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Reem Ghinnagow, Julie de Meester, Luis Javier Cruz, Caroline Aspod, Stéphanie Corgnac, et al.. Co-delivery of the NKT agonist α -galactosylceramide and tumor antigens to cross-priming dendritic cells breaks tolerance to self-antigens and promotes antitumor responses. *OncoImmunology*, 2017, 6 (9), pp.e1339855. 10.1080/2162402X.2017.1339855 . hal-04276618

HAL Id: hal-04276618

<https://hal.science/hal-04276618v1>

Submitted on 30 Aug 2024

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To link to this article: <https://doi.org/10.1080/2162402X.2017.1339855>

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ORIGINAL RESEARCH



Co-delivery of the NKT agonist α -galactosylceramide and tumor antigens to cross-priming dendritic cells breaks tolerance to self-antigens and promotes antitumor responses

Reem Ghinnagow^{a,b,c,d,e}, Julie De Meester^{a,b,c,d,e}, Luis Javier Cruz^f, Caroline Aspod^g, Stéphanie Cornac^h, Elodie Macho-Fernandez^{a,b,c,d,e}, Daphnée Soulard^{a,b,c,d,e}, Josette Fontaine^{a,b,c,d,e}, Laurence Chaperot^g, Julie Charles^{g,i}, Fabrice Soncin^{id d,e,j}, Fathia Mami-Chouaib^h, Joel Plumas^g, Christelle Faveeuw^{a,b,c,d,e}, and François Trottein^{id a,b,c,d,e}

^aUniv. Lille, U1019 - UMR 8204 - CIL - Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France; ^bCentre National de la Recherche Scientifique, UMR 8204, F-59000 Lille, France; ^cInstitut National de la Santé et de la Recherche Médicale U1019, F-59000 Lille, France; ^dCentre Hospitalier Universitaire de Lille, F-59000 Lille, France; ^eInstitut Pasteur de Lille, F-59000 Lille, France; ^fTranslational Nanobiomaterials and Imaging, Department of radiology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; ^gInstitute for Advanced Biosciences (IAB), Team Immunobiology and Immunotherapy in Chronic Diseases, INSERM U1209, CNRS UMR5309, Université Grenoble Alpes, Etablissement Français du Sang-Rhône-Alpes, F-38700 Grenoble, France; ^hINSERM UMR 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, Fac. de médecine - Univ. Paris-Sud, Université Paris-Saclay, 94805, Villejuif, France; ⁱDermatology department, Grenoble Alpes University Hospital, F-38700 Grenoble, France; ^jCentre National de la Recherche Scientifique, UMR 8161, F-59000 Lille, France

ABSTRACT

Vaccines designed to abrogate the tolerance of tumor self-antigens and amplify cytotoxic CD8⁺ T cells (CTLs) have promise for the treatment of cancer. Type I natural killer (NKT) cells have attracted considerable interest in the cancer therapy field. In the current study, we have exploited the unique ability of NKT cells to serve as T-helper cells to license dendritic cells (DCs) for cross priming with the aim to generate efficient CTL antitumor responses. To this end, we designed a nanoparticle-based vaccine to target cross-priming DCs via the Clec9a endocytic pathway. Our results showed for the first time that simultaneous co-delivery of the NKT agonist α -galactosylceramide and tumor self-antigens (Trp2 and gp100) to CD8 α ⁺ DCs promotes strong antitumor responses in prophylactic and therapeutic settings (advanced solid tumor model in the mouse). We attributed the vaccine's therapeutic effects to NKT cells (but not to T-helper lymphocytes) and CD8⁺ T cells. Efficacy was correlated with an elevated ratio between tumor antigen-specific CD8⁺ T cells and regulatory CD4⁺ T lymphocytes within the tumor. The nanoparticle-based vaccine actively targeted human CLEC9A-expressing BDCA3⁺ DCs - the equivalent of murine cross-priming CD8 α ⁺ DCs - and induced a strong expansion of effector memory tumor self-antigen (Melan -A)-specific CD8⁺ T cells from peripheral blood mononuclear cells sourced from healthy donors and melanoma patients. Together, our result shed light on novel therapeutic approaches for controlling tumor development.

Abbreviations: CTLs, cytotoxic CD8⁺ T cells; DCs, dendritic cells; TLR, Toll-like receptors; NKT cells, natural killer T cells; OVA, ovalbumin; NP, nanoparticle; Abs, antibodies; PBMC, peripheral blood mononuclear cells; PD1, programmed cell death protein-1; LN, lymph node; PLGA, Poly(lactic-co-glycolic acid)

ARTICLE HISTORY

Received 28 April 2017
Revised 1 June 2017
Accepted 1 June 2017

KEYWORDS

Cancer; dendritic cells; natural killer T cells; self antigens; targeting; vaccine

Introduction

Although vaccines designed to promote the development of tumor-specific cytotoxic CD8⁺ T cells (CTLs) have promise for cancer treatment, optimization (to abrogate the tolerance of tumor self-antigens) is urgently needed (for reviews,¹⁻³ The development of effective CTLs implies the adequate stimulation of naive CD8⁺ T cells by dendritic cells (DCs). A variety of factors influence the strength and quality of the CTL response, including the nature of the DC subset, the duration of antigen presentation, and the type of costimulatory signals received by the naive CD8⁺ T cells.^{1,3} The BDCA3⁺ DC subset (in humans) and the CD8 α ⁺ DC subset (in the mouse) excel in antigen

cross-presentation and the priming of CD8⁺ T cells.⁴⁻⁶ Many research groups have reported that tumor antigen presentation by cross-priming DCs is essential for an effective antitumor CTL response.^{1,3} This unique property is currently used to enhance the efficacy of tumor vaccines whose delivery systems target cross-priming DCs⁷⁻¹⁰; this approach is now in clinical development.¹¹ Activating/licensing signals are required for the effective induction of CTL responses, and co-stimulatory signals delivered by DC-expressed pattern recognition receptors and/or T-helper cells are critical in this respect.^{3,12,13} Many attempts have been made to optimize the CTL response in tumor settings by using various classes of Toll-like receptor

Table 1. Physicochemical characterization of PLGA NPs containing α -GalCer and OVA or tumor peptides. PLGA NPs were characterized by dynamic light scattering and zeta potential measurements. PLGA NPs size and zeta potential data represent the mean value \pm SD of 10 and 5 readings, respectively. The amount of encapsulated OVA was determined by Coomassie dye protein assay (mean \pm SD, two independent experiments). The amount of encapsulated tumor antigens (gp100, Trp2, Melan A) was determined by reversed-phase high-performance liquid chromatography. The incorporation of α -GalCer into NPs was total due to its hydrophobic nature. The amount of Ab introduced into NPs was determined indirectly by Coomassie Protein Assay Reagent by measuring a small aliquot from the Ab solution at the starting point and at the end of the reaction.

Samples	α -GalCer (μ g/mg NP) (w/w)	OVA (μ g/mg NP) (w/w)	Gp100/Trp2 (μ g/mg NP) (w/w)	Melan A (μ g/mg NP) (w/w)	PLGA NPs diameter \pm S.D. (nm)	Zeta potential (mV) \pm S.D.	Abs (μ g/mg NPs)
NP(α -GalCer/OVA)-Clec9A	0.5	21.0	–	–	234.0 \pm 12.2	–17.3 \pm 1.5	27.9
NP(α -GalCer/OVA)-IgG	0.5	21.0	–	–	221.0 \pm 10.4	–16.7 \pm 1.5	29.0
NP(α -GalCer/gp100/Trp2)-Clec9A	0.5	–	42/38	–	220.6 \pm 12.5	–16.4 \pm 6.2	24.0
NP(α -GalCer/gp100/Trp2)-IgG	0.5	–	42/38	–	208.6 \pm 10.6	–18.6 \pm 6.4	27.0
NP(α -GalCer/MelanA)-CLEC9A	0.5	–	–	48	234.8 \pm 10.9	–12.6 \pm 5.2	22.0
NP(α -GalCer/MelanA)-IgG	0.5	–	–	48	242.2 \pm 14.6	–14.4 \pm 5.1	23.0

(TLR) agonist.^{14,15} Other studies have demonstrated the value of delivering both CTL and T-helper epitopes to the same DC and thus favoring DC/T-helper cell/naive CD8⁺ T cell encounters - the so called “ménage à trois”.^{16,17} In the present study, we chose an alternative strategy based on the recruitment of another type of helper cell for cross-priming DCs - namely type I natural killer T (NKT) cells.

type I NKT cells belong to the “innate-like” T lymphocyte family. Through a semi-invariant T cell receptor, these non-conventional T cells recognize lipid antigens presented by the monomorphic CD1d molecule on antigen-presenting cells, including DCs.^{18–20} In response to the superagonist α -galactosylceramide (α -GalCer), NKT cells swiftly produce immunostimulatory cytokines (including large amounts of IFN- γ) and up-regulate several costimulatory molecules (e.g. CD40); this leads to DC maturation and the downstream activation of critical effectors of antitumor immunity, including NK cells and T lymphocytes.^{21–23} Importantly, Semmling et al. elegantly demonstrated that NKT cells can license DCs for cross-priming through a mechanism other than that used by T-helper cells.²⁴ This alternative cross-priming may lead to a CTL response that differs from classical cross-priming. We noted that Semmling et al. studied the model antigen ovalbumin (OVA), rather than a self-antigen. This observation prompted us to try to co-deliver tumor self-antigens and an NKT cell agonist to CD8 α^+ DCs by using a nanoparticle (NP)-based technology. We hypothesized that this strategy might enhance DC/NKT cell/naive CD8⁺ T cell interactions, abrogate self-tolerance and thus promote effective antitumor CTL responses. To this end, we targeted the C-type lectin Clec9a (also known as DNGR1); this marker is almost exclusively expressed by cross-priming DCs, and is known to confer potent CTL responses.^{7,10,25} By using NPs decorated with antibodies (Abs), we showed that simultaneous, targeted delivery of α -GalCer and tumor self-antigens (Trp2 and gp100) to mouse CD8 α^+ DCs via the Clec9a endocytic pathway is instrumental in inducing a potent CTL response that protects in prophylactic and therapeutic settings against the development of aggressive tumors (melanoma). In the human setting (with peripheral blood mononuclear cells (PBMCs) from healthy donors and melanoma patients), co-delivery of α -GalCer and a tumor antigen (Melan A) to

BDCA3⁺ DCs strongly induced the expansion of tumor-antigen-specific CD8⁺ T cells *in vitro*. Our present findings may have important implications for tumor immunotherapy and vaccine development.

