

Enhanced immunogenicity of mitochondrial localised proteins in cancer cells

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Abstract

Epitopes derived from mutated cancer proteins elicit strong anti-tumor T cell responses that correlate with clinical efficacy in a proportion of patients. However, it remains unclear whether the sub-cellular localisation of mutated proteins influences the efficiency of T cell priming. To address this question we compared the immunogenicity of NY-ESO-1 and OVA localised either in the cytosol or in mitochondria. We showed that tumors expressing mitochondrial localised NY-ESO-1 and OVA proteins elicit significantly higher frequencies of antigen specific CD8⁺ T cells *in vivo*. We also demonstrated that such stronger immune response is dependent on the mitochondrial location of the antigenic proteins, which contributes to their higher steady state amount. Consistent with these findings, we showed that injection of mitochondria purified from B16 melanoma cells can protect mice from a challenge with B16 cells, but not with irrelevant tumors. Finally, we extended these findings to cancer patients by demonstrating the presence of T cell responses specific for mutated mitochondrial localised proteins.

These findings highlight the importance of prioritising epitopes derived from mitochondrial localised mutated proteins as targets for cancer vaccination strategies.

Introduction

T cell responses against human cancers contribute to the control of tumor growth, and targeting of CTLA-4 and the PD-1/PD-L1 axis has been very effective in enhancing anti-tumor immune responses, resulting in clinical objective responses [1, 2], particularly in patients with tumors expressing high mutational burden (high TMB) [1, 2]. These results underscore an unmet clinical need for many cancer patients with low TMB (i.e. the largest proportion of cancer patients), who could receive greater benefit from immune checkpoint inhibition treatment, should optimal neo-epitopes be identified and used in vaccination strategies.

To this end, new strategies need to be developed to identify the most immunogenic cancer-associated neo-epitopes and optimal vaccine platforms to improve immune responses to such predicted epitopes. Current pipelines employed for neoantigen prediction have resulted in a low rate of validation, suggesting that the determinants of peptide immunogenicity are still suboptimal [3-5]. A greater understanding of the biology of the presentation of the cancer mutanome is thus needed in order to improve such algorithms. Several parameters are currently considered when ranking the immunogenicity of mutations in cancer cells [6-8], but it remains unclear whether the subcellular localisation of tumor antigens can modulate the efficiency of priming of anti-tumor specific immune responses and whether such parameter should be considered in algorithms ranking immunogenicity of mutated peptides in cancer cells. This represents a critical knowledge gap, as current prognostic scores for responsiveness to immune checkpoint inhibitors are mainly based on the numbers of mutations, without taking into account whether the sub-cellular localisation of such mutated protein antigens may influence their ability to mount an immune response.

Tumor cells fail to directly prime tumor specific immune responses, likely as a result of low costimulation; instead, DCs function simultaneously as both antigen-presenting cells, capable of taking up tumor debris, and IL-12-producing cells, in a process referred to as 'cross-priming' [9, 10]. Consistent with a role of DC in cross-priming tumor specific T cell responses, recent results have demonstrated the transfer of cellular components from tumor cells to antigen presenting cells [9, 11], and in particular it has been shown that mitochondria can be transferred from tumors to DCs, via cytoplasts [11], raising the possibility that mutations contained within mitochondrial localised proteins in cancer cells could elicit

specific strong T cell responses *in vivo*. Consistent with this possibility, it has been shown that activation of the cGAS-STING pathway in DCs can be driven by tumor mitochondrial DNA, resulting in the induction of a type I IFN response [12]. All these results draw attention to the possible role of tumor-derived mitochondria, and mitochondrial associated antigens, in the generation of tumor specific immune responses.

The mitochondrial DNA only encodes for 13 proteins, which serve as subunits of the respiratory complexes [13]. Overall, mitochondria contain more than 1,200 proteins that are encoded by nuclear DNA and targeted to mitochondria via the expression of specific signalling motifs [14]. In view of the observation that mutations in proteins encoded by mitochondrial genes can lead to the generation of CD8⁺ T cell responses [15, 16], analysis of the antigenicity of the subset of nuclear DNA encoded proteins that are localised in mitochondria warrants further attention.

Previous studies have described the effect of subcellular localisation of protein antigens on direct presentation of T cell epitopes [17-20]. Furthermore, Yamazaki and colleagues have previously demonstrated that different portions of the antigenic protein LIVAT-BP are selected as antigenic epitopes for CD8⁺ T recognition depending on whether LIVAT-BP is located in the cytoplasm or in the mitochondria [20]. While these results are of interest, this paper falls short of distinguishing whether antigen location modulates: i) *in vivo* cross-priming of T cell responses; ii) proteasome-dependent degradation of the antigenic protein; and iii) tumor growth.

Here, we have investigated both *in vitro* and *in vivo* the impact of the mitochondrial location of antigenic proteins on direct- and on cross-priming, as compared with the immunogenicity of the same antigenic proteins expressed in the cytosol. We have extended results obtained in animal models to human samples by demonstrating the presence of CD8⁺ T cell responses specific to mitochondrial localized neoantigens in a cancer patient. Our results provide insights into the ability of mitochondrial localized proteins to induce greater immune responses than proteins localized in the cytosol and provide a rationale for including knowledge of whether or not mutated proteins are localised within mitochondria to improve prediction algorithms for neoepitopes that are effective in priming immune responses.

Material and methods

Mice

STING knockout [21], cGAS knockout [22], MAVS knockout [23], Myd88 knockout, MARCO/SRA knockout, Beta-2-microglobulin knockout and C57BL/6 control mice were bred in the local animal facility under specific pathogen-free conditions and used at 6–10 weeks of age. For tumor immunogenicity experiments, mice were injected subcutaneously with 1×10^6 cells/mouse. For experiments regarding tumor growth, mice were injected subcutaneously with 1.5×10^5 cells/mouse. Animal studies have been conducted in accordance with, and with the approval of the United Kingdom Home Office. All procedures were done under the authority of the appropriate personal and project licenses issued by the United Kingdom Home Office Licence number PBA43A2E4.

Cloning and cell lines

Using standard cloning protocols (supplementary methods), the genes encoding cytosolic NY-ESO-1 and OVA₄₇₋₃₈₆ were fused with an N-terminal HA tag. Mitochondria targeting sequence from human Arg2₁₋₂₂ was fused to their N-terminus to create mitochondrial versions of the proteins. A stable form of the cytosolic OVA₄₇₋₃₈₆ was created by inserting 9 amino acids (MPKKKRVGG) at the N-terminus. This protein was shown experimentally to be cytoplasmic and stable. All of these constructs were inserted into lentiviral vectors which co-translate eGFP protein that is downstream an IRES sequence and used to transduce CT26-, B16f10- and Lewis-lung carcinoma cells. A lentiviral vector containing mitochondrial OVA₄₇₋₃₈₆ protein fused downstream with mCherry protein was used to transduce B16f10 cells.

In vitro and *ex vivo* staining of B16 cell lines

B16 cytoOVA, B16 mtOVA and B16 WT were incubated with IFN γ , TNF α or both (5 ng/ml, Biolegend) for 2 or 5 days and then stained with the anti H-2K^b-OVA₂₅₇₋₂₆₄ antibody ([24]). Stimulated cells were co-cultured with OT-I CD8⁺ T cells (ratio 1:40) overnight and OT-I stained for extracellular markers (CD8 α , CD69, CD25) and viability stain (antibody clones are reported in **Supplementary Table 1**). C57BL/6 mice were injected with B16 mtOVA or B16 cytoOVA and B16 WT. Tumors were collected, pierced and torn in 24-well plates and incubated for 15 min at 37 C in 1 ml of digestion buffer (2 mg/ml collagenase D, and 1

mg/ml DNase I in RPMI-1640 (both from Sigma-Aldrich). After the first 15 minutes of incubation, cells were pipetted up and down repeatedly, then returned for a second 15-min incubation at 37 C. After digestion, B16 cells were stained with anti-H-2K^b-OVA₂₅₇₋₂₆₄ and anti-CD105 antibodies. Samples were acquired on a FACScanto II (BD Biosciences) Flow cytometer and data analysed with Flowjo version 10.4.1.

