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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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This is the author copy of the original published in *Nat. Nanotechnol.* **14**, 891–901 (2019) 1 2 (https://doi.org/10.1038/s41565-019-0512-0 https://www.nature.com/articles/s41565-019-0512-0 Ibrutinib sensitizes melanoma to immune checkpoint modulators following 3 4 immunization with nano-vaccines by relieving immune-suppressing 5 microenvironment **One Sentence Summary:** 6 7 Combination of dendritic cell-targeted nano-vaccines with a myeloid-derived suppressor cell inhibitor and immune checkpoint modulators expands the host 8 antitumor immune cells, restricts tumour growth and prolongs survival in an orthotopic 9 10 melanoma model. João Conniot,<sup>1,2¥</sup> Anna Scomparin,<sup>1,3¥</sup> Carina Peres,<sup>2</sup> Eilam Yeini,<sup>1</sup> Sabina Pozzi,<sup>1</sup> 11 Ana I Matos,<sup>2</sup> Ron Kleiner,<sup>1</sup> Liane IF Moura,<sup>2</sup> Eva Zupančič,<sup>2,4</sup> Ana S Viana,<sup>5</sup> Hila 12 Doron,<sup>6</sup> Pedro MP Gois,<sup>2</sup> Neta Erez,<sup>6</sup> Steffen Jung,<sup>4</sup> Ronit Satchi-Fainaro,<sup>1\*</sup> Helena 13 14 F Florindo<sup>2\*</sup> 15 16 <sup>1</sup>Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel 17 <sup>2</sup> Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, 18 19 Universidade de Lisboa, Lisbon, Portugal 20 <sup>3</sup> Department of Drug Science and Technology, University of Turin, Turin, Italy 21 <sup>4</sup> Department of Immunology, Weizmann Institute of Science, Rehovot, Israel 22 <sup>5</sup> Center of Chemistry and Biochemistry, Faculty of Sciences, Universidade de Lisboa, Lisbon, Portugal 23 24 <sup>6</sup> Department of Pathology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel 25 26 <sup>\*</sup>These authors equally contributed to this work 27 \*Corresponding authors: 28 29 30 Helena F Florindo, hflorindo@ff.ulisboa.pt Ronit Satchi-Fainaro, ronitsf@tauex.tau.ac.il 31

1 Keywords: vaccine, melanoma, ibrutinib, MDSC inhibitor, PD-1, OX40,

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 cancer nano-vaccines with anti-PD-1 for immunosuppression blockade, and anti-6 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 mannosvlated nano-vaccines with anti-PD-1/anti-OX40 (αPD-10 1/αOX40) demonstrate synergism, stimulating T cell infiltration into tumours at early treatment stages. However, treatment at the therapeutic regimen does not result in 11 12 enhanced inhibition of tumor growth compared to  $\alpha PD-1/\alpha OX40$  alone and is accompanied by increased infiltration of myeloid-derived suppressor cells (MDSC) in 13 tumours. Combining the double therapy with ibrutinib, an MDSC-inhibitor, leads to 14 remarkable tumour remission and prolonged survival in treated melanoma-bearing 15 mice. The synergy between the mannosylated nano-vaccines, ibrutinib and aPD-16 17  $1/\alpha OX40$  provides essential insights to devise alternative regimens to improve the efficacy of immune checkpoint modulators in solid tumours by regulating the 18 19 endogenous immune response.

1 2

# INTRODUCTION

3 Advances in immunotherapy have improved the treatment outcomes of melanoma, based on antitumor immune responses elicited by the patient's own 4 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 cellmediated antitumor immunity in clinical practice <sup>1,2</sup>. Since these findings, efforts are 7 being made not only to inhibit negative immune checkpoints but also to stimulate 8 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 proinflammatory cytokine secretion <sup>3,4</sup>. OX40-signaling can also block the inhibitory 13 14 activity of tumour-infiltrating regulatory CD4<sup>+</sup> T cells, by hindering Foxp3, TGF-β, and IL-10 signalling, known to suppress vaccine-induced immune responses <sup>5-7</sup>. 15

16 Despite the promising clinical results, immune checkpoint therapy has been 17 associated with low percentages of effective and durable responses to single agent therapies, due to resistance or relapse <sup>8-10</sup>. The presence of tumour infiltrating 18 lymphocytes (TIL) has been correlated with favourable outcomes of immune 19 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 delivering tumour-associated antigens to dendritic cells (DC) and consequent T-cell 22 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.

