In situ delivery of nanoparticles formulated with micron-sized crystals protects from murine melanoma

Mona O Mohsen, Matthew Heath, Matthias F Kramer, Thalia Carreno Velazquez, Alan Bullimore, Murray A Skinner, Daniel E Speiser, Martin F Bachmann

ABSTRACT

Introduction Intratumoral injections of novel therapeutics can activate tumor antigen-specific T cells for locoregional tumor control and may even induce durable systemic protection (against distant metastases) via recirculating T cells. Here we explored the possibility of a universal immunotherapy that promotes T-cell responses in situ and beyond, upon intratumoral injection of nanoparticles formulated with micron-sized crystals.

Methods Cucumber mosaic virus-like particles containing a tetanus toxin peptide (CuMVₜ) were formulated with microcrystalline tyrosine (MCT) adjuvant and injected directly in B16F10 melanoma tumors. To further enhance immunogenicity, we loaded the nanoparticles with a TLR7/8 ligand and incorporated a universal tetanus toxin T-helper cell peptide. We assessed therapeutic efficacy and induction of local and systemic immune responses, including RNA sequencing, providing broad insight into the tumor microenvironment and correlates of protection.

Results MCT crystals were successfully decorated with CuMVₜ nanoparticles. This ‘immune-enhancer’ formed immunogenic deposits in injected tumors, enhanced polyfunctional CD8⁺ and CD4⁺ T cells, and inhibited B16F10 tumor growth locally and systemically. Local inflammation and immune responses were associated with upregulation of genes involved in complement activation and collagen formation.

Conclusions Our new immune-enhancer turned immunologically cold tumors into hot ones and inhibited local and distant tumor growth. This type of immunotherapy does not require the identification of (patient–individual) relevant tumor antigens. It is well tolerated, non-infectious, and affordable, and can readily be upscaled for future clinical testing and broad application in melanoma and likely other solid tumors.

INTRODUCTION

In situ (intratumoral) immunotherapy aims at generating local and systemic anti-tumor effects. This can mainly be achieved by priming an immune response in a tumor lacking pre-existing immunity or priming and boosting insufficiently immunogenic tumors via activating tumor-infiltrating lymphocytes (TILs). Intratumoral immunotherapy has several advantages such as increasing the local concentration of immunogenic drugs, enabling combination drug therapy, decreasing systemic adverse effects, and enhancing antigenicity and immunogenicity of tumor antigens recognized by T cells.

Therefore, intratumoral immunotherapy can be a versatile approach for concomitant targeting T cells and modulating key components of the tumor microenvironment. In addition, it may be considered as ‘patient individualized’ treatment because tumor antigens are provided by the individual tumor itself.

Malignant melanomas usually arise from melanocytes in skin, mucosa, or retina. The majority of cutaneous or mucosal melanomas
grow in visible and palpable locations. Early-stage surgical excision is the first-line treatment. Melanoma prognosis may be good in case of total tumor resection, but local, regional, or distant progression remains unfortunately relatively frequent. The therapeutic accessibility of melanoma lesions permits increased interest for developing in situ and local therapies. The concept is not new as Dr Coley has made the first attempts in 1891 by performing intratumoral injections of Coley’s toxin consisting of inactivated *Streptococcus pyogenes* and *Serratia marcescens*. In 1975, a case report was published of a patient in his late 70s with 64 cutaneous melanoma lesions as well as several pulmonary metastases who was treated with intratumoral injections of BCG. The patient experienced regression of all treated cutaneous lesions, in addition to 50% regression of distant metastases. Some recent examples of intratumoral treatment in melanoma includes CMP-001, a bacteriophage virus-like particle (VLP) loaded with A-type CpGs (NCT02680184/NCT03084640, formerly called QßG107) without any tumor antigens. This therapy is currently being examined in combination with systemic anti-programmed cell death protein-1 (PD-1) in several clinical trials for patients with advanced melanoma with previous resistance to anti-PD-1 treatment. The recently collected data from the ongoing trials show manageable toxicity and potential to reverse resistance to anti-PD-1.

Preclinically, Lizotte et al have shown that intratumoral injection of cowpea mosaic virus is partially effective in treating dermal B16F10 melanomas, forming central injection sites for days, causing local inflammation. Endotoxin level was measured and was confirmed to be <1000EU/mL. The plant-virus derived CuMV TT VLPs (CuMV TT) are characterized by the triangulation number (T=3), which describes the symmetry of icosahedral particles built up by 180 monomers. CuMV TT lacks genetic material for replication and is accordingly non-infectious. The capsid monomer of CuMV TT has a size of 24 kDa and contains a tetanus toxin (TT)-derived peptide with a universal T-helper (Th) cell epitope to which most people have previously activated memory Th cells. CuMV TT also contain ssRNA that stimulates TLR7/8. It is derived from *Escherichia coli* and is packaged into the VLPs during the expression process. Once CuMV TT assembles into nanoparticles, it is long-term stable at 4°C as well as at 20°C. In several previous studies, we have demonstrated the immunogenicity of these VLPs in several animal models, including mice, cats, dogs, and horses.

Microcrystalline tyrosine (MCT) is a biodegradable depot adjuvant that is being used since decades in allergery immunotherapy (AIT). Specifically, it is formulated with different native or modified allergens for subcutaneous injections in humans. Formulating MCT with different allergoids has been shown to be safe for more than 50 years in millions of people, including vulnerable populations and long-term treatment courses. MCT consists of micron-sized L-tyrosine (L-tyr) natural crystals that cannot readily enter the lymphatics and form a depot which persists at the injection site for days, causing local inflammation. Half-life at subcutaneous injection sites is 48 hours, and biodegradation is completed by roughly 1 week. Generally, depot-forming adjuvants such as MCT may enhance T-cell responses through several mechanisms but mostly through the ability to protect antigens from degradation and clearance and prolong antigen exposure to antigen-presenting cells (APCs), thereby enhancing T-cell activation and clonal expansion. In contrast to its extended use in AIT, MCT remains largely unexplored in cancer immunology. In our previous studies, we have shown that subcutaneous vaccination with CuMV TT-p33 displaying gp33 epitope derived from lymphocytic choriomeningitis virus and formulated with MCT controlled the growth of murine p33-transfected B16F10 tumors. Mechanistically, we observed strong activation of cytotoxic T lymphocytes and favorable changes in T-cell and granulocyte populations in the tumor microenvironment.

In light of the still substantial challenges to improve immunotherapies against cancer, there is a need for effective, affordable, fast, and safe immune therapies that can be widely used for different types of solid tumors. Here, we demonstrate efficient antitumor responses induced by intratumoral injections of our novel ‘immune-enhancer’ consisting of CuMV TT nanoparticles formulated with micron-sized MCT.

**METHODS**

**Expression and production of CuMV TT VLPs**

The expression and production of CuMV TT were carried out as described in Zeltins et al. Endotoxin level was measured and was confirmed to be <1000EU/mL.

**Electron microscopy**

Physical stability and integrity of CuMV TT were visualized by transmission electron microscopy using Philips CM12 EM. For imaging, sample grids were glow discharged and 5 µL of VLP solution was added for 30 s. The grids were then washed three times with ddH2O and negatively stained with 5 µL of 5% uranyl acetate for 30 s. Finally, excess uranyl acetate was removed by pipetting, and the grids were air dried for 10 min. Images were taken with ×84,000 and ×110,000 magnification.

