

# Arginase-1-specific T cells target and modulate tumor-associated macrophages

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## ABSTRACT

**Background** Arginase-1 (Arg1) expressing tumor-associated macrophages (TAMs) may create an immune-suppressive tumor microenvironment (TME), which is a significant challenge for cancer immunotherapy. We previously reported the existence of Arg1-specific memory T cells among peripheral blood mononuclear cells (PBMCs) and described that Arg-1-based immune modulatory vaccines (IMVs) control tumor growth and alter the M1/M2 macrophage ratio in murine models of cancer. In the present study, we investigated how Arg1-specific T cells can directly target TAMs and influence their polarization.

**Methods** Murine Arg1-specific CD4+T cells isolated from splenocytes of animals vaccinated with an Arg1-derived peptide in the adjuvant montanide were co-cultured with either *in vitro* M2-differentiated bone marrow-derived macrophages or *ex vivo* isolated F4/80+TAMs. Human Arg1-specific CD4+T cell clones were co-cultured with Arg1-expressing TAMs generated *in vitro* from either PBMC-derived CD14+cells or the myeloid cell lines MonoMac1 and THP-1. MHC class II-restricted Arg-1 peptide presentation by macrophages was confirmed by immunopeptidomics. T-cell-mediated changes in the macrophage immune phenotype and cytokine microenvironment were examined using flow cytometry, RT-qPCR and multiplex immunoassay. The effect of Arg1-derived peptide IMV on TAMs *in vivo* was assessed by multiplex gene analysis of F4/80+cells.

**Results** We show that Arg1-based IMV-mediated tumor control was linked to a decrease in multiple immunosuppressive pathways in the TAM population of the treated animals. Tumor-conditioned media (TCM) derived from Arg1-vaccinated mice induced significantly higher upregulation of MHC-II on exposed myeloid cells compared with controls. Furthermore, murine CD4+Arg1-specific T cells were able to target TAMs and effectively reprogram their phenotype *ex vivo* by secreting IL2 and IFN $\gamma$ . Next, we established that human Arg1+TAMs present Arg1-derived peptides and are directly recognized by proinflammatory CD4+Arg1-specific T cell clones. These CD4+Arg1-specific T cells were able to reprogram TCM-conditioned macrophages as observed by increased expression of CD80 and HLA-DR.

**Conclusions** TAMs may be directly targeted and modulated by Arg1-specific CD4+T cells. These findings provide a strong rationale for future clinical development of Arg1-based IMVs to alter the immune-suppressive TME

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Arg1-specific T cells are present in the memory T cell compartment of both healthy donors and patients with cancer. In mouse models, immune modulatory vaccination with a class II-restricted Arg1-derived peptide controls tumor growth in different models.

## WHAT THIS STUDY ADDS

⇒ We demonstrate that Arg1-specific CD4+T cells target and modulate the tumor microenvironment by altering the phenotype of tumor-associated macrophages.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings support the development of Arg1-based immune-modulatory vaccines as a promising approach to targeting macrophage-mediated immunosuppression in cancer.

by reprogramming TAMs and promoting a proinflammatory TME.

## INTRODUCTION

Immune-modulatory vaccines (IMVs) offer a novel therapeutic modality that combines the specific targeting of immune regulatory cells and the engagement of T cell-mediated immunity by activating and expanding anti-regulatory T cells (anti-Tregs).<sup>1</sup> Anti-Tregs recognize tumor microenvironment antigens (TMAs)<sup>2</sup> expressed by regulatory cells—for example, IDO1,<sup>3,4</sup> PD-L1<sup>5–7</sup> and Arg1.<sup>8,9</sup> An IDO1-derived and PD-L1-derived peptide-based IMV has shown a significant improvement in the objective response (OR) rate in a phase 1/2 clinical trial involving patients with advanced metastatic melanoma who were treated in combination with anti-PD-1 therapy<sup>10</sup>; 80% of patients achieved an OR, as compared with around 42% in a matched

historical cohort, and 50% of the 30 patients obtained a complete response as recently updated.<sup>11</sup> This approach is currently being investigated in a phase III trial as a first-line treatment for metastatic melanoma (<https://clinicaltrials.gov/>, NCT05155254).

The similar activation of Arg1-specific T cells represents a promising novel approach directed against Arg1-expressing regulatory cells frequently found in the tumor microenvironment (TME). In preclinical mouse models, we demonstrated effective control of tumor growth in animals treated with an Arg1-derived peptide IMV and observed a synergistic effect when combined with anti-PD-1 treatment.<sup>12</sup> Based on these results, we set out to investigate if and how Arg1-specific T cells directly affect Arg1-expressing tumor-associated macrophages (TAMs). TAMs represent one of the most prevalent immune cell subsets found within the TME and are, therefore, a major contributor to both protumor and antitumor immune responses. Within the TME, direct interaction between macrophages and tumor-infiltrating T cells is often viewed in the context of tumor-associated antigen (TAA) presentation by the macrophages. TAMs greatly outnumber dendritic cells in the TME but are known to be less effective at stimulating T-cell proliferation.<sup>13</sup> In recent years, the focus on TAMs has increased since these cells are among the first to encounter the malignant tumor and have been shown to contribute to both the generation and maintenance of the immune-suppressive TME.<sup>14</sup> Here, we report the ability of Arg1-specific CD4<sup>+</sup> T cells to directly alter the phenotypic characteristics of TAMs *ex vivo* and *in vitro*.

## Materials and methods

### Peptides

The peptides ArgLong2 (ISAKDIVYIGLRDVPGEHY ILKTLGIKYFSMTEVDRL), Mart1-Long (RNGYRALM DKSLHVGTCALTRR) and Arg1<sub>261–280</sub> (TEEIYKTGLLSGLDIMEVNP), OVA<sub>323–339</sub> ISQAVHAAHAEINEAGR peptides were produced by Schafer-N (Denmark) at >90% purity.

### Animals and mouse tumor models

Female C57BL/6 J Bom Tac mice (8–12 weeks old) were purchased from Taconic or obtained from own breeding facility (CCIT-DK). Animal experiments were conducted at the animal facility of the Department of Oncology, Copenhagen University Hospital, Herlev, Denmark, following Federation of European Laboratory Animal Science Association guidelines and under a license issued by the Danish Animal Experimentation Inspectorate (2021-15-0201-01001). MC38 (Kerafast, CVCL-B288) and LL (ATCC, CRL-1642) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life technologies, cat. 1020070-106) and 1% penicillin/streptomycin (Gibco, cat. 61870-010). The cell lines were mycoplasma tested. For tumor inoculation,  $5 \times 10^5$  cells were subcutaneously injected into the

left flank. The mice were randomized into different treatment arms and tumor growth was measured three times a week using a vernier digital caliper. Tumor volume was calculated based on the measured perpendicular diameters by applying the following formula: volume ( $\text{mm}^3$ ) = (length  $\times$  width<sup>2</sup> / 2).

### Peptide vaccination

For vaccination, lyophilized peptides were reconstituted to a concentration of 10 mM in dimethyl sulfoxide (DMSO). Mice were vaccinated with 100  $\mu\text{g}$  of peptide emulsified in Montanide ISA 51 VG (Seppic, cat. 36 362Z), containing either Arg1 IMV (Arg1<sub>261–280</sub> peptide) or OVA peptide (OVA<sub>323–339</sub>). The emulsions were prepared by mixing the diluted peptides at a 1:1 ratio with Montanide ISA 51 VG using a double female luer lock connector (Promopla, cat. ODG0015ST). The connector was attached to 1 mL Inject-F syringes (Braun, cat. 9166017V) to ensure thorough mixing.

The administration of the peptide emulsions was performed subcutaneously at the base of the tail using a 27-gauge needle (BD Bioscience, cat. 302200). Mice were vaccinated twice at weekly intervals. Control mice received a peptide-free vaccine composed of the peptide solvent DMSO diluted in water, emulsified with Montanide ISA 51 VG.

### Tissue dissociation

For isolation of splenocyte, the spleens were collected immediately after euthanasia and mechanically disrupted by filtering through a 70  $\mu\text{m}$  strainer followed by treatment with red blood cell lysis buffer (Qiagen, cat. 158904). CD4<sup>+</sup>T cells were isolated using a CD4<sup>+</sup>T cell isolation kit (Miltenyi, cat. 130-104-454) according to the manufacturer's instructions.