In order to modulate the generation and function of myeloid-derived 1 2 suppressor cells (MDSC), we further administered ibrutinib, an irreversible inhibitor of Bruton's tyrosine kinase (BTK). Under cancer-associated inflammation, an 3 4 aberrant myelopoiesis leads to the accumulation of MDSC, compromising the 5 efficient maturation of DC, affecting the antigen presentation process and driving T cell transcriptional programs towards T cell anergy and exhaustion. The presence of 6 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 improve the nanoparticle interactions with DC by targeting the mannose receptor 12 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 acids) melan-A/MART-1 peptides, aiming for the major histocompatibility complex 17 (MHC) class I and class II antigen presentation pathways, respectively. Melan-18 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 both CD4<sup>+</sup> and CD8<sup>+</sup> T cells <sup>15</sup>. 22

Our results demonstrate amplified antitumor immune response mediated by (i)
 targeting DC through vaccination as an active immunotherapy, (ii) inhibiting MDSC,
 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**

To deliver tumour-associated antigens to DC, we developed two different types 6 of biodegradable nanoparticles: PLGA/PLA NP (NP) and mannose-PLGA/PLA NP 7 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 mannosamine <sup>16</sup>. The reaction was confirmed by <sup>1</sup>H NMR of mannose-PLGA 10 11 (Supplementary Fig. 1) compared with the individual <sup>1</sup>H NMR spectra of mannosamine and PLGA. The multiplet signal between 3.2 and 3.8 ppm indicates 12 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** 1), with a low polydispersity index  $(0.13 \pm 0.04 \text{ to } 0.18 \pm 0.04)$ . Electron microscopy 17 and atomic force microscopy showed homogenous spherical particles, with slight 18 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 Assay, using Concanavalin A (Conc A) (Supplementary Fig. 2A). Conc A binds to 21 exposed mannose residues, forming aggregates <sup>15,19</sup>. After incubation of man-NP, 22 Conc A induced particle aggregation, revealed by a 5-fold increase in average size 23 24 (Supplementary Fig. 2B). Particle aggregation (in green) was also confirmed by 25 fluorescence microscopy following incubation of fluorescein isothiocyanate (FITC)-

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

NP and man-NP showed high levels of entrapment efficiency (EE) and loading capacity (LC) (**Table 2**). For the MHCI-ag, EE >97.5  $\pm$  0.2% and LC >48.8  $\pm$  0.1 µg/mg, while for the MHCII-ag EE >74.6  $\pm$  3.5% and LC >37.3  $\pm$  1.7 µg/mg. The EE of CpG was above 80.8  $\pm$  2.5%, which corresponds to LC of 8.1  $\pm$  0.3 µg/mg.

NP and man-NP did not affect DC viability (>90%) in the concentration range
tested (Supplementary Fig. 3A), supporting their physiological biocompatibility. This
was also assessed *ex vivo* by red blood cell (RBC) lysis determination. NP and manNP did not disrupt RBC membranes at concentrations up to 20 mg/ml
(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 also confirmed by confocal microscopy, corroborating the FACS results (Fig. 1F). 18 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 with PVA, including those NP functionalized with mannose (Supplementary Fig. 4). 21 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.

We show that our man-NP (TPGS) was preferentially taken-up *in vivo* by circulating DC (gated as CD11b<sup>+</sup>CD11c<sup>+</sup>), compared to macrophages (CD11b<sup>+</sup>CD11c<sup>-</sup>) and resident DC (CD11b<sup>-</sup>CD11c<sup>+</sup>) (**Fig. 1G, Supplementary Fig.** 5). In addition, our nano-vaccine was able to significantly increase the expression of 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 responses, we performed a 3-time immunization, seven days apart, to C57BL/6J 16 mice. Ten days after the last immunization, splenocytes were harvested and re-17 activated in cell culture for 6 days with anti-CD28 and MHCI-ag alone, or in 18 combination with MHCII-ag (Fig. 2C). The groups treated with NP and man-NP 19 showed an increased secretion of inflammatory cytokines, such as IFN-y, a Th1 20 cytokine, and GM-CSF. The highest levels of IFN-y and GM-CSF were induced by 21 man-NP. Elevated levels of CCL1/TCA-3 and TARC/CCL17 also suggested that our 22 particles played a major role in priming antigen-specific T cells (Fig. 2D). There was 23 24 no evidence of body weight change in all groups, attesting to vaccine tolerability and 25 safety (Supplementary Fig. 6).

Analysis of Th2 cytokines revealed that the administration of NP MHCI-ag/NP
MHCII-ag and man-NP MHCI-ag/man-NP MHCII-ag decreased MIP-1β/CCL4
secretion, whereas both man-NP MHCI-ag and the combination man-NP MHCIag/man-NP MHCII-ag equally reduced the MCP-5/CCL12 levels. These results
indicate the role of nanoparticulate vaccines in the modulation of Th2 chemokine
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 MHCIIag exhibited the highest ex vivo cytotoxicity when co-cultured with mCherry-labelled 11 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 nonmannosylated NP co-entrapping both antigens (P = 0.02), and dramatically stronger 16 than NP MHCI-ag/MHCII-ag ( $P = 9.6 \times 10^{-6}$ ). MHCI-ag/MHCII-ag/CpG/MPLA (free 17 antigen/immune potentiators) or empty particles induced a CTL activity similar to PBS 18 19 (Fig. 2E).

