Small extracellular vesicles containing arginase-1 suppress T-cell responses and promote tumor growth in ovarian carcinoma

Tumor-driven immune suppression is a major barrier to successful immunotherapy in ovarian carcinomas (OvCa). Among various mechanisms responsible for immune suppression, arginase-1 (ARG1)-carrying small extracellular vesicles (EVs) emerge as important contributors to tumor growth and tumor escape from the host immune system. Here, we report that small EVs found in the ascites and plasma of OvCa patients contain ARG1. EVs suppress proliferation of CD4\(^+\) and CD8\(^+\) T-cells in vitro and in vivo in OvCa mouse models. In mice, ARG1-containing EVs are transported to draining lymph nodes, taken up by dendritic cells and inhibit antigen-specific T-cell proliferation. Increased expression of ARG1 in mouse OvCa cells is associated with accelerated tumor progression that can be blocked by an arginase inhibitor. Altogether, our studies show that tumor cells use EVs as vehicles to carry over long distances and deliver to immune cells a metabolic checkpoint molecule – ARG1, mitigating anti-tumor immune responses.
Epithelial ovarian cancer (OvCa) is the most lethal gynecologic cancer in developed countries\(^1\). Initially most OvCa patients respond to standard platinum-based chemotherapy that follows debulking surgery. However, due to resistance of tumor cells to chemotherapy, the majority of patients relapse and die within several years after initial remission\(^2,3\). Novel therapeutic approaches to OvCa are an unmet clinical need. Immunotherapy is considered to be a promising addition to chemotherapy, as OvCa cells express immunogenic tumor-associated antigens, which could be potential targets for specific immune responses\(^4\). However, although immunotherapeutic approaches, especially immune check-point inhibitors proved effective in the treatment of several tumor types including melanoma or non-small cell lung cancer (NSCLC), their antitumor efficacy appears to be modest in OvCa patients\(^5,6\). This limited efficacy might be partly explained by the highly immunosuppressive tumor microenvironment (TME) of ovarian carcinomas\(^7\). A number of immunoregulatory mechanisms have been identified that seem to be responsible for tumor resistance to immunotherapies and for the often unsuccessful clinical responses to anticancer vaccines in OvCa\(^8\). These include downregulation of tumor-associated antigens and antigen-presenting machinery\(^9\), induction of suppressive cells including T regulatory cells\(^10\), B7H4+macrophages\(^11\) and tolerance-inducing plasmacytoid dendritic cells, production of immunosuppressive cytokines, such as IL-10 and TGF\(_β\)\(^12\) and induction of oxidative stress\(^13\), among others. Recent studies have also demonstrated that specific enzymes present in the TME are able to inhibit the immune response by limiting amino acid availability\(^14\). Among them are arginases that catalyze degradation of semi-essential L-arginine to L-ornithine and urea. Besides their fundamental role in the hepatic urea cycle, arginases have been shown to downregulate expression of T-cell receptor (TCR)-associated CD3\(ζ\) and \(ε\) chains, the critical components of the TCR-signaling complex, thereby impairing T-cell functions\(^15,16\). Moreover, depletion of L-arginine from the microenvironment arrests T-cell cycle progression and inhibits IFN-γ production\(^17,18\). Arginase activity also leads to the downmodulation of the expression of MHC class II molecules that are necessary for antigen presentation\(^19\).

There are two arginase isoforms (ARG1 and ARG2), catalyzing the same biochemical reaction, but differing in subcellular localization, expression, and regulation. ARG1 is a cytosolic protein, while ARG2 is mainly localized in the mitochondria\(^20\). High arginase levels, either ARG1 or ARG2, have been reported in several cancer types, including breast cancer\(^21\), NSCLC\(^22\), head and neck squamous cell carcinoma\(^23\), renal carcinoma\(^24\), colorectal cancer\(^25\), skin cancer\(^26\), and cervical cancer\(^27\). Arginases are mainly produced by myeloid-derived suppressor cells (MDCs) that are highly enriched in the TME, and the role of ARG1 expressing MDCs in altering T-cell responses in patients with cancer has been well established\(^28\). Nonetheless, an increasing number of recent studies detected arginases in tumor cell lines or primary tumors, e.g. in prostate cancer\(^29\), neuroblastoma\(^30\) and acute myeloid leukemia\(^31\). However, the expression of arginase and its immunomodulatory effects in OvCa cells have not been described so far.

A recent study reported that nanometer-sized membrane-encapsulated extracellular vesicles (EVs) isolated from the ascites of OvCa patients and identified as exosomes, suppressed TCR-dependent nuclear translocation of NF\(_κB\) and NFAT, CD69 and CD107\(α\) upregulation, and inhibited T-cell proliferation and cytokine production\(^32\). This report and the finding that OvCa-derived membrane vesicles can suppress CD3\(ζ\) levels in T-cells\(^33\) provided the rationale for a more detailed investigation of the arginase expression in OvCa cells, as well as its presence and function in OvCa-derived EVs. The overriding objective was to determine whether OvCa-derived EVs may contain ARG1 and suppress anti-tumor functions of T-cells thus providing the tumor with an advantage to escape from the host immune system.

Here we report that OvCa cells release ARG1 in small EVs and we investigate the influence of ARG1+ EVs on the antitumor effector mechanisms of immune response. We show that EVs distribute ARG1 from tumor cells to antigen-presenting cells in secondary lymphoid organs, suppressing antigen-specific T-cell proliferation and activation. We correlate high ARG1 expression in primary tumors and increased ARG1 activity in plasma with worse prognosis in OvCa patients. In an OvCa mouse model, we show that blocking arginase activity mitigates ARG1-driven tumor progression. Collectively, this study provides the first evidence for the role of ARG1+ EVs in the formation of an immunosuppressive microenvironment in OvCa.

### Results

#### ARG1 expression in primary OvCa

We first evaluated protein levels of ARG1 in established OvCa cell lines. These were relatively high in all tested cell lines as evaluated by immunoblotting (Fig. 1a) and, after cell permeabilization, by flow-cytometry (Fig. 1b). ARG1 was also expressed in tumor cells isolated from the ascites of an OvCa patient (Fig. 1a, b). Next, immunohistochemistry was used to evaluate ARG1 expression in 84 primary ovarian tumors and in normal ovary epithelial tissues that were used as controls. Supplementary Table 1 lists clinicopathologic characteristics of the patient cohort. All tumor specimens showed predominantly cytoplasmic staining for ARG1 of variable intensity (Fig. 1c). Strong staining for ARG1, with ARG scores up to 280 (see “Methods” section for the ARG score definition) was observed in 9 patients (10.71% of the tumor samples; Supplementary Table 2). In 47 patients (55.95% of the samples) a moderate ARG1 expression was observed with ARG scores of 100–180 and 28 (33.33%) of the tumors showed weak ARG1 expression (ARG scores below 100) with up to 50% of tumor cells stained weakly, and rest of the cells scored as negative. No ARG1 expression was detected in epithelial or stromal cells of the normal ovary.