For isolation of TAMs, tumors were transferred to a digestions buffer containing 2 mg/mL of collagenase IV (Millipore, cat. C5138) and 75  $\mu\text{g}$ /mL DNase (Sigma Aldrich, cat. DN25) in RPMI-1640. The tumors were cut into small pieces using scissors and enzymatically digested for 30 min at 37°C and 650 rpm. The cell suspension was filtered through a 70  $\mu\text{m}$  strainer and incubated with red blood cell lysis (Qiagen, cat. 158904). TAMs were isolated using anti-F4/80 MicroBeads UltraPure according to the manufacturer's recommendations (Miltenyi, cat. 130-110-443). The purity of the isolated TAMs was evaluated through flow cytometry following the protocol described in the manuscript. The purity of the samples obtained from isolation was 78%–97% as described in online supplemental figure 1.

### ELISpot

To quantify vaccination-specific T-cell responses in the treated animals,  $9 \times 10^5$  splenocytes were seeded per well into 96-well ELISpot plates previously coated with 6  $\mu\text{g}$ /mL anti-mouse interferon gamma (IFN $\gamma$ ) antibody (AN18 monoclonal capture antibody, Mabtech, Cat. 3321-2-1000). The samples were stimulated with

5  $\mu$ M concanavalin A as a positive control (Sigma-Aldrich, Cat. C5275), DMSO as a negative control or 5  $\mu$ M of peptide. The cells were kept in culture for 18 hours. The plates were then washed with PBS before 2 hour incubation with 1  $\mu$ g/mL of monoclonal anti-mouse IFN $\gamma$  R4-8A2 biotinylated antibody (Mabtech, cat. 3321-6-10), followed by a 1-hour incubation with streptavidin ALP (Mabtech, cat. 3321-10). For detection, the assay was developed using the BCIP/NBT plus substrate (Mabtech, cat. 3650-10). The results spots were analyzed with the CTL ImmunoSpot S6 Ultimate analyzer (Cellular Technology), and Immunospot Software (Cellular Technology).

To evaluate TAM recognition by CD4<sup>+</sup>T cells,  $7.5 \times 10^4$  TAMs were co-incubated with  $4 \times 10^5$  CD4<sup>+</sup> T cells using pre-coated mouse IL2 plates (Mabtech, Cat. 3441-4APW-2) combined with anti-mouse IL-2 biotinylated (5H4, Mabtech, Cat. 3441-6-1000). IL2 ELISpot was performed following the same procedure as described above.

### Proliferation assay

T cells purified from spleens of untreated mice using the Pan T cell isolation kit II mouse (Miltenyi, cat. 130-095-130) were then labeled with CFSE and cultured in a 1:1 dilution of tumor-conditioned media (TCM) and fresh RPMI medium supplemented with 10%FBS and 1% PenStrep. The T cells were stimulated by adding Dynabeads Mouse T-Activator CD3/CD28 (Gibco, cat. 11456D) to induce proliferation in a 1:1 bead-to-cell ratio. After a 72-hour incubation, the cells were harvested and labeled for analysis on NovoCyte Quanteon (Agilent) (see Flow cytometry section).

### Nanostring analysis of gene expression

Gene expression of sorted TAMs was analyzed using the nCounter Mouse Myeloid Innate Immunity V2 Panel (Nanostring technologies, cat. XT-CSO-MM112-12). The data were normalized to the expression of the endogenous genes included in the panel. Quality control and differential gene expression analysis were performed using the ROSALIND software. Genes that displayed a  $\geq 1.5$  fold change and p values  $< 0.05$  (not adjusted for multiple testing) were considered differentially expressed. Gene set enrichment analysis was also performed in the ROSALIND software by using nanostring pathway annotations.

### Murine bone marrow-derived macrophage

Bone marrow was isolated from the femur and tibia of C57Bl/6 J Bom Tac mice by flushing with cold PBS using a 23-gage needle and filtered through a 70  $\mu$ m cell strainer, followed by treatment with blood cell lysis buffer. Cultures were established by plating  $4 \times 10^6$  cells in a 100 mm culture plate containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS, 1% P/S and 20 ng/mL of human macrophage colony-stimulating factor (m-CSF, Peprotech, cat. 300-25-10UG), as human M-CSF is active in murine cells.<sup>15</sup> M2

polarization was induced by treatment with 20 ng/ $\mu$ L of murine IL-4 (Peprotech, cat. 214-14-20UG).

### Murine ex vivo macrophage co-cultures

Bone marrow-derived macrophage (BMDM)-M2 or freshly isolated TAMs derived from MC38-bearing mice were seeded in 96 well UpCell Nunc plate (ThermoFisher, cat. 174897) with CD4<sup>+</sup>T cells at a ratio of 1:5 in medium containing 20 ng/mL m-CSF (Peprotech) overnight.

### Luminex

Cytokine concentrations in co-culture supernatants were quantified using Bio-PlexPro mouse cytokine IL1 $\beta$ , IL2, IL4, IL10, IL12, IL13, IFN $\gamma$ , TNF $\alpha$ , VEGF and human IL2, IFN $\gamma$ , TNF $\alpha$ , IL6, IL8 cytokine assays (Bio-rad) according to manufacturer's instructions. Samples were acquired on Bio-Plex200 and analyzed using Bio-Plex Manager V6.

### Establishment of human Arg1-specific and Mart1-specific T cell clones

ArgLong2 peptide (ISAKDIVYIGLRDVPGEHYILKT LGIKYFSMTEVDRL) specific CD4<sup>+</sup>T cell clones were isolated and expanded from peripheral blood mononuclear cells (PBMCs) of patients with cancer with either melanoma (MM1636.23) or myeloproliferative neoplasms (MPN) (MPN19H2.08). Mart1-Long peptide (RNGYRALMDKSLHVGTQCALTRR) specific CD4<sup>+</sup>T cell clones were isolated and expanded from PBMCs of the cancer patient with MPN. All specific T cell clones were expanded using rapid expansion protocol (REP) with irradiated feeder cells (PBMCs from three different donors), in X-VIVO 15 (Lonza, cat. 02-053Q) +5% Human Serum, 30 ng/mL anti-CD3 antibodies (OKT3, from Janssen-Cilag or Miltenyi Biotec) and 6000 U/mL IL2 (Proleukin, Novartis).

### Flow cytometry

Human co-cultures were treated with Human TruStain FcX Fc receptor blocking solution (Biolegend, cat. 422302) and stained using HLA-DR/APC (cat. 559868), PD-L1/PE (cat. 557924), CD80/PE-Cy7 (cat. 561135) and FVS510 (cat. 564406) from BD Biosciences and CD4/BV510 (cat. 317444) from Biolegend. The samples were analyzed on FACSCanto II (BD Biosciences).

Murine co-cultures were treated with FcR blocking reagent (Miltenyi Biotec, cat. 130-092-575) and stained using antibodies from BioLegend or NordicBiosite: anti-F4/80/FITC (clone BM8, Biolegend, cat. 123108), anti-CD11b/PacificBlue (clone M1/70, Biolegend, cat. 101226), anti-PDL1/APC (clone MIH5, BD Pharmingen, cat. 564715), anti-CD80/BV711 (clone 16–10 A1, BD Pharmingen, cat 104743), anti-CD86/PerCP-Cy5.5 (clone GL-1, Biolegend, cat. 105027), anti-CD206/PE-Cy7 (clone C068C2, Biolegend, cat. 141720), anti-MHCII/APC-Cy7 (clone M5/114.15.2, Biolegend, cat. 107627) and Zombie Aqua fixable viability dye (Biolegend, cat. 423101).

The samples were fixed prior to analysis on NovoCyte Quanteon (Agilent). Data analysis was performed using FlowJo V.10 (Treestar). The gating strategy used

for the identification of macrophages throughout this manuscript is represented in online supplemental figure 1. For proliferation assay, murine splenocytes were stained with CFSE, anti-CD3/AF700 (clone 17A2, Biolegend, cat. 100216), anti-CD4/BV421 (clone GK 1.5, Biolegend, cat. 100437), anti-CD8/BV605 (clone 53–6.7, BD Horizon, cat. 563152), Zombie aqua (Biolegend, cat. 423102). Samples were acquired on NovoCyte Quanteon (Agilent) and analyzed using FlowJo V.10 (Treestar).