Results

Primary and secondary stimulation of NKT cells is enhanced by targeting α -GalCer via NP/Clec9a

We first tested the ability of PLGA NPs containing α -GalCer and OVA and decorated with anti-Clec9a Abs (see Table 1 for information on the NPs’ physicochemical characteristics) to activate NKT cells *in vitro*. NP/ α -GalCer/OVA/Clec9a induced greater cytokine production (including the production of IFN- γ , a critical antitumor cytokine) by spleen cells than the control NP/ α -GalCer/OVA/IgG and (albeit to a lesser extent) soluble, non-targeted (free) α -GalCer (Fig. 1A, left panel and Fig. S1A). Cytokine production was dependent on the antigen-presenting molecule CD1d and on NKT cells (Fig. S1B). Furthermore, cytokine production in response to NP/ α -GalCer/OVA/Clec9a was dependent on CD8 α^+ DCs because spleen cells from *Baft3*^{–/–} mice were much less able to produce IFN- γ (Fig. 1A, right panel). When pulsed *in vitro* with α -GalCer targeted via NP/Clec9a, bone marrow-derived DCs induced higher levels of IL-2 production by the NKT hybridoma DN32.D3 (Fig. 1B, left panel and Fig. S1C for the phenotypic analysis of the DCs), relative to either non-targeted α -GalCer (NP/IgG) or free α -GalCer. Treatment of DCs with anti-Clec9a Abs reduced cytokine production by NKT cells in response to NP/ α -GalCer/OVA/Clec9a, but not NP/ α -GalCer/OVA/IgG (Fig. 1B, right panel). Hence, targeted delivery of α -GalCer via NP/Clec9a effectively activated NKT cells *in vitro*.

We next evaluated the targeted α -GalCer’s ability to activate NKT cells *in vivo*. Whatever the dose used for inoculation, NP/ α -GalCer/OVA/Clec9a induced a higher frequency of IFN- γ expressing NKT cells than NP/ α -GalCer/OVA/IgG or non-targeted α -GalCer plus OVA did (Fig. 1C, left panel and Fig. S2A). It is noteworthy that the inoculation of 5 ng of α -GalCer incorporated into NP/Clec9a or 100 ng of non-targeted α -GalCer resulted in similar proportions of IFN- γ positive NKT cells.

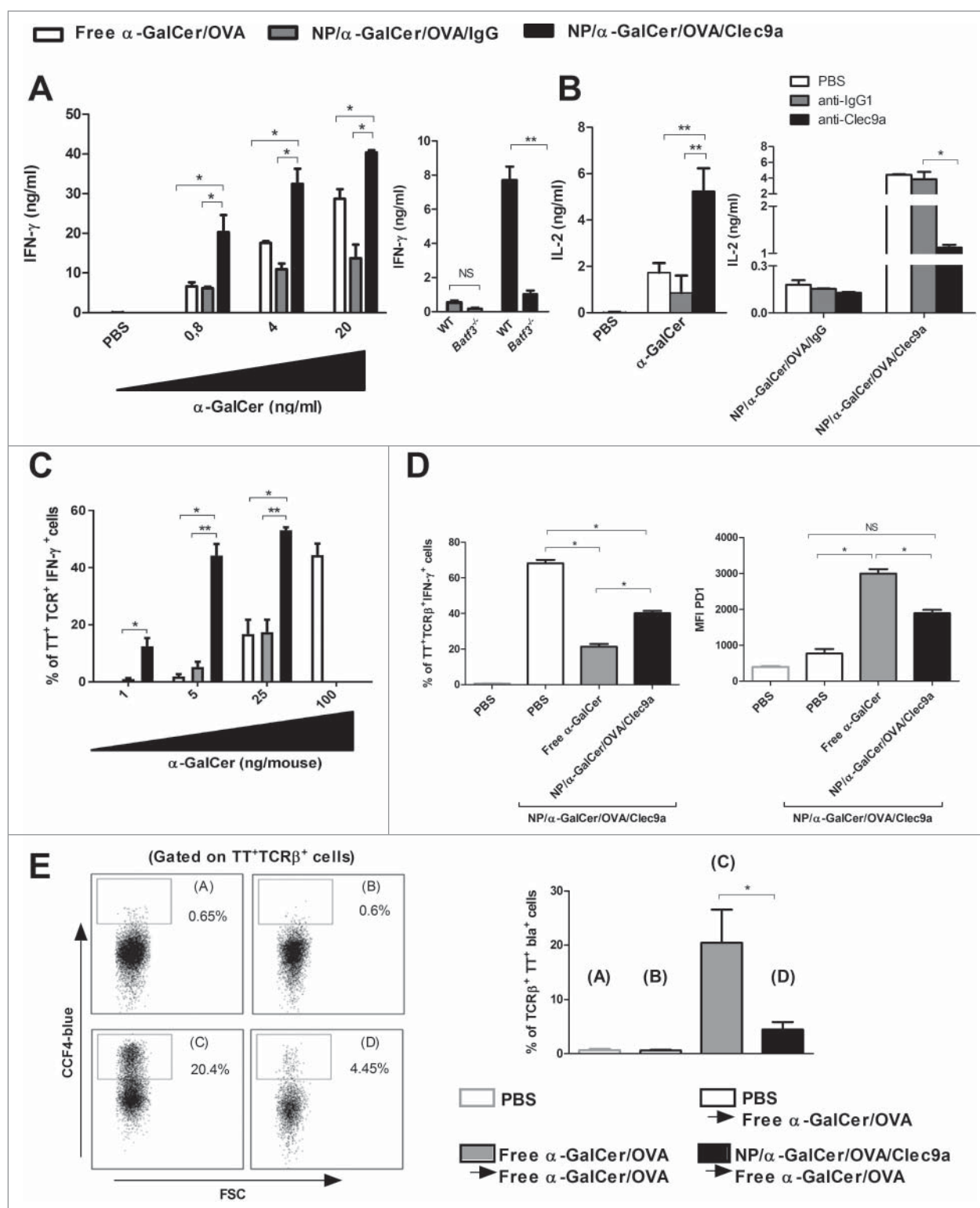


Figure 1. Primary and secondary stimulation of NKT cells is ameliorated by means of α -GalCer vectorization into NP/Clec9a. **A, Left panel,** Spleen cells from WT animals were exposed to grading doses of free α -GalCer plus OVA or with α -GalCer plus OVA encapsulated into PLGA-based NPs armed with anti-Clec9a Abs (NP/ α -GalCer/OVA/Clec9a) or isotype control Abs (NP/ α -GalCer/OVA/IgG). **Right panel,** Spleen cells from WT or *Baft3*^{-/-} were incubated with NP/ α -GalCer/OVA/Clec9a or NP/ α -GalCer/OVA/IgG. Of note, *Baft3* deficiency did not reduce the number of splenic NKT cells (not shown). **B,** BM-DCs were exposed for 2 hours to free or encapsulated α -GalCer and, after washing, DCs were cultured with the NKT cell hybridoma DN32.D3. **Right panel,** Anti-Clec9a or IgG1 control was added (25 μ g/ml) during the antigenic pulse. **(A)** and **B,** IFN- γ and IL-2 production was quantified 48 hours or 24 hours later by ELISA. Data represent a pool of 2 or 3 independent experiments. **C, Left panel,** Mice were i.v. injected with PBS alone or graded doses of α -GalCer either in a free soluble form or encapsulated into NP/Clec9a or NP/IgG. After 3 hours, splenic NKT cells were screened for intracellular IFN- γ production. The average percentages of NKT cells positive for IFN- γ are represented. **D,** Mice were i.v. injected with free α -GalCer (100ng/mouse) or with α -GalCer encapsulated into NP/Clec9a (5 ng/mouse). Seven days later, mice received a second injection of vectorized α -GalCer (NP/ α -GalCer/OVA/Clec9a, 5 ng/mouse) and splenic NKT cells were screened for intracellular IFN- γ production (**left panel**) and cell surface PD1 expression (**right panel**) 3 hours later. **E, Left panel,** Representative dot plots of NKT cells expressing CCF4-blue fluorescence (corresponding to IL-10- β -lactamase⁺ cells). **Right panel,** the percentage of IL-10- β -lactamase⁺ NKT cells among total NKT cells is represented ($n = 5$). **C-E,** Shown is a representative experiment of at least 3 (2 for panel D) performed ($n = 5$). **A-E,** ** $P < 0.01$, * $P < 0.05$ (a Kruskal-Wallis ANOVA).

