Induction of a potent CD4 and CD8 T-cell response against tumor-specific and tumor-associated antigen is critical for eliminating tumor cells. Recent vaccination strategies have been hampered by an inefficacious and low amplitude immune response. Here we describe a self-adjuvanted chimeric protein vaccine platform to address these challenges, characterized by a multidomain construction incorporating (i) a cell penetrating peptide (CPP) allowing internalization of several multiantigenic Major Histocompatibility Complex (MHC)-restricted peptides within (ii) the multiantigenic domain (Mad) and (iii) a TLR2/4 agonist domain (TLRag). Functionality of the resulting chimeric protein is based on the combined effect of the above-mentioned three different domains for simultaneous activation of antigen presenting cells and antigen cross-presentation, leading to an efficacious multiantigenic and multiallelic cellular immune response. Helper and cytotoxic T-cell responses were observed against model-, neo- and self-antigens, and were highly potent in several murine tumor models. The safety and the immunogenicity of a human vaccine candidate designed for colorectal cancer treatment was demonstrated in a non-human primate model. This newly engineered therapeutic vaccine approach is promising for the treatment of poorly infiltrated tumors that do not respond to currently marketed immunotherapies.
Targeting self and neo-epitopes with a modular self-adjuvanting cancer vaccine

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Conflict of interest statement
E.B., J.F.M., W.D.B.B., S.C., E.D., M.L.S.R. and M.D. are employees of AMAL Therapeutics, and M.D. has ownership interests in AMAL Therapeutics. M.D. and P.R.W. are the inventors of patents related to the CPP. M.D. and E.B. are inventors in patents related to KISIMA™. No potential conflicts of interest were disclosed by the other authors.
Abstract

Induction of a potent CD4 and CD8 T-cell response against tumor-specific and tumor-associated antigen is critical for eliminating tumor cells. Recent vaccination strategies have been hampered by an inefficacious and low amplitude immune response. Here we describe a self-adjuvanted chimeric protein vaccine platform to address these challenges, characterized by a multidomain construction incorporating (i) a cell penetrating peptide (CPP) allowing internalization of several multiantigenic Major Histocompatibility Complex (MHC)-restricted peptides within (ii) the multiantigenic domain (Mad) and (iii) a TLR2/4 agonist domain (TLRag). Functionality of the resulting chimeric protein is based on the combined effect of the above-mentioned three different domains for simultaneous activation of antigen presenting cells and antigen cross-presentation, leading to an efficacious multiantigenic and multiallelic cellular immune response. Helper and cytotoxic T-cell responses were observed against model-, neo- and self-antigens, and were highly potent in several murine tumor models. The safety and the immunogenicity of a human vaccine candidate designed for colorectal cancer treatment was demonstrated in a non-human primate model. This newly engineered therapeutic vaccine approach is promising for the treatment of poorly infiltrated tumors that do not respond to currently marketed immunotherapies.
**Introduction**

Although the immune system can recognize and to a certain extent eliminate tumor cells, the anti-tumor immune response often remains inefficient and of low amplitude. Protein-based therapeutic vaccines aim to boost this immune response by inducing a robust and prolonged cellular immune response against specific tumor-associated antigens. However, although supported by extremely promising preclinical data, cancer vaccines have, so far, only achieved moderate clinical efficacy (1, 2). This discrepancy highlights the need to develop a new class of vaccines with improved properties that induce a more potent anti-tumoral response.

One of the four key issues preventing therapeutic cancer vaccines from achieving cancer cell eradication is caused by the heterogenic nature of individual tumors which are composed of multiple cancer cell clones expressing varying levels of different tumor-specific and tumor-associated antigens (TSA and TAA), leading to variable immunogenicity within the same lesion (3, 4). Tumor immunogenicity is also shaped by both naturally-occurring and vaccine-induced immune pressure, through the emergence of antigen loss variants that favor cancer relapse (5). Therefore, in order to achieve a strong and durable immune response, therapeutic cancer vaccines should comprise multiple carefully selected TSA and/or TAA antigens (and preferably multiple epitopes of each TSA/TAA) with multiallelic HLA restriction of epitopes enabling the treatment of a broad range of patients.

A second issue is that many tumors have low levels of infiltrating immune cells which limits anti-tumoral efficacy. Therapeutic vaccines are becoming a key element for increasing immune cell infiltration in poorly infiltrated tumors (6), which offer the additional benefit of potentiating anti-tumor efficacy of immune checkpoint-modulating agents (7).
A third desired property of cancer vaccines is the ability to induce a complete cellular immune response involving both tumor-specific CD8 cytotoxic and CD4 helper T cells (2). However, antigens derived from conventional soluble protein- and peptide-based vaccines are only poorly cross-presented on Major Histocompatibility Complex (MHC) class I molecules by dendritic cells (DCs) and therefore induce only low-level CD8 T-cell responses (8, 9). We have previously demonstrated that this major drawback can be overcome by using cell penetrating peptides (CPP) as delivery vectors (10, 11). These small peptides can cross the plasma membrane even when linked to large multiepitopic cargos, allowing antigens to be delivered into different processing compartments of DCs, so that they are presented on both MHC class I and class II, promoting CD8 and CD4 T-cell activation, respectively (10).

Adjuvanticity represents a fourth critical aspect of therapeutic cancer vaccine development. Crucially, activation of Toll Like Receptors (TLRs) through binding of an appropriate ligand, leads to the maturation of DCs, and consequently, to the initiation of an efficacious antigen-specific T-cell response. Conjugating a vaccine to a TLR ligand was explored with various TLR ligands combined to a peptide or protein vaccine and resulted in improved antigen-specific CD8 T-cell responses (12). Based on these findings we optimized our CPP-based vaccine and recently demonstrated that part of the efficacy of CPP-based therapeutic cancer vaccines relies on their combination with the concomitant administration of the optimal adjuvant (11). A further enhancement of immune activation would be to conjugate the protein vaccine to an appropriate TLR ligand (13) as a state-of-the-art recombinant fusion protein vaccine construct, thereby benefitting from self-adjuvanticity.

We herein report the development of an original chimeric protein vaccine platform, named KISIMA™, to address these challenges. KISIMA™ is composed of three elements: a ZEBRA-
derived CPP, a multiantigenic domain (Mad) with epitopes restricted by multiple MHC alleles, and a self-adjuvanting TLR2/4 agonist. Using a range of preclinical mouse tumor models, we demonstrate that within our improved and unique vaccine construct, the CPP and the TLR agonist (TLRag) act together to elicit both CD8 and CD4 antigen-specific immune responses leading to immunological memory and high vaccine efficacy together with increased intratumoral leukocyte infiltration. Importantly, we show that these newly developed vaccines are safe and elicit a break of tolerance and antigen-specific T-cell immune responses in non-human primates (NHP), attesting to the safety and immune efficacy of the platform for future clinical use.
Results