***Ex vivo* peptide restimulation assay (ICS)**

Splenocytes (2×10^6) were isolated from either naive or tumor bearing C57BL/6 mice 7 days after the injection, and were cultured in presence of OVA₂₅₇₋₂₆₄ peptide (SIINFEKL, Sigma Aldrich) or NY-ESO-1₁₅₇₋₁₆₅ peptide (SLLMWITQC, Sigma Aldrich) peptide in complete RPMI 1,640 medium supplemented with 10% FBS, 2.1 mM ultra-glutamine in presence of brefeldin A and monensin (Biolegend). After 5 hours of incubation cell were stained for extracellular markers (CD8 α , B220, CD44, CD69, CD25) and viability stain (antibody clones are reorted in **Supplementary Table 1**). Cells were then stained for intracellular IFN γ and TNF α using a Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to manufacturer instructions.

Depletion of CD8⁺ T cells

Mice were injected on day 0 and day 2 with 400 μ g/mouse of anti CD8 antibody intraperitoneally (anti-CD8 clone 2.43 or Isotype control (both from Invivomab)). On day 2 mice were injected with the B16 tumor cells subcutaneously (1.5×10^5 cells/mouse), and were then injected every 6 days with 200 μ g/mouse of anti-CD8 antibody intraperitoneally. The depletion of CD8 T cells was confirmed in the blood on day 6 and in the spleen on day 14 after the mice were sacrificed.

Vaccination of mice with isolated mitochondria

Mitochondria from B16 tumor cells or from mouse adult fibroblasts were isolated as previously described [25]. Mice were injected with 50 micrograms of the mitochondrial protein samples. On day 10 after vaccination mice were challenged with B16 or LLC tumor cell lines and the tumor growth was monitored. In some experiments CD8⁺ T cells were depleted before tumor injection. CD8⁺ T cells response was evaluated 14 days after tumor injection: CD8⁺ T cells were isolated from mice spleens with CD8 isolation kit (Miltenyi biotech) and co-cultured overnight with B16 cells that had been previously stimulated 48

hours with IFN γ (10 ng/ml). Brefeldin A and monensin were added for the last 5 hours of co culture and cells were stained for intracellular IFN γ using a Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to manufacturer instructions. Samples were acquired on a Fortessa X50 (BD Biosciences) Flow cytometer and data analysed with Flowjo version 10.4.1.

Results

Cross-priming of CD8⁺ T cells specific for OVA and NY-ESO-1 proteins is enhanced by their targeting to mitochondria

To investigate whether mitochondrial proteins contained within tumor cells could be transferred to antigen presenting cells (APCs) during tumor growth *in vivo*, B16 cells were transduced with lentiviral vectors encoding mitochondrial localised ovalbumin (OVA) fused with the fluorescent protein mCherry (B16 mtOVA-mCherry), which were then injected subcutaneously (SC) in mice. The results of these experiments showed that CD103⁺ and CD11b⁺ migratory DCs had preferentially taken up mCherry protein, compared with CD8 α ⁺ DCs, CD169⁺ macrophages and CD11b⁺ resident DCs (**Supplementary Figure S1A**). In addition, uptake of mitochondrial localised mtOVA-mCherry correlated with enhanced DC maturation, as shown by higher CD86 expression on mtOVA-mCherry⁺ DCs cells compared with mtOVA-mCherry⁻ DCs (**Supplementary Figure S1 B,C**).

The uptake of mitochondrial localised ovalbumin-mCherry fusion protein by migratory DCs raised the possibility that phagocytosis of mitochondria by CD103⁺ DC may elicit cross-priming of T cells specific for antigenic mitochondrial proteins. To address this possibility, the H-2^d tumor cell line CT26 was transduced with a lentiviral vector encoding either NY-ESO-1 or OVA proteins, which were targeted either to mitochondria (hereafter referred to as CT26 mtNY-ESO-1 or CT26 mtOVA) or localised in the cytosol (hereafter referred to as and CT26 cytoNY-ESO-1 or CT26 cytoOVA) and then injected into MHC mismatched mice to assess their ability to induce cross-priming of antigen specific CD8⁺ T cells. Mitochondrial and cytosolic targeting was confirmed by confocal microscopy (**Figure 1A, Supplementary Figure S2A**).

We have previously shown that HLA-A2 transgenic HHD mice can generate HLA-A2 restricted CD8⁺ T cells upon vaccine injection [26, 27]. We therefore challenged HHD

mice with the mismatched H-2^d tumor cell line CT26 mtNY-ESO-1 and CT26 cytoNY-ESO-1 and compared the frequency of HLA-A2 restricted NY-ESO-1₁₅₇₋₁₆₅ specific T cells. Since CT26 cells do not express HLA-A2 molecules, priming of HLA-A2 restricted NY-ESO-1 specific responses is dependent on cross-presentation events controlled by HHD resident HLA-A2⁺ DCs.

Injection of CT26 mtNY-ESO-1 cells into HLA-A2⁺ HHD mice resulted in a significantly higher frequency of NY-ESO-1 specific HLA-A2 restricted CD8⁺ T cells than the injection of CT26 cyto NY-ESO-1 cells, which gave responses only marginally higher than in mice injected with wild type CT26 cells, as defined by staining with HLA-A2/NY-ESO-1₁₅₇₋₁₆₅ tetramers and intracellular staining with IFN γ and TNF α specific antibodies (**Figure 1B**).

These results were confirmed by injecting mismatched CT26 mtOVA cells into C57BL/6 mice, which generated a significantly higher frequency of H-2K^b/SIINFEKL specific CD8⁺ T cell responses compared with CT26 cytoOVA cell line (**Figure 1C**). We have also shown that CT26 mtOVA is superior than CT26 cytoOVA at inducing H-2K^b/SIINFEKL specific CD8⁺ T cell responses, independently from the level of OVA expression and induced a more efficient proliferation of adoptively transferred OT-I splenocytes (**Supplementary Figure S2C-F**).

These findings demonstrate that cross-priming of CD8⁺ T cells specific for NY-ESO-1 and OVA proteins, in HHD and B6 mice, respectively, is enhanced by the targeting of these antigens to mitochondria.

To gain further insights into the mechanisms controlling the enhanced immunogenicity of mitochondrial localised NY-ESO-1 and OVA proteins, we analysed the steady state amount of NY-ESO-1 and OVA proteins and their stability in the presence or absence of proteasome inhibitors (**Figure 1D** and **Supplementary Figure S2G**). We observed that mitochondrial localisation of NY-ESO-1 (**Figure 1D**) and OVA (**Supplementary Figure S2G**) proteins significantly enhanced their steady state amount, as cytosolic NY-ESO-1 or OVA proteins, unlike mitochondrial localised NY-ESO-1 and OVA, were degraded by proteasomes, as defined by their enhanced intensity in the presence of proteasome inhibitor.

In conclusion, these results show that relocation of NY-ESO-1 and OVA proteins from the cytosol to mitochondria increases their stability and steady state amount, due to their protection from proteasome dependent degradation, and hence enhancing their ability to be cross-presented *in vivo*.