These 2 groups of animals did not differ in terms of the serum IFN- γ concentration and the extent of NK cell transactivation (Fig. S2B and data not shown).

It is known that repeated stimulation with non-targeted α -GalCer leads to NKT cell hyporesponsiveness as a result of uncontrolled NKT cell activation.²⁶ This hyporesponsiveness might have a profound impact on the development of NKT cell-based vaccine therapies in cancer because it would limit the prime-boost strategy. We therefore sought to determine whether our delivery system could counter this negative effect. To this end, mice were injected with 100 ng of non-targeted α -GalCer or 5 ng of α -GalCer incorporated into NP/Clec9a, which trigger similar levels of primary NKT cell activation (Fig. 1C). As expected, inoculation of non-targeted α -GalCer led to a much lower frequency of IFN- γ expressing NKT cells than in singly inoculated (NP/ α -GalCer/OVA/Clec9a) mice (Fig. 1D, left panel). Interestingly, primary activation of NKT cells following administration of NP/ α -GalCer/OVA/Clec9a resulted in a lower degree of NKT cell hyporesponsiveness upon the second challenge. Furthermore, this effect was associated with lower cell surface expression of the hyporesponsiveness marker programmed cell death protein-1 (PD-1) on NKT cells²⁶ (Fig. 1D, right panel). Hence, primary activation of NKT cells by NP/ α -GalCer/OVA/Clec9a improves the functionality (as measured by IFN- γ production) of NKT cells upon secondary restimulation. Inoculation of non-targeted α -GalCer has also been shown to induce the expansion of

immunosuppressive, IL-10-producing NKT cells (NKT10 cells),²⁷ which might also pose considerable problems for vaccine-based cancer therapies. Our present study confirmed that the administration of non-targeted α -GalCer led to the expansion of NKT10 cells (Fig. 1E). Remarkably, primary activation of NKT cells with NP/ α -GalCer/OVA/Clec9a dramatically reduced the proportion of NKT10 cells. Together, primary and secondary stimulation of NKT cells is enhanced by targeting α -GalCer via NP/Clec9a.

Co-delivery of particulate protein antigen and α -GalCer via NP/Clec9a induces potent CTL and anti-tumor responses

We next tested our synthetic delivery system's efficacy with regard to the *in vivo* expansion of CD8⁺ T cells. To this end, mice reconstituted with CFSE-labeled OVA_{257–264}-specific CD8⁺ T cells were subcutaneously inoculated with NP/Clec9a containing both α -GalCer and OVA. Relative to controls, NP/ α -GalCer/OVA/Clec9a induced greater expansion of OVA_{257–264}-specific CD8⁺ T cells in the draining lymph nodes and in the spleen (Fig. 2A, right panel, Fig. S3A and S3B). Importantly, expansion of OVA-specific CD8⁺ T cells required the CD1d mode of antigen presentation because only baseline levels of proliferation were found in *Cd1d*^{-/-} mice (Fig. 2A, left panel and Fig. S3B). This finding suggests that help from NKT cells was critical for

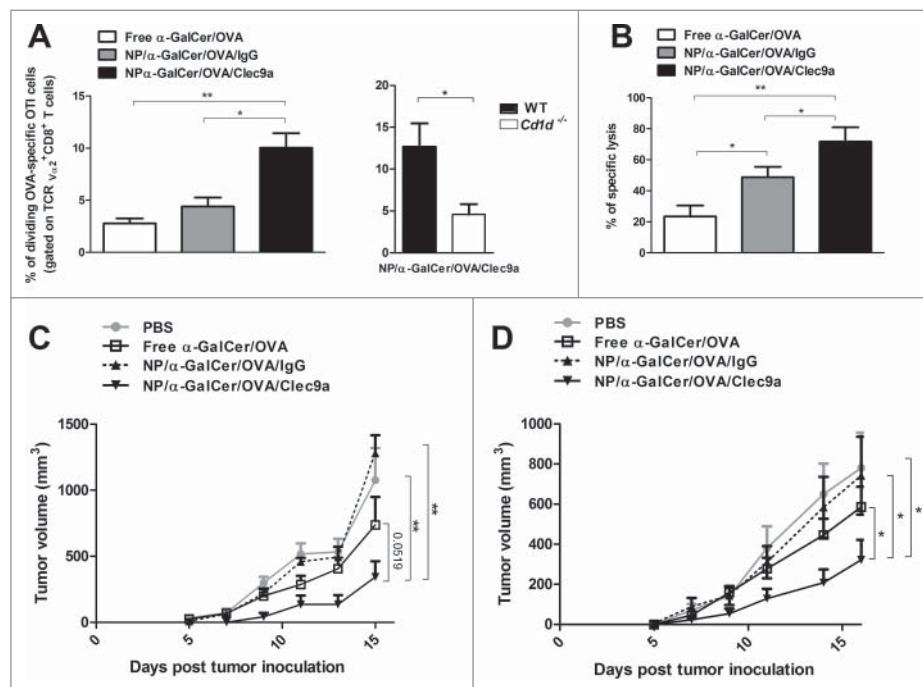


Figure 2. Co-encapsulation of α -GalCer and OVA in NP/Clec9a induces potent CTL and anti-tumor responses. (A) (right panel), WT Mice, previously injected with CFSE-labeled Rag2/OT-I cells, were s.c. inoculated with OVA (210 ng/mouse) and α -GalCer (5ng/mouse) either free or co-encapsulated in NP/IgG or NP/Clec9a. Three days later, the proliferation of CFSE-labeled V α 2 TCR $\gamma\delta^+$ CD8 α^+ in popliteal LNs was determined by flow cytometry ($n = 5-7$). Left panel, The same procedure was repeated but this time WT and *Cd1d*^{-/-} mice were injected with NP/ α -GalCer/OVA/Clec9a ($n = 5$). B, Six days after immunization, mice were transferred with CFSE-labeled SIINFEKL-primed (syngeneic target cells) and PKH-26-labeled unprimed (syngeneic control cells) splenocytes. Data represent the percentage of specific lysis ($n = 5$). C, Free or vectorized OVA (1.26 μ g) and α -GalCer (30 ng) were injected s.c. in mice. Seven days later, mice were inoculated s.c. with OVA-expressing EG7 cells (2×10^5 cells/mouse). D, Mice previously reconstituted with Rag2/OT-I cells (5×10^6 splenocytes/mouse) were inoculated with OVA-expressing EG7 cells. One and 5 d later, mice were injected with free or vectorized OVA (210ng/mouse) plus α -GalCer (5ng/mouse). (C) and D, Tumor size was measured every 2 d ($n = 8$). A-D, One representative experiment out of at least 2 is shown. ** $P < 0.01$, * $P < 0.05$ (a Kruskal–Wallis ANOVA (A, left panel and B) and a Mann–Whitney U test ((A, right panel, C–D, area under curve).