The novel self-adjuvanted KISIMA™ cancer vaccine platform

Different vaccine constructs were generated incorporating newly described TLR agonists (TLRags) along with the CPP and Mad domains (Figure 1A). CD8 and CD4 epitopes of the different multiantigenic domains that were generated with their sequences presented are summarized in Supplemental Table 1. Various constructs were produced in order to define the optimal self-adjuvanting module and to determine whether the position of the module within the construct affects its activity (Figure 1A). For instance, two of the newly generated constructs comprised Z13 CPP, Mad5 and one of the two TLRag (either at the C- or N-terminus): (i) a TLR2-4 peptide agonist called Anaxa which originates from the N-terminus of Annexin II, or (ii) a TLR4 agonist called Extra Domain A (EDA) from fibronectin (14). Upon vaccination, EDA at the N-terminus position elicited the highest T-cell immune response against ovalbumin, whereas Anaxa was more efficacious at the C-terminus position (data not shown). The constructs with the optimal position of TLRag were thus compared for adjuvanting activity in HEK cells expressing human TLR2 or TLR4 and in THP-1-XBlue-MD2-CD14. The Anaxa module showed a higher activation capacity compared to EDA for both TLR2, as expected, and TLR4 activation as observed in Figure 1B. Maximal activation required the presence of both elements, CPP and TLRag, namely, Z13 and Anaxa respectively, when compared with vaccines lacking either TLRag (Z13Mad5 construct) or CPP (Mad5Anaxa construct) demonstrating potentiation of the TLRag activity by the CPP for optimal effect (Figures 1B and 1C) and confirming an activation pathway through both TLR molecules (Figure 1B). Moreover, a complete lack of activation of TLR4-knock-out DCs by Z13Mad5Anaxa showed that the TLR4 pathway was necessary and sufficient for vaccine-mediated DC activation whereas the TLR2 pathway was less critical (Supplemental Figure 1A).
Surface plasmon resonance studies of ATP125, a vaccine containing human antigens (Figure 1A), confirmed affinity of vaccine for TLR4 and TLR2, but not for TLR3 (Figure 1D).

**A new self-adjuvanted cancer vaccine platform eliciting CD8 and CD4 T-cell immune responses**

The constructs were then compared in vivo in a mouse thymoma model EG.7. When compared with EDAZ13Mad5, Z13Mad5Anaxa showed the strongest anti-tumor effect (Figure 2A); therefore, Anaxa was selected as the optimal TLRag for the next steps. Importantly, the anti-tumor activity of the vaccine was decreased when CPP Z13 was removed (Mad5Anaxa construct) (Figure 2B and Supplemental Figure 1B) or replaced by a different ZEBRA-derived CPP (Z14Mad5Anaxa construct) (Supplemental Figure 1B). Concomitant administration of a TLR4 (MPLA) or a TLR2 (Pam3CSK4) agonist with vaccine Z13Mad5 displays less efficacious anti-tumor activity than vaccination with the whole construct Z13Mad5Anaxa (Figure 2B, Supplemental Figure 1C).

The immunogenicity of Z13Mad5Anaxa was also compared to Z13Mad5 administered with or without previously described adjuvants MPLA or Pam3CSK4, or to Mad5 fused to keyhole limpet hemocyanin (KLH) (15). Z13Mad5Anaxa was as immunogenic as MPLA or Pam3CSK4 adjuvanted Z13Mad5 and superior to non-adjuvanted (Figure 2C) or KLH-conjugated vaccines in terms of circulating antigen-specific CD8 T cells (Figure 2D).

Optimization of the vaccination conditions established that a vaccine dose from 2 nmoles was able to elicit a potent CD8 T-cell immune response (Supplemental Figure 2A), with either synthetic peptide or recombinant protein (Supplemental Figure 2B). We identified an optimal vaccination interval of two weeks (Supplemental Figure 2C), and subcutaneous (s.c.) injection as the best route (Supplemental Figure 2D). A maximal immune response was observed after three vaccinations at two-week intervals, with the T-cell response then maintained by monthly
vaccination (Supplemental Figure 2E). Multiepitopic CD8 and CD4 T-cell immune responses were both elicited against different antigens: ovalbumin and self-antigen gp100 (Figure 3A), human papilloma virus antigens (Figure 3B), and glycoprotein 70 (Figure 3C) that is also a self-antigen in the BALB/c strain of mouse (16). Furthermore, a significant proportion of antigen-specific CD4 and CD8 T cells are polyfunctional cells (50% and 25%, respectively), producing at least two different cytokines upon T-cell receptor (TCR) triggering (Supplemental Figure 2F). Interestingly, vaccination elicits circulating memory CD8 T cells, also increased in draining lymph nodes but that mainly home and reside in the bone marrow memory compartment (Supplemental Figure 2G) with a high proportion of central memory cells within various tissues of the mice (Supplemental Figure 2H).

The self-adjuvanted vaccine platform enables the break of self-tolerance, eliciting multiepitopic effector and memory CD8 and CD4 T-cell responses, pre-requisites for efficacious therapeutic cancer vaccines.

Integrated immune responses drive anti-tumor activity with profound modification of the tumor microenvironment

Prophylactic vaccination in the colorectal MC38 tumor model (Figure 3D) led to a 25 days improvement in median survival, which did not reach statistical significance, whereas tumor volume was significantly decreased. Additionally, therapeutic vaccination, starting 10 days after tumor cell implantation, mediated a strong anti-tumor effect in the TC-1 tumor model as shown by the 27 days increased median survival and significantly decreased tumor volume of vaccinated mice (Figure 3E). Furthermore, therapeutic vaccination in the B16-OVA pulmonary metastases tumor model resulted in a significantly decreased number of metastases (Figure 3F).
An increase in proportions of antigen-specific CD8 T cells in the tumor infiltrate was observed within the B16-OVA, EG7, MC38 and TC-1 tumor models following vaccination with Z13Mad5Anaxa (B16-OVA and EG7), Z13Mad12Anaxa (MC38) or Z13Mad10Anaxa (TC-1), respectively (Figure 3G). The percentage of these infiltrated cells was always significantly higher compared to the periphery in both control and vaccinated animals indicating T-cell accumulation and/or proliferation within the tumor. The impact vaccination was the most striking in the poorly immunogenic TC-1 model where the antigen-specific CD8 T-cell infiltrate was increased 30-fold in vaccinated mice compared to the control, in contrast to the 3-fold increase in the immunogenic EG7 model. The specificity of the vaccination was observed by analyzing tumor volume in the TC-1 tumor mouse model, after vaccination with either the corresponding Z13Mad10Anaxa (HPVE7 antigen) vaccine or an irrelevant Z13Mad12Anaxa (MC38-antigens) vaccine. Only Z13Mad10Anaxa vaccination induced an anti-tumor response (data not shown).

The inverse correlation between the antigen-specific CD8 T-cell infiltrate and the tumor size might suggest that anti-tumor activity relies on strong antigen-specific cancer cell elimination (Supplemental Figures 3A and 3B). Alternatively, the data would also be consistent with efficient anti-tumor immunity occurring when tumor burden is low, revealed by production of both TNF-α and IFN-γ by tumor-infiltrating antigen-specific CD8 T cells (Table 1), but which is overwhelmed by the immunosuppressive microenvironment of larger tumors.

