamma$  antibody (R&D Systems Inc.) and incubated for 20 hours with 1 mg/mL of  
4 Melan-A/MART-1 MHC-I-ag peptide. The secreted and captured IFN- $\gamma$  was  
5 subsequently detected using a biotinylated antibody specific for IFN- $\gamma$  and an  
6 alkaline-phosphatase conjugated to streptavidin. Following the addition of substrate  
7 solution, a blue coloured precipitate forms and appears as spots at the sites of  
8 cytokine localization. Automated spot quantification was performed using a UV  
9 ImmunoSpot<sup>®</sup> S6 Ultra-V.

10           For the tetramer staining assay, male C57BL/6J, 8-week-old mice were s.c.  
11 immunized into both right and left inguinal area, 2 times, once a week, with 100  $\mu$ L of  
12 man-NP or NP (100  $\mu$ g of gp 100 antigens/20  $\mu$ g of CpG/20  $\mu$ g of MPLA per mouse;  
13 50  $\mu$ L in each side), free gp 100 antigens with adjuvants CpG and MPLA, or PBS.  
14 Inguinal LN were harvested 10 days after the 2<sup>nd</sup> injection, homogenized in a single-  
15 cell suspension, and plated in 96-well plate for staining. First, the peptide-MHC  
16 tetramer tagged with PE (H-2D<sup>b</sup> – restricted EGSRNQDWL PE-labelled tetramer  
17 (Quimigen S.L., Madrid, Spain)) was added to the single cell suspension, including  
18 FcR blocking, following supplier instructions. After 30 min of incubation at 4°C, the  
19 cells were washed to remove unbound tetramers, and centrifuged at 1300 rpm, for  
20 10 min, 10°C. Cells were resuspended in ice-cold sorter buffer and plated in 96-well  
21 plates. After adding a mix of the antibodies CD3-APC-Cy7 and CD8 $\alpha$ -PE-Cy7, cells  
22 were incubated for 15 min, at 4°C protected from light. Cells were washed,  
23 centrifuged and resuspended in 200  $\mu$ l of ice-cold sorter buffer to determine the  
24 percentages of tumour antigen-specific CD3<sup>+</sup> CD8 $\alpha$ <sup>+</sup> T cells by FACS.

25

1 **Statistical Methods**

2 Data are presented as mean  $\pm$  standard deviation (SD) for *in vitro* assays and  
3 as mean  $\pm$  standard error of the mean (SEM) for *ex vivo* and *in vivo* assays. Statistical  
4 analyses were performed with Student's t-test and one-way analysis of variance  
5 (ANOVA), followed by Bonferroni post hoc test for comparison of multiple groups with  
6 IBM SPSS® Statistics (Version 21, Microsoft). Statistical significance in overall  
7 survival was determined with log-rank test using SigmaPlot software (Systat Software  
8 Inc.) and GraphPad Prism 5® or 7® (GraphPad Software, Inc., La Jolla, CA)  $P < 0.05$   
9 was considered statistically significant.

10  
11  
12

1

2 **Table 1. Particle size, polydispersity index (Pdl) and  $\zeta$ -Potential**

Particles <sup>d</sup>	Size <sup>a</sup> (nm $\pm$ SD <sup>b</sup> )	Pdl <sup>c</sup> $\pm$ SD <sup>b</sup>	$\zeta$ -Potential (mV $\pm$ SD <sup>b</sup> )
NP (empty)	168 $\pm$ 10	0.15 $\pm$ 0.05	-2.17 $\pm$ 0.40
NP MHCI-ag	178 $\pm$ 6	0.16 $\pm$ 0.03	-3.11 $\pm$ 0.50
NP MHCII-ag	170 $\pm$ 5	0.18 $\pm$ 0.03	-2.34 $\pm$ 0.65
man-NP (empty)	169 $\pm$ 16	0.13 $\pm$ 0.05	-2.11 $\pm$ 0.40
man-NP MHCI-ag	181 $\pm$ 8	0.15 $\pm$ 0.04	-3.02 $\pm$ 0.46
man-NP MHCII-ag	166 $\pm$ 5	0.18 $\pm$ 0.04	-1.72 $\pm$ 0.47

3 <sup>a</sup> Z-average hydrodynamic diameter.4 <sup>b</sup> SD, standard deviation.5 <sup>c</sup> Pdl, polydispersity index.6 <sup>d</sup> CpG and MPLA were entrapped in all NP and man-NP, with exception of empty nanoparticulate  
7 systems.8 For each formulation, we prepared 5 batches ( $N = 5$ ) and measured in triplicate ( $n = 3$ ).