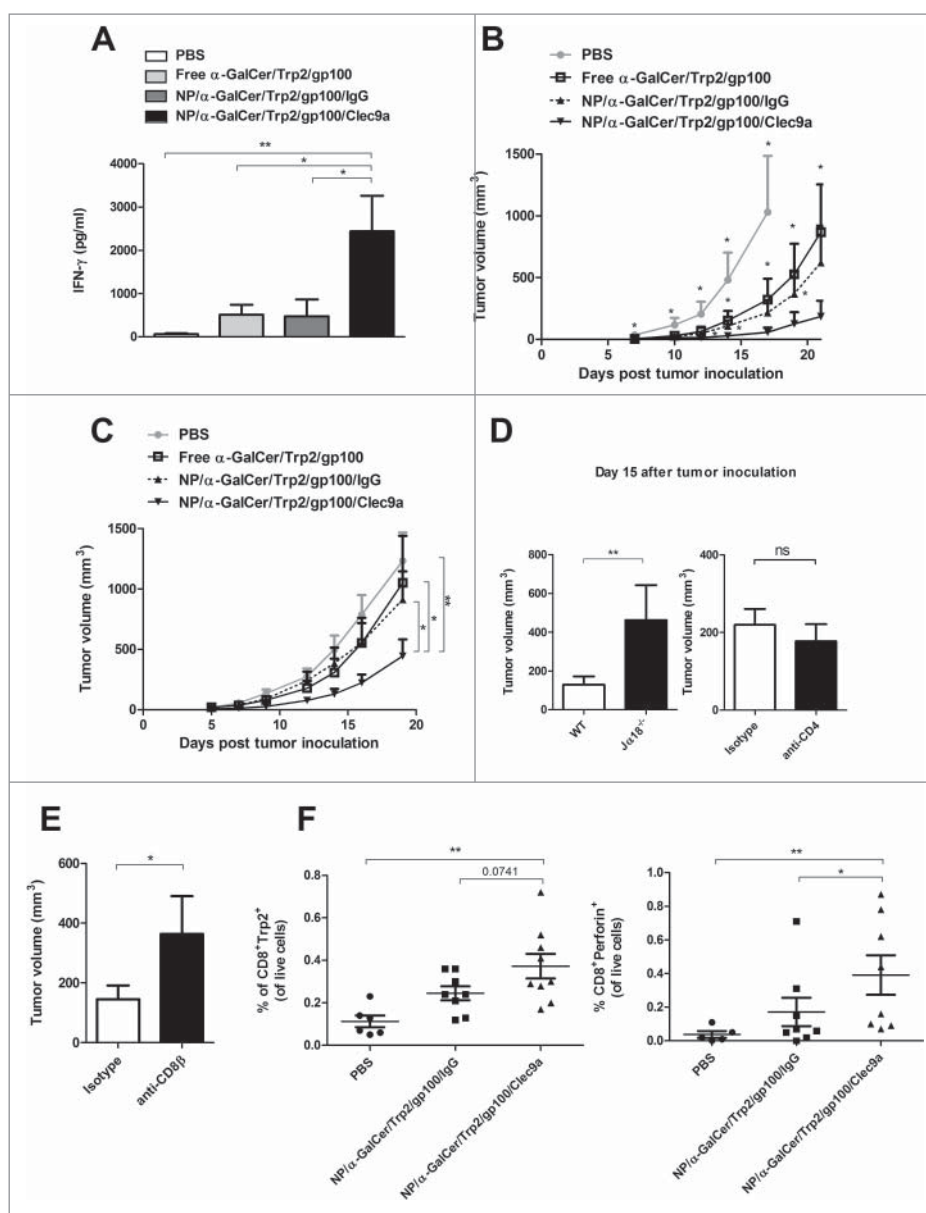


Figure 3. Co-encapsulation of α -GalCer and tumor peptides in NP/Clec9a induces potent CTL and anti-tumor responses. **A**, Mice were s.c. inoculated at day 0, 7 and 14 with Trp2 and gp100 peptides (~ 2.4 μ g each/mouse) and α -GalCer (30ng/mouse) either free or co-encapsulated in NP/IgG or NP/Clec9a. Three days later, skin-draining LN cells were restimulated with a cocktail of Trp2 and gp100 (10 μ g/ml). IFN- γ was measured 48 hours later. Shown is a representative experiment of 2 performed ($n = 5$). **B**, Mice were injected at day 0 and day 7 with peptides (~ 2.4 μ g each/mouse) and α -GalCer (30ng/mouse) either free or co-encapsulated in NP/IgG or NP/Clec9a. Seven days later, mice were inoculated with B16F10 (5×10^5 cells/mouse). **C**, Mice were s.c. implanted with B16F10. Five (tumor volume: 25–50 mm³) and 12 d later, mice were injected with free or vectorized peptides (~ 2.4 μ g each/mouse) and α -GalCer (30ng/mouse). (**B**) and **C**, Tumor volume was measured every 2 d ($n = 16$, 2 pooled experiments). **D**, The same therapeutic protocol (as that described in panel **C**) was performed. *Left panel*, To investigate the role of NKT cells in protection, $J\alpha 18^{-/-}$ mice were vaccinated with NP/ α -GalCer/tumor peptides/Clec9a. *Right panel*, Vaccinated wild type (NKT cell-competent) mice were treated with an anti-CD4 depleting Ab or an isotype control 2 d before the first immunization (day 3 post B16 inoculation) and 2 d before the boost (day 10 post B16 inoculation). **E**, Vaccinated mice were treated with an anti-CD8 β depleting Ab or an isotype control (days 7, 10, 13 and 16). (**D**) and **E**, Tumor volumes are indicated at day 15 post-B16 implantation ($n = 8$). **F**, On day 14, the tumor mass of each mouse was excised and the frequency of Trp2-specific CD8⁺ T cells and perforin-expressing CD8⁺ T cells were determined by flow cytometry (2 pooled independent experiments). ** $P < 0.01$, * $P < 0.05$ (a Kruskal–Wallis ANOVA ((**A**) and **F**) and a Mann–Whitney U test (B–E) (area under curve for panel **C**).

antigen cross presentation in our setting. Six days after immunization, the level of OVA-specific T cell cytotoxicity was measured in an *in vivo* assay. Inoculation of NP/ α -GalCer/OVA/Clec9a elicited a higher CTL response (as assessed by target cell lysis) than in control animals (Fig. 2B). To investigate the effects of targeted antigen and α -GalCer on the control of tumor development, mice were subcutaneously immunized with NP/ α -GalCer/OVA/Clec9a 7 d before the engraftment of EL4 lymphoma cells

expressing OVA (EG7). As shown in Fig. 2C, inoculation of NP/ α -GalCer/OVA/Clec9a delayed the growth of EG7 lymphoma cells, relative to NP/ α -GalCer/OVA/IgG and non-targeted OVA plus α -GalCer. Moreover, NP/ α -GalCer/OVA/Clec9a vaccination of mice bearing pre-established EG7 tumors resulted in a significant delay in tumor overgrowth (Fig. 2D). Taken as a whole, our results show that OVA and α -GalCer targeted via NP/Clec9a have prophylactic and therapeutic effects on solid tumor development.

NPs incorporating α -GalCer and self-tumor antigens protect against tumor development

We next looked at whether our delivery system could abrogate the tolerance of self-tumor antigens. To this end, 2 tumor peptides (corresponding to dominant CD8⁺ T-cell epitopes from the highly malignant B16 melanoma antigens Trp2 and gp100, respectively) were incorporated into NP/ α -GalCer/Clec9a (Table 1). Relative to control groups, the subcutaneous immunization of mice with NP/ α -GalCer/tumor peptides/Clec9a resulted in greater IFN- γ production by draining lymph nodes and spleen cells upon peptide restimulation (Fig. 3A and Fig. S4A). The growth of B16F10 cells was considerably delayed (relative to control groups) in mice having been prophylactically vaccinated with NP/ α -GalCer/tumor peptides/Clec9a (Fig. 3B). We next investigated the constructs' potential therapeutic effects on previously implanted tumors. Inoculation of NP/ α -GalCer/tumor peptides/Clec9a in tumor-bearing mice

was found to strongly delay tumor progression (Fig. 3C). Experiments with $J\alpha 18^{-/-}$ mice were then used to establish whether the protective effect of the NP-based vaccine required help from NKT cells. Indeed, the delay in tumor outgrowth clearly required the presence of NKT cells (Fig. 3D, left panel). On the other hand, depletion of CD4⁺ T cells had no effect on vaccine efficacy (Fig. 3D, right panel and Fig. S4B). Of note, the anti-CD4 Ab also depleted CD4⁺ NKT cells (Fig. S4B) indicating that, in our setting, the help from CD4⁺ NKT cells is sufficient to delay the tumor growth. To prove that the protective effects of the vaccine depended on CTLs, CD8⁺ T cells were depleted by treatment with an anti-CD8 β Ab (Fig. S4C). This procedure indeed abrogated the vaccine's antitumor effect (Fig. 3E). Hence, the therapeutic effect triggered by the vaccine depended on NKT cells (but not T-helper cells) and CD8⁺ T cells. We next quantified the intratumoral infiltration of CD8⁺ T cells. The frequency and cell number of Trp2-specific CD8⁺ T cells (as well as perforin-expressing CD8⁺ T cells) was higher