Immune suppressive cells, such as Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells, regulatory T cells (Tregs) and Tumor-Associated Macrophages-2 (TAM2, unlike the proinflammatary, TAM1), are a major obstacle for
immunotherapy. These cells are all involved in antitumor responses by direct or indirect suppressive mechanisms inducing a tolerogenic and tumor-promoting environment (17, 18). We herein show that vaccination impacted the tumor microenvironment (TME) by decreasing the immunosuppressive granulocytic MDSCs (gMDSCs) in the EG7 model resulting in an increased proportion of functional antigen-specific CD8 T cells producing IFNγ and TNFα. Similarly, in the TC-1 tumor model, we observed enhanced proportion of functional antigen-specific CD8 T cells and a favorable TME characterized by improved CD8/Treg and the TAM-type 1/type 2 ratios (Table 1). These results indicate that this new vaccine increases the tumor infiltration by antigen-specific T cells and can favorably modulate a pro-tumoral microenvironment resulting in diminished tumor size.

_Vaccination generates a humoral immune response that does not affect efficacy of subsequent vaccinations_

A humoral response was detected in mice only after the third vaccination with Z13Mad5Anaxa, with increasing titers following each subsequent vaccination (Supplemental Figure 4A). An increased proportion of OVA257-264-specific CD8 T cells in the blood was still observed after the 4<sup>th</sup>, 5<sup>th</sup> and 6<sup>th</sup> vaccinations despite the accumulation of anti-vaccine antibodies in the serum (Supplemental Figure 4B), with no inverse correlation between T-cell and antibody responses (Supplemental Figure 4C). To confirm that anti-vaccine antibodies do not interfere with vaccine activity, Z13Mad5Anaxa was pre-incubated with serum from Z13Mad5Anaxa immunized mice before vaccination. After two vaccinations, we observed a comparable proportion of OVA257-264-specific CD8 T cells in the blood of mice vaccinated with Z13Mad5Anaxa pre-incubated with
serum from either vaccinated or naive mice (Supplemental Figure 4D). These results indicate that a humoral response against the vaccine does not alter the potency of the induced T-cell response.

**Validation of a new construct encompassing human antigens**

ATP125 vaccine was designed with human sequences of TAA described in colorectal cancer (CRC) (19-22), with a Mad composed of portions of CEA, MUC1, EPCAM and survivin antigens (Supplemental Table 1) and comprising Z13 and Anaxa domains. Blood-derived human DCs were exposed in vitro to ATP125 vaccine and assessed by flow cytometry for extracellular or intracellular staining via the use of an anti-His antibody detecting ATP125. Figure 4A shows an increase of both extracellular and intracellular staining of ATP125 with time, demonstrating its surface exposition and its penetration inside the cells.

Natural processing and presentation of ATP125 on Human Leukocyte Antigen (HLA) molecules by human DCs from six healthy donors was evaluated using mass spectrometry-based HLA ligandome analysis and mapping after overnight loading (Figure 4B and Table 2). For each donor, both class I- and class II-restricted ATP125 Mad-derived peptides were presented by DCs (Supplemental Tables 2 and 3). HLA binding motifs predicted with the NetMHCpan3.0 and SYFPEITHI algorithms were also identified. Peptides derived from all the antigens except MUC1 were identified to be presented on HLA class I molecules (Table 2). The junction of the EPCAM antigen and Anaxa created some class I neo-epitopes of 8 to 12 amino acids in 5/6 donors (Supplemental Table 2). Homology analysis of the spanning region of 17 residues from which the various neo-epitopes originated, showed that this promiscuously HLA-binding neo-epitope has 75% identity with the ComEC/Rec2-related protein and 73% identity with the S-phase kinase-associated protein 2. A higher number of class II-restricted peptides were identified from all the
antigen portions of the vaccine: 6/6 donors presented HLA class II epitopes from CEA and survivin, 5/6 from EPCAM and 3/6 from MUC1 (Table 2). The ligandome analysis showed that more than 4000 different Class I and 5800 Class II peptides were presented as an average per donor by DCs, corresponding to 2700 and 1250 different proteins, respectively. After subtraction of protein found for MPLA-activated DCs, we identified 94 proteins from which derived peptides were presented by at least three donors for the ATP125-loaded condition; these were not presented by non-loaded MPLA activated DCs. To determine whether ATP125-mediated activation can be linked to specific biological processes, GO term enrichment analysis was performed on this set of 94 proteins and revealed processes mainly related to the regulation of the adaptive immune response (Supplemental Figure 5).

In vitro, ATP125 activated blood-derived human DCs, based on analysis of DC specific activation marker overexpression: HLA-DR, CD80, CD83 and CD86 (Figure 4C). The cytokine/chemokine secretion signature of ATP125-loaded DCs or Peripheral Blood Mononuclear Cells (PBMC) was compared to TLR2 (Pam3CSK4), TLR4 (MPLA), and TLR7/8 (R848) ligand loaded DCs and revealed similarity to that of MPLA with secretion of IL-6, IL-8, IL12p70, TNF-α, IP-10, MIP-1α, and MIP-1β by human monocyte-derived DC (moDC), and secretion of IL-6, IL-8, TNF-α, MIP-1α, and MIP-1β by PBMCs (Figure 4D).

Vaccinations are safe in non-human primates
Cynomolgus monkeys received s.c. or intradermal injection of ATP125 at two dose levels (76 µg or 380 µg/vaccination, corresponding to 2 and 10 nmoles respectively), every two weeks for a total of four vaccinations in a toxicology study (Figure 5A). The four vaccinations were well tolerated and no significant outcome was observed on body weight, food consumption, or hematological
and biochemical parameters (data not shown). Very slight, to well-defined erythema was observed on the day after each vaccination for all groups, but spontaneously resolved. No dose-dependent changes in cytokine release or leukocyte sub-population proportions were observed (Supplemental Figures 6A and 6B). Necropsy of treated animals indicated that organ weights were within the normal range for animals of this age, weight, and sex. Microscopic examination of spleen and lymph nodes samples did not show any significant morphologic differences between animals from the four groups (data not shown).

Vaccinations elicit antigen-specific T-cell responses and humoral responses in non-human primates

Immunized cynomolgus macaques were assessed for antigen-specific immune responses elicited by the vaccine in the peripheral blood. For this purpose, we used three complementary methodologies: T-cell intracellular cytokine staining (ICS), IFN-γ ELISPOT and T-cell proliferation performed after short-, mid- or long-term peptide activation. T-cell immune responses were detected in fresh PBMCs for all groups of animals 7 days after the last vaccination (Figure 5B, Table 3, Supplemental Figures 6A and 6B). However, whereas all six animals vaccinated by the s.c. route elicited an immune response against the four antigens, four out of six intradermally vaccinated animals showed an immune response against the four antigens (Supplemental Figure 6C). Furthermore, a multiantigenic CD8 T-cell response was preferentially observed in the high dose group for the s.c. route with 3/3 animals mounting an immune response against at least three antigens, detected by ICS. Similarly, ELISPOT assays highlighted a multiantigenic response in 2/3 animals against all four antigens. Overall, these data showed a preferential multiantigenic T-
cell immune response in the group vaccinated with the high dose of 380 µg (10 nmoles) of ATP125 by the s.c. route.

