Arginase-1 specific CD8+ T cells react toward malignant and regulatory myeloid cells

Glöckner, Hannah Jorinde; Martinenaite, Evelina; Landkildehus Lisle, Thomas; Grauslund, Jacob; Ahmad, Shamaila; Met, Özcan; Thor Straten, Per; Hald Andersen, Mads

Published in:
OncoImmunology

Link to article, DOI:
10.1080/2162402X.2024.2318053

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

OncoImmunology

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/koni20

Arginase-1 specific CD8+ T cells react toward malignant and regulatory myeloid cells

Hannah Jorinde Glöckner, Evelina Martinenaite, Thomas Landkildehus Lisle, Jacob Grauslund, Shamaila Ahmad, Özcan Met, Per Thor Straten & Mads Hald Andersen

To cite this article: Hannah Jorinde Glöckner, Evelina Martinenaite, Thomas Landkildehus Lisle, Jacob Grauslund, Shamaila Ahmad, Özcan Met, Per Thor Straten & Mads Hald Andersen (2024) Arginase-1 specific CD8+ T cells react toward malignant and regulatory myeloid cells, OncoImmunology, 13:1, 2318053, DOI: 10.1080/2162402X.2024.2318053

To link to this article: https://doi.org/10.1080/2162402X.2024.2318053

ONCOIMMUNOLOGY
2024, VOL. 13, NO. 1, 2318053
https://doi.org/10.1080/2162402X.2024.2318053

Taylor & Francis
Taylor & Francis Group

BRIEF REPORT

OPEN ACCESS Check for updates

Arginase-1 specific CD8+ T cells react toward malignant and regulatory myeloid cells

Hannah Jorinde Glöckner[a], Evelina Martinenaite[a,b], Thomas Landkildehus Lisle[a], Jacob Grauslund[a], Shamaila Ahmad[a], Özcan Met[a,c], Per Thor Straten[a], and Mads Hald Andersen[a]

[a]National Center for Cancer Immune Therapy, CCIT-DK, Department of Oncology, Copenhagen University Hospital, Herlev, Denmark; [b]IO Biotech, Research and Development Department, Copenhagen, Denmark; [c]Department of Health Technology, Technical University of Denmark, Lyngby, Denmark

ABSTRACT

Arginase-1 (Arg1) is expressed by regulatory myeloid cells in the tumor microenvironment (TME), where they play a pro-tumorigenic and T-cell suppressive role. Arg1-specific CD4+ and CD8+ memory T cells have been observed in both healthy individuals and cancer patients. However, while the function of anti-regulatory Arg1-specific CD4+ T cells has been characterized, our knowledge of CD8+ Arg1-specific T cells is only scarce. In the current study, we describe the immune-modulatory capabilities of CD8+ Arg1-specific T cells. We generated CD8+ Arg1-specific T cell clones to target Arg1-expressing myeloid cells. Our results demonstrate that these T cells recognize both malignant and nonmalignant regulatory myeloid cells in an Arg1-expression-dependent manner. Notably, Arg1-specific CD8+ T cells possess cytolytic effector capabilities. Immune modulatory vaccines (IMVs) represent a novel treatment modality for cancer. The activation of Arg1-specific CD8+ T cells through Arg1-based IMVs can contribute to the modulatory effects of this treatment strategy.

ARTICLE HISTORY
Received 20 November 2023
Revised 2 February 2024
Accepted 8 February 2024

KEYWORDS
Arginase-1; anti-regulatory T cells; immune modulatory vaccines; myeloid cells; tumor microenvironment

Introduction

Anti-tumor immunity can be significantly hindered by regulatory myeloid cells including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) within the tumor stroma. These regulatory myeloid cells can exert immunosuppressive functions through the upregulation of metabolic enzymes such as indoleamine 2,3-dioxygenase (IDO)[1] and arginase-1 (Arg1),[2,3] or inhibitory checkpoint molecules like programmed death ligand 1 (PD-L1).[4] As the tumor microenvironment (TME) crucially influences the clinical effect of cancer immunotherapy,[5,6] modulation of the TME into a pro-inflammatory tumor hostile environment is an advancing approach to promote anti-tumor immune cell activity.

Immune Modulatory Vaccines (IMVs) provide a novel approach to target regulatory cells in the TME by stimulating anti-regulatory T cells (anti-Tregs).[7] Anti-Tregs are endogenous pro-inflammatory effector T cells specifically recognizing HLA-restricted epitopes derived from tumor microenvironment antigens (TMAs),[8] e.g., Arg1,[9] IDO[10] and PD-L1.[11] Recently, in a phase 2 clinical trial metastatic melanoma patients were treated with an IDO- and PD-L1-derived peptide IMV combined with programmed cell death protein 1 (PD-1) checkpoint inhibitor. An objective response rate of 80% and a complete response rate of 50% was achieved,[12,13] demonstrating the potential of IDO- and PD-L1-specific IMVs.

During the last years we have described Arg1 as a promising new target for IMVs. The metabolic enzyme Arg1 hydrolyses the amino acid L-arginine, which is essential for T-cell survival and functionality.[14–16] In the TME, regulatory myeloid cells, but also cancer cells can express Arg1.[17] Increased Arg1-activity has been described in numerous cancers, e.g., ovarian,[17] breast[18] and colon cancer[19] and is associated with poor survival,[17,19] which emphasizes the impact of Arg1-dependent immune suppression. Arg1-specific T cells target cells with HLA-presented Arg1-epitopes. Arg1-specific T cells are part of the adaptive immune system,[20] and memory Arg1-specific T cells are present in the peripheral blood mononuclear cells (PBMCs) from healthy individuals and cancer patients.[9,21,22]

Recently, two Arg1-peptide based IMV clinical trials (NCT03689192, NCT04051307) have been conducted.[23,24] In the first-in-human clinical trial NCT04051307, myeloproliferative neoplasm (MPN) patients were vaccinated with an Arg1-derived peptide, ArgLong2, together with a PD-L1-derived peptide, PD-L1Long1.[24] Arg1-specific T-cell responses were assessed in the PBMCs and bone marrow aspirates from the patients following the IMV-treatment, revealing enhanced CD4+ and CD8+ Arg1-specific T-cell responses after vaccination.

We have previously characterized CD4+ Arg1-specific T-cell immunity and described the existence of (memory) CD4+ Arg1-specific T cells in both healthy individuals and cancer patients.[21] CD8+ Arg1-specific memory T cells also exist.[21] In addition, expanded CD8+ Arg1-specific T-cell responses were observed following vaccinations in the first-in-human Arg1- and PD-L1-derived peptide IMV trial.[24] In the current study, we therefore aim to characterize

CONTACT Mads Hald Andersen Mads.hald.andersen@regionh.dk Copenhagen University Hospital, Borgmester Ib Juuls Vej 13, Herlev DK-2730, Denmark
Supplemental data for this article can be accessed online at https://doi.org/10.1080/2162402X.2024.2318053.