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

Prophylactic mannosylated nano-vaccines synergize with anti-PD-1/anti-OX40
 (αPD-1/αOX40) treatment, restricting melanoma growth and prolonging
 survival

Given the *in vitro* and *ex vivo* findings, we next asked whether the immune response elicited by the mannosylated nano-vaccines would synergize with the PDblockade and OX40 activation for melanoma therapy. To test this hypothesis, mice were treated according to the schedule in **Fig. 3A**. When man-NP MHCI-ag/man-NP MHCII-ag was combined with  $\alpha$ PD-1/ $\alpha$ OX40, a remarkable tumor growth inhibition was obtained (**Fig. 3B-E, Supplementary Fig. 8**), with minimal systemic toxicity (**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 MHCI-ag/NP MHCII-ag) or mannosylated nano-vaccines (man-NP MHCI-ag/man-NP MHCII-ag) 13 14 with  $\alpha$ PD-1/ $\alpha$ OX40 was much lower compared to animals treated with PBS (P < 0.0016) (Fig. 3D). At day 27, the tumour volume intra-group variability increased for 15 16 all the groups, except for the one treated with the combination of man-NP MHCI-17 ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40, in which the tumours remained small (Fig. **3E.** Supplementary Fig. 8). Empty NP, with and without mannose were used as 18 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 MHCI-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**).

Immunohistochemistry staining revealed high levels of caspase-3 and tumourinfiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in all groups immunized with nano-vaccines (**Supplementary Fig. 9**). The highest level of CD8<sup>+</sup> T cell infiltration was observed in groups treated with mannosylated nano-vaccines, alone or in combination with  $\alpha$ PD-1/ $\alpha$ OX40, and non-mannosylated nano-vaccines alone, suggesting that indeed the nano-vaccines are capable of reactivating cell-mediated cytotoxicity in the TME (**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 nano-vaccines with  $\alpha$ PD-1/ $\alpha$ OX40, we hypothesized that the same antitumor 14 immune-mediated effect could be achieved by treating melanoma-bearing mice at a 15 therapeutic regimen. Therefore, man-NP MHCI-ag/man-NP MHCII-ag were 16 17 combined with  $\alpha$ PD-1/ $\alpha$ OX40 according to the dosing schedule described in Fig. 4A and tested in Ret melanoma cells and mCherry-labelled Ret melanoma (RMS) cells. 18 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-NP MHCI-ag/man-NP MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib, was also similar 22 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

significantly smaller compared to the PBS-treated (P < 0.0012) (Fig. 4B), with</li>
 negligible systemic toxicity observed (Supplementary Fig. 11).

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

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

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

3

# 4 Trivalent combination of mannosylated nano-vaccines with ibrutinib and αPD-

# 5 1/αOX40 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>.

Melanoma-bearing mice were treated according to the schedule in Fig. 5A. On 12 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 nanovaccines +  $\alpha$ PD-1/ $\alpha$ OX40 or  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, however, the tumour volume 16 was significantly reduced by more than 5-folds (P = 0.038 and P = 0.0060, 17 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 onwards (P = 0.005), demonstrating the crucial role of the mannose targeting 20 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 average tumour volume of 54 mm<sup>3</sup>, in contrast to the NP-treated group, which had an 23 average tumour volume of 638 mm<sup>3</sup>. 24

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 response was irrespective of the antigen used (gp100 or melan-A/MART-1) as shown 3 4 by a tetramer staining assay (Fig. 5C, Supplementary Fig. 15C-D). Melan-A/MART-1 specificity was further confirmed by the IFN-γ ELISpot assay (Supplementary Fig. 5 **15E).** The highest levels of antigen-specific intracellular IFN-y<sup>+</sup> stained in tumour-6 7 infiltrating cytotoxic CD8<sup>+</sup> T lymphocytes of RMS-bearing mice measured by FACS, at the 1<sup>st</sup> endpoint, were obtained for animals treated with the mannosylated nano-8 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<sup>3</sup> (**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 of the group  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib (**Fig. 5E**). These animals treated with  $\alpha$ PD-18 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 curves of the triple regimen are statistically different from those obtained for PBS (P 24 25 = 0.0001), man-NP +  $\alpha$ PD-1/ $\alpha$ OX-40 (*P* = 0.0001) and  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib (*P* 

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

4 We determined two endpoints to analyse immune cell infiltrates within the TME. The first endpoint, day 20 after tumour inoculation, corresponds to the day of 5 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.

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

IHC staining of RMS tumour sections for CD4 and CD8 confirmed the flow
 cytometry results, showing a prominent infiltration of CD8<sup>+</sup> T cells at day 20 after
 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 MHCI-ag/man-NP 3 MHCII-ag + ibrutinib and man-NP MHCI-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 MHCI-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 MHCI-ag/man-NP MHCII-ag + ibrutinib, man-NP MHCI-ag/man-NP 10 MHCII-ag +  $\alpha$ PD-1/ $\alpha$ OX40 and  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, although only man-NP 11 MHCI-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**).

In groups treated with  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib, and mannosylated nanovaccines + ibrutinib, we observed a decrease in MDSC infiltrates from the first to the second endpoint. This suggests that ibrutinib was restricting the migration of MDSC 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-

vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 combination *versus* 70% (**Supplementary Fig. 18A**) and 57% (**Supplementary Fig. 18B**) for the  $\alpha$ PD-1/ $\alpha$ OX-40 + ibrutinib combination. The proposed mechanism was confirmed in this second melanoma model by the reduction of infiltrating MDSC and the increase of infiltrating T cells in the TME (**Supplementary Fig. 19**). We selected Day 19 for comparison as this is the last day 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>.