Analysis of publicly available gene expression data sets of primary OvCa tumors from The Cancer Genome Atlas (TCGA) indicated that high ARG1 expression in the tumor corresponded to worse prognosis. In the analysis of a cohort of 215 patients over 50 years old, those with the lowest ARG1 expression (lowest quartile) had a significantly better overall survival (OS) than patients with the highest ARG1 expression (Cox proportional hazards model P = 0.0246, Fig. 1d). Analysis of another transcriptomic data set of 75 patients with serous ovarian carcinoma (Pamula-Pilat-101 MAS 5.0-u133p2; GEO accession #GSE63885) indicated that patients with low ARG1 gene expression had a significantly longer OS (Supplementary Fig. 1a, P = 0.024, log-rank test) and progression-free survival (Supplementary Fig. 1b, P = 0.022, log-rank test) than patients with high ARG1 expression.

Next, we have measured arginase activity in the plasma samples obtained from 81 untreated OvCa patients. Clinicopathologic characteristics of the patient cohort are listed in Supplementary Table 3. Arginase activity was significantly higher in the plasma of patients with stage II and III tumors (mean activity of 9.27 and 10.74 U L\(^{-1}\), respectively) relative to normal controls (mean activity of 2.29 U L\(^{-1}\), P = 0.0024 and P = 0.0001, respectively; Fig. 1e and Supplementary Table 4, Kruskal–Wallis test with Dunn’s multiple comparison test). Furthermore, the arginase activity correlated positively with the tumor grade, increasing from a mean activity of 5.08 U L\(^{-1}\) in grade I tumors...
to 10.74 U L$^{-1}$ in grade III tumors. We used the log-rank test to find the point (cut-off) with the most significant (lowest $P$-value) split in high vs. low ARG1 level group according to OS. Patients with arginase activity $\geq 7.5$ U L$^{-1}$ (high ARG activity) had significantly shorter OS (Fig. 1f, $P = 0.0172$, log-rank test). Altogether, we have found that increased ARG1 expression in the primary tumor, as well as increased ARG activity in plasma correlates with poor prognosis.

ARG1 is carried by OvCa-derived small EVs. We and others have shown that OvCa tumors release EVs, which can be found in large quantities in the patients’ plasma and ascites$^{32-34}$. EVs
ARG1 is expressed in ovarian tumors and its levels correlate with poor prognosis. a, b Arg1 expression in OvCa cell lines and tumor cells obtained from ascites determined by Western blotting and flow cytometry, respectively. The shaded area (in b) represents isotype control staining, whereas the transparent one reflects Arg1 expression. c Representative immunohistochemistry staining of Arg1 in normal ovary (upper left) and ovarian tumor (upper right and lower panels) sections at diagnosis (bar = 50 μm). Images represent no (i), weak (ii), moderate (iii), and strong (iv) staining intensity for Arg1. d Kaplan–Meier curve showing higher survival probability for n = 53 patients demonstrating low Arg1 transcript levels (lower quartile Q1) as compared to n = 53 patients with elevated (upper quartile Q3) expression levels of the gene. P = 0.0246 has been computed with the Cox proportional hazards model with age, clinical stage, and tumor grade included in the analysis. e Arg1 activity in plasma of n = 81 OvCa (staging I–III) patients at the time of diagnosis and of 10 healthy controls (NC) determined in a colorimetric assay. Data show means ± standard deviation (SD). *P = 0.0024; **P = 0.0005; ***P < 0.0001, Kruskal–Wallis test with Dunn’s multiple comparison test. For every patient the mean activity of three independent measurements is shown. f Percent survival of n = 81 OvCa patients with high plasma arginase activity (two upper quartiles, pink) and low plasma arginase activity (two lower quartiles, blue). P = 0.0172 high (≥7.5 U ml⁻¹) vs. low (<7.5 U ml⁻¹) Arg1 activity, log-rank test, blue/pink shading –95% confidence interval. Source data for panels a and e are provided as a Source Data file.

Specifically derived from tumor cells (referred to as tumor-derived EVs, tEVs) are expected to carry a molecular signature that partly reflects that of the parental tumor cells. tEVs are enriched in immunoinhibitory molecules that may inhibit and reduce anti-tumor immune responses. We therefore asked whether Arg1 can be detected in tEVs isolated from cultured OvCa cells. Immunoblotting revealed that Arg1 is present in the lysates of OvCa cell lines, as well as in EVs isolated by sequential centrifugation from supernatants of these cells (Fig. 2a). We performed NanoSight analyses, transmission electron microscopy (TEM), and immunobLOTS for endocytic proteins to closely characterize the obtained EVs. EVs produced by the representative OvCa cell line Skov-3 had the mean particle diameter of 128 nm and their concentration was 9.45 × 10⁹ particles/ml of cell supernatant (Supplementary Fig. 2a). Next, EVs isolated from the ascites of OvCa patients were examined for Arg1 content. We have detected Arg1⁺ EVs in the ascites of OvCa patients but not in the fluid obtained from benign ovarian cysts (normal controls, NC) (Fig. 2b). Furthermore, we confirmed the presence of Tsg101 or CD63 (at least in some cases) in the isolated vesicles. Considering poor quality of immunobLOTS for CD63 with the available antibody we have used beads coated with antibodies targeting either CD9, CD63, or CD81 tetraspans to immune-capture EVs pre-enriched by size-exclusion chromatography (SEC) from ascites of six different OvCa patients and to analyze the presence of the three mentioned tetraspans in the obtained EV-subtypes (Supplementary Fig. 2c). TEM confirmed the typical morphological characteristics of small EVs: round to oval shaped vesicles surrounded by a double membrane and ranging in size from 60 to 120 nm. Immunogold staining for Arg1 confirmed the presence of this enzyme in EVs (Fig. 2c). Examination by NanoSight (Supplementary Fig. 2b) of a representative patient sample gave the mean particle diameter of 125 nm and the concentration of 4.18 × 10⁹ particles per ml of ascites. Since tumor-derived EVs in ascites represent only a small fraction of the total number of EVs present, we have developed an immunoaffinity-based capture method using microbeads coated with anti-EpCAM antibody. EVs directly isolated from ascites with EPCAM-beads were Arg1⁺ and showed markers associated with EVs (Supplementary Fig. 2d). The mean size of these vesicles measured in qNano was comparable to the size of EVs isolated by SEC (Supplementary Fig. 2e). Using sequential centrifugation, we retrieved EVs from 49 patients with grade III OvCa and detected Arg1 in 29 of cases. We also isolated small EVs from benign cysts fluid of 9 patients and used these EVs as NC. OvCa patients had ~2.5 times more EVs (measured as the total protein concentration in the EV lysate, Fig. 2d, left) than normal controls and were characterized by a higher expression of Tsg101, a characteristic marker of small EVs (Fig. 2d, middle), as well as slightly higher, but statistically insignificant (P = 0.1327, unpaired t-test with Welch’s correction) Arg1 levels (Fig. 2d, right).