CD8+ Arg1-specific T cells and assess their functional capabilities in terms of recognizing Arg1-expressing, regulatory myeloid cells.

Materials and methods

Donor material

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors as previously described.[22] The HLA subtypes of healthy donor #117 (from which Arg1-specific T cell cultures and CD8+ Arg1-specific T cell clones were generated) were identified as HLA-A *11:01, HLA-A *23:01, HLA-B * 49:01, HLA-B * 51:01, HLA-C * 07:01, HLA-C * 15 :02, HLA-DRB1 × 11:01, HLA-DRB1 × 15:01, HLA-DQB1 × 03:01, HLA-DQB1 × 06:02, HLA-DPB1 × 04:01, HLA-DPB1 × 04:01.

Cancer cell lines

MonoMac-1 was obtained from DSMZ, German Collection of Microorganisms and Cell Cultures (ACC 252), MDA-MB-231 cells was obtained from Claus Christiansen from the Danish Cancer Society and CAOV3 (HTB-75) and SKOV3 (HTB-77) were both obtained from ATCC. All cancer cell lines were maintained in RPMI-1640 (Life technologies) + 10% FCS and passaged twice a week. Cell lines were confirmed to be negative for mycoplasma.

Peptides

ArgLong2 peptide (ISAKDIVYIGLRDVDPGEHYILKTLGIK YFSMTEVDRL) was synthesized by Schäfer (Denmark) and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 5 mM or 10 mM.

Generation of Arg1-specific T cell cultures

PBMCs from healthy donors were stimulated with 20 µM ArgLong2 peptide and 120 U/mL interleukin-2 (IL-2) the following day. Two weeks after peptide stimulation, cells were restimulated with ArgLong2 peptide for 2.5 h and tumor necrosis factor α (TNFα)-secreting cells were isolated using Miltenyi Biotec's cell enrichment and detection kit according to manufacturer's protocol. The isolated cells were expanded following the rapid expansion protocol (REP) with 20×10^6 irradiated feeder cells (allogenic PBMCs from three different healthy donors), 6000 U/mL IL-2, 0.6 µg αCD3 and 0.5 mL HEPES. ArgLong2 specificity was assessed 14–17 days after REP initiation by intracellular cytokine staining.

CD8+ T cell clones

CD8+ Arg1-specific T cell clones were established by limiting dilution. The CD8+ T cells of an Arg1-specific T cell culture was sorted by positive cell sorting of CD8+ T cells with Miltenyi Biotec's CD8+ MACS microbeads according to manufacturer's protocol. 0.5 or 1.0 CD8+ T cell per well was plated in a 96-well plate and expanded in a small-scale REP culture, see above.

Intracellular cytokine staining

The cytokine production profile of PBMCs and expanded Arg1-specific T cell cultures was characterized in intracellular cytokine staining assays as previously described.[24]

Elispot

Elispot assays were conducted as previously described.[9] Elispot plates were pre-coated with interferon γ (IFNγ)-capture antibody (Ab) (Mabtech, cat. 3420-3-1000), Granzyme B-capture Ab (Mabtech, cat. 3486-3-250) or Perforin-capture Ab (Mabtech, cat. 3465-3-500), and ArgLong2 peptide (5 µM) or target cells at appropriate effector-to-target ratios were added. The following biotin-conjugated secondary antibodies: IFNγ: cat. 3420-6-1000, Granzyme B cat. 3486-6-250, Perforin cat. 3465-6-250, all from Mabtech, were used.

^{51}Chromium-release assay

Conventional ^{51}Chromium-release assays were conducted as previously described.[25] 5×10^5 target cells/condition were labeled with 100 µCi ^{51}Chromium for 1 h. ArgLong2 pulsed target cells were pulsed with 5 µM ArgLong2 peptide for 1 h. ^{51}Chromium release was determined as the counts per minute (cpm) measured with a 2470 Wizard^2 Gamma Counter (Perkin Elmer).

RNA extraction and RT-qPCR

Total RNA extraction and RT-qPCR analysis was performed as described previously.[24] cDNA was synthesized from 1000 ng total RNA using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems) or the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer´s protocol. Quantitative RT-qPCR analysis was assessed in technical triplicates using the TaqMan Gene Expression assay on a QuantStudio 6 Pro thermocycler (Applied Biosystems) or using the LightCycler 480 Probes Master (Roche Diagnostics) on a AriaMx thermocycler (Agilent). The gene expression of interest was normalized to the housekeeping gene, RPLPO, and differential expression was assessed with the dCT method.[26] Primers: hArg1 (ID: Hs00163660_m1, Thermo Fisher Scientific), hRPLPO (ID: Hs99999902_m1, Thermo Fisher Scientific).

Flow cytometry based analysis of HLA ABC expression

MonoMac-1 cells were stained using HLA-ABC-FITC (BD, cat. 555552) and LIVE/DEAD fixable Near-IR (NIR) dead cell stain (Life technologies, cat. L10119) 48 h after culturing in RPMI-1640 (Life technologies) + 10% FCS (control) or RMPI + 10% FCS + 20 U/mL IL-13 (Trichem, cat. 200–13). Sample acquisition was performed on a NovoCyte Quanteon (Agilent) and data was analyzed with FlowJo version 10.6.1.

Electroporation

Cells were electroporated based on previously described protocol.[9] For siRNA transfection experiments, CD14+ monocytes or MonoMac-1 cells were electroporated with three Arg1 siRNA duplexes (Invitrogen, cat. 1299003) or mock. Sequences of the three Arg1 stealth siRNA duplexes: HSS100648 sense:5`-GGGUGGAAGAAGGCCCUACAGUAUU-3´, antisense:5`-AAUACUGUAGGGCCUUCUUCCACCC-3´, HSS100649 sense: 5`-GGGCUACUCUCAGGAUUAGAUAUAA-3´, antisense: 5`-UUAUAUCUAAUCCUGAGAGUAGCCC-3´ HSS100650 sense: 5`-UCGGGAGGGUAAUCACAAGCCUAUU-3´, antisense: 5`-AAUAGGCUUGUGAUUACCCUCCCGA-3´. For mRNA transfection experiments, MonoMac-1 cells were electroporated with 5 µg Arg1 in-house *in vitro* transcribed mRNA. After electroporation, cells were cultured in RPMI-1640 (Life technologies) + 10% FCS for 1 h (mRNA transfection) or 48 h (siRNA transfection) prior to experimental set-up. MonoMac-1 cells were treated with 20 U/mL IL-13 (Trichem, cat. 200–13) 1 h after electroporation, and autologous CD14+ cells were treated with 1 mL TCM directly after electroporation.