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

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These nano-vaccines, as an active immunotherapeutic strategy, have the unique ability to expand host antitumor specific T effector phenotype, improving sensitivity and long-term tumour recognition. The synergistic effect between  $\alpha$ PD-1/ $\alpha$ OX40, already reported in an ovarian cancer model <sup>23</sup>, could be relevant for melanoma <sup>24</sup> and be potentiated by cancer vaccines delivering tumour-associated 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, PdI, 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 adaptive immune responses and vaccine efficacy <sup>25</sup>. The mean average diameter of 12 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 residues on the surface of man-NP, demonstrated by particle aggregation induced by 17 Conc A, increased particle affinity to MR/CD206 of DC, improving particle 18 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.

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

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

Recently, the TLR synergy has been reported <sup>27</sup> showing that their activation 6 on DC is crucial to induce tumour-specific T cell responses <sup>28</sup>. The role of TLR4 7 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+ 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 previously reported that the intra-tumour administration of CpG, in combination with 17 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.

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

mice treated with nano-vaccines. IFN- $\gamma$  and GM-CSF are associated with enhanced antigen priming and subsequent presentation by antigen-presenting cells (APC) <sup>34,35</sup>, as well as cytotoxic T cell activity. IFN- $\gamma$  is extensively produced by CD8<sup>+</sup> T cells and it is a predictor of antigen-specific cytotoxic T cell-mediated responses <sup>36</sup>. The increased activation of CD8<sup>+</sup> T cells in mice treated with nano-vaccines is also supported by the increased secretion of CCL1/TCA-3 and TARC/CCL17, which are 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 cells <sup>39,40</sup>. The suppression of these cells is important for eliciting CTL cell activity 12 13 triggered by our nano-vaccines. Furthermore, the decreased secretion of MIP-14 1B/CCL4 and CCL12, as detected in the splenocytes from animals treated with our nano-vaccines, has also been associated with the inhibition of Treg cells, reduction 15 of tumour angiogenesis and enhanced antitumor efficacy <sup>41,42</sup>. 16

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

enhanced antitumor immunity *in vivo*, when compared to treatment with man-NP
 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 reported for cancer vaccines, due to tumour aggressiveness and the 5 immunosuppressive TME <sup>46</sup>. The combination of prophylactic man-NP MHCI-ag/man-6 NP MHCII-ag with  $\alpha$ PD-1/ $\alpha$ OX40, however, highlights the synergistic antitumor effect 7 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.

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

The tumours collected from mice treated with the combination prophylactic 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 mannosysted nano-vaccines (man-NP MHCI-ag/man-NP MHCII-ag) +  $\alpha$ PD-5 6  $1/\alpha$ OX40 prompted the design of a therapeutic intervention strategy. Therapeutic 7 mannosylated nano-vaccines (man-NP MHCI-ag/man-NP MHCII-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 checkpoint therapy have had promising outcomes in pancreatic cancer <sup>47</sup>, colorectal 10 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>.

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

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

of MDSC into the TME over time. The infiltration of MDSC hindered the early effect of CD8<sup>+</sup> T cell stimulation and proliferation, inhibiting T cell infiltration and CTL activity in the TME. This imbalance promoted an immunosuppressive TME, and the combination of mannosylated nano-vaccines +  $\alpha$ PD-1/ $\alpha$ OX40 failed to show benefit 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 inhibition in two tumor-bearing mouse models, with long-term survival. The  $\alpha$ PD-13 14  $1/\alpha OX40$  + ibrutinib contributed to a homeostasis in the TME ideal for antitumor vaccination. By depleting MDSC, ibrutinib revealed the full potential of our 15 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 addition of ibrutinib also improved DC maturation and CD4<sup>+</sup> T cell proliferation, 18 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 outcomes. These results provide a strong basis to devise novel regimens and 5 6 combination therapies for solid tumours, unravelling the full potential of particulate 7 mannosylated cancer nano-vaccines. 8 9 References 10 11 Topalian, S. L. et al. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in 1 12 Cancer. New England Journal of Medicine 366, 2443-2454, doi:doi:10.1056/NEJMoa1200690 13 (2012). 14 Hodi, F. S. et al. Improved survival with ipilimumab in patients with metastatic 2 15 melanoma. N Engl J Med 363, 711-723, doi:10.1056/NEJMoa1003466 (2010). 16 Gramaglia, I. et al. The OX40 costimulatory receptor determines the development of 3 17 CD4 memory by regulating primary clonal expansion. J Immunol 165, 3043-3050 (2000). 18 4 Arch, R. H. & Thompson, C. B. 4-1BB and Ox40 are members of a tumor necrosis factor 19 (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and 20 activate nuclear factor kappaB. Mol Cell Biol 18, 558-565 (1998). 21 5 Aspeslagh, S. et al. Rationale for anti-OX40 cancer immunotherapy. Eur J Cancer 52, 22 50-66, doi:10.1016/j.ejca.2015.08.021 (2016). 23 Sarff, M. et al. OX40 (CD134) expression in sentinel lymph nodes correlates with 6 24 prognostic features of primary melanomas. Am J Surg 195, 621-625; discussion 625, 25 doi:10.1016/j.amjsurg.2007.12.036 (2008). Vetto, J. T. et al. Presence of the T-cell activation marker OX-40 on tumor infiltrating 26 7 27 lymphocytes and draining lymph node cells from patients with melanoma and head and neck 28 cancers. Am J Surg 174, 258-265 (1997). 29 Spranger, S. et al. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-8 30 L1, or IDO blockade involves restored IL-2 production and proliferation of CD8+ T cells 31 directly within the tumor microenvironment. Journal for ImmunoTherapy of Cancer 2, 3, 32 doi:10.1186/2051-1426-2-3 (2014). 33 Gajewski, T. F. The Next Hurdle in Cancer Immunotherapy: Overcoming the Non-T-9 34 Cell-Inflamed Tumor Microenvironment. Semin Oncol 42, 663-671, 35 doi:10.1053/j.seminoncol.2015.05.011 (2015). Minn, A. J. & Wherry, E. J. Combination Cancer Therapies with Immune Checkpoint 36 10 37 Convergence on Interferon Signaling. Cell 165, 272-275, Blockade: 38 doi:10.1016/j.cell.2016.03.031 (2016). 39 11 Tumeh, P. C. et al. PD-1 blockade induces responses by inhibiting adaptive immune 40 resistance. Nature 515, 568-571, doi:10.1038/nature13954 (2014).