9

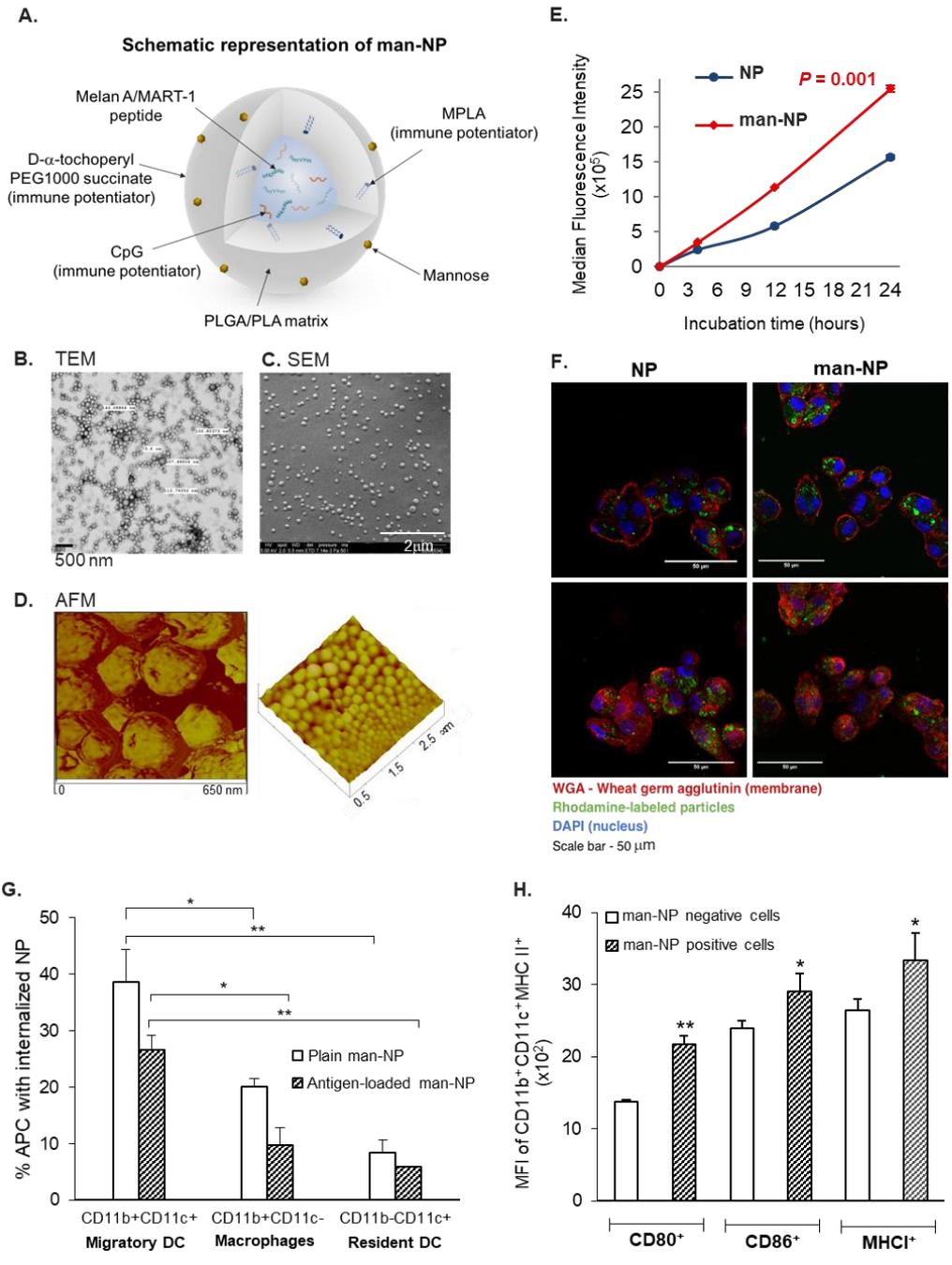
10 **Table 2. Entrapment Efficiency (EE) and Loading Capacity (LC) of antigens in NP**  
11 **and man-NP**

Particles	EE (% $\pm$ SD <sup>a</sup> )	LC ( $\mu$ g/mg $\pm$ SD <sup>a</sup> )
NP MHCI-ag	99.1 $\pm$ 0.1	49.6 $\pm$ 0.05
NP MHCII-ag	82.4 $\pm$ 0.6	41.2 $\pm$ 0.3
man-NP MHCI-ag	97.5 $\pm$ 0.2	48.8 $\pm$ 0.1
man-NP MHCII-ag	74.6 $\pm$ 3.5	37.3 $\pm$ 1.7

12 <sup>a</sup> SD, standard deviation.13 For each formulation, we prepared 4 batches ( $N = 4$ ) and measured in triplicate ( $n = 3$ ).