CD14+ PBMC sorting

Miltenyi Biotec's CD14+ MACS microbeads were used according to manufacturer's protocol to separate CD14+ PBMCs. The sorted CD14+ cells were cultured in 1 mL TCM and 1 mL X-VIVO 15 (Lonza) + 5% human serum for 48 h before experimental setup.

Tumor-conditioned medium

The breast cancer cell line MDA-MB-231 as well as the ovarian cancer cell lines CAOV3 and SKOV3 were cultured in T175 flasks in 35 mL RPMI-1640 (Life technologies) + 10% FCS. When ~90% confluency was achieved, the medium was exchanged to 25 mL X-VIVO 15 (Lonza) + 5% human serum. After 24 h the medium was harvested, spun down to get rid of eventual cells and frozen as tumor-conditioned medium.

Statistical elispot analysis

The distribution free resampling (DFR) method was used to define significant ($p \leq .05$) Elispot assay performed in technical triplicates.[27]

Luminex

IFNγ, IL-2, IL-6, IL-8, IL-10, IL-12, IL-13, TNFα, CCL2, CCL22, and VEGF concentrations in MDA-MB-231 TCM were analyzed using Bio-Plex™ Pro Human Cytokine, Chemokine, and Growth Factor Assays (Bio-Rad) according to manufacturer's protocol. Sample acquisition was performed on Bio-Plex200 and data was analyzed with Bio-Plex™ Manager V6.

Elisa

TGFβ and IL-4 concentration in TCM generated from MDA-MB-231, SKOV3 and CAOV3 was analyzed using the human TGFβ DuoSet Elisa kit (R&D Systems) and the human IL-4 Elisa kit (Thermo Fisher Scientific) according to manufacturer's protocol.

TCRBV 1–24 expresssion assay

T-cell receptor beta variable chain (TCRBV) 1–24 expression assay was performed as previously described.[28] For each T cell clone RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel) and cDNA was synthesized using SuperScript VILO (Invitrogen). cDNA was amplified with primers specific for 24 TCR β variable (BV) regions and a constant region primer using AmpliTaq DNA polymerase (Applied Biosystems). Amplified PCR products were separated in an electrophoresis gel, after electrophoresis the gel was imaged with a UV transilluminator.

Results

Expansion of CD8+ Arg1-specific T cell cultures and clones

To characterize CD8+ Arg1-specific T-cell responses, we first expanded specific T cell cultures and clones. First, we screened for immune responses against the previously described immunogenic Arg1-derived peptide ArgLong2[22] in peripheral blood mononuclear cells (PBMCs) from 13 healthy donors (HD) cultured *in vitro* with ArgLong2 peptide and low-dose IL-2 for two weeks. ArgLong2-specific T-cell responses were identified by evaluating IFNγ and TNFα production using intracellular cytokine staining (ICS) assay. In PBMCs from a healthy donor (HD117, HLA subtypes in Materials) both CD4+ and CD8+ ArgLong2-specific T-cell responses were observed (Figure 1a). To expand these CD4+ and CD8+ ArgLong2-specific T cells, TNFα-producing ArgLong2-peptide reactive T cells were isolated and, subsequently, expanded following a T-cell rapid expansion protocol (details in Methods). A high proportion of CD4+ and CD8+ T-cell reactivity against ArgLong2 was detected in the expanded T-cell culture (Figure 1b). Expression of the cytotoxic marker CD107α on CD8+ ArgLong2-specific T cells was also assessed. Increased CD107α expression was observed in the expanded CD8+ ArgLong2-specific T-cell culture compared to the initially ArgLong2-stimulated CD8+ T cells (Figure 1a,b). CD8+ ArgLong2-specific T cells were isolated by positive CD8 magnetic microbead sorting and expanded by limiting dilution to obtain clones. Arg1-derived peptide-specific reactivity in the obtained CD8+ ArgLong2-specific T cell clones (#18, #20, #23) was confirmed in an ICS assay, with most of the T cells producing both TNFα and IFNγ (Figure 1c). Also, the CD8+ ArgLong2-specific T cell clones #18 and #20 showed high expression of CD107α, whereas lower CD107α expression was detected in the CD8+ ArgLong2-specific T cell clone #23 (Figure 1c). Additionally, a CD8+ T cell clone of unknown specificity was expanded as a control (Figure 1c). The general effector capability of this CD8+ control T cell clone was demonstrated in an IFNγ Elispot assay by stimulation with