### Intracellular cytokine staining

Intracellular cytokine staining for IFN $\gamma$  and TNF $\alpha$  production by human Arg1-specific T cells was performed as previously described.<sup>6</sup> Briefly, T cells were stimulated with 5  $\mu$ M of peptide or co-cultured with the relevant target cells for 5 hours. GolgiPlug was added to all samples after 1 hour of incubation. Following stimulation, cells were stained with surface marker-specific antibodies, then fixed and permeabilized using the Fixation/Permeabilization Kit (eBioscience, cat. 00-5123-43). After fixation and permeabilization, intracellular markers were stained. The following antibodies were used: anti-CD4/PerCP (clone SK3, BD Biosciences, cat. 345770); CD8+/FITC (clone SK1, BD Biosciences, cat. 345772); Anti-CD3/APC-H7 (clone SK7, BD Biosciences, cat. 560275); IFN $\gamma$ -APC (clone 25723.11, BD Biosciences, cat. 341117); TNF $\alpha$ /BV421 (clone Mab11, BD Biosciences, cat. 562783). Dead cells were excluded from analysis using the FVS510 viability dye (BD Biosciences, cat. 564406). The samples were analyzed on a FACSCanto II flow cytometer (BD Biosciences).

### Cancer cell lines and TCM

THP-1 purchased from ATCC (TIB-202) and MonoMac1 cell line obtained from DSMZ, German Collection of Microorganisms and Cell Cultures (ACC 252).

Human TCM was generated from breast cancer cell line MDA-MB-231 (ATCC, CVCL\_0062) or ovarian cancer cell line SKOV-3 (ATCC, CVCL\_0532) based on Benner *et al.*<sup>16</sup> using X-VIVO 15 (Lonza)+5% HS. Harvested TCM was stored at  $-80^{\circ}\text{C}$ . All cell lines were tested and confirmed to be negative for mycoplasma.

Murine TCM was generated using tumor digests from MC38-bearing mice.  $0.6 \times 10^6$  cells were plated in 1.2 mL RPMI in 24-well plates and incubated overnight at  $37^{\circ}\text{C}$ . Harvested TCM was stored at  $-80^{\circ}\text{C}$ .

### Human TCM-differentiated myeloid target generation and co-culture

Human CD14+monocytes magnetically sorted from freshly thawed PBMCs or myeloid cancer cell lines (THP-1 or MonoMac1) were treated with 1 mL of TCM mixed with 1 mL of fresh X-VIVO 15 (Lonza)+5% HS for 48 hours prior to co-culture with T cells. HLA class II compatibility of the target cells and the specific T cell clones is summarized in online supplemental table 1.

### Immunopeptidomics

Immunopeptidomics profiling of the HLA II presented peptides was performed using TrueDiscovery platform performed by Biognosys AG (Switzerland). 235 million snap frozen THP-1 cells differentiated using TCM (TCM-THP1) from breast cancer cell line MDA-MB-231 were lysed with non-denaturing buffer and used for pan-allele HLA class II immunoprecipitation followed by peptide isolation. The eluted peptides were injected on an in-house packed reversed-phase column on a Thermo Scientific EASY-nLC 1200 nano-liquid chromatography system connected to a Thermo Scientific Orbitrap Exploris 480 mass spectrometer equipped with a Nano-spray Flex ion source and a FAIMS Pro ion mobility device (Thermo Scientific). The generated data were analyzed using Spectronaut software V.18.5.231110.55695.

### RT-qPCR

mRNA was isolated using the RNeasy Plus mini kit (Qiagen, cat. 74136) following the manufacturer's guidelines. cDNA was synthesized from 1  $\mu$ g RNA using the Maxima first strand cDNA synthesis kit for RT-qPCR (Thermo Scientific, cat. K1671). Gene expression was assessed by RT-qPCR using TaqMan Fast advanced master mix (Applied Biosystems, cat. 4444963) and TaqMan gene expression probes (Life technologies): *Arg1* (Mm00475988\_m1), and *Hprt* (Mm03024075). Human *RPLP0* (Hs99999902\_m1), *ARG1* (Hs00163660\_m1).

Thermocycling was performed in QuantStudio 6 Pro (Applied Biosystems, cat. A43181). Gene expression was analyzed using the  $2^{-\Delta\text{CT}}$  method<sup>17</sup> and normalized to *HPRT* (murine samples) or *RPLP0* (human samples).

### Statistical analysis

Statistical analysis for comparison between the different treatment groups was performed in the GraphPad Prism. Differences in flow cytometry data or gene expression were analyzed by applying an unpaired t-test while differences in tumor growth were analyzed through a mixed-effect model.<sup>18 19</sup>

## RESULTS

### Arg1 immune modulatory vaccination controls tumor growth by promoting a proinflammatory phenotype of the TAMs

We previously demonstrated that activation of Arg1-specific CD4<sup>+</sup> T cells by an Arg1-derived peptide vaccine resulted in tumor growth control and was associated with increased M1/M2 macrophage ratio in the TME of MC38 tumors.<sup>12</sup> In the present study, we investigated whether this antitumor effect was mediated by the direct targeting and modulation of the Arg1-expressing TAMs. We, therefore, treated mice inoculated with MC38 tumor cells with an Arg1 IMV consisting of Arg1<sub>261–280</sub> peptide vaccine, MHC class II-restricted OVA control peptide (OVA<sub>323–339</sub>), or a peptide-free montanide emulsion. In accordance with the previously published data,<sup>12</sup> a tumor growth delay

was observed in Arg1 peptide-vaccinated animals but not in animals treated with OVA peptide or no-peptide montanide control (figure 1A, online supplemental figure 2A–C). We confirmed that the vaccination with Arg1 IMV and OVA peptides led to comparable CD4<sup>+</sup> T cell responses against the respective peptide epitopes as detected by *ex vivo* IFN $\gamma$  ELISpot (figure 1B). To further characterize the Arg1 IMV vaccine-associated overall changes in TAM phenotype *in vivo*, we performed multiplex gene analysis of the sorted F4/80<sup>+</sup> TAMs. We identified 25 genes with a significantly differential expression in TAMs isolated from Arg1 peptide-vaccinated mice compared with the montanide control (online supplemental figure 3,4A) and 31 genes when compared with the OVA control (figure 1C and online supplemental figure 5). No significant differences were identified between the TAMs from montanide and OVA controls (data not shown). Gene set analysis revealed that Arg1 IMV treatment led to an enrichment of proinflammatory processes in the TAMs, including Th1 activation, TLR signaling and IFN signaling. A reduced enrichment of gene sets associated with overall Th2 activation and extracellular matrix remodeling was also observed (online supplemental figure 4B). Gene expression revealed a significant reduction in *Arg1* expression and an increased *Nos2* expression in TAMs from Arg1 IMV treated animals compared with montanide and OVA<sub>323–339</sub> control groups (figure 1D,E) suggesting a skewing toward iNOS mediated L-arginine metabolism and anti-tumor associated M1-like macrophage function. TAMs from Arg1 IMV treated mice also displayed a significant downregulation of several protumorigenic TAM markers, including *Trem2*, *Mrc1*, *Marco* and *Ccl24* (figure 1F–I).