OVA localisation in mitochondria enhances OVA specific T cell responses in syngeneic mice

Having established the enhanced immunogenicity of mitochondrially localised NY-ESO-1 and OVA proteins upon injection of tumors into mismatched mice, we decided to assess whether a similar observation could be confirmed in a syngeneic mouse model. B16 melanoma cells were transduced with lentiviral vectors encoding either mitochondrial targeted OVA (hereafter referred to as ‘B16 mtOVA’) or cytosolic OVA (hereafter referred to as ‘B16 cytoOVA’). In both cell lines the expression of OVA was linked to the expression of the reporter protein GFP. We observed that cytosolic OVA was rapidly degraded by the proteasome and that proteasome inhibition of B16 cytoOVA rescued the same amount of OVA seen in B16 mtOVA (**Figure 2A and B**). Consistent with equal OVA expression in B16 mtOVA and B16 cytoOVA cells, we showed equal amounts of OVA mRNA (**supplementary Figure S3A**) and GFP expression (**Figure 2A**). B16 mtOVA and B16 cytoOVA cells were injected into C57BL/6 mice and 7 days later the H-2K^b/SIINFEKL specific CD8⁺ T cell response was measured in the spleen. Consistent with our previous results using the mismatched CT26 cell lines, we observed that B16 mtOVA induced a higher OVA-specific CD8⁺ T cell response compared with B16 cytoOVA (**Figure 2C**).

In order to assess whether enhanced priming of the OVA response upon injection of B16 mtOVA cells was due solely to OVA’s increased stability or if the location of OVA in mitochondria had an additional role accounting for its enhanced priming ability, we generated a modified cytosolic OVA (hereafter referred to as ‘mod cytoOVA’) with an N-terminal extension of 9 amino acids (see **Material and Methods**). We showed that when mod cytoOVA was expressed in B16 cells, it resulted in significantly higher steady state levels of OVA compared with wild type cytosolic and mitochondrial OVA (**Figure 2A**) and was less sensitive to proteasomal degradation (**Figure 2A**). Fractionation of B16 mod cytoOVA cell lysates confirmed the enrichment of mod cytoOVA protein in the cytosolic fraction and not mitochondrial (**Figure 2B**). Consistent with the notion that the steady state amount of antigenic proteins determines the potency of their immunogenicity *in vivo* [17, 28, 29], we

observed that the frequency of OVA specific CD8⁺ T cell response following injection of B16 mod cytoOVA was significantly greater than in B6 mice injected with B16 cytoOVA. However, despite the larger amount of steady state OVA in B16 mod cytoOVA cells, priming of OVA specific CD8⁺ T cell response in mice injected with B16 mtOVA was more efficient than in mice primed with B16 mod cytoOVA cells, suggesting that the localisation of OVA into mitochondria confers a distinct priming advantage in addition to its increased steady state amount.

Superior OVA₂₅₇₋₂₆₄ specific T cell priming ability of B16 mtOVA cells over B16 cytoOVA cells results in enhanced tumor control

It is well established that the rate of degradation of antigenic proteins directly correlates with the generation of MHC class I epitopes presented on the surface of antigen presenting cells [30, 31]. Consistent with this notion, the reduced stability of OVA in B16 cytoOVA correlated with a greater amount of the H-2K^b/SIINFEKL epitope generated by B16 cytoOVA both *in vitro* (**Figure 3A**) and *ex vivo* (**Figure 3B**), as defined by FACS staining with the monoclonal antibody specific for H-2K^b/SIINFEKL complex [24] and by *in vitro* T cell proliferation assays (**Figure 3C-D**). Of note, direct presentation of the SIINFEKL epitope *in vivo* on the surface of B16 mtOVA cells was detectable only after 14 days from the injection (**Figure 3B**). Up-regulation of H-2K^b/SIINFEKL complexes on the surface of B16 cells both *in vitro* and *in vivo* was dependent on cytokines stimulation, and in particular on the combined effect of IFN γ and TNF α (**Supplementary Figure S4A**). A greater amount of the H-2K^b/SIINFEKL complex was also observed on the surface of Lewis lung carcinoma cells (LLC) encoding cytoOVA than on LLC encoding mtOVA *in vitro* (**Supplementary Figure S4B**). These results demonstrated that rapid proteasome dependent degradation of cytoplasmic OVA in B16 cells was very efficient in directly presenting the H-2 K^b/SIINFEKL epitope, which resulted in greater activation of SIINFEKL specific CD8⁺ T cells, as compared with mitochondrial localised OVA and more stable cytosolic OVA in B16 cells.

The findings from the above *in vitro* experiments (**Figure 3A,B, C and D**) combined with the results from the *in vivo* immunogenicity experiments (**Figure 2C**), together with previously published studies [28-30], show an inverse relationship between efficient direct antigen presentation of tumor cells and their ability to efficiently prime *in vivo* antigen specific immune response. Since both of these processes are required to generate an efficient

tumor specific immune response capable of controlling tumor growth, we monitored the rate of B16 growth *in vivo* and demonstrated a slower growth of B16 mtOVA than B16 cytoOVA cells (**Figure 3E**), which was dependent on the presence of CD8⁺ T cells (**Figure 3F**). These findings were further confirmed by the observation that B16 mtOVA tumors grew unhindered in β -2 microglobulin knockout mice (**Figure 3G**). As a control, we showed that both tumor cell lines had a similar *in vitro* growth rate (**Supplementary Figure S3B**).

In conclusion, these results demonstrate that direct presentation of the H-2K^b/SIINFEKL epitope by B16 cells is much more efficient in cells expressing cytoplasmic OVA than mitochondrial OVA, but the amount of the directly presented SIINFEKL peptide on the surface of B16 mtOVA *in vivo* is sufficient for optimal tumor control.

The cGAS-STING pathway contributes to the enhanced immunogenicity of B16 cells encoding mitochondrial OVA

Previous studies have demonstrated that the efficiency of priming of tumor specific T cell responses is enhanced by the release of DNA from tumor cells, which acts as a natural adjuvant to activate the STING pathway and induce a type I IFN response [32, 33]. We assessed the role of STING and cGAS in the enhanced immunogenicity of mtOVA B16 cells. We observed a statistical significant reduction of the frequency of H-2K^b/SIINFEKL CD8⁺ T cells in STING knockout mice injected with B16 mtOVA cells as compared with wild type mice (**Figure 4 A**), while no differences observed after the injection of B16 cytoOVA (**Figure 4B**). Similar results were obtained using cGAS knockout mice (**Figure 4C**). As a control, vaccination of cGAS and STING knockout mice with adjuvanted full length OVA protein resulted in a similar frequency of H-2K^b/SIINFEKL CD8⁺ T cells as in wild type mice (**Supplementary figures S5 A-B**). No significant reduction of OVA specific T cell responses was observed in mice lacking expression of Myd88, NLRP3 or MAVS injected B16 mtOVA cells (**Figure 4D**).

In order to further assess the identity of cross-presenting DC responsible for the cross-priming of OVA specific CD8⁺ T cells, we investigated the immunogenicity of mitochondrial localised OVA in BATF3 knockout mice, in which the development of CD103⁺/CD8 α cross-presenting DCs is compromised [34]. Consistent with our earlier observations demonstrating that mtOVA-mCherry is taken up by CD103⁺ DCs (**Supplementary Fig. S1**), injection of B16 mtOVA into BATF3 knockout mice failed to

elicit detectable H-2K^b/SIINFEKL specific CD8⁺ T cell responses as compared with the response seen in wild type C57BL/6 mice, pointing towards a fundamental role of cross-presenting DCs (**Figure 4E**). Next, we sought to identify the receptor involved in the uptake of mtOVA cells, however both in mice lacking the F-actin receptor DNGR-1[35] or in double knockout mice, lacking the expression of both the collagen scavenger receptor MARCO and of the scavenger receptor A (SRA)[36], we observed B16 mtOVA elicited H-2K^b/SIINFEKL specific CD8⁺ T cell responses comparable with the one observed in wild type mice (**Figure 4E and Supplementary Figure S3C**).