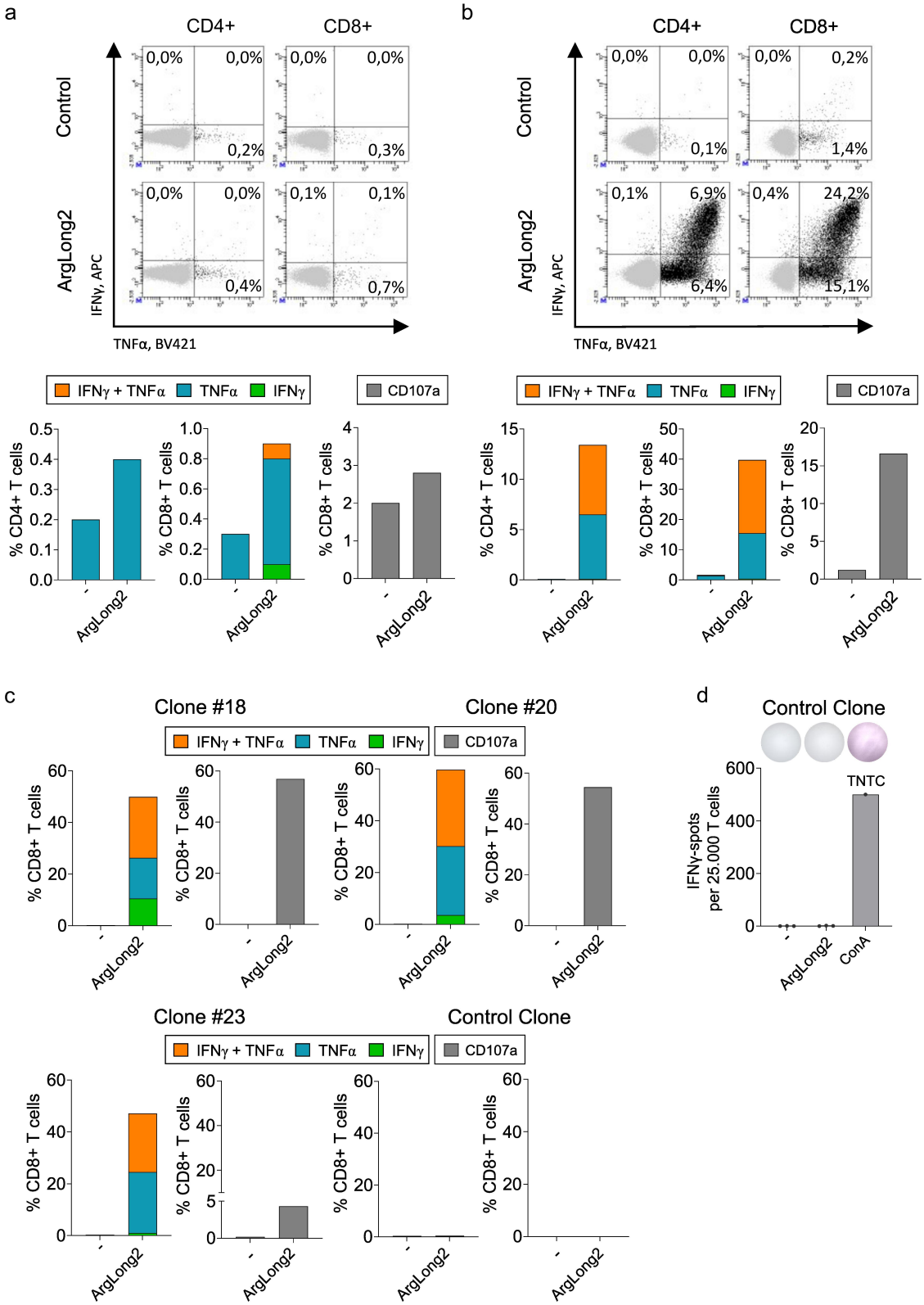

Figure 1. Identification, isolation and expansion of CD8+ Arg1-specific T cell clones. (a) CD4+ and CD8+ Arg1-specific T-cell responses in *in vitro* cultured HD 117 PBMCs after stimulation with ArgLong2 peptide compared to non-stimulated control. (b) Expanded ArgLong2-specific T-cell culture from HD 117 with CD4+ and CD8+ T cells. (c) Expanded CD8+ ArgLong2-specific T cell clones #18, #20 and #23 and CD8+ control T cell clone. (a-c) the specificity of each culture as assessed by CD107α staining (CD8+ T cells) (right) and intracellular staining (ICS) for IFNγ and TNFα (left). (a-b, top) flow cytometry dot-plots of IFNγ+, TNFα+ and IFNγ+/TNFα+ CD4+ and CD8+ ArgLong2-specific T-cell responses. (a-b, bottom) stacked bar charts of CD107α+, IFNγ+, TNFα+ and IFNγ+/TNFα+ CD4+ and CD8+ ArgLong2-specific T-cell responses or (c) CD8+ ArgLong2-specific T cell clones. (d) Reactivity of CD8+ control T cell clone to ArgLong2 peptide or ConA (pos ctrl) stimulation compared to no stimulation (T cells alone) determined in an IFNγ Elispot assay. Representative Elispot wells of responses against non-stimulated cells, ArgLong2-peptide or ConA stimulated cells. Too numerous to count, > 500 IFNγ spots (TNTC).

concanavalin A (ConA) (Figure 1d). The clonal purity of all CD8+ T cell clones (ArgLong2-specific T cell clones #18, #20, #23 and the CD8+ control T cell clone) was verified in a T-cell receptor beta variable chain (TCRBV) expression assay.[28] The ArgLong2-specific T cell clones #18, #20, #23 all express beta variable chain 2 (BV2), while the CD8+ control T cell clone expresses beta variable chain 15 (BV15) (data not shown).

CD8+ Arg1-specific T cells target malignant myeloid cells in an Arg1-expression-dependent manner

To examine the reactivity of the CD8+ Arg1-specific T cell clones, we first assessed their cytolytic activity against an HLA-matched Arg1-expressing malignant myeloid cell line, MonoMac-1. In a ^{51}chromium-release assay the CD8+ Arg1-specific T cell clone was able to lyse MonoMac-1 cells in an effector-to-target dependent manner. Treating MonoMac-1 cells with IL-13 prior to the ^{51}chromium-release assay increased the lysis of MonoMac-1 cells by the CD8+ Arg1-specific T cell clone (Figure 2a, left). Only minimal killing of MonoMac-1 cells was observed regardless of the IL-13 pre-treatment, when the CD8+ control T cell clone was used (Figure 2a, right). IL-13 has previously been demonstrated to increase Arg1 expression,[29,30] and RT-qPCR analysis confirmed the upregulation of Arg1 expression in MonoMac-1 48 h after IL-13 treatment (Figure 2b). Flow cytometry analysis revealed that IL-13 treatment did not cause a major increase in HLA class I expression on MonoMac-1 cells (Figure 2c). To further validate the Arg1-expression-dependent target recognition of CD8+ ArgLong2-specific T cell clones, we transfected MonoMac-1 cells with either Arg1 mRNA or siRNA to increase or silence Arg1 expression, respectively. We observed that Arg1 overexpression significantly increased the target recognition compared to mock transfected MonoMac-1 cells, as detected in an IFNγ Elispot using two CD8+ Arg1-specific T cell clones (#20, #23) (Figure 2d). Increased Arg1 expression in MonoMac-1 cells after Arg1 mRNA transfection (70% Arg1$^+$ among total live cells) compared to mock transfection (0.2% Arg1$^+$ among total live cells) was confirmed by flow cytometry analysis (Figure 2e).

Conversely, downregulation of the Arg1 expression in MonoMac-1 cells by Arg1 siRNA transfection significantly decreased the target recognition by the CD8+ Arg1-specific T cell clone compared to mock transfected MonoMac-1 cells (Figure 2f, left). No reactivity against mock or siRNA transfected target cells was observed from the CD8+ control T cell clone (Figure 2f, right). Furthermore, higher release of both Granzyme B and Perforin in response to mock transfected MonoMac-1 cells compared to Arg1 siRNA transfected MonoMac-1 cells was observed, confirming the cytotoxic function of CD8+ Arg1-specific T cell clones (Figure 2g). RT-qPCR analysis confirmed the downregulation of Arg1 expression in MonoMac-1 cells after Arg1 siRNA transfection (Figure 2h).