**Dynamic light scattering (DLS)**

CuMV TT VLP sample solution (1 mg/mL) was used for DLS analysis on Zetasizer nano ZS instrument (Malvern Instruments, UK). Results of three measurements were analyzed by DTS software (Malvern V.6.32).

**Mass spectrometry (MS)**

MS analysis was performed as described in Zeltins et al.
We used amino acid L-tyr that is a proprietary formulation manufactured under the registered trademark MCT. MCT was kindly provided by Allergy Therapeutics, UK/Bencard Adjuvant Systems. MCT crystals were imaged using bright field on a Zeiss AxioImager A2 microscope with Plan-NEOFLUAR ×20 objective (Zeiss).

CuMVTT VLPs and MCT formulation

Formulating the nanoparticles CuMVTT with MCT adjuvant was carried out by mixing both ingredients in a shaker at 500 rpm in room temperature (RT) for 1 hour. Such procedure allows the nanoparticles CuMVTT to decorate MCT crystals. For visualization of the formulation, CuMVTT VLP samples (3 mg/mL) were labeled with AF488 as per the manufacturer’s instructions, and mixed with MCT, 10 µL was added to a glass slide and covered with a cover slip. The samples were imaged using fluorescent light illumination with a Zeiss AxioImager A2 microscope with Plan-NEOFLUAR ×20 and ×40 objectives (Zeiss).

Mice

Wild-type C57BL/6 female mice (8–12 weeks, Harlan) were used in all experiments. RAG−/− C57BL/6 mice were kindly provided by Professor A Ochsenbein and were bred in our pathogen-free animal facility at University of Bern. All animal experiments were performed in accordance with the Swiss Animal Act (455.109.1 September 5, 2008) under license no. BE10/18 and BE43/21.

Treatment regimen and doses

Wild-type C57BL/6 mice were treated intratumorally on day 3 post B16F10 tumor implantation (2 mm³ fragments). Intratumoral injections were performed three times over 15 days. Treatment doses were 100 µL phosphate buffered saline (PBS) (mock), 50 µg of CuMVTT alone diluted in a final volume of 100 µL PBS/dose, or 50 µg CuMVTT formulated with 4% MCT in a final volume of 100 µL/dose. Intratumoral injections were performed three or four times as indicated in the figure legends. Mice were monitored every 2 days to assess tumor volume and general health score. Tumors in the control groups reached the ethically maximal tolerated size of 1 cm³. Tumors were collected as indicated in the Results section, and mice were monitored every 2 days.

Tumor experiments

One million B16F10 melanoma cells were injected into the flank of RAG−/− C57BL/6 mice. Tumors were allowed to grow into palpable tumors for 15 days until they reached 1 cm³. Tumors were dissected and processed into ~2 mm² fragments and kept in complete medium on ice. A tumor’s fragment was then implanted into a flank of wild-type C57BL/6 mice (8–12 weeks old, Harlan) under full anesthesia. The implanted tumor was allowed to grow for 3 days before treatment start. Implanted tumors were treated intratumorally three times over 13 or 15 days as indicated in the Results section. Tumor growth was monitored every 2 days and measured using calipers. Tumors were collected and weight was recorded. Tumors were kept in Dulbecco’s Modified Eagle Medium (DMEM) medium containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin on ice. TILs were collected as follows: tumors were dissected into pieces, digested with collagenase at 37°C for 30 min and washed using a 70 µM cell strainer. Tumors were washed during the process using DMEM medium containing 10% FBS and 1% penicillin/streptomycin in falcon 50 mL tubes. Collected cells were added to 15 mL tubes containing 2 mL of 35% Percoll slowly. The tubes were centrifuged at 1800 rpm for 25 min at RT to isolate TILs. TILs were then resuspended in 200 µL PBS, 0.1% BSA, and 100 µL was transferred to a 96-well plate v-bottom and centrifuged at 1200 rpm for 5 min. The supernatant was discarded, and red blood cells (RBCs) were lysed using 500 µL Ammonium-Chloride-Potassium (ACK) buffer (Sigma-Aldrich) on ice for 2–3 min. TILs were stained with anti-mouse CD16/CD32 (mouse BD Fc block) mAb clone 2.4G2 (BD Bioscience) for 10 min in the dark, centrifuged as described previously, and stained with live/dead 7-AAD, PE anti-mouse CD8α mAb clone 53–6.7 (BD Bioscience), APC anti-mouse Ly6G mAb clone 1A8 (BioLegend), FITC anti-mouse Ly6C clone HL3.1 (BioLegend) and APC/Cyanine7 anti-mouse CD11b clone M/170 (BioLegend). Plates were centrifuged at 1200 rpm for 5 min; the supernatant was discarded; TILs were resuspended in PBS and 0.1% BSA and were added to 5 mL round-bottom tubes with a cell strainer to remove excess tumor debris. Samples were read by FACSCaliber and analysis was done using GraphPad Prism V.8.4.2 (464). Cell density was measured by dividing the total number of cells in each tumor by its weight.

Costimulation experiments

To perform costimulation experiments, we adapted a similar method as published in van Vloten et al. Briefly, 1×10⁶ cells/well of B16F10 and MC57 (fibrosarcoma) cell lines were plated in U-bottom plates with 10 µg/mL of rIFN-γ for 48 hours. Tumor cells were pelleted by centrifugation for 5 min at 1200 rpm in complete DMEM medium. The supernatant was discarded. TILs were collected from a mock group and another group treated with CuMVTT+MCT as described in the Tumor experiments section. TILs were cocultured with tumor cells (B16F10 or MC57) for 3 days. On day 4, intracellular cytokine (ICS) was performed as explained next for detection of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) production by CD8⁺ or CD4⁺ T cells.

Depletion experiments

Anti-CD8α mAb (clone 53–6.7, 10 µg) or anti-CD4 mAb (clone GK1.5) was administered intravenously 48 hours prior to tumor implantation and subsequently every 2 days using a low dose of 10 µg. Depletion of CD8⁺ or CD4⁺ T cells was confirmed 48 hours post first administration and on day 12. Blood (150 µL) was collected from the tail...
vein in 500 µL 1× PBS containing heparin and kept on ice. Cells were centrifuged at 1200 rpm for 5 min. RBCs were lysed using 500 µL ACK buffer (Sigma-Aldrich) on ice for 2–3 min. Cells were collected by centrifugation for 5 min at 1200 rpm. The supernatant was aspirated and cells were resuspended with 1× PBS containing 0.1% BSA and centrifuged again. Pelleted cells were stained with anti-mouse CD16/CD32 (mouse BD Fc block) mAb clone 2.4G2 (BD Bioscience) for 10 min in the dark, centrifuged as described earlier, and stained with PE-anti-mouse CD8α mAb clone 53–6.7 (BD Bioscience) or FITC-anti-mouse CD4 mAb clone (RM4-5). Samples were read by a FACSCaliber and analysis was done using GraphPad Prism V.8.4.2 (464).