These results demonstrate an important role of the cGAS-STING pathway in contributing to the enhanced immunogenicity of B16 mtOVA cells. In addition these findings demonstrate that priming of H-2K^b/SIINFEKL specific CD8⁺ T cells by the syngeneic B16 mtOVA requires BATF3⁺ DCs, but it is not reduced by lack of DNGR-1, MARCO and SRA receptors.

Vaccination of naïve B6 mice with mitochondria from B16 cells elicits protective CD8⁺ T cell responses

Since mitochondria express more than 1,200 proteins, some of which are known to be mutated from germline sequences [37], we decided to assess whether mitochondria purified from wild type B16 cells (i.e. not expressing OVA) could prime B16 specific immune CD8⁺ T cell responses. To address this hypothesis, mitochondria were purified from B16 cells or from mouse adult fibroblasts (MAFs) from C57BL/6 mice, and were then injected SC into wild type C57BL/6 mice. Purity of the mitochondrial preparation was assessed by western blot, demonstrating an enrichment of the mitochondrial protein TOM20 (the mitochondrial outer membrane translocase) and ATP-B (the mitochondrial inner membrane ATP synthase subunit) in the mitochondrial fraction, while cytosolic GAPDH was almost absent (**Supplementary Figure S6 A-B**). Vaccinated or mock treated mice were then challenged subcutaneously with B16 tumor cells 12 days after vaccination. We observed that vaccination with B16-derived mitochondria, but not with C57BL/6 MAF-derived mitochondria, produced a response capable of controlling tumor growth (**Figure 5A, Supplementary Figure S7A**). This effect was tumor specific, as mice vaccinated with mitochondria isolated from B16 cells and then challenged with the syngeneic, but unrelated Lewis lung carcinoma, failed to control Lewis lung carcinoma growth (**Figure 5B, Supplementary Figure S7B**). Depletion of CD8⁺ T cells resulted in enhanced tumor growth without differences between groups vaccinated

with B16 mitochondria and the control group, showing the importance of CD8⁺ T cell response in this model (**Figure 5C**, **Supplementary Figure S7C**).

To further demonstrate the ability of the CD8⁺ T cells from mitochondria vaccinated mice to recognize *in vitro* B16 cells, mice were vaccinated or mock treated with purified B16 mitochondria and then challenged with B16 cells. Splenocytes from these mice were then co-cultured *in vitro* overnight with B16 cells. As a control, splenocytes from naïve mice were used. The results of these experiments demonstrated that CD8⁺ T cells isolated from mice vaccinated with B16 mitochondria were able to produce an increased frequency of B16-specific CD8⁺ T cell response compared with CD8⁺ T cells isolated from the other two groups (**Figure 5D**).

In conclusion, these results indicate that B16 derived mitochondria are a source of tumor antigens capable of inducing protective CD8⁺ T cell responses *in vivo*.

Identification of CD8⁺ cytotoxic T cell clones specific for mitochondrial localised neoantigens in cancer patients

Finally, we extended our studies to humans by investigating the presence of CD8⁺ T cells specific for mutated mitochondrial localised proteins in human cancers. The frequencies of non-synonymous somatic mutations in mitochondrial proteins that are expressed by genomic DNA from 9,508 samples across 31 cancer types from The Cancer Genome Atlas (TCGA, version 02-04-2018) were investigated. We observed the presence of mutations of mitochondrial localised proteins across different tumor types, with the highest average frequency present in endometrioid cancers (**Figure 6A**). We therefore focused our studies on endometrial cancer patients, and studied the immune response in one patient, with a hypermutated phenotype caused by the loss of function of the proof reading DNA polymerase epsilon (POLE) [38].

By comparing the sequences obtained from tumor and germline DNA, tumor-specific nonsynonymous single-nucleotide variations (SNV) were identified. RNA sequencing was also performed to check the expression of potential neoepitopes. A computational pipeline was used to examine predicted mutant peptide regions for binding to the patient's HLA allele

HLA-A*02:01. We then divided prioritised epitopes based on their mitochondrial or non-mitochondrial localisation and synthesized 60 peptides for each location group. Patient's PBMC were stimulated and expanded in the presence of each peptide and HLA-A2 tetramers loaded with mitochondrial and cytosolic derived peptides were used to assess the presence of neoantigen-specific T cells in the expanded PBMC. We identified CD8⁺ T cells recognising 4 neoantigens derived from 2 mitochondrial localised mutated proteins and 3 neoantigens derived from proteins localised in the cytosol (**Figure 6B and Supplementary Figure S7**), which were capable of specifically killing peptide-pulsed autologous EBV-LCLs (**Figure 6C**) and producing IFN γ (**Figure 6D**).

Discussion

Clinical results have demonstrated a significant correlation between the number of predicted HLA binding peptides derived from mutated proteins in tumor cells and the efficacy of immune checkpoint blocking antibody treatment in cancer patients [1]. Thus, our ability to improve the identification and ranking of immunogenic neoantigens is of importance to optimise the development of cancer vaccines and the effectiveness of checkpoint blockade therapies. Although several strategies are currently pursued to improve algorithms predicting immunogenicity of neoantigens [39, 40], further improvements are still required.

The results of our studies highlight the importance of considering the location of antigenic proteins in mitochondria as an additional criterion to prioritise neoantigen predictions, as we showed an increased immunogenicity of mitochondrial localised OVA and NY-ESO-1 proteins. We demonstrated that their enhanced immunogenicity is due to their increased stability and to the activation of the cGAS/STING pathway. In contrast, cytosolic localised OVA and NY-ESO-1 proteins, which are rapidly degraded by the proteasome and very efficiently directly presented by tumor cells *in vitro*, fail to induce strong antigen specific CD8⁺ T cell responses *in vivo*. These results are supported by previously published papers demonstrating a correlation between protein stability and their cross-priming abilities [17, 30]³². The more efficient cross-priming of mitochondrial localised proteins is likely to be due to the efficient uptake of mitochondria by cross-priming CD103⁺ DCs compared with cytosolic OVA and by a combination of the higher steady state amount and enhanced DC maturation, possibly due to mitochondrial DNA.

The rate at which antigenic proteins are degraded in cross-presenting DC is also a determining factor in controlling the immunogenicity of cross-presented antigens [35, 41], which can be modulated by several factors, including the rate at which antigenic proteins exit from the endosomes/lysosomes to the cytosol in cross-presenting DCs [42], the expression levels of lysosomal proteases [43, 44], and the pH within lysosomes [43, 45, 46], which was shown to be higher in the lysosomes of DC and of importance to improve cross-presentation of antigenic proteins [47]. We found that the two forms of stable OVA (i.e. mod cytoOVA and mtOVA), which were localised in different compartments of B16 cells, displayed different abilities to prime the OVA₂₅₇₋₂₆₄ specific T cell response. Although further

experiments are warranted to dissect the mechanisms controlling these results, it is tempting to speculate that the observed differential priming abilities of B16 mod cytoOVA and B16 mtOVA cells may reflect differences in their processing events in cross-presenting DCs.

Different recognition pathways have been shown to provide the relevant signals for CD8⁺ T cell priming, including extracellular uric acid generated during cell death, which may stimulate an inflammatory response, mediated by NLRP3 inflammasome activation [48]. While we did not observe any difference between wild type mice and NLRP3 deficient mice, we showed that lack of the STING/cGAS pathway significantly reduced priming of CD8⁺ T cells specific for mitochondrial localised proteins [32, 33]. In contrast, DNNGR-1, which was previously shown to be important for the cross presentation of cell associated antigens [35], appeared not to be involved in the cross-presentation of mitochondrial OVA. Previous papers have provided important insights into the mechanisms by which mitochondrial derived proteins may intersect the antigen processing and presentation pathway [19, 20]. However, these papers fail to assess whether mitochondrial antigenic proteins can be efficiently taken up by DCs *in vivo* and cross-presented to antigen specific CD8⁺ T cells.