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6	
7	AUTHOR CONTRIBUTIONS
8	
9	R.S.F. and H.F. conceived and designed all experiments. (ongoing)
10 11 12	XXX and XXX. performed the experiments: G. H. analyzed the data: I. J. contributed materials/analysis tools: A. B. and E. F. co-wrote the paper.
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 <u>www.nature.com/reprints</u> .
25	Correspondence and requests for materials should be addressed to RSF and HF."
26	

#### 1 FIGURE CAPTIONS

Fig. 1. NP and Man-NP are potential delivery systems for vaccination. A. 2 Schematic representation of mannose-PLGA/PLA nanoparticles (man-NP). B. TEM 3 4 image of spherical man-NP. C. SEM image of spherical man-NP. D. AFM images of spherical man-NP, showing narrow size polydispersity. E. Particle internalization by 5 DC determined by FACS. Non-treated cells and non-labeled NP were used as 6 negative controls. Data are presented as mean  $\pm$  SD, N = 4-6, from three independent 7 8 experiments performed in triplicate. F. Confocal images of DC after 3 hours of incubation with NP (left) and man-NP (right). Z-stacks (top) and projections (bottom). 9 10 (N = 3; n = 6). Scale bars = 50  $\mu$ m. **G.** Percentage of NP internalization 17 hours after immunization with empty NP or antigen-loaded NP. man-NP (TPGS) were 11 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 florescence intensity (MFI) of activated DC that internalized NP, present in the lymph nodes (LN), 17 hours after immunization. Mean  $\pm$  SD; N = 3, n = 3, where 15 N denotes the number of independent experiments and n denotes the number of 16 measurements per experiment. Statistics: Two-way ANOVA followed by Tukey Post-17 Hoc test (G.) or Bonferroni test (H.) \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001. 18

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Fig. 2. NP and man-NP vaccines induce splenocyte activation and ex vivo cytotoxicity against melanoma cells. A. Non-invasive intravital fluorescence imaging of C57BL/6J mouse 3 hours and 48 hours following hock immunization with NP (left) and man-NP (right). B. Organ biodistribution according to fluorescence signal (N = 4) with NP and man-NP. Data represent mean ± SD. C. Immunization scheme of C57BL/6J mice and *ex vivo* splenocyte cytotoxic activity timeline. D.

1 Secretion of IFN-γ, GM-CSF, TNF-α, IL-2, IL-6, CCL1/TCA-3, MIP-1β, MCP-5/CCL12 and TARC/CCL17 upon re-stimulation of splenocytes in culture. E. Cytotoxic activity 2 3 of splenocytes harvested from immunized C57BL/6J mice, after incubation with Melan-A/MART-1 and CD28 in solution for 6 days. Data are presented as mean  $\pm$ 4 SEM (N = 6). \* P < 0.05; \*\*\* P < 0.001. F. Images of RMS cells co-cultured with 5 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

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

18

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

endpoint was reached. Quantification was performed by flow cytometry. Data are
 presented as mean ± SD of 3 independent replicates. \* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* <</li>
 0.001.

4

Fig. 5. Trivalent combination of mannosylated nano-vaccines with ibrutinib and 5  $\alpha$ PD-1/ $\alpha$ OX40 strongly restricts melanoma growth, leading to long-term 6 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 day 20 after tumor inoculation. C. Percent of EGSRNQDWL (gp100)-specific CD8<sup>+</sup> T 9 10 cells in the lymph nodes (LN). **D.** ELISpot representative images of IFN- $\gamma$  spot forming cells among splenocytes after ex vivo re-stimulation with melan-A/MART-1 peptides 11 12 on day 22. E. Kaplan-Meier overall survival over time graph, for mice inoculated with  $3 \times 10^5$  RMS (*N* = 13-15). **F.** Individual tumor volume at days 27, 36 and 43 after tumor 13 inoculation, with mean ± SEM represented (N=13-15). All data are presented as 14 mean of 3 independent replicates. \* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* < 0.001. 15