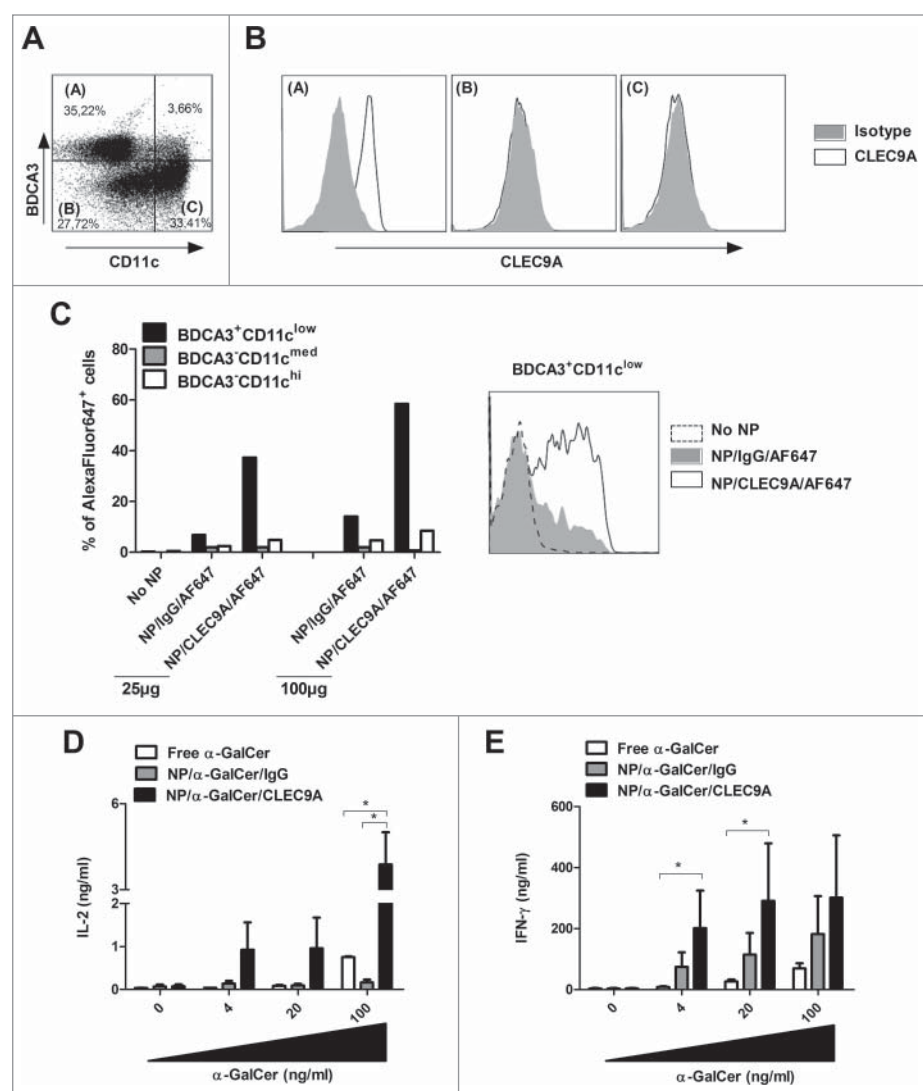


Figure 4. NP/CLEC9A target BDCA3⁺ DCs to activate human NKT cells. A, Live HLA-DR⁺ cells were analyzed to determine the percentage of DC subsets, including BDCA3⁺ DCs. B, BDCA3⁺CD11c^{low} (population A) BDCA3⁻CD11c^{med} (population B) and BDCA3⁻CD11c^{hi} (population C) were analyzed for the expression of CLEC9A. C, 1×10^5 DCs were incubated with 25 or 100 μ g of NP/IgG/AF647 or NP/CLEC9A/AF647 for 15 min. The percentage of AlexaFluor647-positive cells were analyzed by flow cytometry (left panel). Specificity of the targeting in BDCA3⁺CD11c^{low} DCs is shown (right panel). The experiment shown is representative of 3 donors. (D) and E, 1×10^4 DCs were pulsed with graded doses of free or vectorised α -GalCer and were then co-cultured with 5×10^6 NKT hybridoma 55 $\alpha\beta$ (D) or with primary NKT cells (E). Mouse IL-2 and human IFN- γ were measured in the supernatant by ELISA after 24 hours and 48 hours respectively (pool of 3 donors). * $P < 0.05$ (a Kruskal–Wallis ANOVA).

in vaccinated, protected animals than in control groups (Fig. 3F and Fig. S4D, *left panel* and not shown). Of interest, a clear inverse correlate between the percentage of intratumoral perforin-expressing CD8⁺ T cells and tumor size was observed (Fig. S4E). The intratumor ratio of tumor antigen-specific CD8⁺ T cells to regulatory T cells represents an index closely correlated with tumor regression.²⁸ The intratumor ratio of Trp2-specific CD8⁺ T cells (and perforin-expressing CD8⁺ T cells) to Foxp3-expressing CD4⁺ CD25⁺ cells was also higher in animals vaccinated with NP/ α -GalCer/tumor peptides/Clec9a (Fig. S4D, *right panel*). Taken as a whole, our results show that the incorporation of tumor self-antigens and α -GalCer into an NP/Clec9a vector promotes a potent, melanoma-self-antigen-specific CD8⁺ T cell response and has prophylactic and therapeutic effects on the growth of tumor cells.

NP/ α -GalCer/CLEC9A targets BDCA3⁺ DCs and optimizes human NKT cell activation

We next investigated NP/CLEC9A's ability to effectively target human BDCA3⁺ (CD141⁺) DCs. In functional terms, this subset resembles murine CD8 α ⁺ cross-presenting DCs and thus constitutes a very interesting target for cancer immunotherapy.^{5,6,29} To this end, human DCs were differentiated and expanded *in vitro* from cord blood haematopoietic progenitor cells. Several subpopulations of DCs (including CD11c^{low} BDCA3⁺, accounting for around 35% of total DCs, CD11c^{med} BDCA3⁻ and CD11c^{hi} BDCA3⁻ subpopulations) were obtained at the end of the differentiation procedure (Fig. 4A). Flow cytometry analysis indicated that the CD11c^{low} BDCA3⁺ DC subpopulations was the only one to express CLEC9A (Fig. 4B). To further establish whether or not the NP formulation (Table 1) targets BDCA3⁺ DCs, the DCs were incubated with AF647-labeled NPs. As shown in Fig. 4C, incubation of human DCs with NP/CLEC9A revealed that BDCA3⁺ DCs were specifically targeted. In contrast, DCs incorporated low levels of NP/IgG. In co-culture experiments, *in vitro*-generated DCs pulsed with graded doses of NP/ α -GalCer/CLEC9A induced a higher level of cytokine production by NKT cells than control preparations did. Indeed, the mouse NKT cell hybridoma 55 α β (which expresses a human invariant V α 24/V β 11 T cell receptor that binds to the α -GalCer presented by human CD1d) produced a greater amount of IL-2 (Fig. 4D). Similarly, primary NKT cells released greater amounts of IFN- γ when incubated with DCs exposed to NP/ α -GalCer/CLEC9A (Fig. 4E). In contrast, human primary NKT cells produced very little IL-4 and no IL-17A (not shown). In summary, targeting α -GalCer to human BDCA3⁺ CLEC9A⁺ DCs results in a strong NKT cell response.

NP/ α -GalCer/CLEC9A containing the tumor antigen Melan A strongly promotes the expansion of Melan-A-specific CD8⁺ T lymphocytes

Ex vivo or *in vivo* expansion of tumor-antigen-specific CD8⁺ T lymphocytes is an important objective in cancer immunotherapy. To probe the potential impact of targeting BDCA3⁺ DCs on the *in vitro* priming of CD8⁺ T cells, PBMCs from healthy HLA-A2⁺ donors were cultured with α -GalCer and an

antigenic peptide from Melan A (a major target for anti-melanoma therapies). HLA-A2⁺ donors were chosen because they have naïve precursors that are specific for the HLA-A2-restricted Melan A epitope ELAGIGILTV₂₆₋₃₅. For some donors, α -GalCer and Melan A₁₈₋₃₅ encapsulated in NP/CLEC9A strongly promoted the expansion of Melan A-specific CD8⁺ T cells, relative to other groups (Fig. 5A and Fig. S5A). Although the level of response varied from one donor to another, the relative overall increase in expansion was statistically significant (Fig. 5B and data not shown). At day 14 post-priming, most Melan-A-specific CD8⁺ T cells displayed an effector memory phenotype (CD45RA⁻ CCR7⁻) (Fig. S5B). The effect of CLEC9A Ab-armed NPs on the expansion of Melan A-specific CD8⁺ T cells was next tested using PBMCs from melanoma patients. Since the abnormally low frequency of circulating NKT cells in cancer patients is a major hurdle in NKT-cell-based anti-tumor therapy,^{30,31} we considered that it was important to test NP/ α -GalCer/Melan A/CLEC9A's effect on NKT cell expansion. In agreement with the literature data, the melanoma patients had a lower NKT cell count (but enhanced, albeit not significant, BDCA3⁺ DC count, Fig. S5C) than healthy donors on day 0 (Fig. 5C). However, after 14 d of culture, NKT cells from melanoma patients expanded strongly in response to NP/ α -GalCer/Melan A/CLEC9A. It is noteworthy that the fold increase in the frequency of NKT cells from melanoma patients was greater than for healthy donors; however, the difference was not statistically significant. We next investigated NP/ α -GalCer/Melan A/CLEC9A's ability to expand CD8⁺ T lymphocytes from melanoma patients. The formulation strongly induced the expansion of Melan A-specific CD8⁺ T cells (Fig. 5D). The proportion of Melan A-specific CD8⁺ T cells was higher in melanoma patients than in healthy donors (~19.5% and ~6.3%, respectively) (Fig. 5B). Importantly, expanded Melan A-specific CD8⁺ T cells produced IFN- γ and CD107 (a degranulation marker) in response to restimulation with Melan A peptide but not with an irrelevant peptide (Fig. 5E and Fig. S5D). In summary, NPs decorated with anti-CLEC9A Abs strongly induced the expansion of NKT cells and tumor antigen-specific CD8⁺ T cells in healthy donors and in melanoma patients; this augurs well for the potential use of our vaccine formulation in cancer patients.