Importantly, we have extended results obtained with model antigens (i.e. OVA and NY-ESO-1), by using endogenous B16 mitochondria as a source of specific mitochondrial antigens [37] and demonstrated their ability to induce B16 specific CD8⁺ T cell responses capable of controlling B16 growth *in vivo*. These results extend our previous findings obtained with mitochondrial localised OVA and NY-ESO-1 protein by highlighting the strong immunogenicity of endogenous mitochondrial localised antigenic proteins.

Importantly, while cross-presentation events of epitopes derived from mitochondrial localised proteins are of importance for inducing strong priming of tumor specific T cells, such response would not be sufficient to control tumor growth *in vivo*, unless the tumor cells are able to directly present epitopes derived from mitochondrial localised proteins. Our results demonstrate that mtOVA B16 cells can directly present *in vivo* the OVA SIINFEKL epitope, albeit less efficiently than B16 cytoOVA cells, and that combination of the higher frequency of OVA specific T cells and their ability to recognise B16 mtOVA cells *in vivo* accounts for the greater control of tumor growth, as compared with cytoOVA B16 cells. It has been previously shown that direct presentation of epitopes derived from mitochondrial proteins relies on the generation and trafficking of mitochondrial-derived vesicles induced by

heat shock, but not by IFN γ stimulation [19]. We demonstrated that in order to detect the H-2K^b/SIINFEKL complex on the surface of B16 mtOVA and cytoOVA cells *in vitro*, B16 cells needed to be treated with IFN γ and TNF α . Importantly, *in vivo* H-2K^b/SIINFEKL complexes were detectable by flow cytometry on the surface of B16 mtOVA cells, suggesting that the inflammatory tumor microenvironment induces upregulation of the levels of SIINFEKL/H-2 K^b complexes. Indeed, we observed that upon depletion of CD8⁺ T cells, B16 mtOVA cells lack surface expression of H-2K^b/SIINFEKL complexes, suggesting that cytokine expression by infiltrated tumor specific CD8⁺ T cells may be required to induce direct presentation of the SIINFEKL OVA epitope to detectable levels (**Supplementary Figure S4C**).

In conclusion, our results demonstrate that location of antigenic proteins in mitochondria significantly enhances their ability to elicit *in vivo* high frequency CD8⁺ T cell responses. Such enhanced immunogenicity is controlled by cross-priming dependent events, which are facilitated by the steady state amount of mitochondrial localised proteins and by the activation of the STING-cGAS pathway. Our data showed a greater immunogenicity of mitochondrial localised mutated proteins during tumor development; boosting this pre-existing immune response through personalized vaccination would be a novel strategy to enhance the efficacy of cancer immunotherapy treatments.

Acknowledgments

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Figure Legends

Figure 1. Stable mitochondrial localised NY-ESO-1 protein primes higher frequency of NY-ESO-1 specific CD8⁺ T cell responses *in vivo* as compared with rapidly degraded cytosolic localised NY-ESO-1 protein. (A) Representative dual colour confocal images of CT26 cells stably expressing NY-ESO-1 localised in the cytosol (CT26 cytoNY-ESO-1, lower panel) or in the mitochondria (CT26 mtNY-ESO-1, upper panel) with the mitochondrial dye mitotracker (green) and immunostaining (red) for NY-ESO-1. A pixel-wise Pearson's colocalisation test to quantify the colocalisation of mitotracker and NY-ESO-1 has been performed, (values of 1 indicate complete colocalisation, 0 indicates no colocalisation) results are shown in the right panel. Results are representative of 2 independent experiments. (B) HHD mice (n=5 per group) were injected with 1×10^6 mismatched CT26 mtNY-ESO-1, cytoNY-ESO-1 or wild type CT26 cells. Seven days after the injection, splenocytes were stained with HLA-A2 NY-ESO-1₁₅₇₋₁₆₅ tetramers (right panel) or restimulated with the NY-ESO-1₁₅₇₋₁₆₅ peptide (SLLMWITQC) and production of IFN γ (left panel) and TNF α (middle panel) was assessed by intracellular staining (ICS). Data are representative of at least two independent experiments with n=5 and values are expressed as mean \pm SD. (C) C57BL/6 mice (n=5 per group) were injected with 2×10^6 mismatched CT26 mtOVA, cytoOVA or wild type CT26 cells. Seven days after the injections, splenocytes were restimulated with the OVA peptide (SIINFEKL) for 5 hours and the production of IFN γ was assessed by ICS. Data are representative of at least two independent experiments with n=5 and values are expressed as mean \pm SD. Data are representative of at least two independent experiments with n=5 and values are expressed as mean \pm SD. (D). CT26 mtNY-ESO-1 and cytoNY-ESO-1 tumor cell lines were incubated in tissue culture medium containing the proteasome inhibitor MG132 at different concentrations (40, 4 and 0.4 μ M) or DMSO for 3 hours. Western blotting was performed with indicated antibodies. (B-C) **** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test).

Figure 2. Mitochondrial localised OVA primes higher frequency of CD8⁺ T cell responses *in vivo* as compared with cytosolic localised OVA. (A) B16 mtOVA and cytoOVA and mod cytoOVA cell lines were incubated in tissue culture medium containing the proteasome inhibitor epoxomicin (0.2 μ M) or DMSO for 3 hours. Western blotting was performed with indicated antibodies. (B) The localisation of OVA in different compartment was demonstrated by western blot of different cellular fractions. GAPDH is enriched in the

cytosolic fraction, while TOM20 is enriched in the mitochondrial fraction. (C) C57BL/6 mice were injected with 1×10^6 B16 mtOVA, cytoOVA mod cytoOVA or control cells. Seven days after the injection, splenocytes were restimulated with the OVA₂₅₇₋₂₆₄ (SIINFEKL) peptide and the production of IFN γ was assessed by ICS. Bars represent the mean frequencies \pm SD of 14 mice from two independent experiments *** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test).