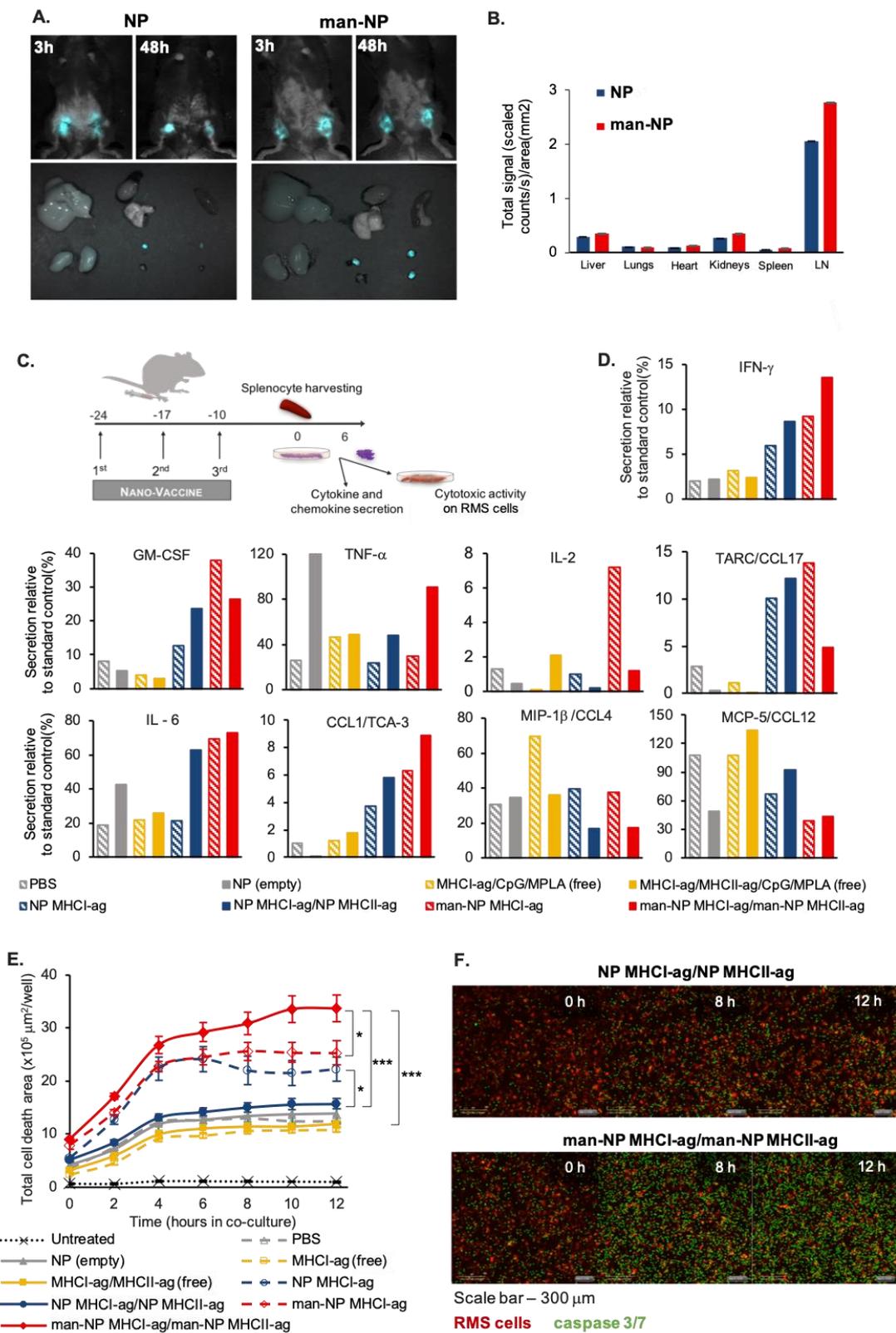
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15



1  
2 **Fig. 1. NP and Man-NP are potential delivery systems for vaccination. A.**  
3 **Schematic representation of mannose-PLGA/PLA nanoparticles (man-NP). B.**  
4 **TEM image of spherical man-NP. C. SEM image of spherical man-NP. D. AFM images of**  
5 **spherical man-NP, showing narrow size polydispersity. E. Particle internalization by**  
6 **DC determined by FACS. Non-treated cells and non-labelled NP were used as**  
7 **negative controls. Data are presented as mean  $\pm$  SD,  $N = 4-6$ , from three independent**