CD8+ Arg1-specific T cells target autologous myeloid cells in an Arg1-expression-dependent manner

After demonstrating Arg1-expression-dependent cytolytic CD8+ T-cell reactivity toward malignant myeloid cells, we next evaluated Arg1-specific CD8+ T-cell reactivity toward autologous monocytes. CD14+ cells were isolated from autologous PBMCs and treated with tumor-conditioned medium (TCM) for 48 h to mimic a TME-like polarization. Treatment with TCM generated from the breast cancer cell line MDA-MB-231 increased the Arg1 expression in both CD14+ myeloid cells and MonoMac-1 cells (Figure 3a), and treatment with TCM generated from the ovarian cancer cell lines CAOV3 and SKOV3 was also able to increase the Arg1 expression in MonoMac-1 cells, as confirmed by RT-qPCR analysis (Figure 3a). Arg1 expression-dependent recognition of either mock or Arg1 siRNA transfected MDA-MB-231 TCM-treated autologous monocytes (CD14+ cells) (Figure 3(b,d)) or CAOV3 TCM-treated autologous monocytes (CD14+ cells) (Figure 3(c,e)) was assessed in IFNγ and Granzyme B Elipot assays. Significantly lower target cell recognition of Arg1 siRNA transfected autologous CD14+ cells compared to mock transfected controls was observed for two CD8+ Arg1-specific T cell clones (#18, #20). These findings show that CD8+ Arg1-specific T cells target autologous myeloid cells in an Arg1-expression-dependent manner.

Discussion

In this study we investigated the functional capabilities of CD8+ Arg1-specific T cells. Our results reveal that when encountering either malignant or nonmalignant Arg1-expressing myeloid cells, CD8+ Arg1-specific T cells can effectively recognize and eliminate them in an Arg1-expression-dependent manner. This finding provides a valuable addition to the existing knowledge regarding CD8+ Arg1-specific T cells.[21]

Arg1-expressing cells, such as MDSCs and TAMs, promote a tumor-favorable milieu and hinder the reactivity of tumor-specific T cells.[2,3] This underlines the rationale for targeting Arg1-expressing cells within the tumor stroma using an Arg1-based IMV. Our previous research has demonstrated significant tumor growth delay and improved overall survival in different *in vivo* syngeneic mouse tumor models treated with Arg1-based IMV.[31] In mice, Arg1-based IMVs stimulate anti-tumor immunity by enhancing the infiltration of immune cells into the tumor and promoting an M1-like TAM phenotype. In the present study we demonstrated CD8+ Arg1-specific T-cell activity against TCM treated myeloid cells in order to mimic a TME-like polarization of these target cells. TCM has been recently described as potent tool for inducing TME-like polarization of myeloid cells *in vitro*.[32] Interestingly, in the present study we could not detect the typical Arg1-inducing cytokines IL-4 (data not shown) and IL-13 in the TCM generated from MDA-MB-231 (Supplementary Figure S1). Instead, we detected intermediate to high levels of IL-6, IL-8, CCL2 and VEGF. Additionally, we observed high concentrations of TGFβ in all TCM tested. Our observations suggest that Arg1 expression typically observed in tumor-associated myeloid cells is a consequence of complex mixture signaling cues present within the TME since both TGFβ[33] and IL-6[34] have been described to increase Arg1 expression in macrophages.

Similar to Arg1, the L-tryptophan metabolizing enzyme IDO is a TMA recognized by anti-regulatory IDO-specific T cells.[8] Intriguingly, the importance of both CD8+ and CD4+