#### Arg1-specific T cell-mediated TAM repolarization contributes to the generation of immune-favorable TME

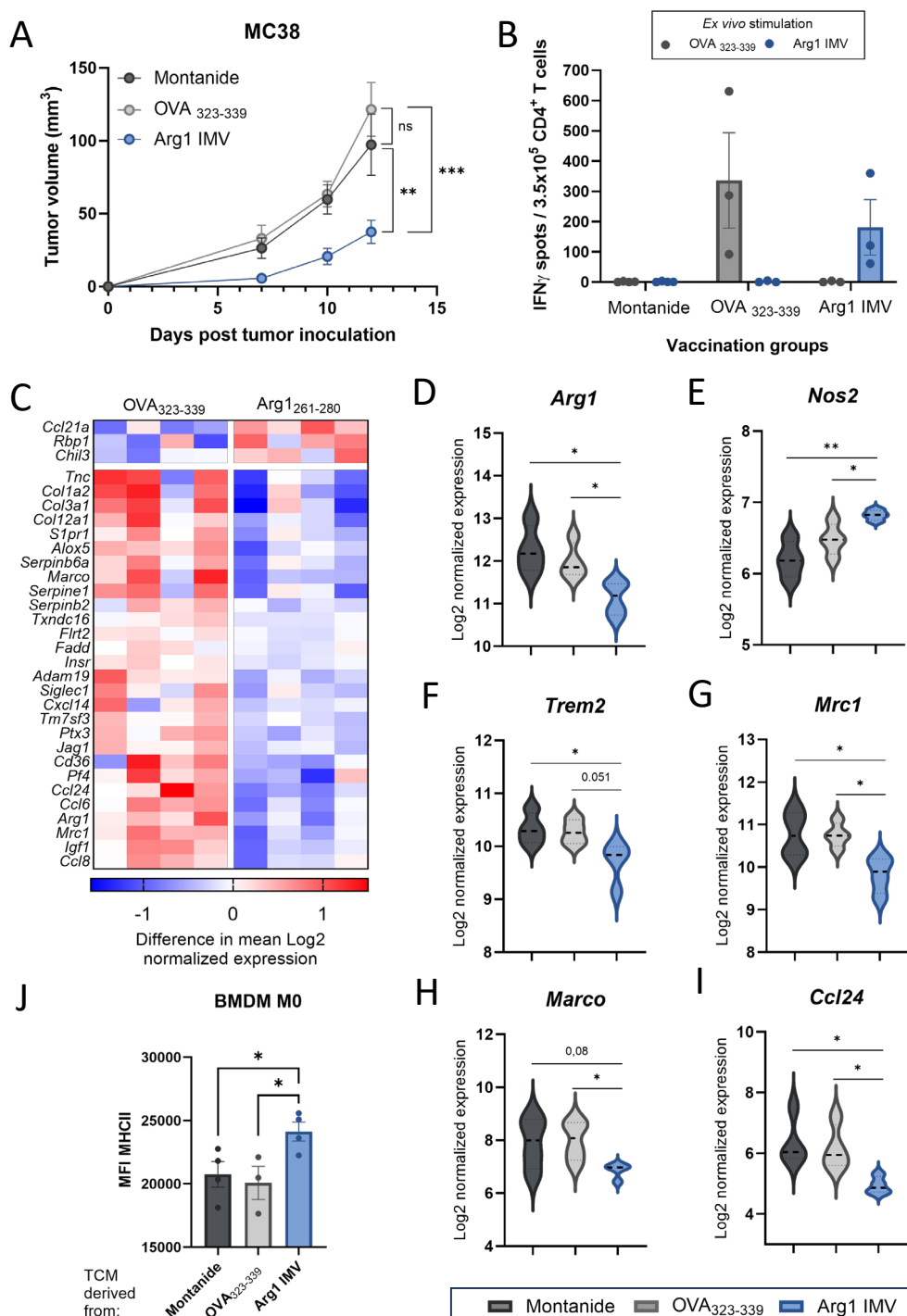
Next, to investigate if changes in the TAM phenotype observed in the Arg1 peptide treated animals were associated with an overall TME, we generated TCM from tumor digests of the different treatment groups and assessed its impact on the *ex vivo* proliferation of spleen-derived T cells and the phenotype of M0-BMDMs. The cytokine composition of the different TCM was analyzed revealing that the TCM consisted of many different cytokines including IL1 $\beta$ , IL2, IL4, IL10, IL12, IL13, IFN $\gamma$ , TNF $\alpha$ , and VEGF (online supplemental figure 6). No significant difference between the control and the Arg1 IMV TCMs was identified. Still, a significantly higher proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to aCD3/aCD28 stimulation was observed in TCM derived from Arg1 IMV-treated animals as compared with montanide control (online supplemental figure 2F,G). We additionally detected that TCM from Arg1 IMV treated mice induced a significant upregulation of MHC-II (figure 1J) and was also associated with a tendency for

a reduced Arg1 and CD206 expression (online supplemental figure 2D,E) compared with M0-BMDMs cultured with TCM derived from montanide control and OVA vaccinated animals. These results suggest that Arg1 IMV therapy supports the immune activation and the proinflammatory phenotype of newly recruited immune cells.

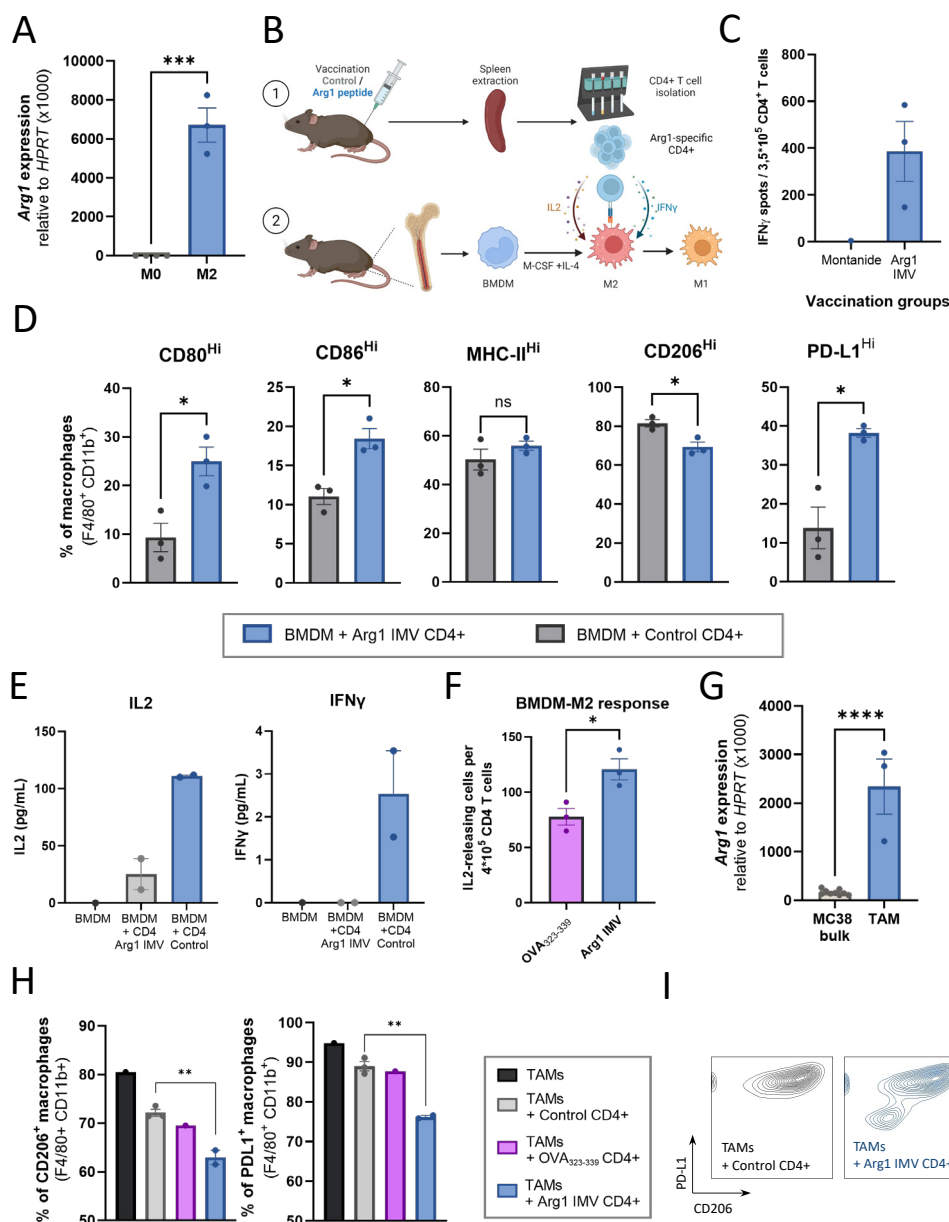
#### Murine CD4<sup>+</sup> Arg1-specific T cells recognize Arg1<sup>+</sup> TAMs and effectively reprogram their phenotype *ex vivo* by secreting IL2 and IFN $\gamma$