A humoral immune response was preferentially detected in animals vaccinated by the s.c. route, demonstrated by higher anti-ATP125 and anti-survivin IgG titers compared to the intradermal route groups (Supplemental Figures 6D and 6E).

*Significant additive effect of self-adjuvanted vaccine in combination with PD1 blockade on tumor growth*

The potential of anti-PD1 antibody combination was investigated in the MC38 colorectal cancer model in which vaccine alone showed a low therapeutic efficacy (Figure 6A), and in TC-1 lung cancer model (Figure 6B). Whereas the anti-PD1 alone showed significant efficacy in the MC38 tumor model compared to controls, the combination of both vaccine and PD1 blockade resulted in a highly significant additive effect with 10 out of 14 mice tumor-free, accompanied by a significant decreased tumor volume (Figure 6A). In the TC-1 tumor model, treatment with anti-PD1 alone did not show efficacy, in contrast to vaccine alone which led to improved median survival and significantly decreased tumor volume. Combinatory treatment significantly improved efficacy as shown by the 31 and 14 days increased median survival in comparison with single treatment with anti-PD1 or vaccine, respectively, and also characterized by significantly decreased tumor volume. These encouraging preliminary data show that the combination is a highly efficacious therapy, and further investigations are required to evaluate tumor infiltration and TME modulation.
**Discussion**

With the combined effect of a CPP and a TLR2/4 peptide agonist for the initiation of an efficacious multiantigenic T-cell response, herein described vaccination strategy addresses several challenges faced by therapeutic cancer vaccines. Namely, the need to overcome the heterogeneity of individual tumors (3, 4), to trigger tumor infiltration (6) and to elicit CD8 and CD4 T-cell immune responses facilitating eradication of tumoral cells and prevention of relapse (23, 24).

The first key finding was the self-adjuvanticity of the vaccine platform, which did not rely on an exogenously administered adjuvant. All DCs presenting vaccine-derived epitopes were activated through the TLR-2/4 pathways and upregulated co-stimulatory molecule expression as previously described for the annexin II protein (25, 26). DCs loaded with self-adjuvanted vaccine secreted MIP1α, MIP1β, IP10 and IL-12p70 that play a crucial role in T-cell recruitment, Th1 response induction and IFN-γ secretion necessary for anti-tumor immunity (27). Moreover, we observed in vitro a significant increase of DC activation when both the CPP and the Anaxa were on the same molecule. This could be a result of longer exposure of the Anaxa to TLR molecules due to CPP binding and/or intracellular activation of TLR4 (28) due to CPP-mediated internalization; further investigation is required to determine the relative importance of these processes. In vivo, the efficacy of vaccine was directly linked to the presence of the CPP and the TLRag in one single protein, since when modules are separated, no therapeutic benefit in any tumor models was observed. Although adjuvant-conjugated vaccines may also be able to activate every DC loaded with the antigen (29), the CPP element brings the additional advantages of intracellular delivery.

Secondly, it was consistently demonstrated, for various multiantigenic constructs, that CPP-mediated antigen uptake, not only with external adjuvant (10, 11) but also within a self-
adjuvanting protein, can promote CD8 and CD4 T-cell immune responses. Furthermore, these immune responses were not limited to model antigens or neoantigens but were also induced for self-antigens both in mice (gp100 and gp70) and in NHP (CEA, survivin, EPCAM and MUC1). This led to efficacious anti-tumor responses as shown in MC38 mouse models upon vaccination against survivin. This was linked to induction of antigen-specific T cells, as vaccination with a non-relevant antigen was ineffective (data not shown). The correct processing and presentation of multiantigenic cargos by human DCs was assessed by analyzing the ligandome on different donors. As in mice and in NHP, we showed here that epitopes were presented on both MHC class I and II molecules. The DC ligandome analysis performed on multiple donors with different HLA haplotypes confirmed the multiallelic binding properties of the vaccine antigens, and also revealed creation of a neo-epitope at the junction between Anaxa and EPCAM, highlighting the interest and strength of using mass spectrometry-based ligandome analysis in the design of chimeric multiantigenic proteins.

Self-adjuvanticity is a key feature of viral or bacterial vectors. However, neutralizing antibodies very often limit the number of administrations to a single injection (30), and clinical development must take into account the essential multiple safety measures necessary for live recombinant vectors. In contrast, humoral responses elicited by the inert vaccine do not negatively impact on T-cell mediated immune responses. Moreover, this humoral immune response is expected to boost T cell response as formation of immune complexes improves both antigen uptake and consequently CD8 T-cell recall responses (31, 32).

The initial study performed in NHP indicated a good safety profile, with no toxicity observed. Overall, we introduced here a new class of self-adjuvanting vaccines, possessing a versatile cargo. Indeed, this platform could be developed either as a synthetic long peptide for
personalized vaccination or as a recombinant multiantigenic vaccine giving rise to responses restricted by multiple HLA alleles as a standardized vaccine approach.

Finally, significant changes in the TME have been observed upon vaccination, including (i) massive tumor infiltration of antigen-specific effector T cells, (ii) reduction of MDSCs and Tregs, (iii) M1 polarization of macrophages. Moreover, combination with PD1 blockade was shown to have an additive effect and to significantly increase the efficacy of our vaccine in cancer models. Altogether, these data indicate that this vaccination strategy is an ideal partner to combine with checkpoint inhibitors (CPI). Indeed, it has been observed that the tumors of patients resistant to PD-1-blockade have limited immune infiltration (33, 34). Therefore, identifying therapies, such the herein presented vaccination strategy, which strikingly enhance T-cell-infiltration, could thereby render tumors sensitive to CPI, one of the current major challenges in cancer immunotherapy.
Methods

Animals
Female C57BL/6J and BALB/cJ mice were purchased from Charles River Laboratories (L’arbresles, France). All mice used in this study were females between 6 and 12 weeks of age at the time of experiments. Male cynomolgus monkeys (Macaca fascicularis) were purchased from Le Tamarinier, (Tamarin, Mauritius) and enrolled in a study conducted by CiToxLab (Evreux, France) and were between 34 and 35 months of age at the time of the study.

Vaccines
Vaccine constructs were designed in-house and produced in E. Coli by Genscript. Vaccines were prepared by dilution in vaccine buffer. In some experiments, synthetic peptides synthetized by Pepscan were used. Vaccinations were mainly performed s.c. with 2 nmoles. However, 0.5, 2 or 10 nmoles of vaccine were used in some experiments. In addition, different routes were also assessed: i.v., i.m., intranodal or i.d. route. During purification process, endotoxins were removed from vaccines through extensive washes with Triton-X114 followed by subsequent affinity chromatography. Endotoxin content was quantified in each vaccine batch using a LAL chromogenic assay. Only the batches with endotoxin level ≤ 10EU/mg protein (according to guidelines) were used for further in vitro/in vivo experiments.