ICS staining for IFN-γ and TNF-α
TILs were isolated as described in the Tumor experiments section and transferred to sterile 96-well flat-bottom plates. TILs were incubated with mouse interleukin (IL)-2 (mIL2-Ref: 11271164001-MERCK) 100 U/mL in DMEM medium containing 10% FBS and 1% penicillin/streptomycin at 37°C for 2 days. TILs were washed three times with DMEM medium containing 10% FBS and 1% penicillin/streptomycin, and a stimulation cocktail was added 1 µg/mL of ionomycin/PMA / Brefeldin and Monensin (1:1000) at 37°C for 6 hours. TILs were washed three times with DMEM medium and then transferred to 96-well v-bottom plates for staining. TILs were stained with anti-mouse CD16/CD32 (mouse BD Fc block) mAb clone 2.4G2 (BD Bioscience) for 10 min in the dark, centrifuged as described previously and stained with PE-anti-mouse CD8α mAb clone 53–6.7 (BD Bioscience). The plate was centrifuged at 1200 rpm for 5 min; the supernatant was discarded; and TILs were fixed using 100 µL of the fixation buffer (BD Cytofix) at 4°C for 15 min. The plate was centrifuged at 1200 rpm for 5 min; the supernatant was discarded; and TILs were washed with 100 µL of 1× diluted permeabilization wash buffer (BioLegend) and centrifuged immediately at 1200 rpm for 5 min; the supernatant was discarded. TILs were then stained with APC-anti-mouse IFN-γ mAb clone XMG1.2 (MERCK) and PerCP-Cyamine5.5 anti-mouse TNF-α mAb clone MP6-XT22 (BioLegend). Plates were centrifuged at 1200 rpm for 5 min; the supernatant was discarded; TILs were resuspended in PBS and 0.1% BSA and added to 5 mL round-bottom tubes with cell strainer to remove excess tumor debris. Samples were read by FACSCaliber and analysis was done using GraphPad Prism V.8.4.2 (464).

Antibody measurements (ELISA)
The induced antibody response after intratumoral injections was assessed by ELISA. Mice were kept warm for up to 15 min; 100 µL of whole blood was collected from mice tail in BD Microtainer tubes. Serum was separated by centrifugation at 8000 rpm for 1 min and stored at −20°C. To determine total IgG antibody titres against CuMVTT proteins; ELISA plates were coated overnight with 2 µg/mL CuMVTT. Plates were washed with PBS 0.01% Tween and blocked using 100 µL PBS–casein 0.15% for 2 hours. Sera from treated mice were diluted 1/20 initially and then serially 1/3. Plates were incubated for 1 hour in RT. After washing with PBS 0.01% Tween, goat anti-mouse IgG conjugated to horseradish peroxidase was added 1/1000 and incubated for 1 hour at RT. Plates were developed and OD reading was performed.

Depot assessment
CuMVTT were labeled with AF488 (Fisher Scientific) for intratumoral injection with or without formulation with MCT in RAG−/− mice. Twelve days later, intratumoral injections with AF488-CuMVTT or AF488-CuMVTT+MCT were performed. Injected tumors were collected 1 and 5 days post injection, and analysis was performed to detect labeled VLPs.

Histology
Histology staining and assessment were performed at the COMPATH Institute at University of Bern. H&E staining was performed on the median-sized tumors preserved in 4% paraformaldehyde for 1 day before sending them to the COMPATH Institute for histological analysis. One full section of each collected tumor was examined after melanin bleach followed by H&E staining. The melanomas were digitally quantified for the extent of microscopic tumor necrosis (software QuPath V.0.1.2). The following parameters were measured digitally and calculated: total area of the tumor cross section, total area of tumor necrosis and percentage of tumor necrosis. Formalin-fixed slides were also assessed by immunohistochemistry staining for CD4 T cells using CD4 (clone 4S95, rat, Thermo Fisher Scientific, 14:9766). One full cross section for each tumor was examined by quantitative evaluation for peritumoral and intratumoral CD4+ T cells using the software QuPath V.0.2.3.

RNA isolation, sequencing and bioinformatics
B16F10 in vivo tumors were collected and stored in RNAlater solution (Sigma-Aldrich) according to the manufacturer’s recommendations. Samples were sent to Microsynth AG for RNA isolation and sequencing. Illumina’s TrueSeq stranded RNA library preparation kit including polyA enrichment was used to construct libraries from total RNA. Subsequently, the Illumina NextSeq 500 and 550 platforms and a high-output V2.5 kit (75 cycles) were used to sequence the libraries. The produced single-end reads which passed Illumina’s chastity filter were subject to demultiplexing with zero mismatches allowed and trimming of Illumina adaptor residues using Illumina’s bcl2fastq software V.2.20.0.422 (no further refinement or selection). Quality of the reads in fastq format was checked with the software FastQC V.0.11.8. The reads were filtered by removing PhiX controls while keeping reads with an average minimum Phred quality score of 20 and 0 ambiguous bases (Ns). The splice aware RNA mapping software STAR V.2.7.7 a was used to map the reads to the reference genome mm10 (UCSC genome browser assembly ID,
based on GRCh38.p6) as downloaded from iGenomes. To count the uniquely mapped reads to annotated genes, the software htsseq-count (HTSeq V.0.13.5) was used. Normalization of the raw counts and differential gene expression analysis were carried out with the help of the R software package DESeq2 V.1.26.0. Based on the differential gene expression results, KEGG pathway and Gene Ontology (GO) genset analyses are carried out with the help of R software package ggplot2 V.3.3.5. Libraries, sequencing and data analysis described in this section were performed by Microsynth AG (Balgach, Switzerland).

Statistics
Data are presented as mean±SEM. Comparisons between more than two groups were performed by one-way analysis of variance; comparisons between two groups were performed by Student’s non-parametric t-test. P values were ****p<0.0001, ***p<0.001, **p<0.01, and *p<0.05.

RESULTS
CuMVₜₜ nanoparticles decorate the surface of MCT micron-sized crystals
CuMVₜₜ consists of icosahedron T₃-nanoparticles and has a size of ~30 nm. Indeed, our CuMVₜₜ comprises pentamers (subunit A) and hexamers (subunits B and C) assembled together into an icosahedron (figure 1A). We have immunologically improved these nanoparticles by incorporating a universal tetanus toxin (TT) T₄ cell epitope (Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu) to the interior surface of the VLPs. This addition may enhance the immune response due to activation of pre-existing TT specific T₄ cells present in most humans, particularly useful in elderly and immunocompromised patients.11 12 Additionally, CuMVₜₜ nanoparticles package ssRNA during the expression process in E. coli, which serves as TLR7/8 ligands facilitating APCs activation. CuMVₜₜ nanoparticles were produced as detailed previously.12 Successful production and confirmation of integrity of the nanoparticles were assessed by SDS-PAGE (figure 1B), electron microscopy (figure 1C) and MS (figure 1D), all demonstrating a homogeneity of the product. Incorporation of ssRNA was visualized by agarose gel (figure 1E). DLS of the nanoparticles confirmed successful assembly of CuMVₜₜ (figure 1F). The average hydrodynamic diameter (Z(ave)) of CuMVₜₜ was ~38 nm.

MCT is an L-tyr-based T₄-polarizing adjuvant15 consisting of micron-sized crystals (figure 1G). MCT is considered a classical, biodegradable depot-forming adjuvant that is used since decades in combination with modified or native allergens for subcutaneous immunotherapy in humans.20 21 Due to their micron size of ~5 μM, MCT cannot readily enter the lymphatics and remain at the injection site and forms a depot causing local inflammation.22 20 Together, we successfully formulated CuMVₜₜ nanoparticles and MCT micro-sized adjuvant, resulting in CuMVₜₜ coated microparticles, our new immune-enhancer (figure 1H–J).