To further investigate how Arg1 IMV therapy can affect TAMs, we assessed the targeting of Arg1<sup>+</sup> myeloid cells by Arg1-specific CD4<sup>+</sup> T cells in murine *ex vivo* and *in vitro* models. The expression of Arg1 in myeloid cells is known to increase in response to Th2 cytokines such as IL4 and/or IL13<sup>20</sup> and is thus primarily associated with an M2-like macrophage phenotype. We confirmed an increased expression of *Arg1* in differentiated murine M2-like BMDM compared with undifferentiated M0 cells (figure 2A). Next, we investigated the direct consequences of the interaction between murine Arg1-specific T cells and Arg1-expressing macrophages by co-culturing splenic CD4<sup>+</sup> T cells from either Arg1-vaccinated or control mice with M2-like BMDMs as illustrated in figure 2B. Before establishing the co-cultures, we confirmed the generation of Arg1-specific T-cell responses induced by vaccination using IFN $\gamma$  ELISpot (figure 2C). A significant increase in the percentage of CD80<sup>Hi</sup> and CD86<sup>Hi</sup> but no significant change in MHC-II<sup>Hi</sup> macrophages was observed in co-culture with CD4<sup>+</sup> splenocytes from Arg1 IMV treated mice compared with CD4<sup>+</sup> splenocytes from control animals (figure 2D), suggesting that the targeted macrophage population had improved antigen-presenting cell function. Concurrently, we detected a significant decrease in CD206<sup>Hi</sup> macrophages, indicating a repolarization away from the M2-like phenotype (figure 2D). In addition, we observed an increase in the percentage of PD-L1<sup>Hi</sup> macrophages (figure 2D), suggesting an involvement of a counter-inflammatory response. The observed phenotypic changes of murine M2-like macrophages were accompanied by an increase in IL2 and IFN $\gamma$  in the co-cultures with CD4<sup>+</sup> splenocytes from Arg1 IMV vaccinated mice compared with co-cultures with control CD4<sup>+</sup> splenocytes or M2-differentiated BMDM alone (figure 2E). Moreover, we confirmed that BMDM-M2 were directly recognized by Arg1-specific CD4<sup>+</sup> T cell as detected by IL2 ELISpot (figure 2F).

We then investigated the reactivity and effect of Arg1-specific CD4<sup>+</sup> T cells on primary tumor-derived F4/80<sup>+</sup> TAMs from murine MC38 tumors as F4/80<sup>+</sup> TAMs showed a considerably higher *Arg1* expression compared with MC38 bulk tumor (figure 2G), therefore, being the likely target for Arg1-specific T cells in Arg1 peptide vaccine treated animals. In co-cultures with CD4<sup>+</sup> T cells, we observed a significant decrease in the CD206<sup>+</sup> TAM population isolated from MC38 after incubation with Arg1-specific CD4<sup>+</sup> T cells as compared with co-culture with



**Figure 1** Arg1 IMV treatment-induced MC38 tumor growth delay is associated with changes in TAM phenotype *in vivo*. (A) MC38 tumor growth curves of mice treated with Arg1 IMV (n=12), OVA (n=9) peptide emulsion in montanide or peptide-free montanide emulsion (n=12). 100  $\mu$ g of each peptide was injected on days 0 and 7 post-tumor inoculation. Statistical differences were identified by applying a mixed model. \*\*p $\leq$ 0.01; \*\*\*p $\leq$ 0.001. (B) Arg1 IMV and Ova<sub>323-339</sub> peptide specific responses quantified by IFN $\gamma$  ELISpot in CD4<sup>+</sup>T cells isolated from splenocytes of treated tumor bearing animals. (C) Heat map of differentially expressed genes in TAMs purified from Arg1 IMV-treated mice, in comparison with gene signature of OVA<sub>323-339</sub>-treated animals. F4/80<sup>+</sup> macrophages were sorted from MC38 tumors 13 days after tumor inoculation. Gene expression is plotted as difference with mean log2 expression. (D–I) Difference individual gene expression for TAMs sorted from peptide-free montanide control, Ova or Arg1 IMV-vaccinated animals. Differences in *Arg1* (D), *Nos2* (E), *Trem2* (F), *Mrc1* (G), *Marco* (H) and *Ccl24* (I) were evaluated by applying an unpaired T test. \*p $\leq$ 0.05, \*\*p $\leq$ 0.01. (J) Expression of MHC-II in bone marrow-derived macrophages cultured overnight with TCM derived Montanide, OVA<sub>323-339</sub> or Arg1 IMV treated MC38-bearing animals. All bars represent mean values $\pm$ SEM. Statistical differences in flow data were analyzed by applying an unpaired student t-test. \*p $\leq$ 0.05. IMV, immune modulatory vaccine; ns, not significant; TAM, tumor-associated macrophage.



**Figure 2** Murine Arg1-specific T cells reprogram Arg1-expressing macrophages ex vivo. (A) Arg1 expression determined through RT-qPCR analysis of murine bone marrow derived macrophages (BMDM) undifferentiated (M0, n=4) or differentiated into M2 (n=3). (B) Diagram representing the experimental set-up: (1) CD4 $^{+}$ T cells were isolated from the spleens of mice vaccinated with either the peptide-free control or Arg1 IMV and co-cultured ON with (2) M2 differentiated BMDM. Created with BioRender.com. (C) Arg1 IMV peptide-specific T-cell responses quantified by IFN $\gamma$  ELISpot in CD4 $^{+}$ T cells isolated from splenocytes of vaccinated mice. (D) Phenotype changes in the macrophage population as determined by changes in the percentage of CD80 $^{Hi}$ , CD86 $^{Hi}$ , MHC-II $^{Hi}$ , CD206 $^{Hi}$  and PD-L1 $^{Hi}$  macrophages in co-cultures with CD4 $^{+}$ T cells isolated from control (gray) or Arg1 IMV (blue) treated animals as determined by flow cytometry. n=3 per group. (E) IL2 and IFN $\gamma$  concentrations in co-culture supernatants from M2 macrophages alone (dark gray) or after ON co-culture with CD4 $^{+}$ T cells from control (light gray) (n=2) or Arg1 IMV-treated mice (blue) (n=2) as measured by Luminex. (F) BMDM-M2-specific responses quantified by IFN $\gamma$  ELISpot using sorted CD4 $^{+}$ T cells from OVA<sub>323-339</sub> (n=3) and Arg1 IMV (n=3) vaccinated mice. BMDM-M2 was co-incubated with CD4 $^{+}$ T cells at a ratio of 1:5. (G) Arg1 expression in unsorted MC38 tumor bulk samples (n=9) and isolated F4/80 $^{+}$ tumor-infiltrating macrophages (n=3) as measured by RT-qPCR. The expression of Arg1 was evaluated relative to hypoxanthine phosphoribosyltransferase (HPRT) expression. (H) Flow cytometry analysis of CD206 and PD-L1 expression on TAMs alone or TAMs after co-culture with CD4 $^{+}$ T cells isolated from either control animals (light gray) (n=3), OVA-vaccinated animals (purple) (n=1) or Arg1 IMV vaccinated mice (blue) (n=2). TAMs were isolated from MC38 tumors. (I) representative contour plot showing PD-L1 and CD206 coexpression on TAMs after co-culture with CD4 $^{+}$ T cells from control (gray) or Arg1 IMV (blue) treated mice. TAMs were isolated from MC38 tumors. Data are displayed as average $\pm$ SEM. Statistical differences were analyzed by applying an unpaired Student's t-test. \*p $\leq$ 0.05; \*\*p $\leq$ 0.01; \*\*\*p $\leq$ 0.001; \*\*\*\*p $\leq$ 0.0001. E:T ratio of 5:1 was used. IMV, immune modulatory vaccines; ns, not significant; TAMs, tumor-associated macrophages.

CD4<sup>+</sup> splenocytes from peptide-free control -vaccinated animals (figure 2H, left). Interestingly, a decrease in PD-L1<sup>+</sup> TAMs (figure 2H, right) was observed within the same population of macrophages displaying a decrease in CD206 expression (figure 2I). Finally, to confirm the universal applicability of our findings, we demonstrated that Arg1-expressing TAMs isolated from Lewis Lung tumors can be recognized by Arg1-specific CD4<sup>+</sup>T cells. This was shown through direct *ex vivo* recognition as detected by IFN $\gamma$  ELISpot (online supplemental figure 2H).

### Human Arg1-specific T cells directly target and modulate HLA-II<sup>Low</sup> Arg1+ myeloid populations

To investigate the translational potential of our findings, we next examined *ARG1* expression in human myeloid cells. Similarly, to the murine setting, we confirmed an increase in *ARG1* expression in a human macrophage cell line THP-1 in response to Th2 cytokines (M2-like), compared with undifferentiated M0 and IFN $\gamma$  polarized M1-like THP-1 cells. Interestingly, we observed the highest elevation in *ARG1* expression in cells treated with tumor-conditioned medium derived from a breast cancer cell line (figure 3A) (termed “TCM-THP1”) and not the Th2 cytokine treatment. The capacity of TCM to act as a potent inducer of a TAM-like phenotype *in vitro* has been described previously,<sup>16</sup> and our data reveal that this phenotype is associated with an increase in *ARG1* expression.