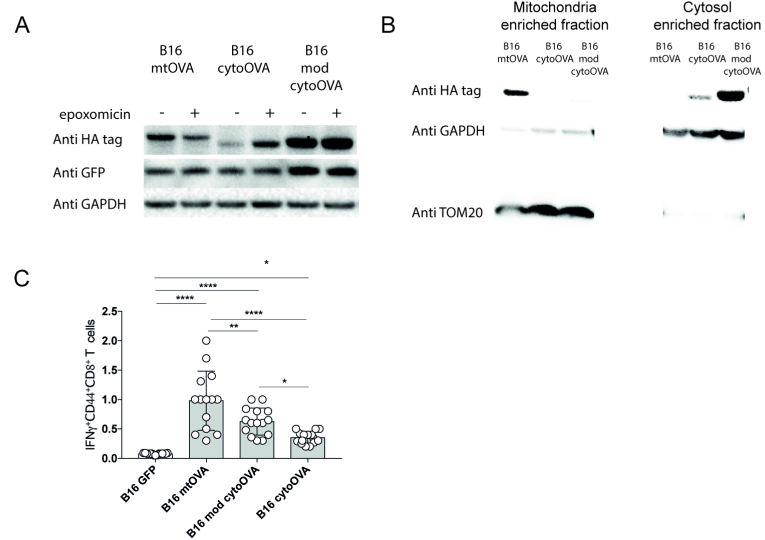
Figure 3. Subcellular location of OVA modulates the efficiency of its direct presentation to OVA specific CD8⁺ T cells. (A) B16 cytoOVA, B16 mtOVA, and B16 GFP were incubated with IFN γ and TNF α (5ng/ml) for 0, 2, or 5 days and then stained with an anti H-2K^b-OVA₂₅₇₋₂₆₄ antibody (results are expressed as MFI). (B) C57BL/6 mice were injected with B16 mtOVA, B16 cytoOVA, and B16 GFP. Tumor cells were isolated on day 7 or day 14 and stained with an anti H-2K^b-OVA₂₅₇₋₂₆₄ antibody, results are shown as MFI. Data are representative of at least two independent experiments with $n=5$ and values are expressed as mean \pm SD. **** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test). (C) B16 cytoOVA, B16 mtOVA and WT B16f10 were treated for 5 days with IFN γ and TNF α and then co-cultured with OT-I T cells in triplicates. Expression of activation markers on OT-I splenocytes was investigated after 24 hours of co-culture. (D) Representative plots are shown. (E) B16 mtOVA and cytoOVA were injected subcutaneously in the flank (1.5×10^5 cells/mouse) tumor growth curves at different time points are shown. Data are representative of at least two independent experiments with $n=8$ and values are expressed as mean \pm SEM. Two-tailed Student's t-test was used for comparing values **** $p < 0.0001$, *** $p < 0.001$. (F) Groups of C57BL/6 mice were injected on days 0, 3 and 8 with an anti CD8 antibody (clone 2.43) in order to deplete CD8⁺ T cells, or with an isotype control. On day 3, mice were injected SC with B16 mtOVA (1.5×10^5 cells/mouse), tumor size on day 16 is shown. Bars represent the mean tumor size \pm SD of 10 mice from two independent experiments. Two-tailed Student's t-test was used for comparing values **** $p < 0.0001$, *** $p < 0.001$. (G) B16 mtOVA cells were injected subcutaneously in the flank (1.5×10^5 cells/mouse) of wild type or beta 2 microglobulin knockout C57BL/6 mice and tumor size at day 14 is shown.

Figure 4. Enhanced cross-priming of OVA specific T cells is reduced in STING and cGAS knockout mice (A-B) Wild type or STING C57BL/6 knockout mice (n=5 per group) were injected with 1×10^6 B16 mtOVA (A) or with 1×10^6 B16 cytoOVA cells (B). Seven days after the injection, splenocytes were restimulated *in vitro* with the OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) and the production of IFN γ was assessed by ICS. Data from at least two independent experiments (n=5) are shown. **(C)** Wild type or cGAS knockout C57BL/6 mice (n=8 per group) were injected with 1×10^6 B16 mtOVA. Seven days after the injection, splenocytes were restimulated with the OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) and the production of IFN γ was assessed by ICS. **(D)** Wild type, MyD88 knockout, NLRP3 knockout or MAVS knockout C57BL/6 mice were injected with 10^6 B16 mtOVA. Seven days after the injections splenocytes were restimulated with the OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) and the production of IFN γ was assessed by ICS. Bars represent the mean frequencies \pm SD of 8 mice from two independent experiments, Two-tailed Student's t-test was used for comparing values $*p < 0.05$. **(E)** Groups of wild type or Batf3 and DNCR-1 C57BL/6 knockout mice (n=5) were injected with B16 mtOVA subcutaneously in the flank (1×10^6 cells/mouse). As a control, wild type mice were injected with B16 without OVA. On day 7, splenocytes were restimulated with OVA₂₅₇₋₂₆₄ peptide (SIINFEKL) for 5 hours and then IFN γ secreting cells were identified by ICS. **** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test). Values are expressed as mean \pm SD.

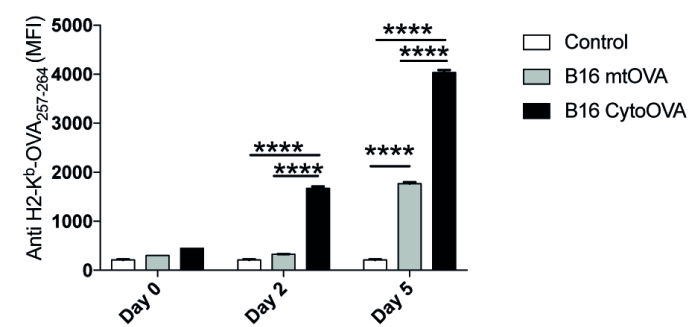
Figure 5. Protective vaccination of naïve C57BL/6 mice with mitochondria purified from B16 tumors. (A) Groups of mice (n=10) were injected on day -12 with mitochondria isolated from B16 or from mouse adult fibroblasts (MAFs) derived from C57BL/6 mice. On day 0, mice were injected subcutaneously with B16 cells (1.5×10^5 cells/mouse). Tumor measurements at day 14 are shown. Data are representative of two independent experiments with n=5 and values are expressed as mean \pm SD **** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test). **(B)** Groups of C57BL/6 mice were injected with mitochondria isolated from B16 cells on day -10. Ten days after injection (on day 0) mice were challenged with B16 cells or Lewis lung carcinoma (1.5×10^5 cells/mouse injected SC in the flank). Tumor measurements at day 14 are shown. Data are representative of two independent experiments with n=5 and values are expressed as mean \pm SD Two-tailed Student's t-test was used for comparing values **** $p < 0.0001$, *** $p < 0.001$. **(C)** Groups of mice (n=5-6) were injected on day -12 with mitochondria isolated from B16 cells or vehicle.

On day -2, 0 and 5 mice were injected with an anti CD8 antibody (clone 2.43) or an isotype control. On day 0, mice were injected subcutaneously with B16 tumor cells (1.5×10^5 cells/mouse). Tumor measurements at day 14 are shown. Data are representative of two independent experiments with $n=5$ and values are expressed as mean \pm SD. Two-tailed Student's t-test was used for comparing values **** $p < 0.0001$, *** $p < 0.001$. **(D)** Groups of mice ($n=8$) were injected on day -12 with mitochondria isolated from B16 or with vehicle. On day 0 mice were injected subcutaneously with B16 tumor cell line (1.5×10^5 cells/mouse). On day 14, CD8⁺ T cells were isolated from the spleen and co cultured with B16 cells and the production of IFN γ was assessed by ICS. Values are expressed as mean \pm SD **** $p < 0.00001$, ** $p < 0.001$, * $p < 0.01$ (one-way ANOVA followed by Tukey's post-test).

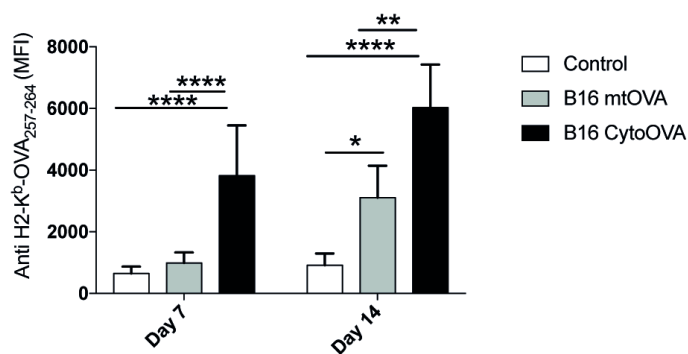
Figure 6. Identification of CD8⁺ T cell clones specific for mitochondrial proteins in a cancer patient. **(A)** Scatter plot shows the disperse frequencies of nonsynonymous somatic mutations in mitochondrial proteins that are expressed by gDNA from 9,508 samples across 31 cancer types from The Cancer Genome Atlas (TCGA, version 02-04-2018). The total number of nonsynonymous somatic mutations in mitochondria-localised proteins is 93,679. The x axis represents the number of such mutations per patient. The top five cancer types with the highest average frequency are endometrioid, colon, melanoma, stomach and rectal in the decreasing order. The somatic mutations are the output of MuTect2[49] with hg38 assembly. **(B)** By comparing the sequences obtained from tumor and normal DNA, tumor-specific nonsynonymous single-nucleotide variations (SNV) were identified. A computational pipeline was used to examine the mutant peptide regions for binding to the patient's HLA alleles. We then divided the epitopes based on whether they derived from proteins localised in mitochondria or cytosol by employing existing databases, and we synthesized 60 peptides for each group of proteins. Peptide–HLA-A2 complexes were generated employing the UV-mediated ligand exchange technology [50]. After restimulation clones specific for mitochondrial localised (left panel) and non mitochondrial localised proteins (right panel) were identified. The ability of identified CD8⁺ T cell clones to kill peptide pulsed autologous EBV immortalised B cell lines **(C)** and to produce IFN γ was investigated **(D)**.