Next, by measuring the ability of EVs to convert l-arginine to urea, we confirmed that Arg1 in EVs derived either from OvCa cell line supernatants or the OvCa patients’ ascites was enzymatically active. Arginase activity in the lysates of all tested OvCa cell lines ranged from 4.82 to 26.9 U/g of total protein (Fig. 2e), while the EVs isolated from the ascites of OvCa patients had enzymatic Arg1 activity in the range of 0.305–6.514 U/ml of ascites (Fig. 2f). The enzymatic Arg1 activity of ascites EVs was higher than the activity in EVs isolated from fluid of benign cysts (Fig. 2f, g).

EVs with Arg1 suppress peripheral T-cells in OvCa patients. Since patients with OvCa have increased arginase activity in the TME and in the peripheral circulation, we questioned whether Arg1⁺ small EVs are detectable in the plasma of OvCa patients. Arg1 was detected in the lysates of plasma EVs by immunoblotting. These EVs contained more Arg1 than EVs obtained from the plasma of patients with benign disease. The relative levels of Arg1 in the plasma EVs of OvCa patients corresponded to increased plasma arginase activity (Fig. 3a).

To determine whether the increased Arg1 in plasma EVs of OvCa patients may associate with the ability of peripheral T-cells to proliferate, we concomitantly measured arginase activity and assayed T-cell proliferation and CD3⁺ levels in 14 patients and 5 controls with benign ovarian cysts. Ex vivo T-cell proliferation was induced with anti-CD3/CD28-coupled beads. Representative histograms of T-cell proliferation are shown in Supplementary Fig. 4a. Only 4/14 OvCa patients had normal T-cell proliferation. In seven patients we observed almost no proliferating CD8⁺ T-cells, and in the remaining three patients, the proliferation was moderately inhibited (Fig. 3b). The observed proliferation impairment of CD8⁺ cells correlated positively with reduced CD3⁺-chain expression in these cells (Fig. 3c and Supplementary Fig. 3b). Reduced CD8⁺ T-cell proliferation and decreased CD3⁺ expression levels correlated with increased Arg1 activity in the plasma (Fig. 3d, e). Similar results were also observed with CD4⁺ T-cells (Supplementary Figs. 3 and 4). In contrast, T-cells of the five patients with benign conditions (endometrial cyst or uterine myoma) showed normal proliferation and stable levels of CD3⁺ expression. Moreover, the measured arginase activity was lower in ascitic fractions after isolation of EVs as compared to full ascites (Supplementary Fig. 5a) and ascitic fractions remaining after EV isolations were less effective in inhibiting T-cell proliferation as compared with full ascites (Supplementary Fig. 5b). Altogether, these results indicate that increased Arg1 content in the plasma EVs of OvCa patients correlates with decreased CD3⁺ levels and impaired proliferation of peripheral T-cells.

ARG1 in EVs suppresses T-cell proliferation in vitro. Then, we sought to investigate the effects of Arg1-containing EVs obtained from OvCa-patients ascites on the proliferation of
**Fig. 2** OvCa-derived EVs contain enzymatically active ARG1.  

(a) ARG1 content in EVs and the parental OvCa cell lines lysates (TUM) as well as in EVs isolated from the cyst fluid and ascites fluid of two OvCa patients (EV1, EV2) determined by Western blotting. Equal amounts of protein (30 μg) were loaded per lane. 

(b) Representative Western blot for ARG1 and typical exosomal markers in EVs isolated from ascites of n = 11 OvCa patients. EVs isolated from ovarian fluid served as normal control (NC). Equal amounts of protein (30 μg) were loaded per lane. 

(c) Representative TEM images of whole-mounted OvCa patient-derived tEVs. Dots indicate immunogold (6 nm gold particles) labeling of ARG1. 

(d) Protein concentration measured with BCA assay, Tsg101 and ARG1 levels in EVs isolated from 2 ml of ascites of OvCa patients (n = 47–50) and from 2 ml of benign cyst fluid (n = 7–9). Relative Tsg101 and ARG1 content was determined by densitometric analysis of Western blots. Data refers to means ± SD, P values were calculated with unpaired t-test with Welch’s correction. 

(e) Arginase activity in OvCa cell line lysates and in the corresponding EVs determined by measuring L-arginine conversion to urea in a colorimetric assay. Data show means ± SD, n = 3. 

(f) Arginase activity in EVs isolated from n = 11 OvCa patients’ ascites (Asc) and benign cyst fluid (NC) calculated per ml of starting fluid volume. Data show means ± SD, n = 3. 

(g) Arginase activity as a function of amount of EVs in n = 2 OvCa patients ascites-isolated tEVs and EVs isolated from n = 2 benign cyst fluid. Data show means ± SD. Source data for data in panels a, b, d-g are provided as a Source Data file.
normal T-cells in vitro. Human peripheral blood CD4+ and CD8+ T-cells were stimulated with anti-CD3/CD28-coupled beads and incubated with EVs isolated from 44 OvCa patients’ ascites. As controls we have used EVs isolated from the fluid of seven benign ovarian cysts (cystic fluid EV, CFEV). CFEVs did not or only marginally suppressed proliferation of the T-cell subsets. In contrast, a significant suppression of T-cell proliferation was observed using EVs isolated from OvCa patients (Fig. 4a, b). Notably, EVs isolated from 18 out of 43 (42%) and 18 out of 44 (43%) of OvCa patients very strongly (by over 70%) inhibited the proliferation of CD4+ and CD8+ T-cells, respectively (Fig. 4b upper panels). EVs from 8 (19%) and 12 (27%) of the patients had no effect (inhibition by <10%) on the proliferation of CD4+ or CD8+ T-cells, respectively. Along with inhibition of T-cell proliferation we also observed a decrease in CD3ζ levels in both T-cell subsets (Fig. 4b lower panels). The observed CD3ζ down-regulation corresponded to the T-cell proliferation inhibition and tended to be stronger with EVs isolated from OvCa patients than with CFEV. EVs suppressed both CD4+ and CD8+ T-cells in a dose-dependent manner (Fig. 4c). Furthermore, we observed that, at least in some cases, the specific inhibition of ARG1 with arginase inhibitor (OAT-1746) or addition of excess l-arginine to the culture medium partially reversed the effects of patients’-derived EVs (Fig. 4d).