In the TME, immune-suppressive Arg1<sup>+</sup> myeloid cell populations such as MDSCs are known to express relatively low HLA class II levels which may hinder their recognition by Arg1-specific CD4<sup>+</sup> T cells. To examine this, we used TCM-THP1 cells as these cells express low HLA-DR levels. We observed that despite low HLA-II expression, an Arg1-specific CD4<sup>+</sup> T-cell clone was able to recognize and react against M2-like THP1 (online supplemental figure 7A) and TCM-THP1 (figure 3B). Furthermore, we confirmed by a mass spectrometry-based immunopeptidome profiling that Arg1 peptides derived from the protein region recognized by the Arg1-specific CD4<sup>+</sup> T-cell clone (ArgLong2 peptide) were present on the HLA class II molecules of TCM-THP1 cells despite general low class II expression levels (figure 3C).

Having confirmed the direct recognition of TCM-treated Arg1+myeloid cells by Arg1-specific T cells, we set out to delineate the impact of these T cells on the phenotype of the targeted myeloid cells in a series of *in vitro* experiments. First, we observed that Arg1-specific CD4<sup>+</sup> T-cell clone induced an upregulation of HLA-DR and PD-L1 on the surface of TCM-THP1 cells (figure 3D). These changes were associated with an increase in the proinflammatory cytokines IFN $\gamma$  and TNF $\alpha$ , as well as IL2, IL6, and IL8, in the microenvironment compared with HLA-DR<sup>Low</sup> TCM-THP1 and CD4+T cells cultured separately (figure 3E). The same CD4+Arg1-specific T cell clone was also confirmed to target and induce phenotypic changes in another HLA-matched myeloid cell model,

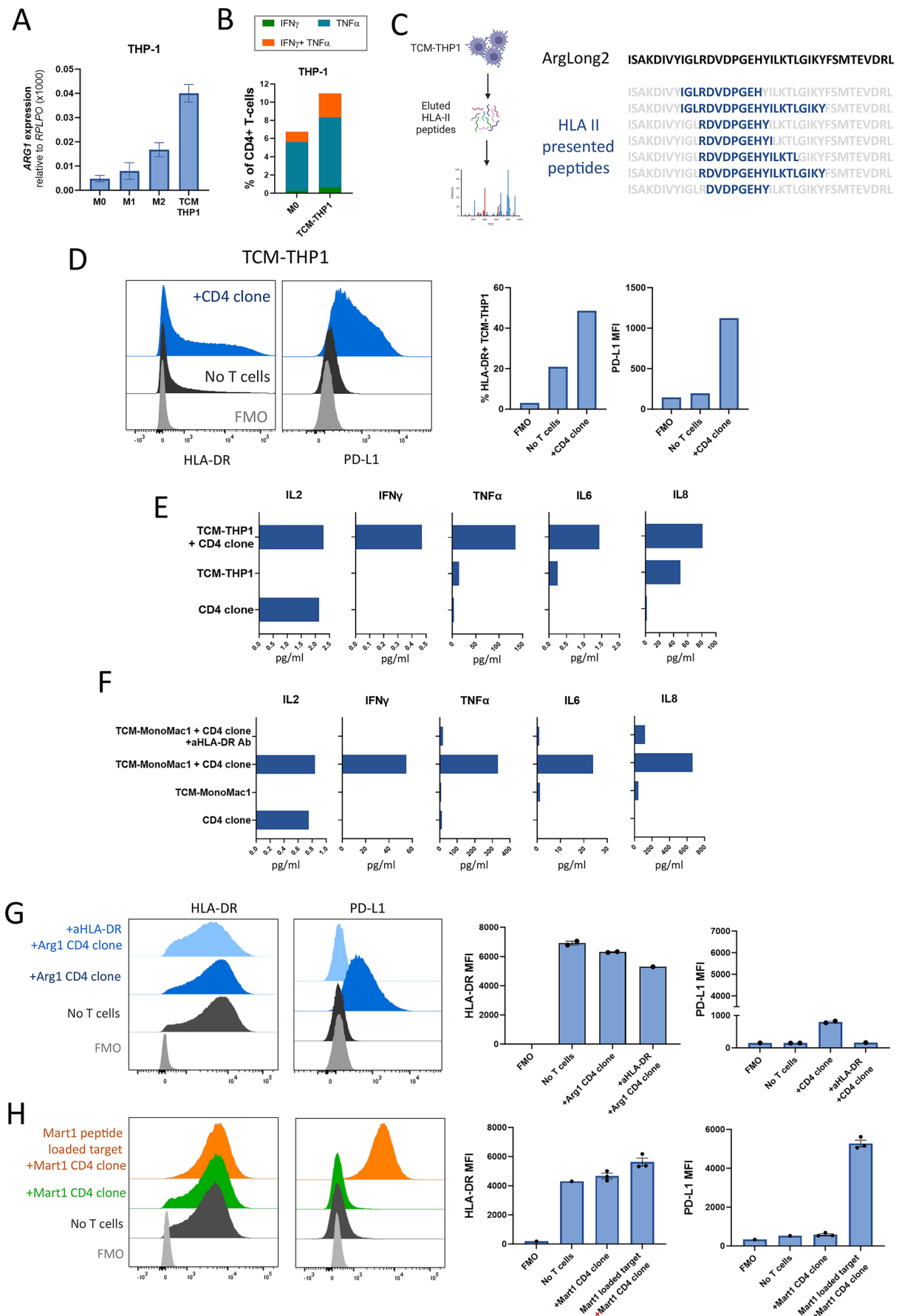
the MonoMac1 cell line, treated with TCM derived from ovarian and breast cancer cell lines (online supplemental figure 8A,B).

Next, we confirmed that the proinflammatory cytokine production patterns were dependent on direct HLA-restricted interaction between Arg1-specific T cells and Arg1+myeloid cells using an HLA-DR<sup>high</sup> HLA-matched myeloid cancer cell line MonoMac1 treated with TCM (TCM-MonoMac1). Hence, we blocked HLA-II presentation in co-cultures with TCM-MonoMac1 myeloid cancer cells and HLA-matched CD4+Arg1-specific T cell clones. No increase in IL2, IFN $\gamma$ , TNF $\alpha$ , IL6, or IL8 concentration was observed when TCR:HLA-DR interaction was blocked (figure 3F) verifying that Arg1-specific T cells require Arg1-derived peptide presentation in the context of HLA-II to modulate the cytokine microenvironment. Similarly, we did not observe a change in HLA-DR and PD-L1 expression when HLA-DR peptide presentation of TCM-MonoMac1 cells was blocked (figure 3G). The importance of HLA-peptide presentation was further confirmed in co-culture experiments with the Arg1-specific CD4+T cell clone and IL-13-treated MonoMac1 cells. The upregulation of surface marker expression and alterations in the cytokine environment was abrogated on HLA-DR blockade (online supplemental figure 9A).

Within the TME, other proinflammatory T cells specific against, for example, tumor associated antigens (TAAs) would not be able to target or modulate TAMs as these myeloid cells do not endogenously express their corresponding antigens.<sup>13</sup> To test this hypothesis, we established Mart1-specific CD4+T cell clones and co-cultured these with HLA-matched (see online supplemental table 1) TCM-MonoMac1-myeloid cells. We observed that co-culture with Mart1-specific CD4+T cells did not result in modulation of the TCM-MonoMac1 cells as measured by the lack of upregulation of either HLA-DR or PD-L1 on the surface (figure 3H). Unlike the effect seen in co-cultures with Arg1-specific CD4 clones, the modulation of the HLA-DR or PD-L1 (figure 3H) and CD80 (online supplemental figure 7B) by the Mart1-specific CD4 T cells was only observed when the minimal HLA class II Mart1 peptide epitope were externally preloaded on the TCM-MonoMac1 cells. This further demonstrates an inherent difference in the impact of Arg1-specific CD4+T cells compared with TAA-specific T cells on the reprogramming TAMs as illustrated in online supplemental figure 7C.