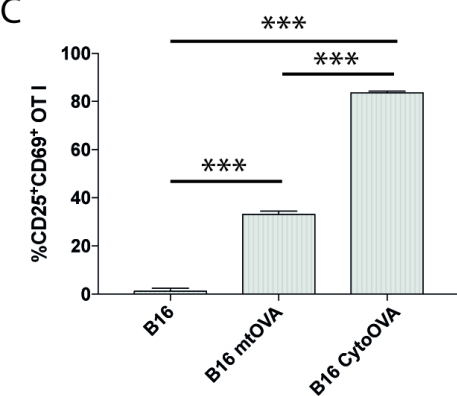
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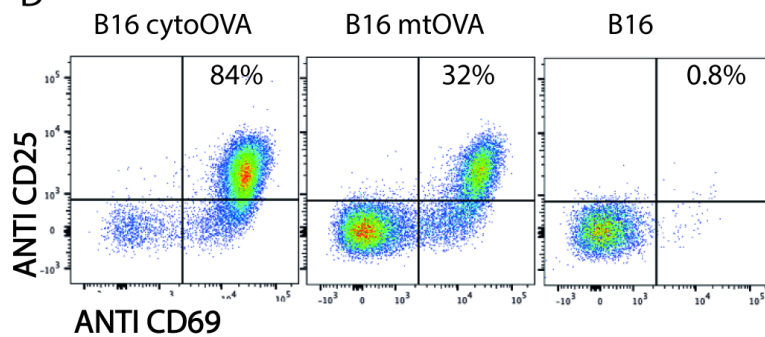
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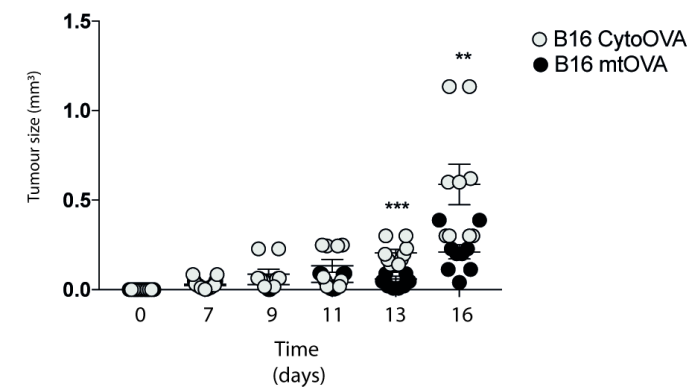
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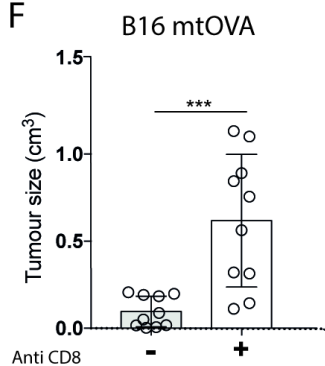
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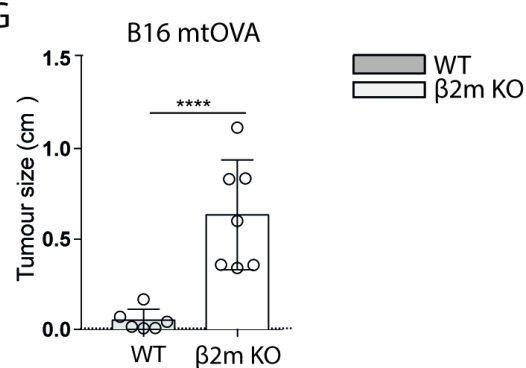
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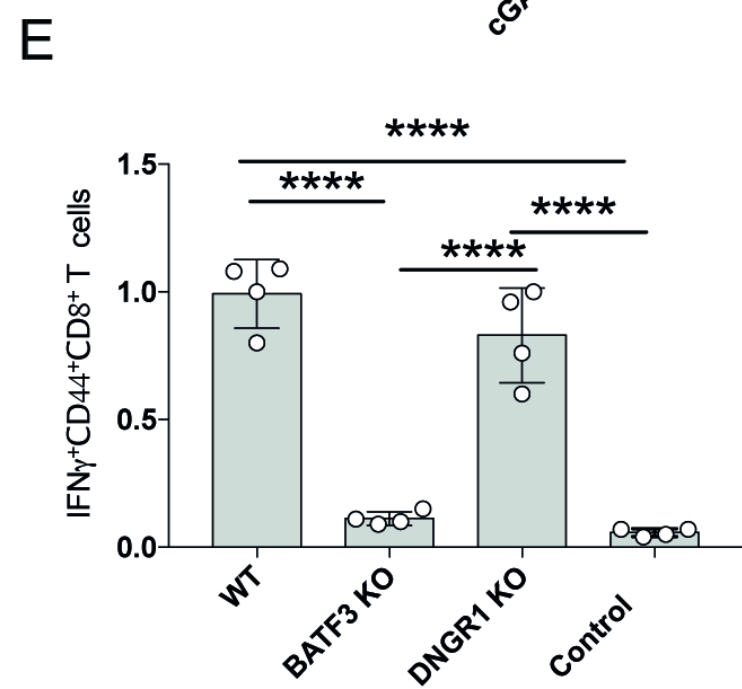
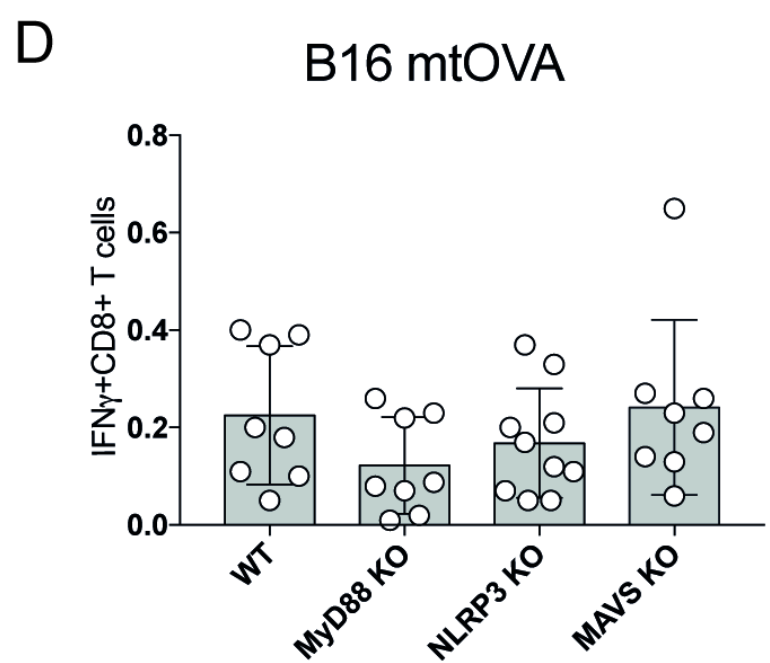
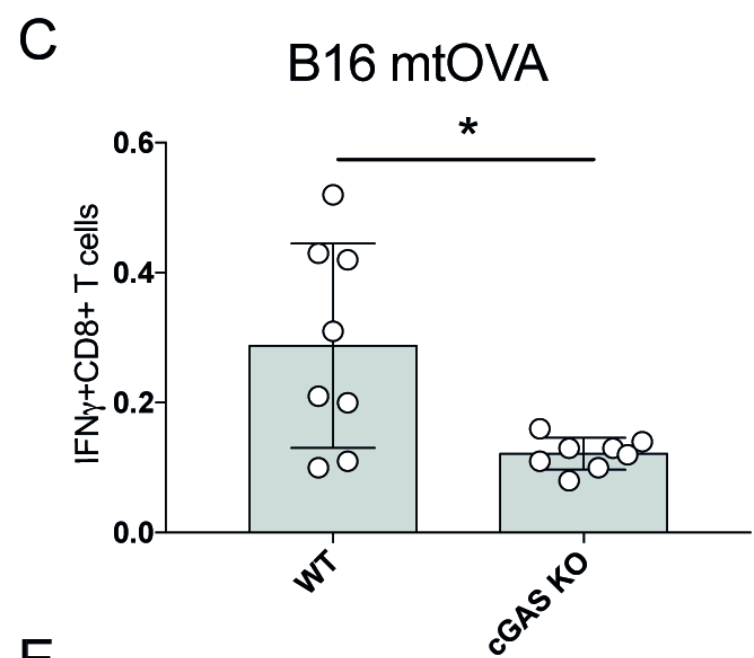
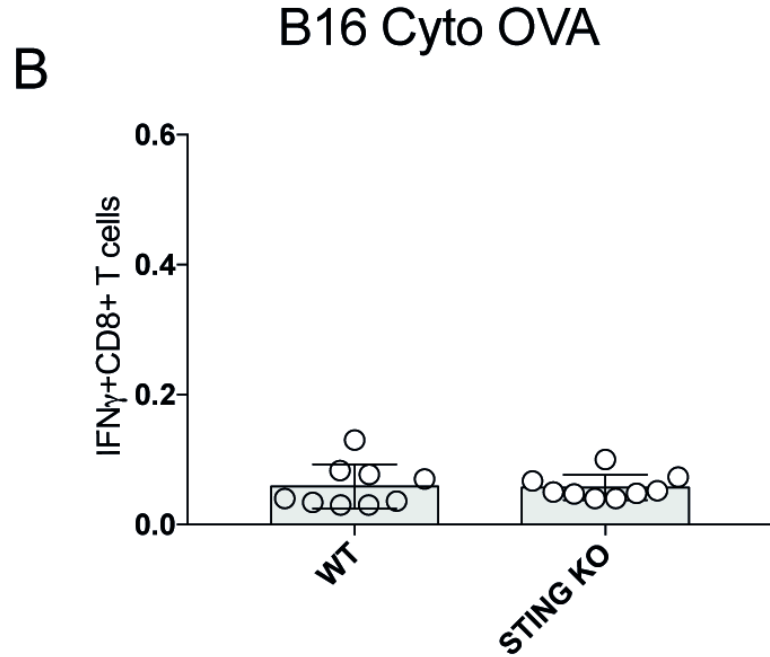
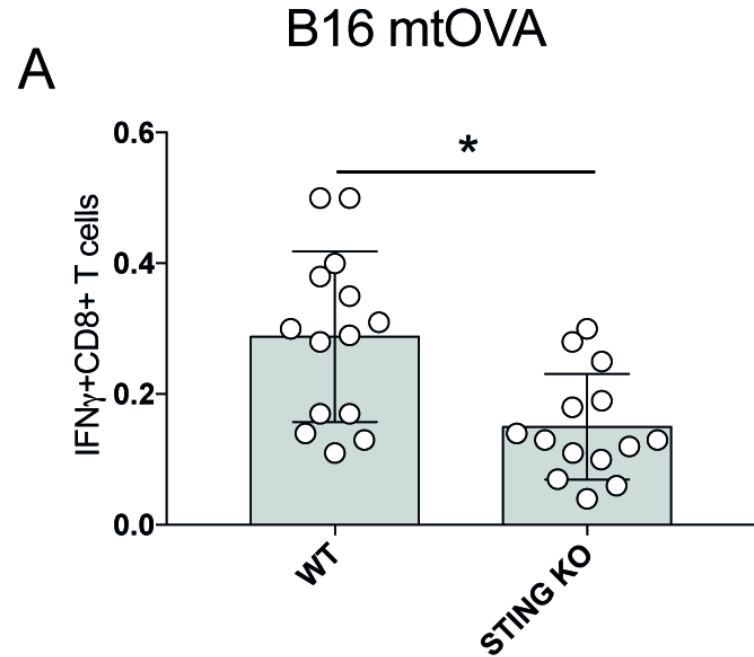