16

Fig. 6. High CD8<sup>+</sup>/Treg ratio and inhibition of MDSC are associated with 17 18 improved therapeutic efficacy. A.-H. Tumor-infiltrating immune cell populations. Tumors were isolated on the first endpoint, day 20 after tumor cell inoculation ( $N \ge$ 19 3), and at the tumor size-matched second endpoint: day 27 for PBS, ibrutinib only 20 and nano-vaccines + ibrutinib; day 35 for  $\alpha$ PD-1/ $\alpha$ OX40 + ibrutinib and nano-21 vaccines +  $\alpha$ PD-1/ $\alpha$ OX40. Quantification was performed by flow cytometry. Data are 22 presented as mean ± SD of 3 independent replicates. \* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* < 23 0.001. 24

1

# 2 METHODS 3019 words

#### **3 MATERIALS AND METHODS**

4

#### 5 Materials

6 Poly(L-lactic acid) (PLA, 2000 Da) with an average molecular weight (Mw) of 2000 was purchased from PolySciences, Inc., UK. Tumour-associated peptides 7 8 MHCI-restricted Melan-A:26-35(L27), ELAGIGILTV, (MHCI-aq), and MHCII-9 restricted Melan-A:51-73(RR-23), RNGYRALMDKSLHVGTQCALTRR (MHCII-ag) 10 were purchased to GeneCust Europe. Alternatively, the synthesis and purification of 11 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 12 13 (MHCI-restricted gp100:25-33 (EGSRNQDWL) and MHCII-restricted gp100:44-59 14 (WNRQLYPEWTEAQRLD) was performed by the Blavatnik Center for Drug 15 Discovery at Tel Aviv University. H-2Db-restricted EGSRNQDWL PE-labelled 16 tetramer was supplied by Quimigen S.L. (Madrid, Spain). CpG ODN 1826 (TCCATGACGTTCCTGACGTT) was purchased from InvivoGen (San Diego, CA, 17 USA). MEM  $\alpha$ , nucleosides, ascorbic acid was purchased from Invitrogen. RPMI 18 19 1640, heat inactivated foetal bovine serum (FBS), trypsin EDTA 0.05%, penicillin (10,000 Unit/ml) and streptomycin (10,000 µg/ml), sodium pyruvate (100 mM), GM-20 21 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 22 Conjugate, Hoechst® 33342 and AlamarBlue<sup>®</sup> reagent were purchased from Thermo 23 24 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 25

purchased from BD Biosciences - Discovery Labware, Erembodegem, Europe.
 IncuCyte<sup>®</sup> Caspase-3/7 Apoptosis Assay Reagent was acquired from Biological
 Industries Israel Beit-Haemek Ltd. PLGA (Resomer 503H; Mw 24 000 – 38 000),
 mannosamine · HCI, dimtehylformamide, 4-dimethylaminopyridine , *N*,*N*-

5 Dicyclohexylcarbodiimide, methanol, anhydrous sodium sulphate, Concanavalin A, 6 FITC-labelled Concanavalin A from Canavalia ensiformis (Jack bean) Type IV lyophilized powder, Lipid A monophosphoryl from Salmonella enterica serotype 7 minnesota Re 595 (Re mutant) (MPLA), bovine serum albumin, d-α-tocopherol 8 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 from Bio X cell. Rabbit monoclonal anti-caspase 3 was purchased from Epitomics 12 (CA, USA). Antibodies anti-mouse CD4 (clone: CK1.5), CD8a (clone: 53-6.7) and 13 14 DAPI acquired from BioLegend (San CA, were Diego, 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 Biotech, unless otherwise specified. ELISpot kit was purchased by R&D Systems Inc. (Minneapolis, USA). 18

19

# 20 Synthesis and characterization of mannose-PLGA polymer

Mannose-PLGA (man-PLGA) was synthesized from PLGA (Resomer 503H;
Mw 24,000 – 38,000 Da). Carboxylic acid terminal groups of PLGA were modified
with mannosamine under nitrogen atmosphere in mild conditions <sup>17</sup>. Briefly,
Mannosamine •HCI (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 mannosamine. PLGA (Resomer 503H; Mw 24 000 - 38 000) (400 mg, 0.0133 mmol, 3 4 1 eq.) was added to the previous solution and stirred for 10 minutes at room temperature. N,N-Dicyclohexylcarbodiimide (5.5 mg, 0.027 mmol, 2 eq.) was added 5 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 and weighed after 24 hours (186.7 mg;  $\eta = 47\%$ ). 13

14

#### 15 Nuclear Magnetic resonance

16 Details are described in the Supplementary Methods.

17

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

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

amplitude. TPGS aqueous solution 2.5% (m/v) (400 µl) was added and the second 1 2 emulsion was formed using the same conditions. The double emulsion was added dropwise into a 0.25% (m/v) PVA aqueous solution and stirred for 1 hour at room 3 temperature. Particle suspension was collected by centrifugation at 20,000 g for 45 4 minutes, 4°C (SORVALL<sup>®</sup> RC-5B PLUS Superspeed centrifuge). Particles were 5 washed with ultrapure water, collected by centrifugation and finally resuspended in 6 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