**ARG1+ EVs are internalized by DCs.** To further delineate immunoregulatory mechanisms of ARG1+ EVs, we have used a murine ID8 OvCa model. Since parental ID8 tumor cells do not express ARG1, we overexpressed V5-tagged murine ARG1 (ID8-ARG1-V5) using lentiviral transduction system. As controls we used empty vector-transduced ID8 cells (ID8-pLVX). Small EVs isolated from the supernatants of ID8-ARG1-V5 cells (EVs-ARG1) contained enzymatically active ARG1 (Supplementary Fig. 6a), the presence of which could be specifically detected by immunoblotting with an anti-V5-tag antibody (Supplementary Fig. 6b). Confocal microscopy showed that PKH67-stained EVs-ARG1 were internalized by murine bone marrow-derived dendritic cells (BMDCs) at 37 °C, but not at 4 °C (Fig. 5a) and EV-derived ARG1 was specifically detected by Western blotting in DCs lysates (Fig. 5b). The EV-associated ARG1 was functionally active, as induction of CD8+ and CD4+ T-cell proliferation with anti-CD3/CD28-coupled beads was inhibited by DCs co-incubated with EVs-ARG1 (Fig. 5c). EVs-ARG1 also reduced the expression of CD3ζ in both CD8+ and CD4+ T-cells (Fig. 5d). The control EVs isolated from ID8-pLVX cells (EVs-pLVX) had no influence on T-cell proliferation. Similarly, EVs-ARG1 inhibited the OVA-peptide-specific proliferation of DC-primed OT-1 T-cells (Fig. 5e). In all experimental settings, the suppressive effects of EVs-ARG1 were completely abrogated by addition of the arginase inhibitor OAT-1746, confirming the role of ARG1 in EV-mediated suppression of T-cell proliferation.

**ARG1+ EVs suppress antigen-specific T-cells in vivo.** Antigen-specific T-cell proliferation is triggered in secondary lymphoid organs. Thus, we have developed an animal model to investigate the effects of OvCa-derived tEVs on T-cell proliferation in the local lymph nodes (LN). To this end, control EVs-pLVX as well as EVs-ARG1 were subcutaneously inoculated into C57BL/6
mice. Immunoblotting of lysates of local LNs demonstrated the presence of V5 tag at 4 and 24 h post tEVs inoculation (Fig. 6a), indicating that the injected tEVs were being transported to the draining LNs. Subcutaneously inoculated EVs-ARG1 significantly inhibited OVA-specific proliferation of adoptively transferred OT-I T-cells ($P = 0.0275$, Kruskal–Wallis with Dunn’s multiple comparison test), whereas inoculation of control EVs-pLVX had no effect on T cell proliferation (Fig. 6b). Notably, inoculation of recombinant mouse ARG1 (rmARG1) followed by adoptive transfer of OT-I T-cells also failed to inhibit OT-I T-cell proliferation (Supplementary Fig. 7). To further investigate whether the suppressive effects are mediated by ARG1, we performed the in vivo proliferation experiment in OAT-1746-treated mice. Pharmacological ARG1 inhibition partially reversed
**Fig. 4** EV-ARG1 is involved in the suppression of T-cell proliferation in vitro. **a** Representative proliferation histograms of αCD3/αCD28-stimulated CD8+ and CD4+ T-cells incubated for 6 days with tumor ascites-derived EVs isolated from n = 5 OvCa patients (left panel, tEVs 1–5) and control EVs (CFEV1-5, right panel), isolated from n = 5 patients with benign cyst of the ovary. **b** Inhibition of proliferation (upper) and decrease in CD3ε levels (lower) of peripheral blood CD4+ (left) or CD8+ T-cells (right) by OvCa ascitic fluid-isolated EVs (n = 43–44). Data show means ± SD, P values for OvCa ascites vs. benign cyst fluid-isolated EVs treated group (n = 6–7) were calculated with Mann–Whitney U-test. The amount of added EVs corresponded to 2 ml of starting material. **c** Representative proliferation histograms of αCD3/αCD28-stimulated CD4+ and CD8+ T-cells incubated with increasing amounts of tEVs, isolated from 2 ml (8 µl), 1 ml (4 µl), 0.5 ml (2 µl), or 0.25 ml (1 µl) of ascites, respectively. **d** Representative proliferation histograms of peripheral blood CD4+ and CD8+ T-cells incubated for 6 days with tEVs (corresponding to 2 ml of ascites) and indicated concentrations of an arginase inhibitor, OAT-1746. Cells incubated for 6 days with tEVs and 2 mM l-arginine served as a positive control for l-arginine-dependent reversal of T-cells proliferation inhibition. Source data for panels **b** and **d** are provided as a Source Data file.