Cell lines
The EG.7-OVA cell line (EG7, CRL-2113; American Type Culture Collection), a stable transfectant of the murine OVA-expressing EL-4 thymoma (H-2b), was maintained in complete
RPMI 1640 medium with 0.4 mg/ml geneticin (Life Technologies). The B16-OVA cell line (kindly provided by Bertrand Huard, University of Grenoble-Alpes), an OVA-transfected clone derived from the murine melanoma cell line B16 was maintained in complete RPMI 1640 medium with 1 mg/ml geneticin. The TC-1 cell line (The Johns Hopkins University), a lung epithelial cell line with HPV16 E6 and E7 and c-H-ras oncogenes was maintained in complete RPMI 1640 with 0.4 mg/ml geneticin. The MC38 cell line (kindly provided by Bertrand Huard, University of Grenoble-Alpes), a murine colorectal cancer cell line, was maintained in complete DMEM containing 5% of gentamicin. The HEK-Blue™ hTLR2 and HEK-Blue™hTLR4 cell lines (InvivoGen) were maintained in DMEM Glutamax with 100 µg/mL Normocin™ (InvivoGen). Medium was supplemented with HEK-Blue™selection 1X (InvivoGen) after cells had been passaged twice. THP1-XBlue™-MD2-CD14 cells (InvivoGen) were maintained in RPMI 1640 Glutamax with 25 mM HEPES (Gibco) and 100 µg/mL Normocin™ (InvivoGen). Medium was supplemented with 200 µg/mL Zeocin™ and 250 µg/mL G418 (InvivoGen) after cells have been passaged twice.

In vivo tumor experiments

C57BL/6 mice were implanted s.c. with 3x10^5 EG7-OVA tumor cells in the left flank and vaccinated two times (d5 and d13) by s.c. injection of 2 nmoles of vaccine in the right flank. Tumor size was measured with a caliper. Mice were euthanized when their tumor reached a diameter of 13 mm. For tumor-infiltrating lymphocytes, tumors were harvested, chopped, digested in collagenase D/DNAse enzyme mix for 30 min at 37°C, and filtered through a 70 µm cell strainer. TILs were then purified by Ficoll separation.
C57BL/6 mice were implanted intravenously with $1 \times 10^5$ B16-OVA tumor cells and vaccinated twice (d0 and d10) by s.c. injection of 2 nmoles of vaccine. At day 17 post-implantation, lungs were perfused with Ringer solution, recovered and metastases counted using microscopy.

C57BL/6 mice were implanted s.c. with $1 \times 10^5$ TC-1 tumor cells in the back and vaccinated two times (d10 and d17) by s.c. injection of 2 nmoles of vaccine at the tail base. Tumor size was measured with a caliper. Mice were euthanized when tumor reached a diameter of 13 mm.

C57BL/6 mice were implanted s.c. with $2 \times 10^5$ MC38 tumor cells in the back. Vaccination schedule was either prophylactic (3 weeks and 1 week before tumor implantation) or therapeutic (d3, d10 and d17 post-tumor implantation) by s.c. injection of 2 nmoles of vaccine at the tail base. Tumor size was measured with a caliper. Mice were euthanized when tumor reached a diameter of 13 mm.

In vivo non-human primate toxicological study

Cynomolgus monkeys received 4 injections of ATP125 every two weeks via s.c. or intradermal route at 2 doses level: 76 µg or 380 µg/vaccination, corresponding to 2 and 10 nmoles respectively. Local reactions at the injection sites were evaluated before each administration, then 24, 48 and 72h after injection. Local reaction recording was prolonged until recovery when findings were present. The animals were checked twice daily for clinical signs. Rectal temperature was recorded before each vaccination and 1, 8 and 24h post-vaccination. Body weight was recorded twice before the beginning of the treatment period, on the first 3 days of the study and at least once a week until the end of the study. Food consumption was checked daily.

Hematology and blood biochemistry investigations were performed before the beginning of the treatment period, on Day 22 and at the end of the study (Day 50). Blood collections were
performed for anti-ATP125 antibodies titration and immune response evaluation before the first vaccination and on Days 22, 36 and 50. Determination of cytokines blood levels was performed once before the beginning of the treatment period, before vaccination (on Day 43 only) and then 1, 4 and 8h post-vaccination on Days 1 and 43.

On completion of the treatment period, the animals were euthanized and a full macroscopic post-mortem examination was performed. Brain, cecum, colon, duodenum, heart, ileum, jejunum, kidneys, liver, lungs with bronchi, inguinal and mesenteric lymph nodes, spleen, testes, thymus and injection sites were weighed and selected tissues were preserved. A microscopic examination was performed on injection sites, inguinal lymph nodes and spleen from all animals on completion of the study. A single-cell suspension was prepared from spleen and depth inguinal draining lymph nodes sampled at necropsy in order to perform ELISPOT analysis.

Cell preparation
Bone marrow-derived DCs (BMDCs) were prepared from C57BL/6 mice as previously described (35), and used at day 6 of culture. Human monocyte derived dendritic cells (MoDCs) were prepared from buffy coats of normal blood donors. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll density gradient (GE Healthcare) and resuspended into RPMI-1640 glutamax medium. Monocytes were then isolated by aggregation at cold temperature and contaminating T cells were removed by formation of rosettes after incubation overnight at 4°C with an equal volume of sheep red blood cells. Another Ficoll density gradient was then performed to isolate enriched monocytes, which were resuspended and maintained in complete RPMI medium supplemented with 1000 U/ml GM-CSF and 400 U/ml IL-4 (Miltenyi Biotech). Fresh
complete medium was replaced after 4 days, and immature MoDCs were obtained after 7 days of culture.

Tumor-infiltrating leucocytes were purified from collected tumor tissues. Briefly, tumor tissues were cut into small pieces, and resuspended in HBSS medium containing 2 mg/ml collagenase D (Roche) and 100 µg/ml DNase (Sigma). Tumors were digested for 1h at RT with magnetic stirring. Foetal calf serum (FCS) was then added to stop the enzymatic action and tumor tissues were transferred to ice. A mechanic dissociation of the tumor was then performed on a 70 µm cell strainer with a syringe plunger. Next, Percoll gradient was performed to obtain a single cell suspension.

Peripheral blood and spleen mononuclear cell suspensions from mice were isolated using Ficoll-Paque gradient (GE Healthcare) before flow cytometry analysis or ELISPOT assay.

PBMC suspension from non-human primates was isolated using Ficoll-Paque gradient 90% with PBS 10%.

*In vitro dendritic cells activation*

Human immature MoDCs plated in 24-wells plates were loaded with the vaccine, MPLA (Avanti Polar Lipids) as a positive control (300nM) or with buffer and were incubated over-night at 37°C. After cell scraping and washes, cells were stained for the monitoring of HLA-DR, CD80, CD83 and CD86 expression by flow cytometry. An activation index was calculated as follow: the geometric mean fluorescence intensity for each marker was reported and transformed as a percentage of the expression monitored for the positive control after s.c. of the value observed for buffer-incubated cells. An average of the values obtained for each activation marker was calculated and used as the activation index.
Murine BMDC were loaded with vaccine, MPLA (300nM) as a positive control or buffer and incubated overnight. Cell activation was monitored by flow cytometry after staining with anti CD11c, anti-IA-IE, anti-CD86 and anti-CD40. An activation index was also calculated based on the expression level of IA-IE, CD86 and CD40 on CD11c+ cells.