Administering the novel immune-enhancer intratumorally hinders B16F10 progression
We tested the effects of this novel immune-enhancer (CuMVₜₜ+MCT) in an aggressive melanoma murine model which we have previously established in our laboratory.26 30 This model facilitates intratumoral injections as it is based on tumor fragment implantation rather than injection of cell suspensions. Intratumoral injections with the immune-enhancer started few days post tumor implantation (figure 2A), allowing physiological stroma and tumor vascularization to be established before initiating the treatment. Appropriate control treatments were included such as mock (PBS), CuMVₜₜ (alone) and MCT (alone).

The tumor weight on day 15 showed that the treatments with MCT or CuMVₜₜ alone significantly hindered B16F10 tumor progression (p=0.0018 and 0.0012, respectively). However, the best response was achieved with the immune-enhancer (p<0.0001) (figure 2B–D). Since CD₈ T-cell infiltration often correlates with favorable therapeutic outcome and prognosis,31–33 we determined the density and total number of tumor-infiltrating CD₈ T cells. No significant increase in CD₈ T-cell density was found in the groups treated with MCT or CuMVₜₜ alone. Interestingly, T-cell infiltration was increased in mice treated with our immune-enhancer (figure 2E–F), significantly correlating with reduced tumor weight (p=0.0025) (figure 2G). IFN-γ producing CD₈ T cells were also significantly increased after treatment with the immune-enhancer (p=0.0046) (figure 2H). The density of TNF-α CD₈ T cells producing cells in tumors showed no significant increase in CuMVₜₜ+MCT; however, the polynuclear CD₈ T cells producing both IFN-γ and TNF-α were significantly increased (p=0.0054) in this group (figure 2I–J).

To evaluate specific CD₈ T-cell reactivity against the B16F10 melanoma cell line, we performed a coculture experiment. Briefly, TILs were isolated from mock-treated mice as well as from mice treated with the immune-enhancer. The isolated TILs were cocultured with the B16F10 cell line or the irrelevant HMC-matched MC57 fibrosarcoma cell line for 3 days. To induce MHC-I expression on tumor cells, B16F10 and MC57 cells were incubated with rIFN-γ for 48 hours prior co-culturing. Online supplemental figure 1A shows tumor weight on day 15 from both groups. TILs isolated from the mock group did not show reactivity against B16F10 nor MC57. TILs from the group treated with CuMVₜₜ+MCT could induce MHC-I expression on tumor cells, B16F10 and MC57 cells were incubated with rIFN-γ for 48 hours prior coculturing. These data confirm the specific reactivity of CD₈ T cells following intratumoral treatment.
Figure 1  
CuMV\textsubscript{TT} nanoparticles decorate the surface of MCT micron-sized crystals. (A) Geometry of the icosahedral CuMV\textsubscript{TT} VLPs illustrating the capsid protein in \textit{T}=3 configuration. VLPs are formed of 60 of each subunits A, B and C (illustrated here in shades for clarity). (B) SDS-PAGE analysis of CuMV\textsubscript{TT} VLPs, lane 1: protein size marker (Thermo Scientific), lane 2: 6 µg VLPs. A monomer of CuMV-protein has a size of \~[25–30 kDa]. (C) Electron microscopy image of purified VLPs, VLP sample (1.5 mg/mL) examined by JEM-100C electron microscopy, VLP’s size between 26 and 28 nm. (D) Mass spectrometry analysis confirmed a major peak of \~24,477.586. (E) Agarose gel analysis of the VLPs, lane 1: DNA marker (Thermo Fisher), lane 2: 10 µg purified VLPs, VLP sample (1.5 mg/mL). (F) Dynamic light scattering analysis of the VLP nanoparticles, VLP sample (1.5 mg/mL). Three measurements were performed and analyzed by DTS software. (G) MCT crystals visualized with light microscopy, exhibiting a micron size of ca=4.5 µm in length. (H) CuMV\textsubscript{TT} VLPs labeled with AF488 and formulated with MCT micron-sized adjuvant, referred to as immune-enhancer, \times40 objective scale bar 20 µm. (I) CuMV\textsubscript{TT} VLPs labeled with AF488 and formulated with MCT micron-sized adjuvant, \times40 objective scale bar 20 µm. (J) A cartoon illustrating CuMV\textsubscript{TT} VLPs; packaging ssRNA (in red) and decorating MCT crystals. CuMV\textsubscript{TT}, cucumber mosaic virus-like particles incorporating a tetanus toxin peptide; MCT, microcrystalline tyrosine; VLP, virus-like particle.
Figure 2  Administering the novel immune-enhancer intratumorally hinders B16F10 progression. (A) A cartoon illustrating the treatment regimen and the groups of mice treated with intratumoral injections. (B) Tumor weight (mg) measured on day 15 post B16F10 (2 mm³) implantation; each dot represents an individual tumor. Combined (C) and individual (D) tumor growth curves of B16F10 melanoma in the different mouse groups measured by caliper, as tumor volume mm³. (E) Density of CD8⁺ T cells in tumors as determined by flow cytometry. The densities were determined by dividing the total number of CD8⁺ T cells in each tumor by the tumor weight (in mg), pregated on TILs. Using FS/SS (F) FACS plots showing the total numbers of CD8⁺ T cells in a tumor, pregated on TILs. (G) Correlation between density of CD8⁺ T cells and tumor weight (mg). (H) Density of IFN-γ (I) and IFN-γ/TNF-α producing CD8⁺ T cells in tumors as determined by flow cytometry upon stimulation with 1 µg/mL PMA/ionomycin. The densities were determined by dividing the total number of CD8⁺ T cells in each tumor by the tumor weight (in mg). (J) Representative FACS plots showing the total numbers of IFN-γ/TNF-α producing CD8⁺ T cells in tumors. Statistical analysis (B,C,E,G–I) by ordinary one-way analysis of variance (multiple comparison) (mean±SEM). Statistical analysis in F by simple linear regression. n=12 (B–G) and n=5 (H,I). One representative of two similar experiments is shown. ****p<0.0001; ***p<0.001; **p<0.01; and *p<0.05. APC, antigen-presenting cell; IFN-γ, interferon gamma; MCT, microcrystalline tyrosine; ns, not significant; TIL, tumor-infiltrating lymphocyte; TNF-α, tumor necrosis factor alpha.
In a separate experiment, we measured the density of CD4+ T cells. Results showed that intratumoral treatment with the immune-enhancer increased CD4+ T-cell density in comparison to the control group (online supplemental figure 2A–C). We performed H&E staining to assess intratumoral and peritumoral CD4+ T cells. Histological sections showed a significant increase (p=0.0248 and 0.0046, respectively) in the treated group (online supplemental figure 2D–F).

### CD8+ T cells play an essential role in the antitumor impact of the immune-enhancer

To better understand the role of CD8+ T cells in the antitumor impact of the immune-enhancer (CuMV TT +MCT), we depleted CD8+ T cells. Briefly, anti-CD8α mAb was administered intravenously 48 hours prior to tumor implantation and subsequently every 2 days. Effective depletion of CD8+ T cells in the blood was confirmed before tumor implantation and on day 12 post tumor implantation. Tumor-bearing mice were treated intratumorally with mock (PBS), CuMV TT +MCT alone or in combination with anti-CD8α mAb.