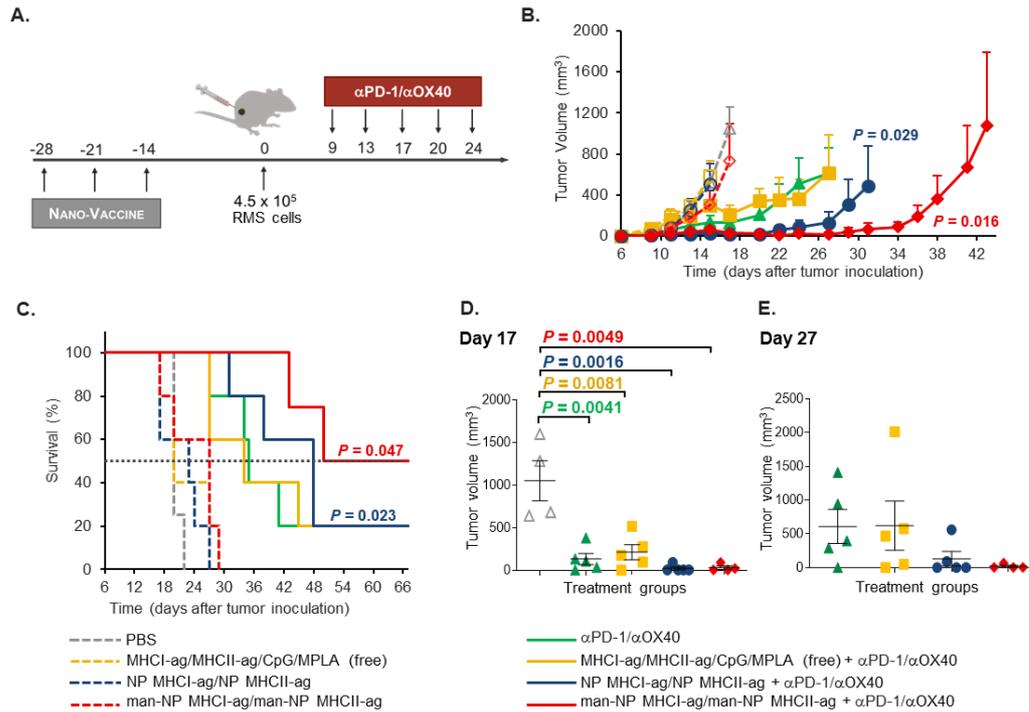
1 experiments performed in triplicate. **F.** Confocal images of DC after 3 hours of  
2 incubation with NP (left) and man-NP (right). Z-stacks (top) and projections (bottom).  
3 ( $N = 3$ ;  $n = 6$ ). Scale bars = 50  $\mu\text{m}$ . **G.** Percentage of NP internalization 17 hours after  
4 immunization with empty NP or antigen-loaded NP. man-NP (TPGS) were  
5 preferentially internalized by circulating DC in immunized C57BL/6J mice and  
6 increased the expression of the activation and maturation markers of these APC. **H.**  
7 Median fluorescence intensity (MFI) of activated DC that internalized NP, present in  
8 the lymph nodes (LN), 17 hours after immunization. Mean  $\pm$  SD;  $N = 3$ ,  $n = 3$ , where  
9  $N$  denotes the number of independent experiments and  $n$  denotes the number of  
10 measurements per experiment. Statistics: Two-way ANOVA followed by Tukey Post-  
11 Hoc test (**G.**) or Bonferroni test (**H.**)  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$ .  
12



1

2 **Fig. 2. NP and man-NP vaccines induce splenocyte activation and ex vivo**  
 3 **cytotoxicity against melanoma cells. A. Non-invasive intravital fluorescence**