Discussion

Results from preclinical and clinical studies have highlighted the value of using the NKT cell agonist α -GalCer in cancer treatments.^{32,33} In the clinic, α -GalCer has been inoculated alone as a soluble agent or loaded onto PBMCs or DCs.^{34,35} Although animal studies have clearly revealed that the adjuvant functions of NKT cells can be exploited by the co-administration of soluble α -GalCer and antigens,^{19,22,36} the NKT cells' helper functions have yet to be tested in a human setting. In the mouse system, there is evidence to suggest that NKT cells can license DCs for cross-priming; the resulting CTL response may differ from that generated by DCs licensed by TLR activation or by T-helper lymphocytes.^{22,21,23,24,37} Importantly, Semmling and al. demonstrated that the CD1d-restricted lipid and protein antigens (free α -GalCer and OVA) must be presented by the same DC.²⁴ To generate an

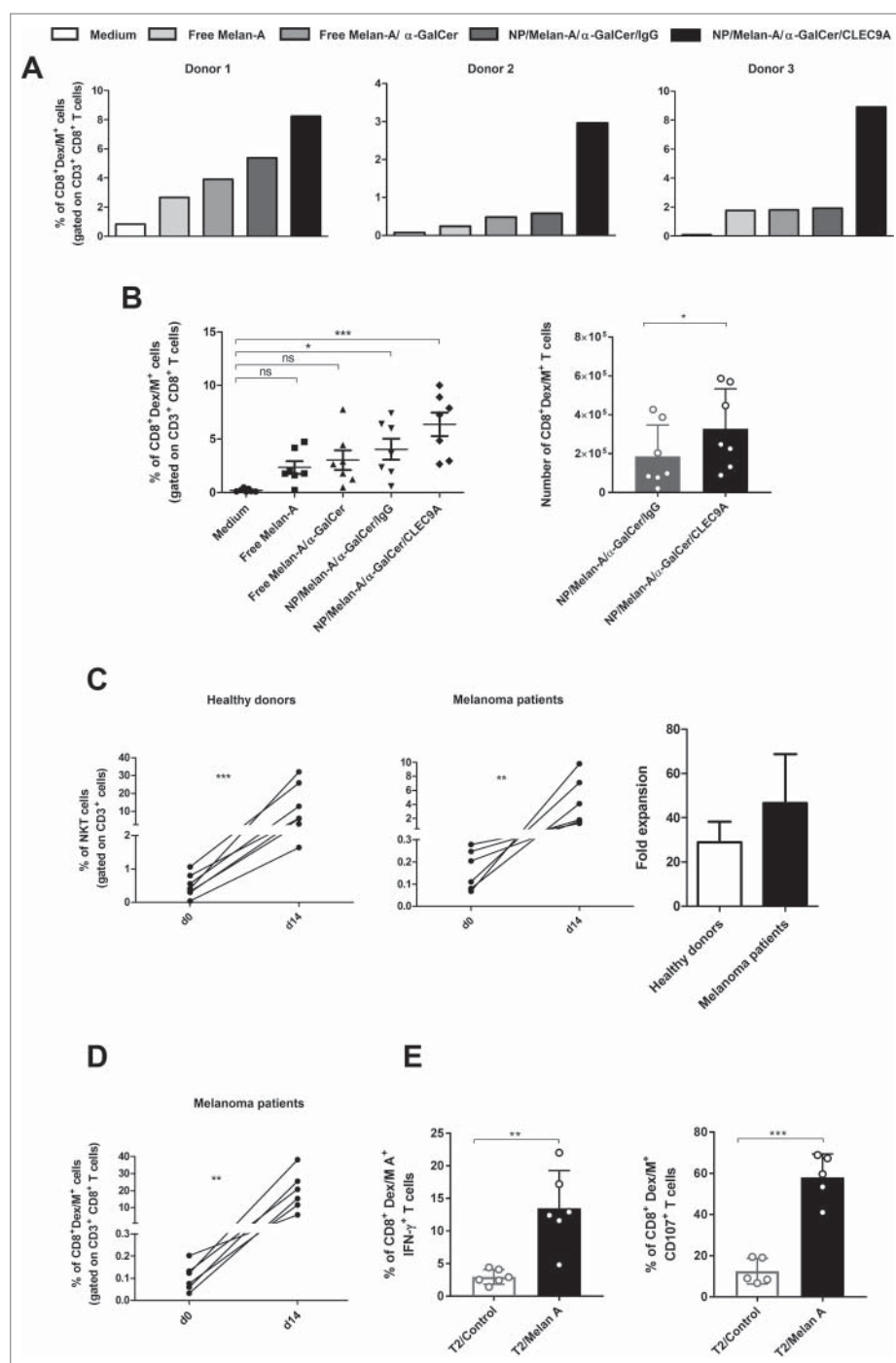


Figure 5. Co-encapsulation of α -GalCer and Melan A in NP/CLEC9A augments the proliferation of Melan A-specific CD8⁺ T cells *in vitro*. A, PBMCs from HLA-A2⁺ donors were cultured with Melan A peptide alone (0.9 μ g/ml) or supplemented with α -GalCer (10ng/ml), either free or encapsulated in NP/Clec9a or NP/IgG. Fourteen days later, the proportion of Melan A-specific (dextramer A2/Melan A_{26–35}-specific cells, Dex/M⁺) CD8⁺ T cells were determined by flow cytometry. Data from 3 donors are shown. B, The magnitude (frequency and cell number/well) of the Melan A-specific CD8⁺ T cell populations from a pool of 7 healthy donors is shown. Horizontal bars indicate mean values. C, At the end of the culture, gated CD3⁺ expanded cells were analyzed for 6B11 labeling. The frequency of human NKT cells (CD3⁺ 6B11⁺) from healthy donors and from melanoma patients is shown at day 0 and day 14. The fold expansion of NKT cells is presented in the *right panel*. (D) and E, PBMCs from melanoma donors were cultured with NP/ α -GalCer/Melan A/Clec9a. D, Proportions of Melan A-specific CD8⁺ T cells are shown at day 0 and day 14. E, The proportion of Melan A-specific CD8⁺ T cells (day 14) expressing IFN- γ or CD107 upon activation with T2 cells pulsed with the Melan A peptide or with an irrelevant peptide is shown. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (a Wilcoxon test (B, *right panel*), a Mann–Whitney U test (C, (D) and E) or a Kruskal–Wallis ANOVA (B, *left panel*)).

effective CTL and antitumor response, we exploited the NKT cells' unique ability to license cross-priming DCs. To this end, we developed a targeted strategy based on the use of PLGA NPs decorated with anti-clec9a Abs. This formulation was designed to simultaneously deliver tumor antigens and α -GalCer to the same cross-priming DCs, giving a cognate

interaction. The biodegradable polymer PLGA has been widely used for drug delivery in general and in cancer therapy in particular. PLGA NP systems have several advantages, including (i) a high antigen density, (ii) the ability to incorporate various classes of molecules (including proteins and lipids), (iii) the ability to reach MHC I pathway after uptake by

DCs, and (iv) slow release kinetics.³⁸ By using PLGA NPs armed with anti-clec9a Abs, we clearly demonstrated that active co-delivery of tumor antigens and α -GalCer to cross-priming DCs promotes better CTL and antitumor responses than free antigen and α -GalCer do. These data extend our previous findings in experiments with DEC205 (a marker that is not exclusively expressed by cross-priming DCs) and the non-self-antigen OVA.⁹ After checking that our NP-based anti-Clec9a formulation also triggered cross-priming of OVA and protected against the growth of aggressive, OVA-expressing EG7 tumors, we then showed that it can abrogate the immune tolerance of tumor self-antigens (Trp2, gp100). The resulting potent antitumor CTL response was able to prophylactically and therapeutically control solid tumor outgrowth in a model of aggressive melanoma. We showed that antigen cross-presentation *in vivo* has an absolute requirement for help from NKT cells, but not from conventional T-helper cells. With the abrogation of tolerance, our data extend Semmling et al.'s pioneering work and shed light on novel therapeutic approaches for controlling tumor development.

With a view to optimizing immune responses (especially in the context of cancer), vaccination usually involves several prime-boost immunizations. Our NP formulation's ability to promote strong CTL and antitumor responses (relative to free tumor antigens and α -GalCer) may be rooted in the priming and boost steps. Firstly, the NKT cells' primary activation threshold (i.e. the IFN- γ production) is higher following direct activation by cross-priming DCs. As a result, the enhancement of the NKT cells' helper function is likely to prime naïve CTLs to a greater extent. Secondly, the cross-talk between DCs, NKT cells and naïve CD8⁺ T cells elicited by our co-delivery system is likely to lead to a CTL response that differs both quantitatively and qualitatively from that induced by more classical activation pathways (e.g., DCs matured with TLR agonists or licensed by T-helper cells). Interestingly, Dölen and colleagues observed that the co-encapsulation of antigen (OVA) and α -GalCer in NPs triggers stronger CTL and antitumor responses than co-encapsulation of antigen and a TLR agonist does.³⁷ Although the latter researchers did not specifically target DCs, their results suggested that cognate licensing of DCs by NKT cells is more effective than conventional, TLR-based adjuvants. Several lines of research indicate that α -GalCer and TLR agonists can act cooperatively to promote DC maturation and T cell priming.^{19,39-41} In the future, we intend to look at whether or not the incorporation of α -GalCer and a TLR ligand in our NP-based formulation will cooperatively enhance the licensing of DCs and improve the outcome of antitumor vaccination. Thirdly, our data clearly show that the use of our NP formulation was associated with stronger secondary stimulation of NKT cells (IFN- γ production) and a greater ability to boost CTLs. The [cross-priming] DCs' unique ability to limit the appearance of unresponsive (PD1-expressing) NKT cells has positive implications for the development of NKT cell-based cancer therapies. Fourthly, our formulation strongly limited the expansion of IL-10-expressing NKT cells. A recent study showed that mice pretreated with α -GalCer are less able to reject tumors (due to the role of NKT10 cells).²⁷ Therefore, the reduced expansion of

NKT10 seen in mice vaccinated with anti-Clec9a Ab-bearing NPs might contribute to the observed antitumor effects. Overall, there are many reasons for co-delivering protein antigen and α -GalCer to cross-priming DCs. In view of the promising proof-of-concept results, we considered that it was important to analyze the co-delivery strategy's potential added value in a human setting.