Treatment with anti-CD8α mAb depleted ~95% of CD8+ T cells (figure 3A). Interestingly, intratumoral injection of CuMV TT +MCT resulted in a significant increase in the systemic (blood) percentage of CD8+ T cells, which was abolished after CD8+ T-cell depletion (figure 3B,D), as well as granulocytic cells characterized by CD11bHiLy6GHi 12 days post tumor implantation (after two injections) (figure 3C,E). Tumors were collected on day 15 post tumor implantation. Results were consistent with the aforementioned findings, and tumors treated with the immune-enhancer showed significant tumor weight reduction (p=0.0037) in comparison to the control group or the group with CD8+ T-cell depletion (p=0.0072) (figure 3F). The CD8+ T-cell density was increased only in the group treated with CuMV TT +MCT (figure 3G). In the next step, we depleted CD4+ T cells to determine their possible contribution to the induced antitumor effect (online supplemental figure 3A,E). Tumors collected on day 15 showed a small trend of weight increase which was, however, not statistically significant (p=0.2857) (online supplemental figure 3B). The density of CD8+ T cells was higher in the groups treated with the immune-enhancer with or without CD4+ T cell depletion than the mock (online supplemental figure 3C) but not in the groups treated with CuMV TT +MCT. When assessing the density of CD4+ T cells in the tumor microenvironment, the results showed a significant increase in the group treated with the immune-enhancer in comparison to the mock (online supplemental figure 3D). As expected, there were no intratumoral CD4+ T cells upon depletion (online supplemental figure 3D). Collectively, these data confirm that CD8+ but not CD4+ T cells are key for the induced antitumor effects.

### Intratumoral administration of CuMV TT +MCT forms a depot, enhances inflammation, and reduces tumor necrosis

The observed strong T-cell responses may be enabled by the capability of MCT to form depots, prolonging immune stimulation and antigen exposure to APCs, thereby enhancing T-cell activation and clonal expansion. To explore this possibility in vivo, we formulated fluorescently labeled AF488-CuMV TT with and without MCT and performed intratumoral injections in RAG2/− mice harboring B16F10 melanoma (online supplemental figure 4A). Tumors were collected 1 and 5 days post treatment, and flow cytometry was performed to assess the percentage of CD11b+ cells positive for AF488-CuMV TT. The labeled VLPs could be detected 1 day after intratumoral injection in both groups at similar levels. However, 5 days later, the percentages of positive cells in the group injected with CuMV TT +MCT were significantly higher compared with the group injected with CuMV TT alone (p=0.0047) (online supplemental figure 4B). These data confirm that MCT promotes depot persistence in situ.

To assess whether intratumoral administration of the immune-enhancer (CuMV TT +MCT) impacts on immunosuppressive cells, we measured the percentages of granulocytic and monocytic myeloid-derived cells in the tumor by focusing on the CD11bαLy6GHi and CD11bαLy6CHi populations. Due to the short life span of neutrophils, we collected the treated tumors 1 day after the third intratumoral injection (day 13). Interestingly, we found a significant increase in the granulocytic population (p=0.0286) (figure 4A,B), while the monocytic population showed a significant reduction (p=0.0286) (figure 4C,D) compared with the mock (PBS) group. Both the increase of granulocytic cells and the decrease of monocytic cells correlated significantly with the tumor weight (p=0.0413 and 0.0041, respectively) (figure 4E,F). We were also interested in examining the histological changes of the treated tumors on day 15 after third intratumoral injections. We quantified necrotic tumor areas by digital histological assessment. Several studies have shown that tumor necrosis reflects the presence of hypoxia in the tumor, which can indicate an aggressive phenotype of rapid cellular proliferation.34 We have found that the intratumoral administration of the immune-enhancer could significantly reduce (p=0.0159) the total number of necrosis in the tumor in comparison to the mock group (figure 4G,H), which is likely related to the reduced size of the tumors.

### Systemic therapeutic effects induced by the treatment with the immune-enhancer

We have shown previously that, when injected subcutaneously in the footpad of mice, the VLPs drain readily into the lymphatic system, as expected for such particles with a size of ~30 nm.35 Furthermore, CuMV TT p33 displaying p33 T-cell epitope formulated with MCT crystals formed depots at subcutaneous injection sites. These depots facilitated slow release and were detectable for at least 9 days.26 Accordingly, to confirm that in situ injections of CuMV TT +MCT can induce a systemic response, we first measured anti-CuMV TT VLP antibodies in the sera of treated mice after two intratumoral injections. As expected, the treatment with CuMV TT or CuMV TT +MCT readily induced anti-CuMV TT antibodies (figure 5A). No
Figure 3  CD8+ T cells play an essential role in the antitumor impact of the immune-enhancer. (A,B) Percentage (%) of CD8+ T cells in peripheral blood 48 hours post anti-CD8+ mAb administration and on day 12 post tumor implantation; each dot represents an individual tumor. Depletion efficacy was ~95%. (C) Percentage (%) of CD11b+Ly6G+ cells in peripheral blood; each dot represents an individual tumor. (D) Representative FACS plots showing the percentage of CD8+ T cells in peripheral blood. (E) Representative FACS plots showing the percentage of CD11b+Ly6G+ cells in peripheral blood. (F) Tumor weight (in mg) measured on day 15 post B16F10 (2 mm3) implantation; each dot represents an individual tumor. (G) Density of CD8+ T cells in tumors as determined by flow cytometry. The densities were determined by dividing the total number of CD8+ T cells in each tumor by the tumor weight (in mg), pregated on TILs. Statistical analysis by ordinary one-way analysis of variance (multiple comparison) (mean±SEM) n=5 or 6, one representative of two similar experiments is shown. ****p<0.0001; ***p<0.001; **p<0.01; and *p<0.05. MCT, microcrystalline tyrosine; ns, not significant. TIL, tumor-infiltrating lymphocyte.
Figure 4  Intratumoral administration of CuVM_{T\!T}+MCT forms a depot, enhances inflammation and reduces tumor necrosis. (A) Percentage (%) of CD11b^{hi} Ly6G^{hi} cells in tumors; each dot represents an individual tumor. (B) Representative FACS plots showing the percentage (%) of CD11b^{hi} Ly6G^{hi} cells in a tumor. (C) Percentage (%) of CD11b^{hi} Ly6C^{hi} cells in tumors; each dot represents an individual tumor. (D) Representative FACS plots showing the percentage (%) of CD11b^{hi} Ly6C^{hi} cells in a tumor. (E) Correlation between percentage (%) of CD11b^{hi} Ly6G^{hi} cells and tumor weight (in mg). (F) Correlation between percentage (%) of CD11b^{hi} Ly6C^{hi} cells and tumor weight (in mg). (G) Total number of necrosis (mm^2); each dot represents an individual tumor. (H) Example of a mock tumor (left): necrosis comprises 30% of the tumor section, example of a treated tumor with the immune-enhancer (right): necrosis comprises 2% of the tumor section. Necrosis is indicated in yellow (*). The entire tumor cross section was assessed for necrosis, H&E staining. Statistical analysis (A,C,G) by Student's t-test (mean±SEM). Statistical analysis (E,F) by linear regression. n=4 (A–C) and n=5 (E,F,G), one representative of two similar experiments is shown. MCT, microcrystalline tyrosine.
Figure 5  Systemic therapeutic effects induced by the treatment with CuMVTT+MCT. (A) Log OD50 of CuMVTT specific IgG antibodies in serum of treated mice. (B) A cartoon illustrating the treatment regimen and mouse groups using intratumoral injections. (C) Tumor weight (mg) measured on day 14 post B16F10 (2 mm³) implantation; each dot represents an individual tumor. (D) Representative images of B16F10 tumors on both flanks in a mock and a treated mouse; right treated tumors are marked with yellow stars and left untreated tumors with green stars. (E) Density of CD8⁺ T cells in treated and untreated tumors in each group, as determined by flow cytometry. The densities were determined by dividing the total number of CD8⁺ T cells in each tumor by the tumor weight (in mg), pregated on TILs. (F) Representative FACS plots showing the total number of CD8⁺ T cells in treated and untreated tumors in each group, pregated on TILs. Statistical analysis (A) by one-way analysis of variance (multiple comparison) (mean±SEM). Statistical analysis (C,E) by Student’s t-test (mean±SEM) n=5. One representative of two similar experiments is shown. MCT, microcrystalline tyrosine; TIL, tumor-infiltrating lymphocyte.
antibodies were detected in the groups after mock or MCT-alone treatments. Together with the increased T-cell frequencies in blood (figure 3B), these results indicate that intratumoral treatment with VLPs can effectively induce a systemic immune response.