### Arg1-specific T cells directly modulate the phenotype of ARG1-expressing autologous myeloid cells through the secretion of proinflammatory cytokines

The elevation of *ARG1* was likewise observed in autologous CD14+monocytes isolated from PBMCs differentiated *in vitro* into a TAM-like phenotype using TCM from an ovarian cancer cell line (TCM-CD14+) (figure 4A) compared with undifferentiated M0 cells. Next, we verified that TCM-CD14+ myeloid cells with elevated *ARG1* expression were recognized by Arg1-specific CD4<sup>+</sup> T-cell



**Figure 3** (Continued)

**Figure 3** Human Arg1-specific CD4<sup>+</sup> T cells but not tumor antigen-specific T cells impact Arg-1 expressing myeloid cells and promote a proinflammatory microenvironment. (A) *ARG1* expression in human THP-1 cells as determined by RT-qPCR. THP-1 cells were either undifferentiated (M0), treated with 200 U/mL IFN $\gamma$  (M1-like), IL-13 20 U/mL (M2-like), or tumor-conditioned media (TCM-THP1) for 48 hours prior to expression analysis. All bars represent average values  $\pm$ SEM. (B) Intracellular cytokine staining of IFN $\gamma$  and TNF $\alpha$  production by Arg1-specific CD4<sup>+</sup> T cell clone reacting against HLA-matched myeloid cancer cell line THP-1. Myeloid cells were either used undifferentiated (M0) or differentiated with TCM derived from ovarian cancer cells (TCM-THP-1) for 48 hours. E:T ratio of 4:1 was used. (C) Arg1-derived peptide sequences presented by the THP-1 cells differentiated *in vitro* into a TAM-like phenotype using TCM (blue) aligned with the ArgLong2 peptide (black) as identified by mass spectrometry-based immunopeptidome profiling. Illustration of the experimental workflow created with BioRender.com. (D) representative histograms (left) and bar plots (right) representing changes in % of HLA-DR<sup>+</sup> cells and PD-L1 MFI on TCM-THP-1 after co-culture with an HLA-matched Arg1-specific CD4<sup>+</sup> T cell clone as determined by flow cytometry. (E) IL2, IFN $\gamma$ , TNF $\alpha$ , IL6 and IL8 cytokine concentrations in culture supernatants of either an Arg1-specific T cell clone co-cultured with HLA-matched TCM-THP1, TCM-THP1 alone, or the Arg1-specific CD4<sup>+</sup> T cell clone alone. (F) IL2, IFN $\gamma$ , TNF $\alpha$ , IL6 and IL8 cytokine concentrations in culture supernatants of either an Arg1-specific T cell clone co-cultured with TCM-MonoMac1 cells with or without HLA-DR blockade, TCM-MonoMac1 cells alone, or an Arg1-specific CD4<sup>+</sup> T cell clone alone. (G) Representative histograms (left) and bar plot representation (right) of changes in MFI of HLA-DR and PD-L1 on TCM-MonoMac1 cells after co-culture with HLA-matched Arg1-specific CD4<sup>+</sup> T cell clone as determined by flow cytometry. TCM-MonoMac1 cells were co-cultured with the Arg1-specific clone directly or after pre-treatment with HLA-DR blocking antibody. All T-cell and myeloid co-cultures were set up at a E:T ratio of 1:10. Error bars represent SEM of two technical replicates. (H) Representative histograms (left) and bar plot representation (right) of changes in MFI of HLA-DR and PD-L1 on TCM-MonoMac1 cells after co-culture with HLA-matched Mart1-specific CD4<sup>+</sup> T cell clone as determined by flow cytometry. Mart1-peptide loaded TCM-MonoMac1 cells (orange histograms) were used as positive control. All T-cell and myeloid co-cultures were set up at a E:T ratio of 1:10.

clones. We observed a higher production of IFN $\gamma$  and TNF $\alpha$  against TCM-CD14<sup>+</sup> compared with to the reactivity seen against their undifferentiated (M0) counterparts expressing lower levels of *ARG1* (figure 4B). Finally, we co-cultured TCM-CD14<sup>+</sup> cells with autologous Arg1-specific CD4<sup>+</sup> T-cell clones (derived from two different cancer patients) overnight and observed an increase in expression of HLA class II and CD80 compared with TCM-CD14<sup>+</sup> cultured without the addition of Arg1-specific T cells (figure 4C,D and online supplemental figure 9B). Interestingly, we also observed an increase in PD-L1 expression on TCM-CD14<sup>+</sup> in co-culture with Arg1-specific T cells. We further observed that the phenotypical changes in the TCM-CD14<sup>+</sup> polarization were accompanied by an increase in the levels of secreted IL2, IFN $\gamma$ , TNF $\alpha$ , IL6, and IL8 in the co-culture supernatants compared with T cells and TCM-CD14<sup>+</sup> cultured alone (figure 4E, online supplemental figure 9B), suggesting an overall shift to a proinflammatory environment as a consequence of the immune attack by the Arg1-specific T cells.

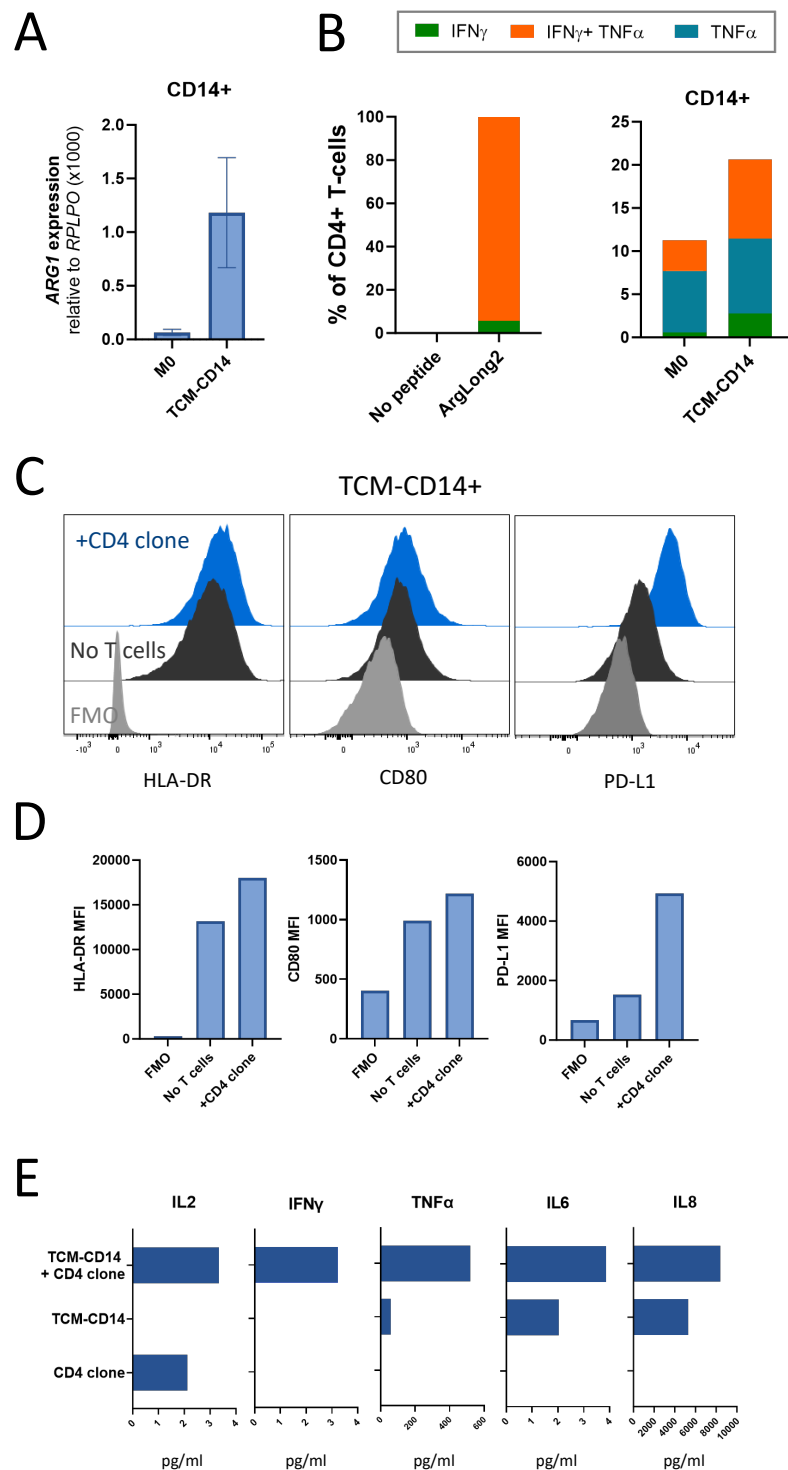
## DISCUSSION

While immune-suppressive myeloid cells comprise a heterogeneous population, TAMs represent one of the most abundant immune cell subsets found within the TME. TAMs play a significant role in inducing and maintaining the immunosuppressive TME and their infiltration into tumors has been correlated with a poor prognosis and limited response to therapies, including checkpoint inhibitor therapies.<sup>21 22</sup> Consequently, TAMs have emerged as important therapeutic targets as they can either contribute to tumor progression or promote tumor elimination based on the balance of the regulatory and counter-regulatory signals present in their micro-environment. TAMs are not terminally differentiated

cells, and their immunosuppressive polarization can be reversed toward M1-like phenotype in response to proinflammatory stimuli.<sup>23</sup>