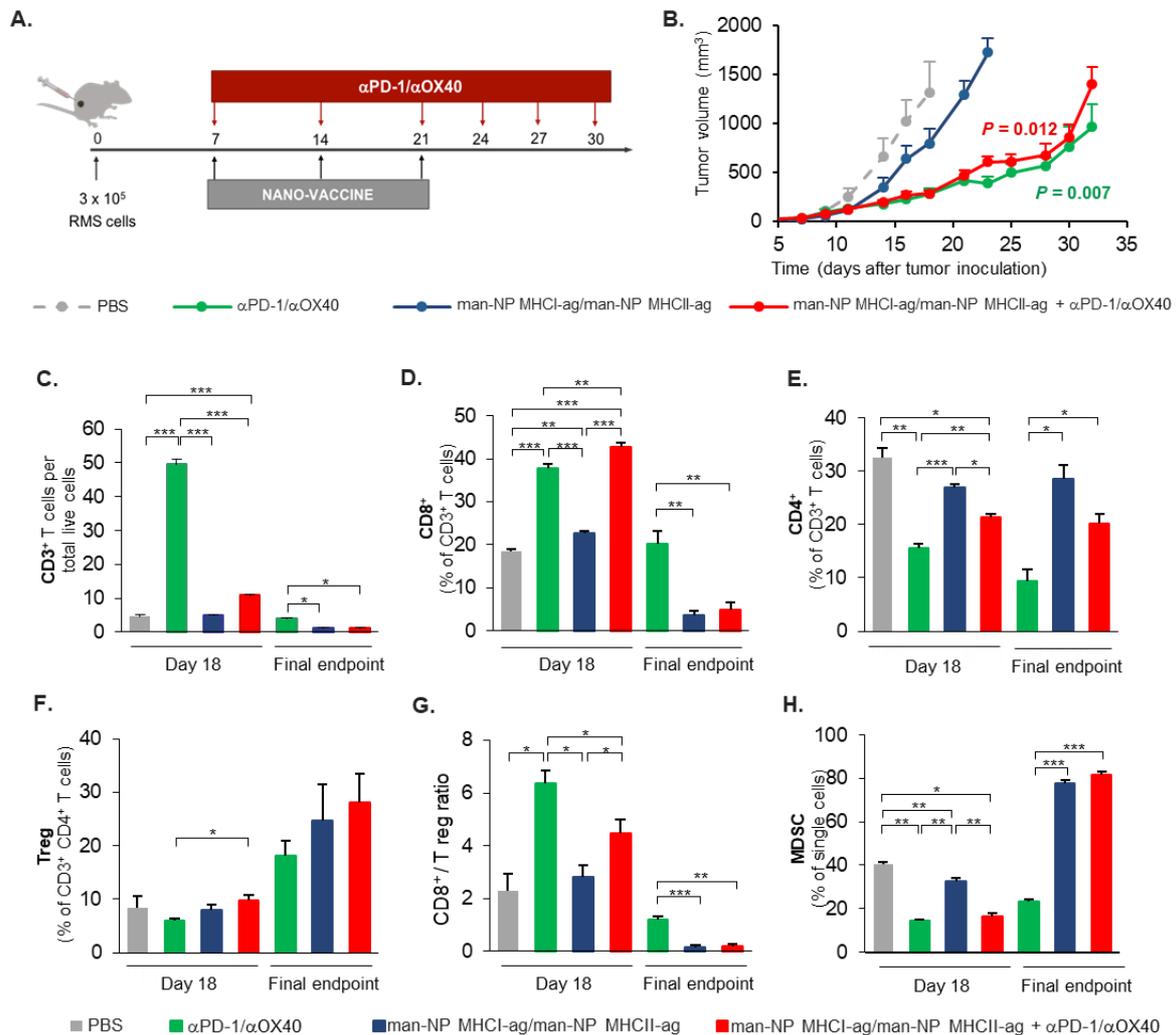
1 imaging of C57BL/6J mouse 3 hours and 48 hours following hock immunization with  
2 NP (left) and man-NP (right). **B.** Organ biodistribution according to fluorescence  
3 signal ( $N = 4$ ) with NP and man-NP. Data represent mean  $\pm$  SD. **C.** Immunization  
4 scheme of C57BL/6J mice and *ex vivo* splenocyte cytotoxic activity timeline. **D.**  
5 Secretion of IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , IL-2, IL-6, CCL1/TCA-3, MIP-1 $\beta$ , MCP-5/CCL12  
6 and TARC/CCL17 upon re-stimulation of splenocytes in culture. **E.** Cytotoxic activity  
7 of splenocytes harvested from immunized C57BL/6J mice, after incubation with  
8 Melan-A/MART-1 and CD28 in solution for 6 days. Data are presented as mean  $\pm$   
9 SEM ( $N = 6$ ). \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ . **F.** Images of RMS cells co-cultured with  
10 reactivated splenocytes from the group immunized with NP MHCI-ag and MHCII-ag  
11 (top) and the group immunized with man-NP MHCI-ag/man-NP MHCII-ag (bottom).  
12 Cell death was detected with an apoptosis reagent that couples to activated caspase-  
13 3/7 recognition motif and quantifies apoptosis (in green).  
14