**In vitro T cell stimulation**

PBMCs from non-human primates were counted and 2x10^6 cells were plated per condition. Cells were incubated with a pool of overlapping peptides for each of the 4 antigens of ATP125, with PHA as a positive control or without any stimulant as a negative control, in presence of brefeldin A and anti-CD107a for 5h. After washing, cells were stained for cells surface antigens CD3, CD4, CD8 and fixable viability dye, then, after fixation and permeabilization, cells were stained for intracellular TNF-α, IL-2 and IFN-γ. In parallel, 2x10^6 cells were plated in presence of overlapping peptides from the 4 antigens of ATP125, or with 2 concentrations of concanavalin A (20 µg/ml and 4 µg/ml) and incubated for 6 days. Cells were then stained for surface antigens CD3, CD4, CD8 and fixable viability dye. After fixation and permeabilization, cells were stained for Ki67 expression.

**TLR activation experiment**

HEK-Blue™ hTLR2 cells and HEK-Blue™ hTLR4 cells were incubated with 1µM of vaccine construct. After 24h, supernatants were recovered and IL-8 measured by ELISA (R&D Systems, Minneapolis, MN). BMDCs were incubated with 1 µM of construct. After 24h, supernatants were harvested and IL-6 measured by ELISA (Biolegend, San Diego). THP1-XBlue™-MD2-CD14 cells were stimulated with various concentrations of vaccine constructs. Upon TLR stimulation,
these cells produce the secreted embryonic alkaline phosphatase (SEAP) which is detectable using QUANTI-Blue™ Solution (InvivoGen). After 18h, cell supernatants were recovered and incubated with QUANTI-Blue™ Solution at 37°C according to manufacturer’s instructions. SEAP activity was assessed by reading the optical density at 620 nm.

**Antibodies and Flow Cytometry**

For surface staining of murine blood cells, the following monoclonal antibodies (mAbs) were used: CD4 FITC (RMA4-4), CD11b FITC (M1/70), CD19 FITC (6D5), CD8 APC-H7 (53-6.7), KLRG1 BV421 (2F1), PD-1 PE-Cy7 (29F.1A12) all from BD Biosciences (San Jose, CA) except PD-1 (Biolegend). Dead cells were identified with LIVE/DEAD yellow fluorescent reactive dye (L34959) from Life Technologies and were excluded from analyses. Murine MHC-peptide PE-conjugated multimers were from Proimmune (Oxford, UK) or from Immudex (Copenhagen, Denmark). Multimer gating strategy used a dump gate (CD4, CD11b, CD19), a CD8 gate and excluded dead cells.

Intracellular staining of murine spleen cells was performed after stimulation with the indicated peptides and in presence of CD107a BV711 mAb (1D4B, BD Biosciences) for 6h in the presence of Brefeldin A (GolgiPlug, BD Biosciences). Intracellular staining was done with mAbs to IFN-γ APC (XMG1.2, BD Biosciences), tumor necrosis factor α PE (MP6-XT22, BD Biosciences), IL2 AlexaFluor488 (JES6-5H4, BD Biosciences), and corresponding isotype controls (BD Biosciences). Fixation and permeabilization was carried out using the BD Bioscience kit according to manufacturer’s instructions.

BMDCs were stained with CD11c PE (HL3), IA-IE BV421 (M5/114.15.2), CD40 APC (3/23) and CD86 PE-Cy7 (GL1) and Live Dead Yellow (ThermoFisher Scientific).
Human monocyte-derived DCs were stained with CD1a PE (H149), HLA-DR PE-Cy5 (BB7.2), CD80 BV605 (L307.4), CD83 FITC (HB15e), CD86 APC (2331) (all from BD Biosciences) and Live Dead Yellow.

For ATP125 internalization monitoring, cells were stained with anti-6His antibody (clone 6His, BioLegend) unconjugated or FITC-conjugated on cell surface and following fixation-permeabilization step using the CytoFix-CytoPerm kit from BD Biosciences according to manufacturer’s instructions. For the extracellular only condition, cell surface was stained with the FITC-conjugated anti-6His, for the intracellular only condition, cell surface was stained with the unconjugated anti-6His, and the intracellular compartment stained with the FITC-conjugated anti-6His. For the extra and intracellular condition, both the cell surface and the intracellular compartment were stained with the FITC-conjugated anti-6His.

For staining of non-human primate cells, the following antibodies were used: CD45 FITC (D058-1283), CD4 BV605 (L200), CD123 BV786 (7G3), CD14 BV650 (M5E2), CD20 V450 (L27), CD8 PerCP-Cy5.5 (RPA-T8), CD16 APC-H7 (3G8), CD3 PE-Cy7 (SP34-2), HLA-DR PE-Cy5 (G46-6), CD28 APC (CD28.2), CD95 BV421 (DX2), CD56 PE (MY31), CD123 BV786 (7G3), CD107a FITC (H4A3), IL-2 FITC (MQ1-17H12), Ki67 AlexaFluor488 (B56) and TNF-α PerCP-Cy5.5 (MAb11) from BD Biosciences, and PD1 APC-Cy7 (EH12.2H7), IFN-γ APC (4S.B3) and CD11c APC (S-HCL-3), from Biolegend.

Fixation and permeabilization was performed using the CytoFix-CytoPerm kit from BD Biosciences according to manufacturer’s instructions. Cells were analyzed using a Gallios flow cytometer (Beckman Coulter) or an LSR II flow cytometer (BD Biosciences) and results were processed with FlowJo (TreeStar, Inc.) or Kaluza (Beckman Coulter) software.
**ELISPOT assay**

The enzyme-linked immunospot (ELISPOT) assay for detection of peptide-specific gamma interferon (IFN-γ)-secreting T cells was performed essentially as described previously (36). Briefly, mouse splenocytes were incubated overnight in coated ELISPOT plates (Diaclone) in the presence or absence of 5 µM of peptide epitope. In the same manner, cynomolgus splenocytes were incubated over-night in pre-coated Elispot plates (MabTeck) in the presence or absence of a pool of 15 amino acid-long peptides (5 µM each), with an 11 amino acid-long overlapping region for the four antigens, since cynomolgus animals were outbred with unknown HLA types. The number of peptide-specific IFN-γ-producing cells was calculated by subtracting the number of IFN-γ-secreting cells cultured without peptide from that obtained with cells cultured with peptide.

**Quantification of cytokine production**

The production of IL-8 cytokine was measured in supernatants from HEK-Blue™ hTLR2 cells and HEK-Blue™ hTLR4 cells, using commercial ELISA kits according to the manufacturer's recommendations (R&D Systems). IL-6 in the supernatant of BMDCs cultures was measured by commercial Biolegend ELISA kit following the manufacturer's recommendations. A multiplex ProCartaPlex kit, including IFN-γ, IL-1b, IL-12p40, IL-2, IL-6, IL-8, MCP-1, TNF-α and IL-10 was used to measured cytokines from the cynomolgus monkey plasma samples.