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**Fig. 5** ARG1-EVs are internalized by murine bone marrow-derived DCs (BMDCs) and block DCs-primed T-cell proliferation. **a** Representative image of EVs endocytosed by BMDCs from confocal microscopy. BMDCs were incubated with 50 µg of PKH-67-stained ARG1-EVs isolated from the supernatants of ID8-ARG1-V5 murine ovarian carcinoma cell line for 4 h at 37 or 4 °C (inhibited internalization, negative control), washed and fixed. Green—PKH67-stained EVs, blue—DAPI nuclear stain. **b** Western blotting for V5-tag labeled ARG1 in DCs lysates. BMDCs were incubated with 50 µg of ARG1-EVs isolated from supernatants of ID8-ARG1-V5 murine OvCa cell line for 4 h at 37 °C, washed and lysed. ID8-ARG1-V5 (ID8-ARG1) cells lysate was used as a positive control for V5-tagged Arg1 detection. Beta-actin served as equal protein loading control. **c** Representative proliferation histograms of αCD3/αCD28-stimulated CD4+ and CD8+ T-cells co-cultured with BMDCs pre-incubated with 100 µg EVs isolated from the supernatants of ID8-ARG1 cells (EVs-ARG1) or ID8-pLVX (EVs-pLVX) cells for 3 days. EVs-ARG1 or EVs-pLVX with no BMDCs and/or ARG inhibitor OAT-1746 (200 nM) were added to some groups as indicated in the figure. **d** Relative CD3ε expression evaluated with flow cytometry in αCD3/αCD28-stimulated CD4+ (upper graph) and CD8+ (lower graph) T-cells after pre-incubation with EVs-ARG1 or EVs-pLVX (90 µg) and/or ARG inhibitor OAT-1746 (400 nM). Data show MFI of two technical repeats from n = 3 mice ± SD. **e** Representative proliferation histograms of SIINFEKL-specific CD8+ T-cells (OT-I T-cells) primed with SIINFEKL peptide pulsed BMDCs. Where indicated, DCs were pre-incubated with EVs-ARG1 or EVs-pLVX (100 µg) in the presence of 200 nM ARG inhibitor OAT-1746. Source data for panels **b** and **d** are provided as a Source Data file.
tEVs-mediated suppression of T cell proliferation (Fig. 6c). Flow cytometry analysis of OT-I cells from the draining LNs showed that ARG1+ EVs significantly decreased the percentage of activated (CD69+) OT-I T-cells and downregulated CD3ε expression in these cells. Upon ARG1 inhibition the CD3ε levels in OT-I cells were restored and the percentage of activated (CD69+) OT-I cells and their expression of CD69 significantly increased (P = 0.0391, P = 0.0041, P = 0.0433, respectively, Kruskal–Wallis with Dunn’s multiple comparison test), exceeding even the levels in control mice (Fig. 6d).
ARG1 promotes OvCa progression. Since human OvCa cells express ARG1, we questioned if ARG1 is involved in the regulation of tumor progression. Mice were inoculated i.p. with control (ID8-pLVX) or ARG1-transduced (ID8-ARG1) tumor cells and were treated with OAT-1746 or PBS starting from day 15 after inoculation of tumor cells. Tumor progression was monitored by measuring weight and waist circumference gains. Mice inoculated with ID8-ARG1 cells showed faster tumor growth as compared with control ID8-pLVX tumors (Fig. 7a). In these animals ascites formed at an earlier time point and accumulated markedly faster than in mice bearing ID8-pLVX tumors. Diffuse peritoneal dissemination of tumor cells consisting of multiple tumor nodules of 0.5–5 mm, which were dispersed on the parietal and visceral surfaces of the peritoneal cavity at 28–34 days post tumor cells inoculation was observed. Tumor nodules were particularly noticeable in the diaphragmatic peritoneum resembling human ovarian carcinoma. Control animals bearing ID8-pLVX tumors, as well as OAT-1746-treated mice displayed occasional small (0.5–2 mm) nodules on the diaphragmatic peritoneum. Serum ARG1 levels in ID8-ARG1 tumor-bearing mice increased concomitantly with the tumor growth (Supplementary Fig. 8a). The mean arginase activity in the small EV fraction obtained from the ascitic fluid collected from ID8-ARG1 tumor-bearing mice at weeks 3 (n = 3) and 7 (n = 4) was 2.08 mU/mI of ascites (Source Data File—Table 1), which is within the range of arginase activities observed in OvCa patients (Fig. 2f). Cells highly expressing ARG1-V5-tag were detected by immunoblotting in ascites 28 days post inoculation of tumor cells (Supplementary Fig. 8b). Furthermore, ARG1-V5-positive EVs were isolated from ascites of these mice (Supplementary Fig. 8c). ARG1 inhibition with OAT-1746 significantly reduced the growth of ID8-ARG1 tumors (Fig. 7a, for weight P = 0.0130, for waist circumference P = 0.020, unpaired t-test). A less pronounced, but nevertheless significant reduction of tumor growth upon OAT-1746 treatment was also observed in the control group with ARG1-negative tumors (Fig. 7a, for weight P = 0.0065, for waist circumference P = 0.0093, unpaired t-test), indicating potential blockade of arginase activity in MDSCs and TAMs, the other known sources of ARG1 in the TME. Consistent with the results obtained in vivo with the EVs-ARG1 in the adoptive transfer model in non-tumor settings, ID8-ARG1 bearing-mice had a significantly reduced percentage of activated CD69-positive CD4+ (Fig. 7b left) and CD8+ (Fig. 7b middle) T-cells in the peritoneal cavity relative to mice bearing control tumor cells. OAT-1746 treatment noticeably increased the percentage of activated CD4+ and CD8+ cells (P = 0.0007, P = 0.0030, respectively, unpaired t-test). Furthermore, in ID8-ARG1 bearing-mice up to 1% of peritoneal activated CD11c+ dendritic cells stained positive for V5-tag indicating an uptake of tumor-derived ARG1 (Fig. 7b right). As we have shown in the in vitro and in vivo assays, upon internalization of ARG1 antigen-presenting DCs lose their activating potential and instead become suppressive. Due to very limited cell numbers we were not able to check the activating capacity of the V5-positive DCs in this experimental setting. Upon ARG1 inhibition the percentage of V5-positive, potentially suppressive CD11c+ dendritic cells in the peritoneal cavity has decreased. Taken together, these data indicate that ARG1 expressed by tumor cells accelerates tumor progression and is a potential therapeutic target in ovarian carcinoma.

Discussion

Arginine metabolism is one of the metabolic pathways responsible for tumor progression38. Since ARG1 regulates the availability of L-arginine, it is under extensive investigation as an anticaner therapeutic target39. However, the potential involvement of ARG1 in OvCa has received little attention to date, and in other tumors it was mostly considered in association with myeloid cells and MDSCs. Murine and human OvCa-induced MDSCs were reported to express ARG1, and murine MDSCs were shown to require ARG1 for suppression of T-cells40. Furthermore, higher arginase activity as compared to normal controls was detected in the plasma of OvCa patients41 and it decreased after chemotherapy42.

Our study is the first to report that ARG1 is expressed in OvCa and that it becomes distributed far beyond the local TME by EVs. We observed that increased arginase activity was not only confined to the TME, but was also detectable in the peripheral circulation of OvCa patients and correlated with poor survival. Therefore, we hypothesized that ARG1 can be released from tumor cells in EVs and found ARG1+ small EVs in ascites, as well as in the plasma of OvCa patients. Remarkably, high arginase activity in patients’ plasma along with high ARG1 content in plasma EVs correlated with lower CD3ζ expression levels and poor proliferative capacity of circulating T-cells in our patient cohort. In the following in vitro studies, we showed that patient-derived as well as OvCa cell line-derived ARG1+ EVs impair the functions of human and murine T-cells by blocking their proliferation, and reducing expression levels of the CD3ζ and CD3ε chains. This transmembrane components of the TCR complex serve as essential signaling molecules in T lymphocytes, and their appropriate expression and phosphorylation are critical for T lymphocyte activities, such as proliferation and cytokine production. Numerous studies have previously demonstrated
alterations in expression and function of CD3ζ in both tumor-infiltrating lymphocytes (TILs) and peripheral blood T-cells [16,43]. While transient decrease in the CD3ζ expression level occurs normally during antigenic stimulation [44], its persistent loss, as often observed in TILs, has been correlated with reduced T-cell proliferation and cytokine production [15,45]. These alterations, along with other mechanisms, are responsible for deficient immune responsiveness of T-cells in cancer patients, including OvCa [46,47]. Importantly, the CD3ζ chain loss in T-cells of cancer patients is biologically significant, as it correlates with worse prognosis and shorter OS, as reported for OvCa [33], head and neck cancer [48], and breast cancer [49]. We and others have shown previously that tumor-derived microvesicles, including exosomes in OvCa, suppress CD3ζ chain expression of T-cells [50,51], but identified no responsible mechanism. We observed an induction of apoptosis by tumor EVs and linked this finding with the presence of Fasl or TRAIL on the exosomes surface [50,51]. Further, we have shown that EV-induced apoptosis is accompanied by caspase-3 cleavage, cytochrome c release, mitochondrial membrane potential, DNA-fragmentation, and inactivation of the PI3K/Akt pathway with concomitant downregulation of anti-apoptotic proteins [50,52]. The present study defined ARG1 as an additional EV-derived component that is responsible for CD3ζ and CD3ζ chain downregulation and T-cell suppression. Furthermore, we demonstrate that ARG1+ EVs deliver the active enzyme to the draining LNs in vivo, where vesicle ARG1 either directly inhibits T-cell proliferation by decreasing l-arginine levels or is taken up by DCs impairing their activating potential. In contrast, recombinant ARG1 even at high doses failed to inhibit specific T-cell proliferation, probably due to the fast degradation of the free

| Weight (g) | Waist (cm) | % of CD4+ CD69+ cells | % of CD11c+ MHC IIhigh V5+ cells |
|-----------|------------|-----------------------|-------------------------------|
| 10        | 10         | 5                     | 5                             |
| 20        | 20         | 10                    | 10                            |
| 30        | 30         | 15                    | 15                            |