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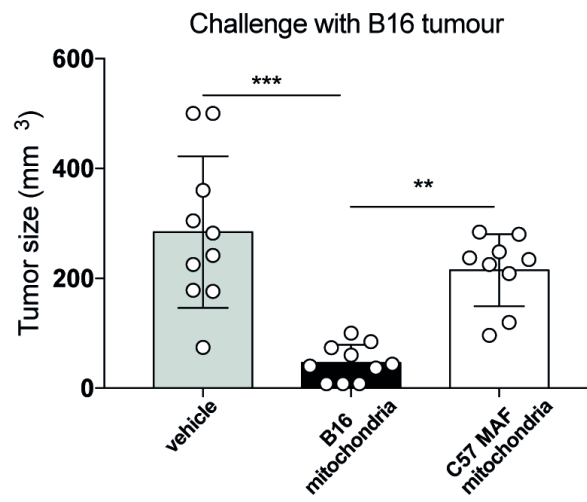


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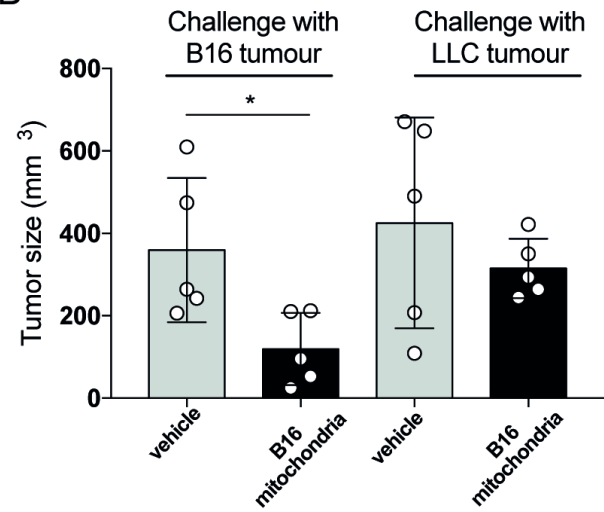




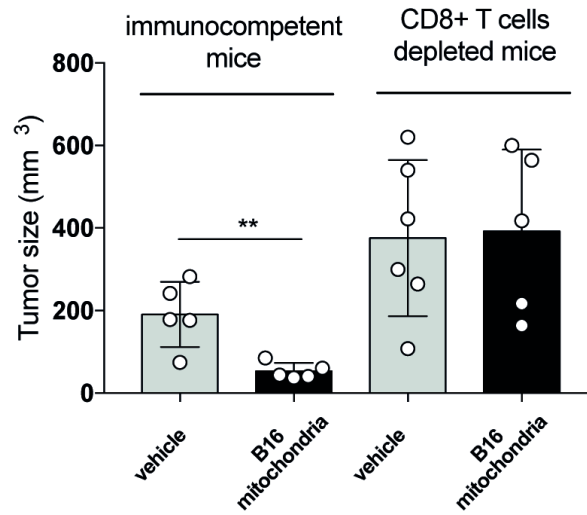
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