Given that the NKT/CD1d axis is conserved in humans (with no HLA restriction), NKT cell therapy has attracted interest. Adoptive transfer of *in-vitro*-expanded, tumor-specific CD8⁺ T cells is an interesting immunotherapeutic option. Hence, the NKT cells' licensing function might be worth exploiting (either alone or in combination with vaccine strategies) in cell-based allogeneic immunotherapy. Targeting antigens to human BDCA3⁺ DCs via the CLEC9A endocytic pathway favors antigen cross-presentation and the priming of CD8⁺ T cell responses.²⁵ To assess the targeting properties of anti-CLEC9A Ab-armed NPs, we studied DCs expanded from cord blood haematopoietic progenitor cells. Under our experimental conditions, the *ex vivo* generated DCs resembled blood DCs.⁴² The NP formulation (i) specifically targets cross-priming, CLEC9A-expressing DCs, and (ii) triggers a strong NKT cell response. Targeted α -GalCer not only improves the *in vitro* expansion of human NKT cells (data not shown) but also enhances their cytokine response relative to experiments with free α -GalCer or control NPs. Finally, using the clinically relevant tumor antigen Melan A, we show that NKT cell-licensed human DCs trigger the expansion of functional Melan A-specific CD8⁺ T cells both in healthy donors and - more importantly - in melanoma patients. To the best of our knowledge, our study is the first to have implemented Semmling et al.'s concept in a human system. An *in vivo* demonstration will require the use of humanized mice. Relative to cancer-free individuals, melanoma patients have lower NKT cell counts and/or impaired NKT cell functions.^{30,31} We showed that targeted α -GalCer strongly expands NKT cells from melanoma patients. Taken as a whole, our results suggest that this NP-based formulation may have promise in the treatment of cancer. One concern with the clinical implementing of this type of vaccine is the relatively low NKT cell count in humans (relative to mice). Various means of circumventing this potential drawback are being developed, and the transfer of autologous, *ex-vivo*-expanded NKT cells appears to be a reliable strategy for enhancing anticancer strategies based on NKT cells.⁴³ Moreover, the reprogramming of NKT cells to induced, pluripotent stem cells and their subsequent re-differentiation into more functional NKT cells (compared with the parental cells) is opening up new avenues in this field.^{44,45}

In conclusion, we have developed an experimental strategy that emulates NKT cell help and analyze the corresponding formulation's effect on the expansion of tumor antigen CD8⁺ T cells (in mouse and human systems) and the control of tumor development. With regard to improving vaccine-based cancer treatments, our data highlight the potential benefits of delivering tumor antigens and α -GalCer into cross-presenting DCs. The present preclinical results constitute the first proof of concept for the clinical use of this NP delivery system in cancer immunotherapy.

Materials and methods

Mice

Male C57BL/6 mice (8-week old) were purchased from Janvier (France). Rag2/OT-I transgenic mice (enriched in OVA_{257–264}-specific CD8⁺ T cells) were from Jackson Laboratory (France). The generation of *Cd1d*^{-/-}, *Ja18*^{-/-} (which lacks NKT cells) and *Baft3*^{-/-} (which have a strongly reduced proportion of CD8 α ⁺ DCs) mice was described in.^{46–48} The IL-10- β -lactamase reporter mouse (the ITIB mouse) was described in.⁴⁹ All animal work conformed with the Lille Pasteur Institute's regulations on animal care and use guidelines and was approved by the local Animal Care Committee (CEAA75 and 00357.03).

Reagents and peptides

α -GalCer was synthesized as described.⁹ OVA was purchased from InvoGen (France), vybrant CFDA SE Cell Tracer Kit from Invitrogen (France) and the PKH26 Red Fluorescent Cell Linker Kit from Sigma Aldrich (France). All cytokines for expansion and differentiation assays were from Peprotech (France). The following peptides were synthesized to a purity >95–99% by high-performance liquid chromatography (GL Biochem, China): SIINFEKL, spanning amino acids 257–264 of the mouse OVA; SVYDFVWL, spanning amino acids 180–188 of the mouse melanoma antigen tyrosinase related protein-2 (Trp2); KVPRNQDWL, spanning amino acids 25–33 of the human melanoma glycoprotein 100 (gp100) and HSYTTAEELAGIGILTV, spanning amino acids 18–35 of the human Melan A.

Description of Abs, tetramers and dextramers

Allophycocyanin-conjugated mAbs against mouse Siglec H, PE-Cy7 conjugated CD11c, anti-NK1.1, anti-PD1, anti V α 2-TCR, FITC-conjugated anti-TCR β , anti-CD8 α , CD172 α , PerCp-CY5.5-conjugated CD24, Alexa Fluor647-conjugated IFN- γ and isotype control, Pacific blue-conjugated I-A/I-E, PE-conjugated anti-CD8 α , anti-Clec9a (also termed DNGR-1) were all purchased from BD Biosciences (France) or Ozyme/Biolegend (France). Antibodies used to analyze CD45⁺-enriched tumor cells were as follows: CD3-APCCy7, CD8-Alexa Fluor 700, CD4-Pacific blue, CD25-FITC, FoxP3-PeCy7, perforin-APC (Biolegend or eBiosciences). Mouse anti-human Abs were as follows: APCCy7-conjugated HLA-DR, AF700-conjugated CD11c and PercpCy5.5 anti-NKT (clone 6B11), FITC anti-human Lineage Cocktail were from Biolegend. V500-conjugated CD8, AF700-conjugated CD3, AF647-conjugated CCR7, FITC-conjugated CD3, V450-conjugated (CD3, CD14, CD19, CD20, CD56), FITC-conjugated IFN- γ , APC-H7-conjugated CD3, V421-conjugated HLA-DR, PECy7-conjugated CD45RA and FITC-conjugated CCR7 were all purchased from BD Biosciences. APC-conjugated BDCA1, PE-conjugated CLEC9A, FITC-conjugated BDCA3 and PE-conjugated anti-NKT (clone 6B11), APC-conjugated BDCA3 were from Miltenyi Biotec (France). PE-conjugated dextramer H-2K^b/Trp2_{180–188} and dextramer A2/Melan A_{26–35} were from Immudex (Denmark). PE-conjugated α -GalCer-loaded CD1d tetramer was from the National Institute

of Allergy and Infectious Diseases Tetramer Facility. Flow cytometric analyses were performed with a Fortessa cytometer (BD Biosciences) and the acquired data were processed using FlowJo software (Tree Star Inc.). Pure anti-mouse Clec9a (clone 7H11), anti-human CLEC9A (clone 8F9) and isotype rat IgG1 (clone HRPN) and mouse IgG2a (clone C1.18.4) used for the NP formulation were from Miltenyi Biotec and from Bio X Cell (USA), respectively. The neutralizing rat anti-mouse CD8 β Ab (YTS-156.7, IgG2b) was described in⁵⁰ and the anti-mouse CD4 Ab (GK1.5, IgG2b) was from Dr L. Fend (Transgene, France). The isotype controls were from Bio X Cell.

Preparation and characterization of PLGA-based NPs

Poly(lactic-co-glycolic acid) (PLGA)-based NPs coated with lipid-polyethylene glycol and carrying Abs were generated using the copolymer PLGA essentially as described.^{9,51,52,44} In brief, endotoxin-free full OVA (5 mg) or tumor peptides (20 mg) and α -GalCer (50 μ g) were co-encapsulated to 100 mg of PLGA. Mouse or human anti-Clec9A/CLEC9A Abs or their isotype controls were attached to the lipid-polyethylene glycol layer.^{9,51,52} PLGA-based NPs were characterized by dynamic light scattering and zeta potential (see details in Table 1).^{9,51} Incorporation of OVA or peptides in NPs was quantified by Coomassie dye protein assay (Thermo Fischer scientific, USA) or by reversed-phase high-performance liquid chromatography.⁵³ The presence of Abs on the particle surface was quantified by Coomassie dye protein assay.

Cell lines and mouse hybridomas

EG7 and B16F10 cells were obtained from the American type culture collection. The V α 14/V β 8.2⁺ NKT cell hybridoma DN32.D3 was from A. Bendelac (USA). The mouse T cells hybridoma 55 α / β cells transfected with a human invariant V α 24/V β 11 TCR (which recognizes α -GalCer presented by human CD1d) was described by Thedrez and colleagues.⁵⁴

In vitro validation of the NPs

Spleen cells were incubated with grading doses of free or encapsulated α -GalCer plus OVA for 48 hours. Bone marrow-derived DCs (BM-DCs) generated in the presence of fms-like tyrosine kinase 3 ligand were incubated during 2 hours with free or vectorized α -GalCer (1 \times 10⁵/well) in the presence or absence of anti-Clec9a or anti-IgG1 (25 μ g/ml). After washes, NKT hybridoma (DN32.D3, 1 \times 10⁵ cells/well) were added to pulsed BM-DCs for 24 hours.