# **Size Distribution and** *ζ***-Potential Measurements**

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

18

# **19** Particle morphology

Atomic Force Microscopy (AFM). Particles were diluted at 5 mg/ml in ultrapure water. A drop of sample was placed onto freshly cleaved mica for 20 minutes and dried with pure N<sub>2</sub>. Samples were analysed by AFM in tapping mode in air at room temperature, using a Nanoscope IIIa Multimode Atomic Force Microscope (Digital Instruments, Veeco), and etched silicon tips (ca. 300 kHz), at a scan rate of ca. 1.6 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 -20°C for 14 hours and then at 20°C for 2 hours. Dried specimens were coated with 3 4 gold on a Peltier-cold stage sputter-coater and examined using a FEI Quanta 200 FEG ESEM Phillips 500 scanning electron microscope at 5 kV accelerating voltage. 5 Transmission Electron Microscopy. Particles were diluted in PBS and placed on a 6 7 carbon-coated copper grid and dried. Samples were analysed by Philips CM 120 Bio-8 Twin TEM.

9

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

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

- 22
- 23

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

1	Loading Capacity (LC $\mu g/mg$ ) =
2	initial amount of agent - amount of agent in the supernatant (2)
3	total amount of polymer
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 (5x10 <sup>4</sup> 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	$\mu$ g/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® $\mu\text{-Slide}$ microscopy chambers were incubated with 300 $\mu\text{L}$
25	fibronectin (10 $\mu$ g/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 (5x10 <sup>4</sup> cells/well)

1 were seeded in 8-well Ibidi® µ-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 man-NP (500 µg/ml) for 3 hours. Non-treated cells and non-labelled NP were used 3 4 as negative controls. Cells were washed and fixed at room temperature in 4% paraformaldehyde, containing Hoechst<sup>®</sup>332 at 2 µg/mL and WGA Alexa Fluor 633 at 5 6 5 µg/mL to stain the nucleus and the cell membrane, respectively. Each well was 7 washed three times with PBS and 100 µL 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 µm Z-stacks and processed using the Leica Application Suite-Advanced Fluorescence (LAS-AF) 11 12 software.

13

# 14 Animal studies

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

weight loss (above 10% within a few days or 20% from the initial weight). Tumourbearing mice were euthanized in case the tumour size exceeded 2000 mm<sup>3</sup> or if the tumour was necrotic or ulcerative. Mice were perfused intracardially with PBS, immediately after euthanasia, tumours were dissected and incubated in 4% PFA and 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 flank by the s.c. hock immunization with one of the Cy5.5 fluorescently-labelled plain 9 10 and antigen-loaded NP formulations (2 mg of NP/mouse). Left non-injected flank served as negative control. All formulations contained MART-1 antigens and TLR 11 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

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

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

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

#### 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 (n = 6) and concentrated in concentration tubes (Amicon). Each sample was applied 17 on a nitrocellulose membrane containing capture antibodies that bind to specific 18 19 target protein. After overnight incubation at 4°C, streptavidin-HRP and 20 chemiluminescent reagents were added, and incubation steps were performed according to the protocol provided by the analysis kit. Membranes were exposed to 21 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

Immune cell cytotoxic activity was assessed using IncuCyte<sup>®</sup> ZOOM live-cell 2 instrument. RMS cells (6x10<sup>3</sup> cells/well) were seeded in 96-well plates in complete 3 4 RMPI without phenol red, on the day before the addition of splenocytes. Prestimulated splenocytes were added to RMS cells in a 1:100 ratio. IncuCyte™ 5 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 µM. 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 volume12 measurements

13 Male C57BL/6J mice (8 weeks old) were randomized into different groups (N = 4-5) according to **Supplementary Table 2** for the prophylactic combination study. 14 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, respectively). For the prophylactic study, on day 0, 50 µl of cell suspension containing 17 4.5x10<sup>5</sup> murine RMS cells mixed with growth-factor reduced matrigel (1:1) were 18 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 on day 0 was 3x10<sup>5</sup> instead. For the intervention study on the second melanoma 21 model, 100 µl of saline with 1.5x10<sup>5</sup> B16-F10 cells in suspension were inoculated 22 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 depilatory cream before the injection.  $\alpha$ PD-1 and  $\alpha$ OX40 were administered 25

intraperitoneally at 10 mg/kg. Ibrutinib was also administered intraperitoneally at 6 mg/kg. Tumour size was measured every 3 days with a calliper. Tumour volume was determined by  $X^2 \cdot Y \cdot 0.5$  (X – small diameter; Y – large diameter) and body weight 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 single-cell suspensions were obtained by mechanical disruption of the tissues and 11 enzymatic digestion in RMPI medium with 0.5% BSA, 0.1% collagenase type II 12 13 (LS004177, Worington), 0.1% dispase (LS02109, Worthington) and DNase (LS002007, Worington) for 1 hour at 37°C. After digestion, the suspension was 14 15 filtered through a 70 µm filter (BD Biosciences) to remove the debris. For the RMS cells, the obtained single-cell suspension was then stained with fluorochrome labelled 16 antibodies and analysed using an LSR Fortessa (BD Biosciences) and FlowJo 17 software (Tree Star Inc.). Intracellular staining was performed with the Inside stain kit 18 (Miltenvi Biotec, Cat.# 130-090-477), according to the manufacturer's protocol. 19