**Humoral immune response**

Mice were bled at different time points after vaccination with Z13Mad5Anaxa. Antibody titers against Z13Mad5Anaxa were measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated with 10 µg/ml of Z13Mad5Anaxa in PBS, overnight at 4°C. After washing with PBS-
0.05% Tween 20 and blocking with PBS-0.05% Tween 20 containing 1% bovine serum albumin, serial dilutions of sera were added to the plates and incubated at 37°C for 2 h. After washing, the plates were incubated with peroxidase-conjugated goat anti-mouse IgG (Catalog A90-131P, Bethyl, Montgomery). Cut-off was determined as the optical density (OD) mean plus 4SD of non-vaccinated mice. Antibody titer was determined as the last dilution factor for which the OD was the upper the cut-off value.

Cynomolgus monkeys were bled at different time points after vaccination with ATP125. A similar ELISA assay to that described above was performed but ELISA plates were coated with 10 µg/ml of ATP125 in PBS and HRP-Conjugated Goat anti-monkey IgG heavy and light chain was used for detection (Catalog A140-102P, Bethyl, Montgomery).

**ATP125-loaded human DC ligandome analysis**

DCs from HLA-A2⁺ donors were prepared from leukapheresis products, cultured in complete RPMI medium with granulocyte–macrophage colony-stimulating factor (GM–CSF; 2500 U/ml) (Miltenyi) and interleukin-4 (IL-4; 1000 U/ml) (Miltenyi). The immature DCs were loaded with 300 nM ATP125, stimulated with MPLA (1.4 µM, Avanti Polar Lipids) or control buffer overnight at 37°C before being scraped and pelleted for further ligandome analysis. A small number of cells were kept for activation state analysis by flow cytometry. HLA class I and class II molecules were isolated using standard immunoaffinity purification as described before (37, 38). In brief, the pan-HLA class I-specific mAb W6/32, the pan-HLA class II-specific mAb Tü-39, and the HLA-DR-specific mAb L243 (all produced in-house) were used to extract HLA ligands.

Peptide samples were separated by nanoflow high-performance liquid chromatography (RSCLnano, Thermo Fisher Scientific) using a 50µm x 25cm PepMap rapid separation liquid
chromatography column (Thermo Fisher Scientific) and a gradient ranging from 2.4% to 32.0% acetonitrile over 90 min. Eluting peptides were analyzed in an online-coupled LTQ Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) using a top speed collision-induced dissociation (CID) fragmentation method for HLA class I or a higher-energy collisional dissociation (HCD) fragmentation method for HLA class II. For data processing the Proteome Discoverer 1.4 (Thermo Fisher Scientific) was used to integrate the search results of the SEQUEST HT search engine (University of Washington) (39) against the human proteome as catalogued in the Swiss-Prot database (20,279 reviewed protein sequences, September 27th 2013) combined with the construct ATP125 without enzymatic restriction. Precursor mass tolerance was set to 5 ppm, and fragment mass tolerance to 0.02 Da. Oxidized methionine was allowed as a dynamic modification. The false discovery rate (FDR, estimated by the Percolator algorithm 2.04) (40) was limited to 5% for HLA class I and 1% for HLA class II. Peptide lengths were restricted to 8-12 amino acids for HLA class I and 8-25 amino acids for HLA class II. Protein inference was disabled, allowing for multiple protein annotations of peptides. To determine HLA class I binding peptides, the NetMHCpan-3.0 algorithm (41, 42) with a percentile binding rank below 2% and the SYFPEITHI (43) algorithm with a threshold of 50% of the maximum score were used.

Statistical Analysis

Statistical analyses were performed using Prism software (GraphPad) and considered statistically significant if p < 0.05.

Study approval
Mouse studies have been reviewed and approved by the institutional and cantonal veterinary authorities in accordance with Swiss Federal law on animal protection. Monkeys were enrolled in compliance with Animal Health regulation, in particular: Council Directive No. 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes.

**Author contributions**

E.B. and M.D. designed the vaccine platform, E.B. and M.D. designed the syngeneic mouse experiments, J.F.M. designed the in vitro experiments, A.N. and S.S. performed the ligandome analysis, E.B., J.F.M, S.C., W.D.B.B., E.D. and C.V. performed the in vivo and in vitro experiments, E.B., J.F.M, M.L.S.R., P.R.W. and M.D. analyzed the data and wrote the manuscript.