To test for a potential abscopal effect, two tumors were implanted in each mouse flank as illustrated in figure 5B. Only the right tumor was treated twice intratumorally, while the left one was left untreated. As the total tumor burden was higher in this experiment, we increased the treatment regimen to two injections weekly.

The obtained results revealed that intratumoral treatment with the immune-enhancer in one implanted tumor can induce an antitumor response in the untreated side of the tumor as well. This was shown by the decreased tumor weight in both right and left implanted tumors in the treated group in comparison to the mock group (figure 5C,D). The density of CD8+ T cells in the right (treated) tumor was significantly higher than that in the right tumor in the control group (p=0.0317). The density of CD8+ T cells in the left untreated tumor of the treated mice showed a trend but not a statistically significant increase (p=0.0556) likely related to relatively large heterogeneity (figure 5E,F).

Treatment with the novel immune-enhancer inhibits tumor recurrence

The observed antitumor immune response in vivo of the immune-enhancer promoted us to determine whether it could prevent recurrence after primary melanoma resection, an endpoint with clinical relevance. To this end, a similar experimental setting as described in the section (Administering the novel immune-enhancer intratumorally hinders B16F10 progression) was used, and primary tumors were resected on day 15 as illustrated in figure 6A. The tumor weight in the group treated with the immune-enhancer was significantly lower (p=0.0038) (figure 6B). The mice were subsequently monitored for tumor local and metastatic recurrence. We found that 75% of mice treated with the immune-enhancer had disease-free survival, while only 25% had local melanoma recurrence (figure 6C). In contrast, all of the non-treated animals succumbed to lethal tumor recurrence. Thus, intratumoral treatment with the immune-enhancer was capable

Figure 6  Treatment with the novel immune-enhancer inhibits tumor recurrence. (A) Mice were implanted with B16F10 tumor fragment on day 0 and treated three times intratumorally over 15 days. The primary tumor was surgically resected on day 15 under isoflurane anesthesia. (B) Tumor weight (in mg) measured on day 15 post B16F10 (2 mm³) implantation; each dot represents an individual tumor. Statistical analysis by Student’s t-test (mean±SE). (C) Mouse survival; mice were euthanized when the volume of recurrent tumors reached 1 cm³, statistical analysis by log-rank test. n=4, one representative of two similar experiments is shown. MCT, microcrystalline tyrosine.
of inducing a systemic effect that protected against tumor recurrence.

**Transcriptional changes induced by the intratumoral treatment**

To identify the molecular changes in the tumor microenvironment following intratumoral administration of the immune-enhancer and to obtain broad insight in the transcriptional landscape, we performed RNA sequencing (RNA-Seq) profiling. We compared B16F10 tumors treated intratumorally with the immune-enhancer (CuMV_{TT}+MCT) versus mock (PBS). Differential gene expression was analyzed using the first principal component (PC), revealing a 70% distinct gene expression on intratumoral treatment compared with mock. In situ treatment with the immune-enhancer also formed gene groups that were most distant from mock-treated tumors for PC2 (23% variance) and PC3 (4% variance) (figure 7A,B).

Differential gene expression showed an overexpression of several genes involved in complement activation as well as collagen formation. The overexpressed genes of the complement pathway were C1qa, C1ab, C1qc, C1s, C3, and C6, while the overexpressed genes associated with collagen formation included Col6a2, Col6a1, and Col5a1 (figure 7C,D).

GO and KEGG pathway analyses were performed to identify the biological processes significantly enriched with differentially upregulated and downregulated genes in both treated tumors. We report here the enrichment of the top 15 GO biological processes and KEGG pathways in the tumors treated with CuMV_{TT}+MCT (figure 7E,F, and online supplemental figure 5A,B). Upregulated GO biological processes were collagen-containing extracellular matrix, leukocyte migration, cytokine production, cell chemotaxis, myeloid leukocyte activation and phagocytosis. KEGG pathway analysis showed significant upregulation in genes involved in cytokine receptor interaction, cell adhesion molecules, complement and coagulation pathways, as well as chemokine signaling pathways and leukocyte migration.

**DISCUSSION**

In this study, we developed an immune-enhancer for intratumoral administration with the aim to promote immunity and reverse immune suppression in the tumor microenvironment. Our immune-enhancer consists of the modified plant-derived CuMV_{TT} and packaged with ssRNA (TLR7/8 ligand), formulated together with a micron-sized MCT adjuvant. The novel immune-enhancer is applicable to individuals with solid tumors without the need to know tumor-specific antigens. Our data show efficient local and systemic tumor control of aggressive implanted B16F10 tumors through activation of CD8+ T cells and reduction of immunosuppressive myeloid cells.

Intratumoral injection of immunostimulatory adjuvants such as CpG-oligomucleotides, a TLR-9 ligand, has shown promising therapeutic effects through activation of both innate and adaptive immune system components. In murine models, TLR-9 is expressed by almost all dendritic cells (DCs); however, in humans, its expression is much more limited, essentially to plasmacytoid DCs and B cells. In contrast, targeting of TLR7/8 also leads to the highly desired activation of myeloid DCs in both mice and humans. We took advantage of the fact that ssRNA (a TLR7/8 ligand) is naturally packaged during expression of VLP proteins such as our CuMV_{TT} in *E. coli*. Our newly optimized nanoparticles also incorporate a TT peptide in its interior surface which enhances T_{c}c-cell activation, which is particularly powerful in TT-immune elderly people and individuals with otherwise weak immune responses. B cells are also activated by our immune-enhancer, likely further enhancing tumor inflammation by local immune complex formation. Several recent studies have pointed out the potential importance of B cells and tertiary lymphoid structures (TLSs) for the activation of tumor-specific CD8+ T cells in patients with melanoma, correlating with patient survival. We are currently investigating whether our novel immune-enhancer is capable of inducing TLS formation.