Assessment of primary and secondary NKT cell activation in vivo

Mice were administrated intravenously (i.v.) with different doses of free or vectorized α -GalCer. Three hours later, sera were collected for IFN- γ quantification and spleen cells were assessed for IFN- γ intracellular staining by flow cytometry. To study NKT cell hyporesponsiveness, mice received a second challenge of vectorized α -GalCer (NP/ α -GalCer/OVA/Clec9a) 7 d after the primo-activation. To quantify the frequency of IL-10-producing NKT cells, ITIB (IL-10-reporter) mice were i.v.

administrated with free or vectorized α -GalCer and one month later the expression of β -lactamase (IL-10) by NKT cells was assessed by flow cytometry.⁴⁹ Briefly, spleen cells were resuspended in PBS containing CCF4-AM substrate (1 μ M) supplemented with probenecid (2.5 mM), and then incubated in the dark for 45 min at room temperature. The cells were then washed, labeled with appropriate Abs, α -GalCer-loaded CD1d tetramer and propidium iodide and analyzed by flow cytometry.

Analysis of the CD8⁺ T cell and anti-tumor response to OVA or OVA-expressing tumor cells

To assess the *in vivo* OVA-specific CD8⁺ T cell response, CFSE-labeled Rag2/OT-I spleen cells (2×10^7) were adoptively transferred (i.v.) to naïve C57BL/6 mice at day 0. One day later, mice were immunized into the footpads with free or vectorized OVA plus α -GalCer (200 ng OVA and 5ng α -GalCer/mouse). The frequency of CFSE-labeled OVA-specific CD8⁺ T cells were analyzed in popliteal lymph nodes (LNs) and spleen cells at day 4 by flow cytometry. To this end, cells were stained with anti-CD8-PE and anti-TCRV α 2-PECy7 Abs. For the *in vivo* CTL assay, 6 d after immunization with NPs, mice were i.v injected with both CFSE-labeled SIINFEKL-pulsed splenocytes and PKH-26-labeled non pulsed splenocytes (2×10^7 cells/mouse). Two days later, spleen cells were harvested and the number of CFSE-labeled and PKH-26-labeled cells were determined by flow cytometry. The percentage of specific lysis was calculated as follows: $(1 - \{\text{ratio non pulsed}/\text{ratio pulsed}\}) \times 100$, where the ratio is equal to number of PKH-26-labeled cells/CFSE-labeled cells. Mice were s.c. injected with free or vectorized OVA and α -GalCer. Seven days later, mice received s.c. OVA-expressing EL-4 lymphoma cells (EG7 cells, 2×10^5 cells/mouse). In the therapeutic context, mice were first reconstituted (i.v.) with Rag2/OT-I cells (5×10^6 spleen cells/mouse) and then inoculated with EG7 cells. Mice were immunized with free or vectorized OVA and α -GalCer 1 day and 5 d after the inoculation of EG7 cells. Tumor volume (mm³) was calculated using the formula: length (mm) \times (width² (mm²) \times 3.14/6. For ethical reasons, mice were sacrificed before tumor size reached 1500 mm³.

Analysis of the CD8⁺ T cell response to self-antigens and assessment of anti-tumor responses

To analyze the immune response to tumor self-antigens, the formulations were injected subcutaneously (s.c.) in the back of the mouse 3 times with an interval of 7d. Three days after the last injection, cytokine production by skin-draining lymph node (LN) cells and/or splenocytes was quantified after restimulation with Trp2 and gp100 peptides (10 μ g/ml). To study the effect of the vaccine on the anti-tumor response, mice were injected twice (day 0 and day 7) and were implanted with 5×10^5 B16F10 cells at day 14. For the therapeutic setting, mice were injected with 5×10^5 B16F10 cells (day 0) before the 2 immunizations (day 5 and day 12). Tumor size was measured using a caliper every 2 d. Infiltration of T cells in tumors was analyzed at day 14 (therapeutic protocol). Briefly, single cell suspensions from tumors were enriched for CD45⁺ cells using the multiMACS separation

system (Miltenyi Biotec). Cells were stained with the PE-conjugated dextramer H-2K^b/Trp2₁₈₀₋₁₈₈ at RT for 30 min and then surface stained for CD3 and CD8. For regulatory CD4⁺ T cell labeling, tumor cells were surface stained for CD3, CD4 and CD25 and fixed, permeabilized and labeled with anti-Foxp3 Ab.

Generation of BDCA-3⁺ CLEC9A⁺ DCs and NKT cells

Cord blood haematopoietic progenitor CD34⁺ cells were from ABCell-Bio (Paris, France) or from the EFS Grenoble. To amplify haematopoietic progenitor cells, cells were cultured in the stemspan medium in the presence of fms-like tyrosine kinase 3 ligand (100ng/ml), stem cell factor (100ng/ml), IL-3 (20ng/ml) and thrombopoietin (50ng/ml) for one week. To induce differentiation of the expanded cells into DCs, cells were cultured in complete RPMI 1640 medium (round-bottom 96 well plate, $1-2 \times 10^4$ cells/well) containing stem cell factor (20ng/ml), granulocyte-macrophage colony-stimulating factor (20ng/ml), IL-4 (20ng/ml) and fms-like tyrosine kinase 3 ligand (100ng/ml). To optimize the differentiation step and to increase the yield of DCs, 1mM stemregenin-1 (Calbiochem, France) was added.⁴² The milieu plus differentiation factors were renewed after 5 d. Dendritic cells were harvested between day 10 and day 14. NKT cells were expanded from human PBMCs. Briefly, free or vectorized α -GalCer (10ng/ml) was added at day 0 and IL-2 (50ng/ml) at day 1. At day 7, the percentage of NKT cells (CD3⁺ 6B11⁺ cells) was determined by flow cytometry. To enrich NKT cells, cells positive for 6B11 (a mAb that recognizes a unique determinant in the CDR3 region of the invariant (V α 24-J α Q) TCR chain of NKT cells) were selected with immune-magnetic beads according to the manufacturer's instructions (MACS separator, Miltenyi Biotec).

Targeting of BDCA-3⁺ CLEC9A⁺ DCs and co-cultures with NKT cells

To study the specificity of the targeting, total DCs (1×10^5 cells/well) were incubated with Alexa Fluor647-labeled NPs. After 15 minutes, the frequency of Alexa Fluor647 positive cells was analyzed within the different DC subpopulations by flow cytometry. To study NKT cell activation, total DCs were pulsed with free or vectorized α -GalCer and co-cultured for 24 or 48 hours with NKT hybridoma or with primary NKT cells (purity >95%) (ratio 1:5).

Origin of PBMCs

Blood samples from healthy donors and from melanoma patients (stage I to IV) were obtained from the EFS Grenoble. Approval to conduct the study was given by the local ethics committee of Grenoble University Hospital. The procedures are in accordance with the Helsinki Declaration of 1975 and all participants gave their written consent. PBMC samples were obtained via standard protocols.

Expansion of Melan A₂₆₋₃₅-specific CD8⁺ T cells

To expand Melan A₂₆₋₃₅-specific CD8⁺ T cells, PBMCs (2×10^6 cells/well) from HLA-A2⁺ individuals were cultured with

free or vectorized Melan A_{18–35} peptide (0.9 μg/ml) plus α-GalCer (10 ng/ml) in the presence of IL-2 (50 ng/ml) for 14 d. Dextramer A2/Melan A_{26–35}-specific cells were analyzed by flow cytometry. To analyze IFN-γ and CD107 expression, cells were first labeled with PE-conjugated dextramer A2/Melan A_{26–35} washed and restimulated with Melan A peptide-pulsed TAP-deficient T2 cells (10:1 ratio) for 5 hours. Cells were then surface-labeled with anti-CD3 and anti-CD8 Abs and submitted to IFN-γ intracellular staining. For CD107 detection, anti-CD107a+b Abs were added in the medium at the beginning of the restimulation in the presence of Golgi-STOP for 4 hours. Cells were then labeled with anti-CD3 and anti-CD8 Abs.

Statistical analysis

Data are expressed as the mean ± SEM. A Mann-Whitney U test was used to compare 2 groups, unless otherwise specified. Comparisons of more than 2 groups with each other were analyzed by one-way analysis of variance (ANOVA) Kruskal-Wallis test (nonparametric), followed by Dunn's post-test (PRISM v6 software; Graphpad). For tumor size, means of "area under the curve" of each individual were compared using a Mann-Whitney U test unless otherwise specified. A *P* value <0.05 was considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Drs A. Bendelac (University of Chicago, IL) and M. Casorati and M. Della-bona (San Raffaele scientific institute, Milan, Italy) are acknowledged for the gift of the NKT cell hybridoma DN32.D3 and 55αβ, respectively. We are grateful to Drs H. Bouabe (Babraham Institute, Cambridge, United Kingdom) and T. Torrès (ULB, Brussels, Belgium) for the gift of ITIB and *Baft3*^{-/-} mice. Dr L. Fend (Transgene, Strasbourg, France) and Drs S. Cobbold and A. Kok (Oxford, United Kingdom) are acknowledged for the gift of the neutralizing anti-mouse CD4 and CD8β Ab, respectively. We gratefully acknowledge the generous support from the NIAID Tetramer Facility (Emory University, Atlanta, GA) in supplying α-GalCer-loaded CD1d tetramer. Dr L. Poulin (CIIL, Institut Pasteur de Lille, France) is acknowledged for its advices on the expansion of human DCs.

Funding

This work was supported by Institut National du Cancer (INCa) grant INCA_6699.

ORCID

Fabrice Soncin  <http://orcid.org/0000-0001-6312-0673>
François Trottein  <http://orcid.org/0000-0003-3373-1814>

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