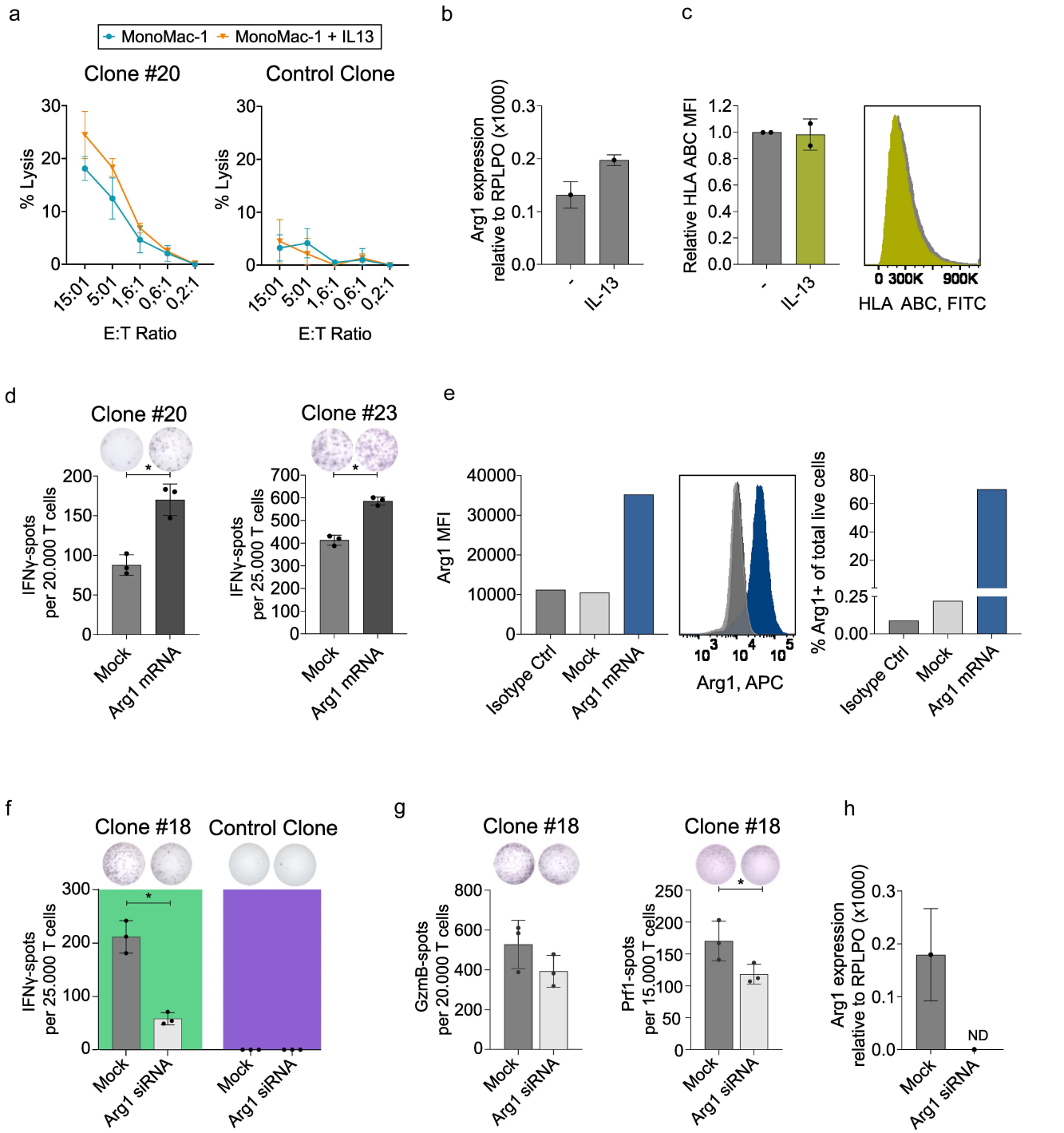

Figure 2. CD8+ Arg1-specific T cells target malignant myeloid cells in an Arg1-expression-dependent manner. (a) ^{51}Chromium-release assay of CD8+ Arg1-specific T cell clone #20 (left) or CD8+ control T cell clone (right) against MonoMac-1 cells ± IL-13 (20 U/mL, 48 h) in technical duplicates. 7.5×10^4 effector cells at E:T ratio of 15:1. Error bars represent mean ± SD. (b) Arg1 expression in MonoMac-1 ± IL-13 (20 U/mL, 48 h) evaluated by RT-qPCR analysis. (b, h) Arg1 expression was measured relative to large ribosomal protein (RPLPO) expression in technical triplicates. (c) Bar charts (left) and histogram plots (right) of flow cytometry determined relative MFI values for HLA ABC expression in MonoMac-1 cells treated with IL-13 (20 U/mL, 48 h) (green) compared to untreated MonoMac-1 cells (grey) in biological duplicates. (d) IFNγ Elispot responses of Arg1-specific T cell clone #20 and #23 to Arg1 mRNA or mock transfected MonoMac-1 cells. E:T ratio of 1:1. Representative Elispot wells of responses against (d) Arg1 mRNA or mock or (f-g) Arg1 siRNA or mock transfected MonoMac-1 cells. (e) Bar charts (left) and histogram plots (center) of flow cytometry determined MFI values for Arg1 expression in MonoMac-1 cells and bar charts (right) of the frequency of Arg1+ cells among total live MonoMac-1 cells, 24 h after mock (light grey) or Arg1 mRNA (blue) transfection. Isotype ctrl of mock transfected cells shown in dark grey. (f) IFNγ Elispot responses of Arg1-specific T cell clone #18 (green background) and CD8+ control T cell clone (purple background) to Arg1 siRNA or mock transfected MonoMac-1 cells treated with IL-13 (20 U/mL, 48 h). E:T ratio of 2:1. (g) Granzyme B (GzmB) and perforin (Prf1) Elispot responses of Arg1-specific T cell clone #18 to Arg1 siRNA or mock transfected MonoMac-1 cells treated with IL-13 (20 U/mL, 48 h). E:T ratio of 2:1. (h) RT-qPCR analysis of Arg1 expression in MonoMac-1 after mock or Arg1 siRNA transfection ± IL-13 (20 U/mL, 48 h). Not detected (ND). *$p \leq .05$. All bars represent the mean values ± SD.