**Fig. 7** ARG1 promotes tumor growth in vivo. C57BL/6 mice were inoculated i.p. with 4 × 10^6 ID8-VegfA/Defb29 cells transduced with V5-tagged murine ARG1 (ID8-ARG1-V5) or the control vector (ID8-pLVX). **a** Mice were treated from day 14th after tumor inoculation with OAT-1746 or PBS i.p. twice daily and monitored for tumor development until first mice met the humane endpoint criteria described in the “Methods” section. Increase in mice weight (upper left) and waist circumference (lower left) in time compared to day 0 (day of tumor cells i.p. inoculation) as a measure of ovarian cancer progression/ascites development. Measurements of gained weight (upper right) and percentage of gained waist circumference (lower right) on day 34 after inoculation of tumor cells. Each experimental group consisted of n = 5–9 mice. Data show means ± SE. **b** Mice were treated from day 14th after tumor inoculation with OAT-1746 ARG inhibitor or PBS i.p. twice daily for consecutive 2 weeks. After 2 weeks of treatment cells from ascites were isolated and analyzed by flow cytometry. Percentage of ascitic activated (identified as CD69+) CD4+ (left panel) or CD8+ T-cells (middle panel), as well as percentage of activated (MHCII[high]) CD11c+ DCs positive for V5-tag (right panel) on day 28th after inoculation of tumor cells. Each experimental group consisted of n = 3–6 mice. Data show means ± SD; P values were calculated with unpaired t-test. Source data are provided as a Source Data file.
enzyme in vivo. Arginase can be produced and released from normal cells, but in the extracellular milieu, the enzyme is unstable and its circulating half-life in humans is <30 min. Our results indicate, that ARG1 in EVs, in contrast to free ARG1, remains stable, possibly protected from degradation by the EVs membrane.

It has been well established that a special subpopulation of endosome-derived and tetraspanins-enriched small EVs called exosomes are involved in multiple facets of tumorigenesis. For example, tumor-derived exosomes (TEX) were shown to induce bone marrow progenitor cell differentiation into provasculogenic cells facilitating development of the pre-metastatic niche. TEX can modulate immune response by delivering activated epidermal growth factor receptor (EGFR) to host macrophages rendering these cells less efficient in type I interferon production and compromising host antiviral immunity. We previously showed that EVs contribute indirectly to immune suppression through expansion of T regulatory cells. Moreover, recent studies reported that TEX carry PD-L1 molecules that contribute to immune suppression and that PD-L1 levels on exosomes correlate with cancer activity and progression. It was previously shown that OvCa exosomes can be transported from the periphery to the LNs in a process actively enhanced by lymphatic endothelial cells. Also, a selective uptake of exosomes by various types of target T-cells has been described.

To the best of our knowledge we are the first to report ARG1 presence in tumor-derived EVs, although modulating activity of ARG1 in EVs has been recently reported in non-tumor settings. In a mouse model of the autoimmune disease alopecia areata, MDSC-derived exosomes containing ARG1 were preferentially taken up by activated T-cells and suppressed the proliferation, as well as CD3 and CD69 expression in activated LN cells and skin-infiltrating lymphocytes. Similarly, exosomes with enzymatically active ARG1 that were isolated from granulocytic MDSCs of tumor-bearing mice attenuated DSS-induced murine colitis by suppressing Th1 cells and promoting Treg differentiation.

In diabetic mice, ARG1 was found to be enriched in serum exosomes and ARG1+ exosomes were effectively taken up by endothelial cell contributing to development of diabetic endothelial dysfunction resulting from inhibition of NO production. Increased secretion of active ARG1 in exosomes was also observed after drug-induced liver injury. ARG+ EVs secreted by hepatocytes were shown to modulate the blood metabolome associated with oxidative stress and endothelial regulation, and inhibited the acetylcholine-induced relaxation of isolated pulmonary arteries.

In light of these findings ARG1 carried by small EVs emerges as a potent metabolic and immunomodulatory factor, since it remains stable, may easily cross tissue barriers and quickly reaches secondary lymphoid organs. Vascular ARG1 can act beyond the TME, being distributed in the peritoneal cavity by the ascites fluid and systemically through the peripheral circulation, leading to a systemic T-cell suppression. Therefore, although MDSCs and TAMs, in comparison to ARG1+ EVs, are the predominant sources of ARG1 within TME, ARG1 in EVs may exert systemic biological effects.

By using a small molecule arginase inhibitor, we have shown that the negative effects of this enzyme can be mitigated. Several previous studies indicated that blocking arginase activity is an attractive therapeutic target to promote anticancer immune responses. A number of synthetic as well as natural arginase inhibitors are under intense preclinical and clinical evaluation. Treatment with the small-molecule inhibitor nor-NOHA abrogated the arresting effects of arginase on T-cell proliferation and led to lymphocyte-dependent reduction of tumor growth, and similar effects were achieved in a murine OvCa model. Arginase inhibition also nearly completely abrogated the immunosuppressive effects of ARG2-positive circulating AML blasts. Treatment with the small-molecule arginase inhibitor CB-1158 was shown to reduce tumor growth in several mouse models of cancer by increasing the number of tumor-infiltrating CD8+ T-cells and NK cells, and production of TH1-associated inflammatory cytokines. CB-1158 also significantly improved the efficacy of checkpoint blockade (anti-PD1 and anti-CTLA-4), adoptive T-cell or NK cell therapy or chemotherapy with gemcitabine and is currently investigated in phase I clinical trial as a single agent or in combination with immune checkpoint therapy in patients with advanced or metastatic solid tumors. Additionally, arginase activity was shown to impair CAR-T-cell therapy of neuroblastoma, emphasizing the significant clinical implications of arginase as a potential target for T-cell immunotherapy.

Altogether, we provide the first evidence for the role of ARG1 in the formation of an immunosuppressive microenvironment in OvCa. Moreover, we show that the effects of ARG1 are not only confined to the tumor site, but are disseminated through the release of ARG1-containing small EVs. These EVs transfer functionally active ARG1 as metabolic checkpoint molecules over a long distance to antigen-presenting cells and mitigate antitumor immune response, leading to an enhanced tumor growth in vivo. We identify hereby a novel mechanism of tumor-induced systemic T-cell dysfunction based on the activity of tumor-derived ARG1+ EVs, that may also apply to other arginase-expressing tumor types and may have significant clinical implications for T-cell immunotherapy approaches.