1  
2 **Fig. 3. Prophylactic nano-vaccines have synergistic effect with PD-1 blockade**  
3 **and OX40 activation, restricting melanoma growth and prolonging survival. A.**  
4 **Timeline of immunization, tumour inoculation and immune checkpoint therapy. B.**  
5 **Tumour growth curve. *P* values correspond to tumour volume at day 17. Data are**  
6 **presented as mean  $\pm$  SEM (*N*=4-5). C. Kaplan-Meier overall survival over time graph,**  
7 **for mice inoculated with  $4.5 \times 10^5$  RMS cells (*N* = 4-5). D. Individual tumour volume**  
8 **at day 17 and E. Day 27 following tumour inoculation.**

9

1 endpoint and, therefore, corroborates higher tumour growth rate.

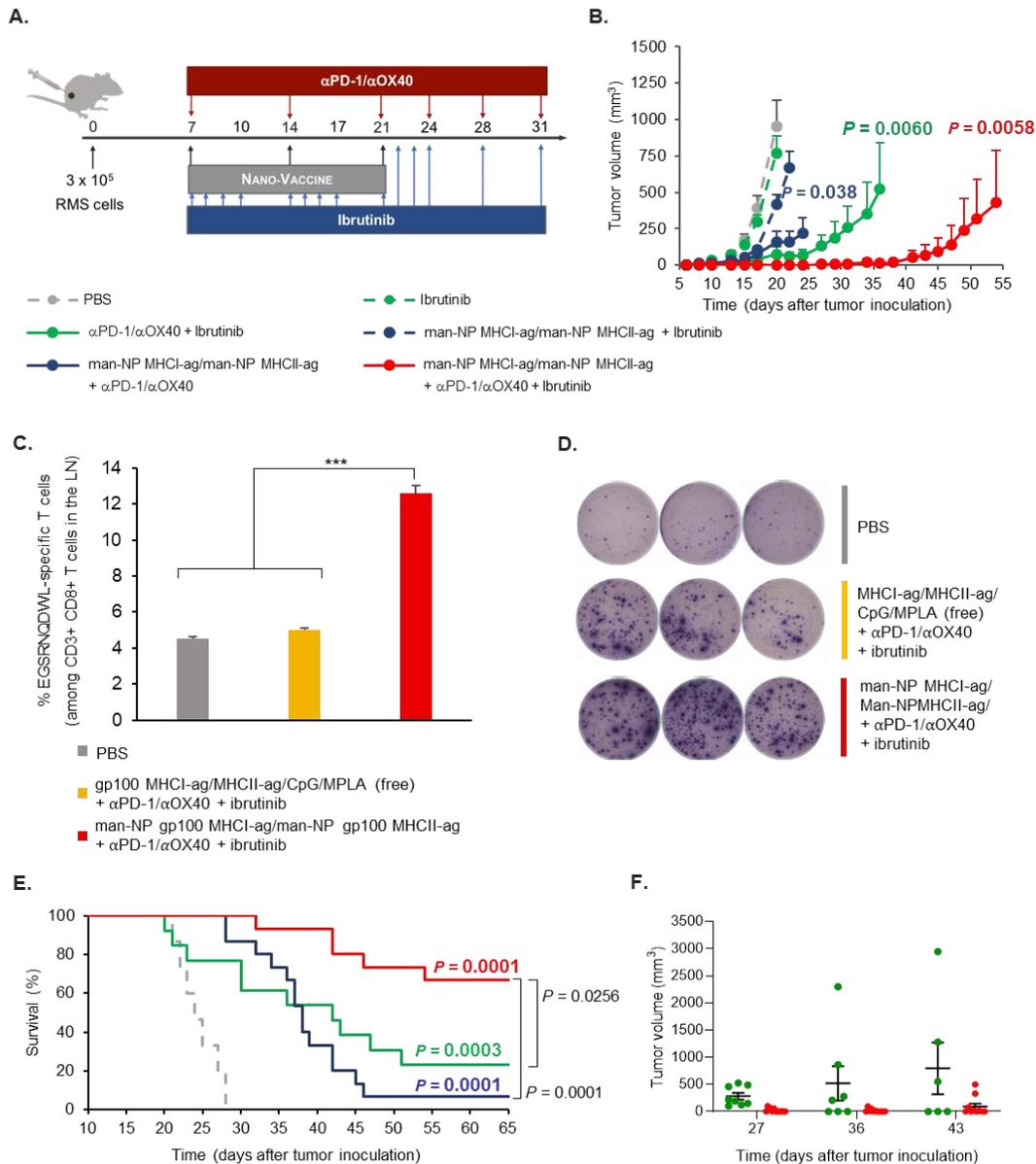


2 **Fig. 4. Low CD8<sup>+</sup>/Treg ratio and high infiltration of Myeloid-derived Suppressor**  
 3 **Cells (CD11b<sup>+</sup>Gr-1<sup>+</sup>MDSC) compromise the therapeutic efficacy of the**  
 4 **combination of mannosylated nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40. A.** Timeline of  
 5 tumor inoculation and treatments. **B.** Tumour growth curve. Data are presented as  