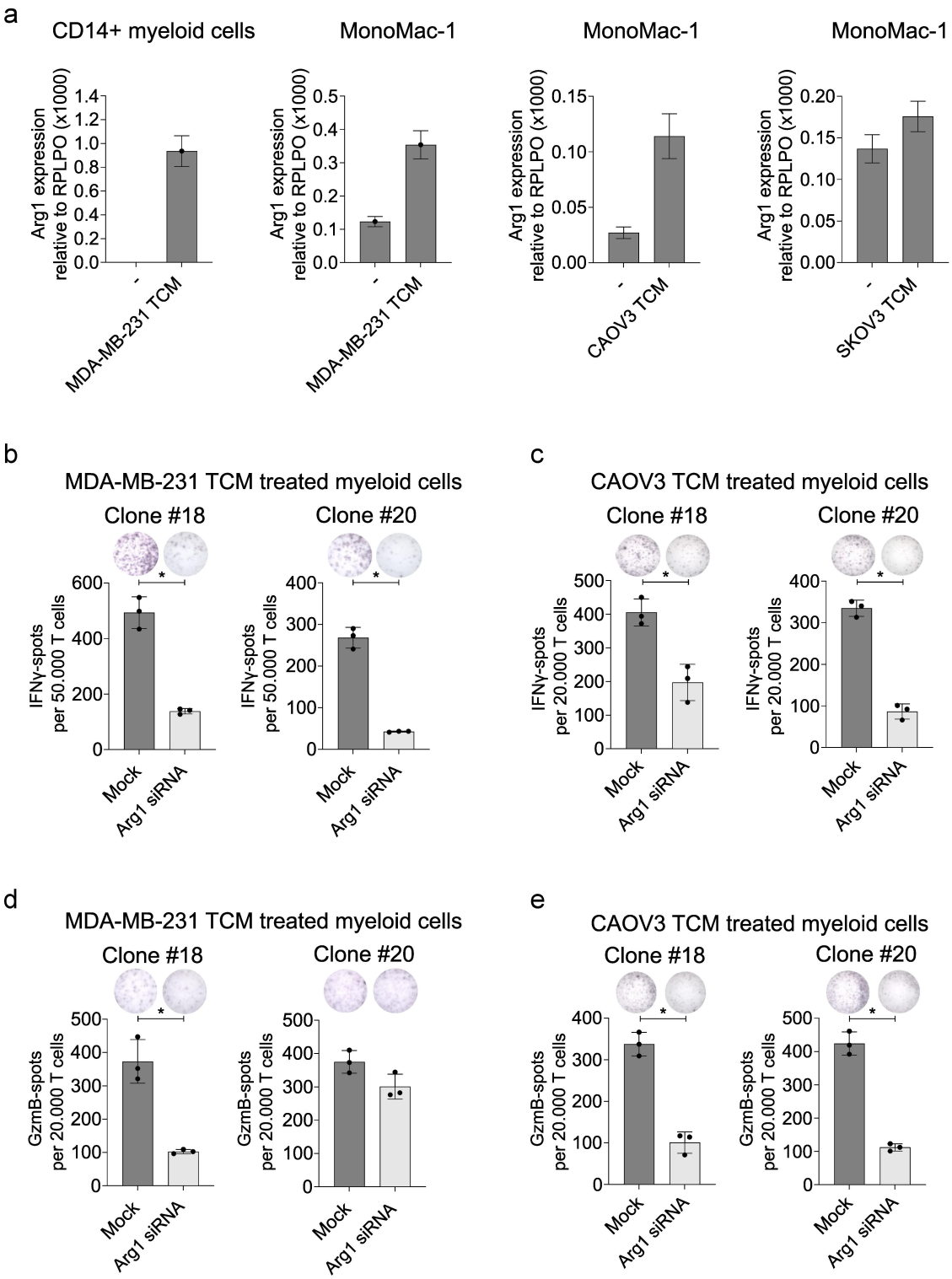

Figure 3. CD8+ Arg1-specific T cells target autologous myeloid cells in an Arg1-expression-dependent manner. (a) Arg1 expression in CD14+ monocytes from a random healthy donor ± MDA-MB-231 TCM treatment (1 mL, 48 hrs) and MonoMac-1 ± MDA-MB-231 TCM, CAOV3 TCM or SKOV3 TCM treatment (1 mL, 48 hrs) evaluated by RT-qPCR analysis. Arg1 expression was measured relative to large ribosomal protein (RPLPO) expression in technical triplicates. (b-e) Responses of Arg1-specific T cell clone #18 and #20 to (b,d) MDA-MB-231 TCM treated or (c,e) CAOV3 TCM treated Arg1 siRNA or mock transfected autologous CD14+ cells as assessed in (b-c) IFNγ Elispot and (d-e) Granzyme B (GzmB) Elispot. E:T ratio of (b,d) 2:1 and (c,e) 4:1. Representative Elispot wells of responses against Arg1 siRNA or mock transfected autologous CD14+ cells. *$p \leq .05$. All bars represent the mean values ± SD.

anti-Tregs in IMVs has been demonstrated in a murine cancer model utilizing an IDO-based IMV.[35] Mice were vaccinated with either an MHC class I or II-directed IDO-based IMV, which stimulated CD8+ and CD4+ IDO-specific T cells, respectively. Each IMV demonstrated a comparable delay in tumor growth on its own. However, when the MHC class I and II-directed IDO-based IMVs were combined, there was a more pronounced delay in tumor growth, indicating

a synergistic reactivity of CD4+ and CD8+ IDO-specific T cells. This study underscores the potential of CD8+ Arg1-specific T cells to induce immune-modulation independently and in conjunction with CD4+ Arg1-specific T cells. Moreover, the observed expansion of CD8+ and CD4+ Arg1-specific T cells in the first-in-human Arg1- and PD-L1-derived peptide IMV trial[24] suggests the capacity of an Arg1-based IMV to induce immune modulation through stimulation of both CD4+ and CD8+ Arg1-specific T-cell activity.

In summary, the findings presented in this paper suggest that CD8+ Arg1-specific T cells possess the capability to directly influence the TME by targeting and depleting Arg1-expressing malignant and regulatory myeloid cells. Additionally, these CD8+ Arg1-specific T cells secrete pro-inflammatory cytokines such as IFNγ and TNFα, which further aid in reshaping the suppressive TME into a pro-inflammatory milieu. CD8+ Arg1-specific T cells can contribute to reprograming of a suppressive microenvironment and therefore support the modulation of the TME by CD4+ T cells.

Acknowledgments

We would like to thank Merete Jonassen, Anette Højgaard Andersen and Tina Seremet for excellent technical assistance. This work was supported by Herlev and Gentofte Hospital, and through a research funding agreement between IO Biotech ApS and National Center for Cancer Immune Therapy (CCIT-DK).

Disclosure statement

Mads Hald Andersen is named as an inventor on various patent applications relating to therapeutic uses of arginase peptides. These patent applications are assigned to the company IO Biotech ApS, which is developing immune-modulating cancer treatments. Mads Hald Andersen is founder, shareholder and advisor of IO Biotech ApS. Evelina Martinenaite is an employee at IO Biotech ApS. The other authors declare "no conflict of interest".

Funding

The work was supported by the National Center for Cancer Immune Therapy, CCIT-DK.

ORCID

Mads Hald Andersen http://orcid.org/0000-0002-2914-9605

Data availability statement

The data that support the findings of this study are available from the corresponding author, MHA, upon reasonable request.

References

1. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med. 1999;189(9):1363–1372. doi:10.1084/jem.189.9.1363.
2. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–268. doi:10.1038/nri3175.
3. Bronte V, Serafini P, De Santo C, Marigo I, Tosello V, Mazzoni A, Segal DM, Staib C, Lowel M, Sutter G. et al. IL-4-Induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol. 2003;170(1):270–278. doi:10.4049/jimmunol.170.1.270.
4. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, Bronte V, Chouaib S. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. J Exp Med. 2014;211(5):781–790. doi:10.1084/jem.20131916.
5. Yu Y, Cui J. Present and future of cancer immunotherapy: a tumor microenvironmental perspective (review). Oncol Lett. 2018. doi:10.3892/ol.2018.9219.
6. Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The tumor microenvironment: a milieu hindering and obstructing antitumor immune responses. Front Immunol. 2020;11:940. doi:10.3389/fimmu.2020.00940.
7. Andersen MH. Anti-regulatory T cells. Semin Immunopathol. 2017;39(3):317–326. doi:10.1007/s00281-016-0593-x.
8. Andersen MH. Tumor microenvironment antigens. Semin Immunopathol. 2022;45(2):253–264. doi:10.1007/s00281-022-00966-0.
9. Martinenaite E, Mortensen REJ, Hansen M, Orebo Holmström M, Munir Ahmad S, Grønne Dahlager Jørgensen N, Met Ö, Donia M, Svane IM, Andersen MH. et al. Frequent adaptive immune responses against arginase-1. Oncoimmunology. 2017;7(3):e1404215. doi:10.1080/2162402X.2017.1404215.
10. Sørensen RB, Berge-Hansen L, Junker N, Hansen CA, Hadrup SR, Schumacher TNM, Svane IM, Becker JC, Straten PT, Andersen MH. et al. The immune system strikes back: cellular immune responses against Indoleamine 2,3-dioxygenase. PloS One. 2009;4(9):e6910. doi:10.1371/journal.pone.0006910.
11. Munir S, Andersen GH, Svane IM, Andersen MH. The immune checkpoint regulator PD-L1 is a specific target for naturally occurring CD4 + T cells. Oncoimmunology. 2013;2(4):e23991. doi:10.4161/onci.23991.
12. Kjeldsen JW, Lorentzen CL, Martinenaite E, Ellebaek E, Donia M, Holmstroem RB, Klausen TW, Madsen CO, Ahmed SM, Weis-Banke SE. et al. A phase 1/2 trial of an immune-modulatory vaccine against IDO/PD-L1 in combination with nivolumab in metastatic melanoma. Nat Med. 2021;27(12):2212–2223. doi:10.1038/s41591-021-01544-x.
13. Lorentzen CL, Kjeldsen JW, Ehrnrooth E, Andersen MH, Marie Svane I. Long-term follow-up of anti-PD-1 naïve patients with metastatic melanoma treated with IDO/PD-L1 targeting peptide vaccine and nivolumab. J Immunother Cancer. 2023;11(5):e006755. doi:10.1136/jitc-2023-006755.
14. Mondanelli G, Ugel S, Grohmann U, Bronte V. The immune regulation in cancer by the amino acid metabolizing enzymes ARG and IDO. Curr Opin Pharmacol. 2017;35:30–39. doi:10.1016/j.coph.2017.05.002.
15. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. 2003;24(6):301–305. doi:10.1016/S1471-4906(03)00132-7.
16. Canè S, Barouni RM, Fabbi M, Cuozzo J, Fracasso G, Adamo A, Ugel S, Trovato R, De Sanctis F, Giacca M. et al. Neutralization of NET-associated human ARG1 enhances cancer immunotherapy. Sci Transl Med. 2023;15(687). doi:10.1126/scitranslmed.abq6221.
17. Czystowska-Kuzmicz M, Sosnowska A, Nowis D, Ramji K, Szajnik M, Chlebowska-Tuz J, Wolinska E, Gaj P, Grazul M, Pilch Z. et al. Small extracellular vesicles containing arginase-1 suppress T-cell responses and promote tumor growth in ovarian carcinoma. Nat Commun. 2019;10(1):3000. doi:10.1038/s41467-019-10979-3.
18. de Boniface J, Mao Y, Schmidt-Mende J, Kiessling R, Poschke I. Expression patterns of the immunomodulatory enzyme arginase 1 in blood, lymph nodes and tumor tissue of early-stage breast cancer patients. Oncoimmunology. 2012;1(8):1305–1312. doi:10.4161/onci.21678.