In the tumor microenvironment, neutrophils represent a heterogenous population with different phenotypes and opposing functions. Mouse models were used to classify tumor-associated neutrophils in antitumor neutrophils (N1) and tumor-promoting neutrophils (N2). The antitumorogenic function of N1 cells is exerted by direct cytotoxicity, antibody-dependent cytotoxicity, and/or activation of different innate and adaptive immune cells including DCs, natural killer cells, and T and B cells. Lizotte et al have demonstrated that in situ immunotherapy with cowpea mosaic virus by inhalation resulted in activation of Ly6G+ CD11b+ neutrophils 24 hours later. Previous studies have shown the ability of MCT to induce the secretion of caspase-dependent IL-1β from human monocytes in vitro, which was inhibited by zVAD, indicating that MCT can cause an acute transient inflammatory response that recruits granulocytes. This is supported by the finding that intraperitoneal injections of MCT in murine models promoted an inflammatory response of Ly6G+ CD11b+ cells which was inflammmasome independent and lasted for 24 hours. Neutrophils can be attracted to tumors by several mechanisms including chemokines produced by tumor cells. This is consistent with RNA-Seq data, suggesting that intratumoral administration of the immune-enhancer supports neutrophil infiltration through upregulation of several neutrophil attracting chemokines such as CXCL1 (KC), CXCL2 (MIP-2), and CXCL16. Several studies have shown that the prognostic impact of tumor necrosis may represent a paradoxical relationship whereby evidence of increased tumor cell death indicates a more aggressive cancer and decreased necrosis may predict a more benign situation. This relationship can be explained by rapid tumor growth that has outgrown its own blood supply, creating a hypoxic microenvironment
Figure 7  Transcriptional changes induced by the intratumoral treatment. (A,B) Principal component analysis (PC1, PC2 and PC3—together representing 97% of the variance in the data) of genes differently expressed between the two biological groups (mice with B16F10 tumors treated with immune-enhancer CuMV_TT+MCT vs mock group). (C) Volcano plot of differentially expressed genes (treated vs mock). The plot illustrates the log2 fold change threshold and the threshold for p values adjusted for multiple testing (Benjamini-Hochberg procedure). Upregulated genes are presented in red and downregulated in green. (D) Heat map of differential gene expression of mice treated with immune-enhancer versus mock group. RNA-Seq analysis was performed on RNA isolated from triplicate samples for each biological group. (E,F) Analyses of GO and KEGG pathway enrichment were performed to identify biological processes and pathways significantly enriched with upregulated and downregulated genes in tumors for each biological group. Enrichment scores show gene count and statistical significance determined with Fisher’s exact test and presented for the top 15 biological process-related GO terms and the top 15 KEGG pathways. FDR, false discovery rate; GO, gene ontology; MCT, microcrystalline tyrosine; RNA-Seq, RNA sequencing.
and subsequently causing tumor cell death. The presence of necrosis was significantly associated with advanced stage, poor differentiation, vascular invasion, and large tumor size.46 We found reduced tumor necrosis in the treated tumors, likely reflecting reduced growth and, in particular, size of these tumors.

We found that our immune-enhancer induced upregulation of several collagen genes such as COL6A1, COL6A2, as well as COL5a1. Interestingly, a recent study has shown that type VI-α collagens such as COL6A1 and COL6A2 may act as classical collagens in bladder carcinoma and form a physical barrier to inhibit tumor growth and invasion.47 COL6A1 and COL6A2 could effectively inhibit human bladder carcinoma BCaEJ cells proliferation in vitro, induced cell cycle arrest, and inhibited wound healing and invasion by suppressing matrix metalloproteinase MMP-2 and MMP-9.48 It remains to be determined whether similar mechanisms apply in our model.

The complement system is considered a phylogenetically conserved branch of the innate humoral immune system, which rapidly responds to microbial intruders and consequent systemic T-cell activation.49-50 Given the strong immunogenicity of our novel therapy and the consequent systemic T-cell activation, it was perhaps not surprising that distant tumors were also inhibited. Even after surgical removal of the primary tumor, we found an efficient systemic effect preventing tumor recurrence, a finding of direct potential clinical relevance.

Together, our new immune-enhancer triggers multiple immune pathways likely explaining its considerable antitumor effects. As good manufacturing practice GMP-grade MCT is available and production of GMP-grade CuMV Tt is planned for the near future, this novel approach will be readily translated for clinical application for the treatment of the increasing numbers of tumor types that are accessible for intratumoral injection.

Acknowledgements We thank Marc Gasser from Microsynth for RNA sequencing data and pathway enrichment analyses, and Dr Simone Danielle De Brot at COMPATH, University of Bern, for histology.

REFERENCES
1 Marabelle A, Tseliakas L, de Baere T, et al. Intratumoral immunotherapy: using the tumor as the remedy. Ann Oncol 2017;28:xii33–43.
2 Nobukou D, Yoshikawa T, Fujisawa T, et al. Peptide intra-tumor injection for cancer immunotherapy: enhancement of tumor cell antigenicity is a novel and attractive strategy. Hum Vacc Immunother 2013;9:1234–6.
3 Bommareddy PK, Silk AW, Kaufman HL. Intratumoral approaches for the treatment of melanoma. Cancer J 2017;23:40–7.
4 Middleton MR, Hoeller C, Michielin O, et al. Intratumoral immunotherapies for unseetable and metastatic melanoma: current status and future perspectives. Br J Cancer 2020;123:885–97.
5 McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. Iowa Orthop J 2006;26:154–8.
6 Mastrandelo MJ, Bellet RE, Berkelhammer J, et al. Regression of pulmonary metastatic disease associated with intrallesional BCG therapy of intracutaneous melanoma metastases. Cancer 1975;36:1305–8.
7 Sabree SA, Voigt AP, Blackwell SE, et al. Direct and indirect immune effects of CMP-001, a virus-like particle containing a TLR9 agonist. J Immunother Cancer 2021;9.
8 Ribas A, Medina T, Kirkwood JM, et al. Overcoming PD-1 blockade resistance with CpG-A Toll-like receptor 9 agonist Vidutolimod in patients with metastatic melanoma. Cancer Discov 2021;11:2998–3007.
9 Lizotte PH, Wen AM, Sheen MR, et al. In situ vaccination with cowpea mosaic virus nanoparticles suppresses metastatic cancer. Nat Nanotechnol 2016;11:295–303.
10 Lee KL, Murray AA, Le DHT, et al. Combination of plant virus nanoparticle-based in situ vaccination with chemotherapy potentiates antitumor response. Nano Lett 2017;17:4019–28.
11 Mohsen MO, Augusto G, Bachmann MF. The 3Ds in virus-like particle based-vaccines: “Design, Delivery and Dynamics”. Immunol Rev 2020;296:155–68.
12 Zeltins A, West J, Zabel F, et al. Incorporation of tetanus-epitope into virus-like particles achieves vaccine responses even in older
Open access

recipients in models of psoriasis, Alzheimer’s and cat allergy. Npj Immunov 2017;2.

13 Mohsen MO, Balke I, Zinkhan S, et al. A scalable and highly immunogenic virus-like particle-based vaccine against SARS-CoV-2. Allergy 2022;7:154–66.

14 Cabral-Miranda G, M Salman A, O Mohsen M, et al. Dpis adjuvant confers enhanced protection against malaria for VLP-TRAP based vaccines. Diseases 2018;6. doi:10.3935/diseases6040107. [Epub ahead of print: 21 11 2018].

15 Cabral-Miranda G, M Salman A, O Mohsen M, et al. Correction: Zika Virus-Derived E-DIII Protein Displayed on Immunologically Optimized VLPs Induces Neutralizing Antibodies without Causing Enhancement of Dengue Virus Infection. Vaccines 2019, 7, 72. Vaccines 2020;8. doi:10.3390/vaccines8010094. [Epub ahead of print: 20 02 2020].