### Methods

#### Reagents

Recombinant human ARG1 was obtained from Biolegend (San Diego, CA, USA), recombinant human and murine IL-2, murine IL-4, and GM-CSF were purchased from Peprotech, arginase inhibitor OAT-1746 was synthesized at OncoArendi Therapeutics, Warsaw, Poland. All other reagents, if not otherwise stated, were obtained from Sigma-Aldrich.

#### Cell lines

Human ovarian cancer cell lines used in this study are listed in Supplementary Table 5. The murine ovarian cancer cell line ID8, derived from spontaneous malignant transformation of C57BL/6 MOSE cells in vitro was kindly provided by Kathy Roby from University of Kansas. For in vivo tumor models, VegFα/Defb29 transduced ID8 cells (ID8-VegFα/Defb29) kindly obtained from Jose R. Conejo-Garcia and Kathy Roby (University of Kansas Medical Center, KS, USA) were used. All cell lines were cultured in RPMI 1640 or in Dulbecco’s modified Eagle’s (DMEM) media supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin at 37 °C in an atmosphere of 5% CO2 in air. Tumor cell lines were regularly tested for Mycoplasma and confirmed to be negative.

#### OvCa patient samples

Primary ovarian carcinoma lesions were collected from 84 previously untreated patients with epithelial OvCa who were admitted to the Gynecologic Oncology Clinic at the University of Medical Sciences in Poznan, Poland. Histological diagnoses including tumor grade were determined by WHO criteria and were confirmed by a second review of the original H&E tissue sections. Normal ovarian tissues obtained from patients who underwent radical hysterectomy (e.g. to non-ovarian disease) or to non-ovarian disease (e.g. to non-uterine fibroids) were used as controls. Plasma arginase activity was measured in 81 OvCa patients and 10 normal controls (Supplementary Table 3). All OvCa patients underwent cytoreductive surgery and received subsequently standard first-line platinum-based chemotherapy. Ascites and/or plasma were collected during a routine medical procedure from 49 women diagnosed with stage III serous ovarian carcinoma at the Gynecologic Oncology Clinic at the University of Medical Sciences in Poznan, Poland or at the Department of Obstetrics and Gynecology of the Praski Hospital in Warsaw, Poland. In brief, 5–20 ml of ovarian cyst fluid was collected after removal of the cyst from the abdomen by puncturing the cyst wall with an 18-gauge needle mounted on a 10-ml syringe. Controls (n = 9), with histologically benign gynecological conditions including fibromas, endometriosis, and mucinous and serous cystadenomas were selected based on age-matching to patients with ovarian cancer. Normal control peripheral blood mononuclear cells (PBMC) were obtained by Histopaque-1077 (Sigma Aldrich) or Lymphoprep (Stemcell Technologies) separation from buffy coats from healthy volunteers, commercially obtained from the Regional Blood Centre in Warsaw, Poland. The approval for these studies was obtained from the Institutional Bioethical Review Board of the Medical University of Warsaw.
Isothermal titration calorimetry (ITC) experiments were performed in a Microcal 
 vice versa. The final value for the thermodynamic parameters were obtained for the 

**Characterization of isolated EVs.** EVs were evaluated for morphology by TEM and for particle distribution and size using tunable resistive pulse sensor (TRPS) technology (qNano instrument, Izon Science) or dynamic light scattering (NanoSight NS300, Malvern). For TEM the EV pellet obtained from 25 ml of tumor supernatant was fixed in 2% glutaraldehyde for 5 min and washed in PBS. Next, the EVs were adsorbed and the grid was next embedded in a mixture of 2% uranyl acetate (UA) and 1% methyl cellulose and for TEM the EV pellet obtained from 25 ml of tumor supernatant was fixed in 1% glutaraldehyde for 5 min and washed in PBS. Next, the EVs were adsorbed and the grid was next embedded in a mixture of 2% uranyl acetate (UA) and 1% methyl cellulose.

**Western blotting.** Lysed EVs or viable cell pellets were sonicated 2× for 15 s in an ultrasonic bath (BD ProbeTec ET). Subsequently, samples were boiled in Laemmli loading buffer, separated using SDS–PAGE and transferred to nitrocellulose membranes (Amersham) by semi-dry blotting. After blocking with 5% (w/v) non-fat dry milk in Tris-buffered saline/1% Tween-20 membranes were probed with primary antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies. Antibody conditions were optimized for immunoblotting as described in Supplementary Table 2. Bands were revealed using Western Bright Quantum Kit (Advansta). Stela Imaging System (Raytest Isopenmessgeraete) or the Chemidoc Touch System (Bio-Rad) were used for image acquisition. ImageLab software version 5.2.1 (Bio-Rad) was used for densitometric analysis of the blots. For evaluation of EV-associated proteins standard curve of 25 ng/ml recombinant human ARGI (set as 1). Relative Tsg101 expression was calculated by division of the optical density of the band corresponding to 10 ng of recombinant human ARGI (set as 1). Relative Tsg101 expression was calculated by division of the optical density of the band corresponding to 10 ng of recombinant human ARGI (set as 1).

**Arginase activity assay.** The activity of arginase in OvCa patients’ plasma was determined by measuring the conversion of L-arginine to L-ornithine in a colorimetric assay. Briefly, 2.5 µl MmGlu was added to 25 µl of plasma (10 mM end concentration) and the enzyme was activated at 37°C for 5 min. Then, 50 µl of 0.5 M sulfuric acid/1.1% phosphoric acid/0.75% ninhydrin, and heating the samples for 15 min at 100°C. Absorbance was measured at 515 nm (Asys UVM 340 Plate Reader) and compared with L-ornithine monochloride standard curve. For the measurements of arginase activity in cells and EV fractions, a colorimetric assay for urea detection was used. For activity measurements in cell line supernatants 1 × 10⁶ cells were incubated in 80 µl OptiMEM medium in a 96-well microplate and the presence of 20 µl L-arginine at 37°C for 24 h. For activity measurements in EV lysates, 20 µg of patient-derived EVs were lysed in 1% (v/v) Triton X-100 in PBS in an end-volume of 50 µl. Then 50 µl of the cell supernatant or of EV lysates, respectively, were added to 150 µl of freshly prepared mixture (1:1) of 4 mM o-phenylaldehyde in 50 mM boric acid/1 M sulfuric acid/0.03% Brij-35, and 4 mM N-(1-Naphyl) ethylene-diamine dihydrochloride in 50 mM boric acid/1 M sulfuric acid/0.03% Brij-35 and incubated for 30 min at RT. Absorbance was measured at 540 nm (Asys UVM 340 Plate Reader).

**Generation of ID8 cells stably expressing murine ARGI.** ID8 cells stably expressing murine ARGI tagged at C-terminus with V5-tag (ID8-ARG1-V5) were generated using lentiviral transduction. Murine full length ARGI cDNA was amplified from the pCMV6-Kan/Neo plasmid encoding murine ARGI (Origene Technologies) using the following primers: forward: 5′-GGCGCAATTCACC ATGAGCTCCAAAAGCCAAAGTCC-3′ and reverse: 5′-GGCGGCGCGCGCTCA GTGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCTTAG GTTGTTTAAAGGTATCAGTCC-3′ resulting in addition of C-terminal V5-tag. The tagged ARGI cDNA was inserted into the multi cloning site of plPX-IREs-Puro—the mammalian expression vector for bi-cistronic expression of a gene-of-interest, together with a puromycin-resistance marker (Thermo Fisher Scientific). Transduced ID8 cells were selected with 1 µg ml−1 puromycin. ID8 cells transduced with the empty plPX-IREs-Puro vector served as control cell line.