 6 mean  $\pm$  SEM ( $N = 7$ ).  $P$  values correspond to tumour volume at day 18 after tumour  
 7 inoculation. **C.-H.** Tumour-infiltrating immune cell populations. Tumours were isolated  
 8 on day 18 after tumour cell inoculation ( $N \geq 3$ ) and when the tumour volume for final  
 9 endpoint was reached. Quantification was performed by flow cytometry. Data are

1 presented as mean  $\pm$  SD of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P <$

2 0.001.

3



1

2

**Fig. 5. Trivalent combination of mannosylated nano-vaccines with**

3

**ibrutinib and αPD-1/αOX40 strongly restricts melanoma growth, leading to**

4

**long-term survival. A.** Timeline of tumour inoculation and treatments. **B.** Tumour

5

growth curve. Data are presented as mean ± SEM ( $N = 7$ ).  $P$  values correspond to

6

tumour volume at day 20 after tumour inoculation. **C.** Percent of EGSRNQDWL

7

(gp100)-specific CD8<sup>+</sup> T cells in the lymph nodes (LN). **D.** ELISpot representative

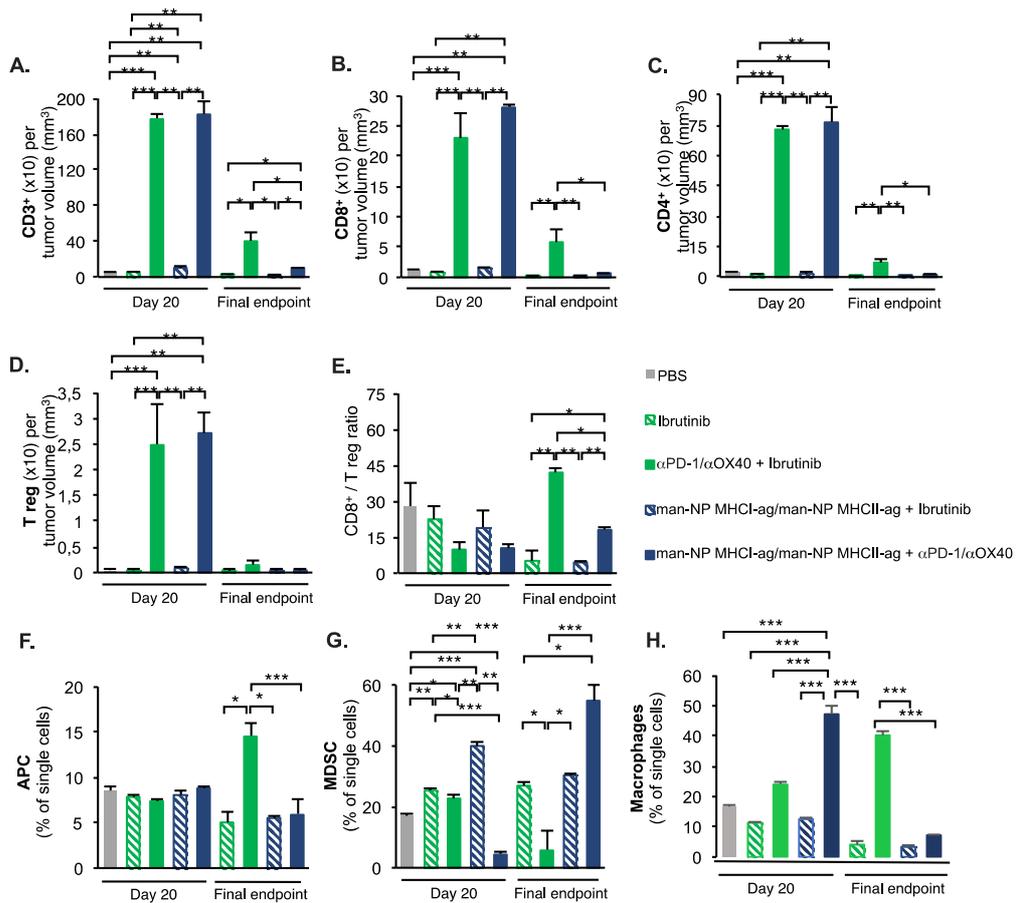
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images of IFN- $\gamma$  spot forming cells among splenocytes after *ex vivo* re-stimulation with