- 20 Details of the anitbodies used in each panel are in Supplementary information.
- 21

### 22 Functional assessment of T-cells

For the ELISpot assay, on day 0, 50 μl of cell suspension containing 4.5x10<sup>5</sup>
 murine RMS cells were inoculated subdermally. Starting on day 7, mice were treated
 with two weekly doses as reported in **Supplementary Table 3.**

1 On day 21, mice were euthanised and spleens harvested and splenocytes isolated. Splenocytes were seeded at 2x10<sup>5</sup> cells per well in 96-well plates coated 2 with IFN-y antibody (R&D Systems Inc.) and incubated for 20 hours with 1 mg/mL of 3 4 Melan-A/MART-1 MHCI-ag peptide. The secreted and captured IFN-y was subsequently detected using a biotinylated antibody specific for IFN-y and an 5 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 ImmunoSpot<sup>®</sup> S6 Ultra-V. 9

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 µL of man-NP or NP (100 µg of gp 100 antigens/20 µg of CpG/20 µg of MPLA per mouse; 12 50 µL in each side), free gp 100 antigens with adjuvants CpG and MPLA, or PBS. 13 Inguinal LN were harvested 10 days after the 2<sup>nd</sup> injection, homogenized in a single-14 15 cell suspension, and plated in 96-well plate for staining. First, the peptide-MHC tetramer tagged with PE (H-2D<sup>b</sup> - restricted EGSRNQDWL PE-labelled tetramer 16 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 cells were washed to remove unbound tetramers, and centrifuged at 1300 rpm, for 19 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 CD8α-PE-Cy7, cells 22 were incubated for 15 min, at 4°C protected from light. Cells were washed, 23 centrifuged and resuspended in 200 µl of ice-cold sorter buffer to determine the percentages of tumour antigen-specific CD3<sup>+</sup> CD8 $\alpha$ <sup>+</sup> T cells by FACS. 24

25

# **1** Statistical Methods

2 Data are presented as mean ± standard deviation (SD) for in vitro assays and 3 as mean ± 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 Inc.) and GraphPad Prism 5<sup>®</sup> or 7<sup>®</sup> (GraphPad Software, Inc., La Jolla, CA) P < 0.058 was considered statistically significant. 9

10 11

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

# 2 Table 1. Particle size, polydispersity index (PdI) and ζ-Potential

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

14



Fig. 1. NP and Man-NP are potential delivery systems for vaccination. A. Schematic representation of mannose-PLGA/PLA nanoparticles (man-NP). B. TEM image of spherical man-NP. C. SEM image of spherical man-NP. D. AFM images of spherical man-NP, showing narrow size polydispersity. E. Particle internalization by DC determined by FACS. Non-treated cells and non-labelled NP were used as 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 incubation with NP (left) and man-NP (right). Z-stacks (top) and projections (bottom). 2 (N = 3; n = 6). Scale bars = 50  $\mu$ m. **G.** Percentage of NP internalization 17 hours after 3 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 increased the expression of the activation and maturation markers of these APC. H. 6 7 Median florescence 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-Hoc test (G.) or Bonferroni test (H.) \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001. 11



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

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





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



Fig. 4. Low CD8<sup>+</sup>/Treg ratio and high infiltration of Myeloid-derived Suppressor 2 Cells (CD11b<sup>+</sup>Gr-1<sup>+</sup>MDSC) compromise the therapeutic efficacy of the 3 combination of mannosylated nano-vaccines with aPD-1/aOX40. A. Timeline of 4 tumor inoculation and treatments. **B.** Tumour growth curve. Data are presented as 5 mean  $\pm$  SEM (*N* = 7). *P* values correspond to tumour volume at day 18 after tumour 6 7 inoculation. C.-H. Tumour-infiltrating immune cell populations. Tumours were isolated 8 on day 18 after tumour cell inoculation ( $N \ge 3$ ) and when the tumour volume for final endpoint was reached. Quantification was performed by flow cytometry. Data are 9

- 1 presented as mean ± SD of 3 independent replicates. \* *P* < 0.05; \*\* *P* < 0.01; \*\*\* *P* <
- 2 0.001.





Fig. 5. Trivalent combination of mannosylated nano-vaccines with 2 3 ibrutinib and  $\alpha$ PD-1/ $\alpha$ OX40 strongly restricts melanoma growth, leading to long-term survival. A. Timeline of tumour inoculation and treatments. B. Tumour 4 growth curve. Data are presented as mean  $\pm$  SEM (N = 7). P values correspond to 5 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 8 images of IFN-γ spot forming cells among splenocytes after ex vivo re-stimulation with melan-A/MART-1 peptides on day 22. E. Kaplan-Meier overall survival over time 9

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



2 Fig. 6. High CD8<sup>+</sup>/Treg ratio and inhibition of MDSC are associated with improved therapeutic efficacy. A.-H. Tumour-infiltrating immune cell populations. 3 Tumours were isolated on the first endpoint, day 20 after tumour cell inoculation ( $N \ge$ 4 3), and at the tumour size-matched second endpoint: day 27 for PBS, ibrutinib only 5 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 presented as mean ± SD of 3 independent replicates. \* P < 0.05; \*\* P < 0.01; \*\*\* P < 8 9 0.001.

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

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