**EV endocytosis.** BMDMs precursors were isolated from bone marrow by flushing femur, tibia, and humerus bones of 6-week-old female C57BL/6 mice with cold PBS. After lysis of RBCs with ammonium chloride. Cells (1 × 10⁶ cells in 5 ml per well) were cultured in six-well plates in non-essential amino acid supplemented
Supernatant after EVs isolation by sequential centrifugation (90% v/v). After other stated. The arginase inhibitor OAT-1746 was added as indicated in the

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In vitro T-cell proliferation assay. Freshly isolated PBMC were counted in Türk’s solution and immediately used for experiments. CD8+ and CD4+ T cell subsets were separated using EasySep Human CD4+ T-Cell Enrichment Kit or EasySep Human CD8+ T-Cell Enrichment Kit (StemCell Technologies) according to the manufacturer’s protocols. Purity of each fraction was >92%, with >98% viability. For further experiments CD8+ and CD4+ T cells were labeled with PE-Cy5.1/7 violet dye (5 μM final concentration, Thermofisher Scientific) according to manufacturer’s manual. Next, the labeled T-cells were plated in round-bottomed 96-well plates (1–2 × 10^6 cell per well) in 1-arginine-free RPMI medium (SILAC RPMI-medium, Thermofisher) supplemented with 10% FBS, penicillin/streptomycin, 2% glutamine and 2% L-lysine, 150 μg/ml L-arginine and stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermofisher Scientific) in the presence of 30 μg/ml recombinant human IL-2 (Peprotech). Patient-derived EVs pre-enriched by sequential centrifugation and purified by SEC were added in amount corresponding to 2 ml of starting material (ascites or cyt fluid), if not otherwise stated. The cognate inhibitor OAT-1746 was added as indicated in the figures. For some experiments T-cells were cultured in full ascs or in the ascitic supernatant after EVs isolation by sequential centrifugation (90% v/v). After incubation for 3−6 days at 37°C, 5% CO2, T-cells were harvested, stained with corresponding anti-CD3, anti-CD8, or anti-CD4 antibodies (Supplementary Table 6) and analyzed by flow cytometry (FACSCanto II, BD Biosciences). Percentages of proliferating cells were calculated using the FlowJo Software v7.6.5 (Tree Star) and the percentage of proliferation inhibition in the presence of EVs was calculated relative to bead-stimulated control T-cells (Supplementary Fig. 8).

Bioinformatics analysis. Survival analysis according to ARG1 gene expression has been carried out for two transcriptional data sets: (1) data set of 489 type II–IV serous ovarian published by TCGA® and (2) Tumor Ovarian-Pam- 

Pilat-101-MASS-0.1133p2 [GEO accession no. GSE63885] data set of 75 ovarian cancer tumors available through the R2 Genomics Analysis and Visualization Platform [an Affymetrix analysis and visualization platform developed in the Department of Oncogenetics at the Academic Medical Center at the University of Amsterdam (http://r2.amc.nl)]. OS analysis in the TCGA data set (1) has been carried out in R for the group of ovarian cancer patients based on primary tumor samples gene expression data. To make the analyzed patient group comparable to our IHC patient cohort according to age and ethnicity, the patient group has been filtered to exclude patients below 50 years of age and of other than white ethnicity. Subsets of the patients demonstrating either upper quartile Q3 (n = 53) or lower quartile Q1 (n = 53) of ARG1 expression level were compared. Significance of the observed effects has been determined using the Cox proportional hazards model with age, clinical stage, and tumor grade included in the analysis. In order to visualize the survival difference in R2 dataset (2), we used the log-rank test to find the point (cut-off) with the most significant (lowest P-value) split in high vs. low ARG1 level groups. Survival curves were derived by Kaplan–Meier method for these cut-offs.

Statistical analysis. Data are presented as means ± SD of at least three independent experiments. All analyses were done using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). Normal distribution of data was tested using the Shapiro–Wilks test. Differences between two groups were calculated using the unpaired two-tailed Student’s t-test with Welch’s correction or the nonparametric Mann–Whitney test for not normally distributed data. Statistical analyses of three or more groups were compared using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test. When the sample size was too small for test normality, the Kruskal–Wallis test was used. P < 0.05 was considered statistically significant. Survival estimates were computed using the Kaplan–Meier plot and comparisons between groups were analyzed using the log- rank test.

Data availability. Data are available within the article and supplementary files. The source data underlying Figs. 1a, 2a, b, d–g, 3a–c, 4b, 5d, 6a–d, 7a, b, Supplementary Figs. 2d, 3a–d, 5a, 6a, 7b, c, 8a are provided as a Source Data file. The TCGA microarray data are available in

from the Jackson Laboratories. The experiments were performed in accordance with the guidelines approved by the first Local Ethics Committee of the University of Warsaw and were carried out in accordance with the rules of EU (Directive 2010/63/EU) and Polish (Dz. U. poz. 266/15.01.2015) legislation.
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Author contributions
M.C.-K. designed and supervised the study, conducted the experiments, analyzed the data, and wrote the manuscript. A. Sosnowska performed in vivo studies, in vitro proliferation assays, and Western blot analysis. K.R. performed in vitro proliferation assays and Western blot analysis. D.N. designed the vector constructs, performed confocal microscopy, and wrote the manuscript. J.C.-T. participated in in vivo studies. M.S. supervised the collection of patient material and clinical data, performed tissue staining, and participated in EVs isolation. E.W. performed electron microscopy. P.G. performed bioinformatic analysis of expression data. M.G. participated in vitro and in vivo experiments, and EV and PBMC isolation. Z.P. participated in in vivo experiments. A.Z. participated in in vitro experiments. K.S. performed Western blot analysis, arginase activity assays, and PBMC and EV isolations. A.G.-J. performed confocal microscopy. S.C., R.K., and E.E. prepared immunohistochemistry images and analyzed the tissue staining. S.G. performed statistical analyses. A. Stefanowicz collected blood and ascites samples. R.B., B.B., and A.G. designed and synthesized ARG1 inhibitor. T.W. participated in manuscript preparation and data interpretation and provided scientific advice. J.G. conceived the hypothesis, designed the study and wrote the manuscript. All authors edited and approved the final manuscript.

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Competing interests: J.G. is a shareholder and Scientific Advisory Board member in OncoArendi Therapeutics, R.B., B.B., and A.G. are employees of OncoArendi Therapeutics, Warsaw, Poland. The remaining authors declare no competing interests.

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