**Acknowledgments**

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References

Figure 1. Optimization of the vaccine construct based on self-adjuvanticity. (A) Scheme of KISIMA™ vaccination platform consisting of 3 modules: the cell-penetrating peptide, the multiantigenic domain and the TLR agonist. (B) HEK-Blue™ hTLR2 cells and HEK-Blue™ hTLR4 cells were incubated with 1μM of vaccine construct or adjuvant (MPLA or Pam3Cys), medium or buffer. After 24h, supernatants were harvested and IL-8 measured by ELISA. One experiment shown representative of 2 (mean ± SEM). *, p<0.05; **, p<0.01 (Unpaired T test). (C) THP1-XBlue™-MD2-CD14 cells were incubated with various concentrations of vaccine constructs, medium or buffer. After 18h, supernatants were recovered and SEAP activity was measured by QUANTI-Blue™ assay (InvivoGen). The EC50 of the Z13Mad5Anaxa and Mad5Anaxa was calculated from the obtained dose-response curves using Prism software. (D) The binding of ATP125 to TLR4, TLR2 and TLR3 was measured by surface plasmon resonance analysis for different concentrations of ATP125: 100, 200, 300 (in duplicate), 400 and 500nM and sensorgrams were obtained. All curves indicate the response after subtraction of non-specific binding of molecules to a control channel.
Figure 2. **Z13Mad5Anaxa showed the strongest anti-tumor effect.** (A) Tumor growth curve of C57BL/6 mice (n=7 mice/group) implanted s.c. with EG7-OVA cells and vaccinated twice (d5 and d13) with EDAZ13Mad5 or Z13Mad5Anaxa proteins. Values are represented as the mean ± SEM. One experiment shown representative of 2. *, p<0.05; ****, p<0.0001 (2-way ANOVA). (B) Tumor growth curve of C57BL/6 mice (n=7 to 14 mice/group) implanted s.c. with EG7-OVA cells and vaccinated twice (d5 and d13) with Z13Mad5Anaxa, Mad5Anaxa or Z13Mad5 with MPLA. Values are represented as the mean ± SEM. A pool of 2 independent experiments is shown. *, p<0.05; **, p<0.01, ****, p<0.0001 (2-way ANOVA). (C) Mice were vaccinated twice (d0 and d14) with different constructs with or without adjuvants. One week after the last vaccination, multimer staining was performed on blood cells for detecting OVA257-264-specific CD8 T cells. A pool of 3 independent experiments is shown (mean ± SEM, n=4 to 6 mice/group). *, p<0.05 (Kruskal-Wallis test). (D) Mice were vaccinated three times (d0, d14, d28) with two different constructs. One week after the last vaccination, multimer staining was performed on blood cells for detecting OVA257-264-specific CD8 T cells (mean ± SEM, n=2 to 4 mice/group). *, p<0.05 (Kruskal-Wallis test).
**Figure 3.** KISIMA™ vaccine induces antigen-specific T cell response in several tumor models. (A) Mice were vaccinated 6 times with Z13Mad5Anaxa, (B) 4 times with Z13Mad10Anaxa, (C) 4 times with Z13Mad8Anaxa as recombinant protein or synthetic peptide. One week after the last vaccination, ELISPOT assays were performed on spleen cells for detecting (A) IFN-γ-producing OVA<sub>257-264</sub>-specific CD8 T cells (OVACD8), OVA<sub>323-339</sub>–specific CD4 T cells (OVACD4) and gp100<sub>25-33</sub>-specific CD8 T cells (gp100CD8). A pool of 2 independent experiments is shown (n=4 to 10 mice/group). **, p<0.01 (Mann-Whitney test).
(B) IFN-γ-producing HPV16E7\textsubscript{48-57}-specific CD8 T cells and HPV16E7\textsubscript{48-57}-specific CD4 T cells (n=4 mice/group). *, p<0.05 (Mann-Whitney test). (C) IFN-γ-producing gp70\textsubscript{427-435}-specific CD8 T cells (Gp70-CD8) and gp70\textsubscript{609-622}-specific CD4 T cells (Gp70-CD4) (n=2 to 4 mice/group). *, p<0.05 (Mann-Whitney test). (D) Survival curve and mean tumor volume of C57BL/6 mice (n=7 mice/group) vaccinated with Z13Mad11Anaxa 21 and 7 days before s.c. implantation with MC38 cells. Median survival is indicated on the graph (m.s.). (E) Survival curve and mean tumor volume of C57BL/6 mice (n=7 mice/group) implanted s.c. with TC-1 cells. Mice were vaccinated at d10, d17, d28 and d49 with Z13Mad10Anaxa. Median survival is indicated on the graph (m.s.). One experiment shown representative of 3. *, p<0.05, **, p<0.001, ****, p<0.0001 (Log-Rang test). (F) Mice were implanted i.v. with B16-OVA cells and vaccinated twice (d0 and d10) with Z13Mad5Anaxa, Mad5Anaxa or Z13Mad5 with Pam3CSK4. On day 17, mice were euthanized and the number of lung metastatic foci was counted (n=5 to 7 mice/group). A pool of 2 independent experiments is shown. *, p<0.05 (Kruskal-Wallis test). (G) For different tumor models, blood cells and lung (LILs) or tumor-infiltrating leukocytes (TILs) from control and vaccinated mice were analyzed for antigen-specific CD8 T cells. C57BL/6 mice were implanted i) i.v. with B16-OVA tumor cells, vaccinated at d0 and d10 and analyzed 2 weeks later (n=5 mice/group); ii) s.c. with EG7 cells, vaccinated at d5 and d13 and analyzed 1 week later (n=4 mice/group); iii) s.c. with MC38 colorectal tumor cells, vaccinated at d3, d10 and d17 and analyzed 1 week later; a pool of 3 independent experiments is shown (n=10 to 14 mice/group) or iv) s.c. with TC-1 tumor cells, vaccinated at day 7 and day 14 and analyzed 2 weeks later; a pool of 2 independent experiments is shown (n=7 mice/group). * p<0.05, ** p<0.01, *** p<0.001 (Mann-Whitney test). (A-G) Mean ± SEM are shown.
Figure 4. ATP125 is processed and presented by human DCs. (A) Representative intracellular and extracellular anti-His staining of human DCs stimulated with 300nM of ATP125. One experiment shown representative of 3. (B) Identification of naturally presented HLA class I and HLA class II-restricted peptides by ATP125-loaded human DCs using a mass spectrometry-based immunopeptidomics approach. The bold lines indicate regions of the recombinant protein containing HLA class I or class II-presented peptides. (C) Human DCs were cultured overnight with 300nM of ATP125 before cytometry analysis. Histograms of activation markers are shown. One experiment shown representative of 3. (D) Heatmap of secreted cytokines/chemokines (pg/ml) by 24h activated human DCs (n=3) or human PBMCs (n=3) with 300nM of ATP125, MPLA (TLR4 ligand), Pam3CSK4 (TLR2 ligand) or R848 (TLR7/8 ligand), equivalent volume of ATP125 buffer and control cells.
Figure 5. ATP125 elicits T-cell responses in non-human primates. (A) Vaccination and assessment schedule of *Cynomolgus fascicularis* non-human primates. (B) Cellular immune response against CEA, MUC-1, survivin and EPCAM was monitored by IFN-γ ELISPOT on PBMC prior to any vaccination (left panel) or 1 week after the last vaccination on day 50 (left panel) in animals vaccinated with 76 or 380 µg of ATP125 s.c. or i.d. Representative ELISPOT IFN-γ wells for one animal out of three for each treatment group are shown.
Figure 6. Self-adjuvanting efficacy of KISIMA™ vaccines further enhanced with anti-PD1 therapy. (A) Survival curve and mean tumor volume of C57BL/6 mice (n=14 mice/group) implanted s.c. with MC38 cells. One group was used as control. In the other groups, mice were vaccinated with Z13Mad12Anaxa s.c. on d3, d10 and d17, injected with a mouse-specific anti-PD1 i.p. from d6 to d31, or received the combination treatment. A pool of 2 independent experiments is shown. (B) Survival curve and mean tumor volume of C57BL/6 mice (n=7 mice/group) implanted s.c. with TC-1 cells. One group was used as control. In the other groups, mice were vaccinated with Z13Mad10Anaxa s.c. on d7, d14, d28 and d49, injected with a mouse-specific anti-PD1 i.v. on the same days, d7, d14, d28 and d4, or received the combination treatment. One experiment shown representative of 3. Median survival is indicated on the survival graph (m.s.). *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001 (Log-Rang test). *, p<0.05, **, p<0.01, ****, p<0.0001 (2-way ANOVA for tumor growth curves).
Table 1. Tumor size and tumor-infiltrating leucocytes

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<th>p value</th>
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<td>Tumor size (mm$^3$)</td>
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<td>0.099 ± 0.036</td>
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<td>Tumor-infiltrating IFN-γ and TNF-α-producing HPVE7$^{49-57}$-specific among CD8 T cells (%)</td>
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<td>Z13Mad10Anaxa</td>
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<td>Tumor-infiltrating Tregs among CD4$^+$ T cells (%)</td>
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<td>16.4 ± 1.7</td>
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Table 2. HLA haplotype for each donor and total number of ATP125-derived HLA class I or II-presented peptides. The number of peptides showing a canonical HLA binding motif and thereby predicted by NetMHCpan 3.0 or SYFPEITHI as HLA class I ligands is indicated in bold.

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<th>CEA</th>
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<th>MUC1</th>
<th>EPCAM</th>
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<td>0</td>
<td>1</td>
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<td>A<em>02:02, A</em>34:02, B<em>18, B</em>44</td>
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<th>Donor</th>
<th>HLA class II haplotype</th>
<th>CEA</th>
<th>survivin</th>
<th>MUC1</th>
<th>EPCAM</th>
</tr>
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<td>1</td>
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Table 3. Spots/million of blood CD3\(^+\) cells from NHP vaccinated with 76 or 380µg of ATP125 s.c. or i.d.

<table>
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<tr>
<th>Treatment</th>
<th>Animal #</th>
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<th>MUC1</th>
<th>EPCAM</th>
<th>survivin</th>
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<td>Pre Vac</td>
<td>Post-Vac4</td>
<td>Pre Vac</td>
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