16 Fettelschoss-Gabriel A, Fettelschoss V, Olomski F, et al. Active vaccination against interleukin-5 as long-term treatment for insect-borne hypersensitivity in horses. Allergy 2019;74:572–82.

17 Bachmann MF, Zeltins A, Kalnins G, et al. Vaccination against IL-31 for the treatment of atopic dermatitis in dogs. J Allergy Clin Immunol 2018;142:279–81.

18 Olomski F, Fettelschoss V, Jonsdottir S, et al. Interleukin 31 in insect bite hypersensitivity—Alleviating clinical symptoms by active vaccination against IL-31. Allergy 2019;144:193–203.

19 Thom S, Jennings GT, Maudrich M, et al. Immunization of cats to induce neutralizing antibodies against Fel d 1, the major feline allergen in human subjects. J Allergy Clin Immunol 2019;2019:144;193–203.

20 Baldick R, Johnson D, Wheeler AW. Review of L-tyrosine confirming its safe human use as an adjuvant. J Appl Toxicol 2002;22:333–44.

21 Heath MD, Mohsen MO, de Kam P-J, et al. Shaping Modern Vaccines: Adjuvant Systems Using MicroCrystalline Tyrosine (MCT®). Front Immunol 2019;10:414.

22 Bell AJ, Heath MD, Hewings SJ, et al. The adsorption of allergoids and 3-O-desacyl-4′-monophosphoryl lipid A (MPL®) to microcrystalline tyrosine (MCT) in formulations for use in allergy immunotherapy. J Inorg Biochem 2015;152:147–53.

23 Leuthard DS, Dull A, Freiberger SN, et al. Microcrystalline tyrosine and aluminum as adjuvants in allergen-specific immunotherapy protect from IgE-mediated reactivity in mouse models and act independently of inflammamome and TLR signaling. Journal of Immunology 2018;200:3151–8.

24 Sharpow E, Ezell C. The size of micro-crystalline tyrosine (MCT®) influences its recognition and uptake by THP-1 macrophages in vitro. RSC Adv 2019;9:24505–18.

25 Awate S, Babliuk LA, Mutwiri G. Mechanisms of action of adjuvants. Front Immunol 2019;10:154–66.

26 Mohsen MO, Heath MD, Cabral-Miranda G, et al. Correction to: vaccination with nanoparticles combined with micro-adjuvants protects against cancer. J Immunother Cancer 2019;7:137.

27 Zeltins A, West J, Zabel F, et al. Incorporation of tetanus-epitope into virus-like particles achieves vaccine responses even in older recipients in models of psoriasis, Alzheimer’s and cat allergy. NPJ Vaccines 2017;2;10.

28 van Vloten JP, Santry LA, McAusland TM, et al. Quantifying antigen-specific T cell responses when using Antigen-Agnostic immunotherapies. Mol Ther Methods Clin Dev 2019;13:154–66.

29 Leuthard DS, Duda A, Freiberger SN, et al. Microcrystalline tyrosine and aluminum as adjuvants in allergen-specific immunotherapy protect from IgE-mediated reactivity in mouse models and act independently of inflammamome and TLR signaling. J Immunol 2018;200:3151–9.

30 Mohsen MO, Vogel M, Riether C, et al. Targeting mutated plus germ line epitopes confers pre-clinical efficacy of an instantly formulated cancer Nano-Vaccine. Front Immunol 2019;10:1015.

31 Tumeh PC, Harvie CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515:568–71.

32 Edwards J, Wilmott JS, Madore J, et al. CD103+ Tumor-Resident CD8+ T Cells Are Associated with Improved Survival in Immunotherapy-Naïve Melanoma Patients and Expand Significantly During Anti-PD-1 Treatment. Clin Cancer Res 2018;24:3036–45.

33 Matsumoto H, Thike AA, Li H, et al. Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer. Breast Cancer Res Treat 2016;156:237–47.

34 Hiraoka N, Ino Y, Sekine S, et al. Tumour necrosis is a postoperative prognostic marker for pancreatic cancer patients with a high interobserver reproducibility in histological evaluation. Br J Cancer 2010;103:1057–65.

35 Mohsen MO, Gomes AC, Cabral-Miranda G, et al. Delivering adjuvants and antigens in separate nanoparticles eliminates the need of physical linkage for effective vaccination. J Control Release 2017;251:92–100.

36 Koster BD, van den Hout MCM, Sluijter BJ, et al. Local adjuvant treatment with low-dose CpG-B offers durable protection against disease recurrence in clinical stage II-I melanoma: data from two randomized phase II trials. Clin Cancer Res 2017;23:5679–86.

37 Mohsen MO, Rothen D, Balke I, et al. Neutralization of MERS coronavirus through a scalable nanoparticle vaccine. NPJ Vaccines 2021;6:107.

38 Cabrita R, Lauss M, Sanna A, et al. Author correction: tertiary lymphoid structures improve immunotherapy and survival in melanoma. Nature 2020;580:E1.

39 Fridlender Z, Rabin R, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 2009;16:183–94.

40 Mantovani A, Cassatella MA, Costantini C, et al. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol 2011;11:319–31.

41 Uribe-Querol E, Rosales C. Neutrophils in cancer: two sides of the same coin. J Immunol Res 2015;2015:983698.

42 Masucci MT, Minopoli M, Carriero MV. Tumor associated neutrophils, their role in tumorigenesis, metastasis, prognosis and therapy. Front Immunol 2019;10:146.

43 Zhang L, Ran L, Garcia GE, et al. Chemokine CXCL16 regulates neutrophil and macrophage infiltration into injured muscle, promoting muscle regeneration. Am J Pathol 2009;175:2518–27.

44 Bachmann IM, Ladstein RG, Straume O, et al. PD-L1 associates with hypoxia, angiogenesis and inflammation responses. Int J Oncol 2008;8:

45 Bredholt G, Mannelqvist M, Stefansson IM, et al. Tumor necrosis is an important hallmark of aggressive endometrial cancer and associates with hypoxia, angiogenesis and inflammation responses. Oncotarget 2015;6:39676–91.

46 Pollheimer MJ, Komprat P, Lindth RA, et al. Tumor necrosis is a new promising prognostic factor in colorectal cancer. Hum Pathol 2010;41:1749–57.

47 Piao X-M, Hwang B, Jeeo P, et al. Collagen type VIα1 and 2 repress the proliferation, migration and invasion of bladder cancer cells. Int J Oncol 2021;59. doi:10.3892/ijo.2021.5217. [Epub ahead of print: 13 05 2021].

48 Mohsen MO, Gomes AC, Vogel M, et al. Interaction of viral Capsid-Derived virus-like particles (VLPs) with the innate immune system. Vaccines 2018;6. doi:10.3390/vaccines6030037. [Epub ahead of print: 02 07 2018].

49 Reis ES, Mastellos DC, Ricklin D, et al. Complement in cancer: untangling an intricate relationship. Nat Rev Immunol 2018;18:5–18.

50 Revel M, Daugen MV, Sautès-Fridman C, et al. Complement system: promoter or suppressor of cancer progression? Antibodies 2020;9. doi:10.3390/antib9040057. [Epub ahead of print: 25 Oct 2020].