19. Ma Z, Lian J, Yang M, Wuyang J, Zhao C, Chen W, Liu C, Zhao Q, Lou C, Han J. et al. Overexpression of arginase-1 is an indicator of poor prognosis in patients with colorectal cancer. Pathol Res Pract. 2019;215(6):152383. doi:10.1016/j.prp.2019.03.012.
20. Andersen MH. The balance players of the adaptive immune system. Cancer Res. 2018;78(6):1379–1382. doi:10.1158/0008-5472.CAN-17-3607.
21. Martinenaite E, Ahmad SM, Svane IM, Andersen MH. Peripheral memory T cells specific for arginase-1. Cell Mol Immunol. 2019;168(8):718–719. doi:10.1038/s41423-019-0231-3.
22. Martinenaite E, Ahmad SM, Bendtsen SK, Jørgensen MA, Weis-Banke SE, Svane IM, Andersen MH. Arginase-1-based vaccination against the tumor microenvironment: the identification of an optimal T-cell epitope. Cancer Immunol Immunother. 2019;68 (11):1901–1907. doi:10.1007/s00262-019-02425-6.
23. Lorentzen CL, Martinenaite E, Kjeldsen JW, Holmstroem RB, Mørk SK, Pedersen AW, Ehrnrooth E, Andersen MH, Svane IM. Arginase-1 targeting peptide vaccine in patients with metastatic solid tumors – a phase I trial. Front Immunol. 2022;13. doi:10.3389/fimmu.2022.1023023.
24. Grauslund JH, Holmström MO, Martinenaite E, Lisle TL, Glöckner HJ, El Fassi D, Klausen U, Mortensen REJ, Jørgensen N, Kjær L. et al. An arginase1- and PD-L1-derived peptide-based vaccine for myeloproliferative neoplasms: a first-in-man clinical trial. Front Immunol. 2023;14. doi:10.3389/fimmu.2023.1117466.
25. Andersen MH, Bonfill JE, Neisig A, Arsequell G, Søndergaard I, Neefjes J, Zeuthen J, Elliott T, Haurum JS. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. J Immunol. 1999;163(7):3812–3818. doi:10.4049/jimmunol.163.7.3812.
26. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF. High□Throughput Real□Time Quantitative Reverse Transcription PCR. Curr. Protoc. Mol. Biol. 2006;73(1):15.8.1–15.8.28. doi:10.1002/0471142727.mb1508s73.
27. Moodie Z, Price L, Janetzki S, Britten CM. Response determination criteria for ELISPOT: toward a standard that can be applied across laboratories. Methods Mol Biol. 2012;792:185–196.
28. Thor Straten P, Guldberg P, Grønbæk K, Hansen MR, Kirkin AF, Seremet T, Zeuthen J, Becker JC. In situ T cell responses against melanoma comprise high numbers of locally expanded T cell clonotypes. J Immunol. 1999;163(1):443–447. doi:10.4049/jimmunol.163.1.443.
29. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J Immunol. 1998;160(11):5347–5354. doi:10.4049/jimmunol.160.11.5347.
30. Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol. 1999;163 (7):3771–3777. doi:10.4049/jimmunol.163.7.3771.
31. Jørgensen MA. Ugel S, Linder Hübbe M, Carretta M, Perez-Penco M, Weis-Banke, SE, Martinenaite E, Kopp K, Chapellier M, Adamo A, De Sanctis F. et al. Arginase 1–based immune modulatory vaccines induce anticancer immunity and synergize with anti–PD-1 checkpoint blockade. Cancer Immunol Res. 2021. doi:10.1158/2326-6066.CIR-21-0280.
32. Benner B, Scarberry L, Suarez-Kelly LP, Duggan MC, Campbell AR, Smith E, Lapurga G, Jiang K, Butchar JP, Tridandapani S. et al. Generation of monocyte-derived tumor-associated macrophages using tumor-conditioned media provides a novel method to study tumor-associated macrophages in vitro. J Immunother Cancer. 2019;7(1):140. doi:10.1186/s40425-019-0622-0.
33. Boutard V, Havouis R, Fouqueray B, Philippe C, Moulinoux JP, Baud L. Transforming growth factor-beta stimulates arginase activity in macrophages. Implications for the regulation of macrophage cytotoxicity. J Immunol. 1995;155(4):2077–2084. doi:10.4049/jimmunol.155.4.2077.
34. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM, Wallace J. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. PLoS One. 2014;9(4):e94188. doi:10.1371/journal.pone.0094188.
35. Dey S, Sutanto-Ward E, Kopp KL, DuHadaway J, Mondal A, Ghaban D, Lecoq I, Zocca M-B, Merlo LMF, Mandik-Nayak L. et al. Peptide vaccination directed against IDO1-expressing immune cells elicits CD8 + and CD4 + T-cell-mediated antitumor immunity and enhanced anti-PD1 responses. J Immunother Cancer. 2020;8(2):e000605. doi:10.1136/jitc-2020-000605.