9

melan-A/MART-1 peptides on day 22. **E.** Kaplan-Meier overall survival over time

1 graph, for mice inoculated with  $3 \times 10^5$  RMS ( $N = 13-15$ ). **F.** Individual tumour volume  
2 at days 27, 36 and 43 after tumour inoculation, with mean  $\pm$  SEM represented ( $N=13-$   
3 15). All data are presented as mean of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P <$   
4 0.01; \*\*\*  $P < 0.001$ .  
5



1

2 **Fig. 6. High CD8<sup>+</sup>/Treg ratio and inhibition of MDSC are associated with**

3 **improved therapeutic efficacy. A.-H. Tumour-infiltrating immune cell populations.**

4 Tumours were isolated on the first endpoint, day 20 after tumour cell inoculation ( $N \geq$

5 3), and at the tumour size-matched second endpoint: day 27 for PBS, ibrutinib only

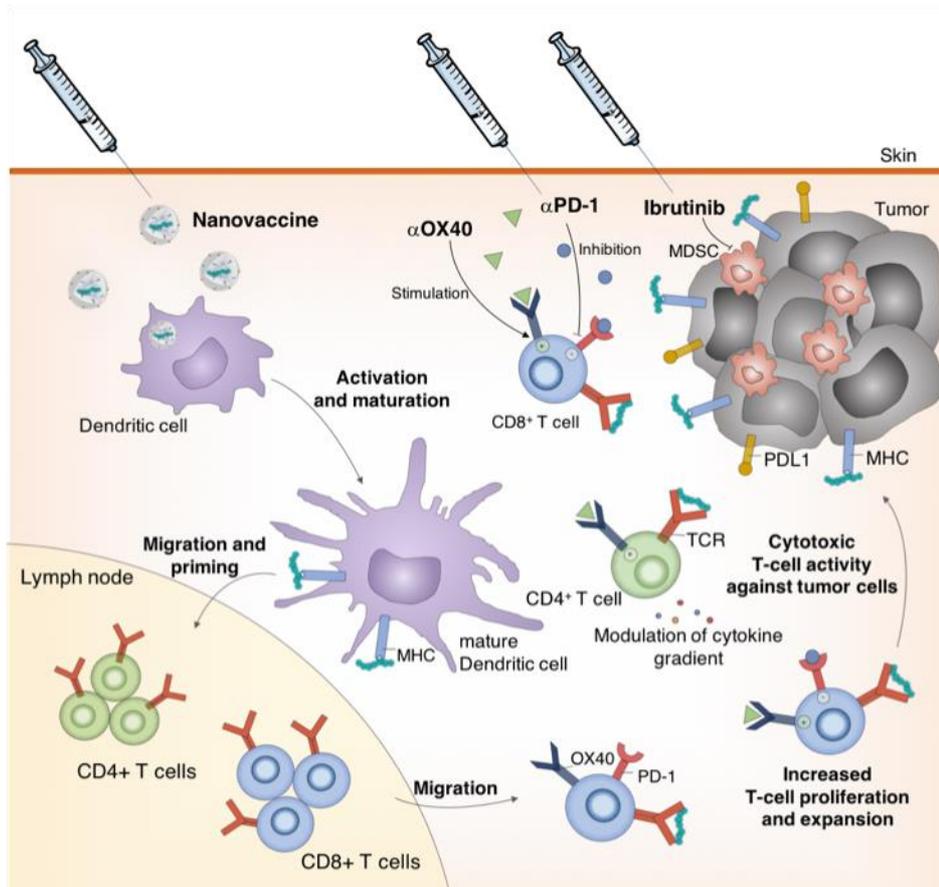
6 and nano-vaccines + ibrutinib; day 35 for  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib and nano-

7 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40. Quantification was performed by flow cytometry. Data are

8 presented as mean  $\pm$  SD of 3 independent replicates. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P <$

9 0.001.

10



1

2 **Fig. 7. Proposed model for the trivalent therapeutic strategy combining**  
 3 **mannosylated nano-vaccines with ibrutinib and  $\alpha$ PD-1/ $\alpha$ OX40.**

4