In the present study, we show that targeting Arg1-expressing TAMs by CD4<sup>+</sup>Arg1-specific T cells in a vaccination setting resulted in a decrease in *Arg1* expression, along with an increase in the *Nos2* expression indicating a transition away from an immunosuppressive phenotype and toward an antitumor functionality associated with M1-like polarization. Concomitantly, we observed a downregulation of genes associated with protumorigenic macrophages, such as *Mrc1* (CD206), *Marco*<sup>24</sup> and *Ccl24*,<sup>25</sup> in Arg1 IMV-treated mice. Interestingly, we also observed a significant reduction in the expression of *Trem2*, a marker that has been associated with an immunosuppressive TME and resistance to checkpoint inhibitors in both humans and mice.<sup>26</sup> Casanova-Acebes *et al* have identified through single-cell RNA sequencing that Arg1 and TREM2 are expressed by the same subset of TAMs in a model of lung cancer<sup>14</sup> and our findings highlight that by targeting Arg1+TAMs with Arg1-specific T cells other TAM-associated immunosuppressive pathways are also modulated in the TME. A decrease in the number of suppressive macrophages was found to be crucial for the immune remodeling associated with successful anti-CTLA-4 antibody therapy.<sup>27</sup> This further supports the rationale for combining an Arg1-based IMV with immune checkpoint blockade.

Our results further demonstrate that Arg1-specific CD4<sup>+</sup>T cells can directly recognize and modulate TAMs. They act as potent cytokine producers and can thereby provide the necessary cytokine support for the repolarization of Arg1 expressing TAMs. While other T cell subsets, such as Th2 and Th17, could play a role in macrophage polarization, our experimental design specifically focused on Th1 phenotype Arg1-specific T cells. We



**Figure 4** Human Arg1-specific CD4+ T cell clones recognize and polarize CD14+ monocyte derived Arg-1 expressing myeloid cells to promote a proinflammatory microenvironment. (A) ARG1 expression as determined by RT-qPCR in undifferentiated human CD14+ cells or CD14+ cells differentiated to a TAM-like phenotype by *in vitro* treatment with ovarian cancer cell-derived tumor-conditioned media (TCM-CD14) for 48 hours. All bars represent average values  $\pm$  SEM. (B) Intracellular cytokine staining of IFN $\gamma$  and TNF $\alpha$  production by Arg1-specific CD4+ T cell clone reacting against the Arg1-derived epitope (ArgLong2) (left) or autologous PBMC derived myeloid cell (right). Myeloid cells were either used undifferentiated (MO) or differentiated with TCM derived from breast cancer cells (TCM-CD14) for 48 hours. E:T ratio of 4:1 was used. (C) Representative histograms and (D) bar plot representation of changes in MFI of HLA-DR, CD80 and PD-L1 on TCM-CD14 cells after co-culture with autologous Arg1-specific CD4+ T cell clone as determined by flow cytometry. (E) IL2, IFN $\gamma$ , TNF $\alpha$ , IL6 and IL8 cytokine concentrations in culture supernatants of either an Arg1-specific T cell clone co-cultured with autologous TCM-CD14, TCM-CD14 alone or the Arg1-specific CD4+ T cell clone alone. PBMC, peripheral blood mononuclear cell.

isolated and expanded IFN $\gamma$  and TNF $\alpha$ -producing Arg1-derived peptide-reactive T cells, as these were the dominant phenotype observed in our studies thus far. IFN $\gamma$  produced as a result of the immune interaction between TAMs and Arg1-specific Th1 cells may not only act directly on Arg1-expressing TAMs but also influence other antigen-presenting cells in the TME. Recent evidence has shown that IFN $\gamma$  production induces the expression of IL12 by tumor-resident dendritic cells, which is essential for the efficacy of anti-PD-1 therapy.<sup>28</sup>

Our data support the direct targeting of different TAM subtypes by Arg1-specific CD4<sup>+</sup>T cells. We, however, observed a different inherent response of primary TAMs to proinflammatory signaling and targeting by Arg1-specific CD4<sup>+</sup> T cells compared with targeting of M2-like BMDMs in which an increase in PD-L1 expression was observed. The inherent difference between the two Arg1-expressing macrophage types used in our experiments was apparent, as *ex vivo* TAMs were almost uniformly PD-L1<sup>high</sup> compared with M2-like BMDMs, where high expression of PD-L1 was seen only in a fraction of the population. Our results further provide evidence that targeting Arg1-expressing TAMs through this approach can also lead to the amplification of proinflammatory signaling within the microenvironment, as indicated by increased secretion of IFN $\gamma$ , TNF $\alpha$ , IL2, IL6, and IL8 in the microenvironment of the experiments. These changes cannot be efficiently induced by tumor antigen-specific T cells due to the limited external antigen presentation capacity of TAMs.<sup>13</sup> Our *in vivo* models showed that Arg1-based IMV promotes the generation of a proinflammatory TME, which supported T cell proliferation and was capable of inducing a proinflammatory phenotype in non-polarized (M0) macrophages. Interestingly, our *in vivo* data showed that the overall TME modulation by Arg1 IMV *in vivo* did not result in a clear increase in Th1-associated cytokine concentrations and thus suggests a complex interplay between the proinflammatory cues introduced by Arg1-specific T cells and the immunosuppressive TME.

In recent phase 1 clinical trials involving patients with MPN and advanced solid tumors, we observed that vaccination with Arg1 peptides and *in vivo* expansion of Arg1-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations was safe.<sup>29,30</sup> Moreover, in patients with MPN, an expansion of Arg1-specific T-cell responses was correlated with a reduction in Arg1 expression in the bone marrow. When considered alongside the results of the current study, this evidence suggests that targeting the Arg1+TME with Arg1-specific T cells holds promising therapeutic potential. Unlike most cancer vaccine strategies targeting neoantigens aim to induce cancer-specific CD8 cytotoxic T cells, the activation of anti-Tregs serves the purpose of converting an immunosuppressive environment into a proinflammatory one. This is particularly relevant in solid cancers where T-cell activity is often excluded from the TME due to the presence of Arg1-expressing TAMs. Such an environment can potentially be altered by the activation of

pro-inflammatory Arg1-specific T cells, as described here, thereby enhancing the effects of T-cell-boosting immunotherapies, such as immune checkpoint inhibitors.

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**Contributors** Design of the project (MHA and EM), performing experiments (EM, IL, MA-J, SMA, HJG, MP-P, MC and LLdIT), data analysis (EM and IL), interpretation of the data (EM, IL and MHA), providing reagents/materials (AMAR), writing of the manuscript (EM, IL, MHA and AWP) and supervision and conceptualization (MHA). MHA is the guarantor for this work. All authors have read and approved the final manuscript.

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**Competing interests** MHA has made an invention based on the use of Arg1 for vaccinations. The rights of the invention have been transferred to Copenhagen University Hospital Herlev, according to the Danish Law of Public Inventions at Public Research Institutions. The capital region has licensed the rights to the company IO Biotech ApS. The patent application was filed by IO Biotech ApS. MHA is an advisor and shareholder at IO Biotech. EM, IL, MC, and AWP are employees at IO Biotech. The additional authors do not declare any competing financial interests.

**Patient consent for publication** Not applicable.

**Ethics approval** This study involves human participants and was approved by the ethical committee of the Capital region of Denmark (H-17000988) and the ethical committee of Region Zealand (SJ-456). Participants provided informed consent to participate in the study before taking part.

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