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Oncolytic Adenovirus-Loaded Menstrual Blood Stem Cells Overcome the Blockade of Viral Activity Exerted by Ovarian Cancer Ascites

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Patients with ovarian cancer present peritoneal ascites at recurrence as a marker of disseminated disease and dismal prognosis. Oncolytic immunotherapy is an emerging approach for the treatment of disseminated cancer. In the present work, we constructed a novel oncolytic adenovirus, AR2011, to target malignant ovarian tumors. AR2011 exhibited a clear lytic effect in vitro in human ovarian cancer cell lines and malignant cells obtained from ascitic fluids (AFs) of patients with ovarian cancer. AR2011 activity was neutralized by antibodies present in 31 samples of patient-derived AFs. However, this blockade was overridden by preloading menstrual blood stem cells (MenSCs) with AR2011 (MenSC-AR), since AFs exerted no in vitro inhibitory effect on viral lytic activity under these conditions. Moreover, soluble factors present in AFs act as MenSC chemoattractants. MenSC-AR treatment of nude mice carrying established peritoneal carcinomatosis following administration of human ovarian cancer cells was able to inhibit tumor growth at levels similar to those observed with AR2011 alone. This study demonstrates that MenSCs can be used to override the blockade that AFs exert on viral oncolytic effects.

INTRODUCTION

Most patients with ovarian cancer present with advanced disease stages IIIC or IV at diagnosis.[1] Almost 20% of patients with stage I/II and 90% of patients with stages III/IV ovarian cancer present malignant ascites as a manifestation of disseminated disease,[2] which is a feature of short life expectancy.[3] Current treatments for advanced stages of ovarian cancer include either primary cytoreductive surgery followed by adjuvant chemotherapy or neoadjuvant chemotherapy followed by interval cytoreductive surgery and adjuvant chemotherapy.[4,5] The use of neoadjuvant treatments remains controversial,[1] although the American Society of Clinical Oncology recently recommended their use.[3] The use of antiangiogenic agents such as bevacizumab, a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody, is another possibility for treating ovarian cancer.[6] The recent approval of anti-poly(ADP-ribose) polymerase (PARP) inhibitors by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat a specific subgroup of 10%–15% of patients with ovarian cancer carrying mutations in BRCA1/2 adds novel paradigms to treat this disease, which can be eventually combined with mainstay drugs and immune checkpoint inhibitors.[7] Overall, current treatments either as single agents or their combinations are inefficient to treat advanced stages of ovarian cancer and new therapies are urgently needed.

Oncolytic immunotherapy is a promising approach for the treatment of advanced cancer. This approach consists of the use of an oncolytic virus to directly target the tumor mass, followed by a secondary immune attack on remnant or disseminated tumors.[8] This approach gained momentum following the recent approval of talimogene laherparepvec (T-VEC), a herpes simplex virus, to treat advanced melanoma.[9] Although there are currently still no commercial viral-based immunotherapies for ovarian cancer treatment, data from a few early clinical trials using different oncolytic viruses have been released. Measles virus, vaccinia virus, and reovirus of serotype 3 have been used in early trials in ovarian cancer.[10–12] Among oncolytic adenoviruses (Ads), the first clinical trial in recurrent ovarian cancer included the intraperitoneal (i.p.) administration of dl1520 with no clear-cut evidence of clinical or radiologic response in any patient.[13] Another phase I study was performed in 21 patients with the conditionally replicative Ad (CRAd) Ad5-Δ24-Arg-Gly-Asp (RGD); although the virus demonstrated promising clinical activity (seven patients had a decrease in the biomarker CA-125), no objective responses were observed.[14] A similar CRAd expressing granulocyte macrophage colony-stimulating factor (GM-CSF) was also assessed; of 16 radiologically evaluable patients, 2 had complete responses, 1 had a minor response, and 5 had disease stabilization. Responses were frequently seen in injected and non-injected tumors demonstrating the outcome of a secondary immune response.[15] A further modification of the virus that included a chimeric fiber was assayed in 21 patients. Evidence

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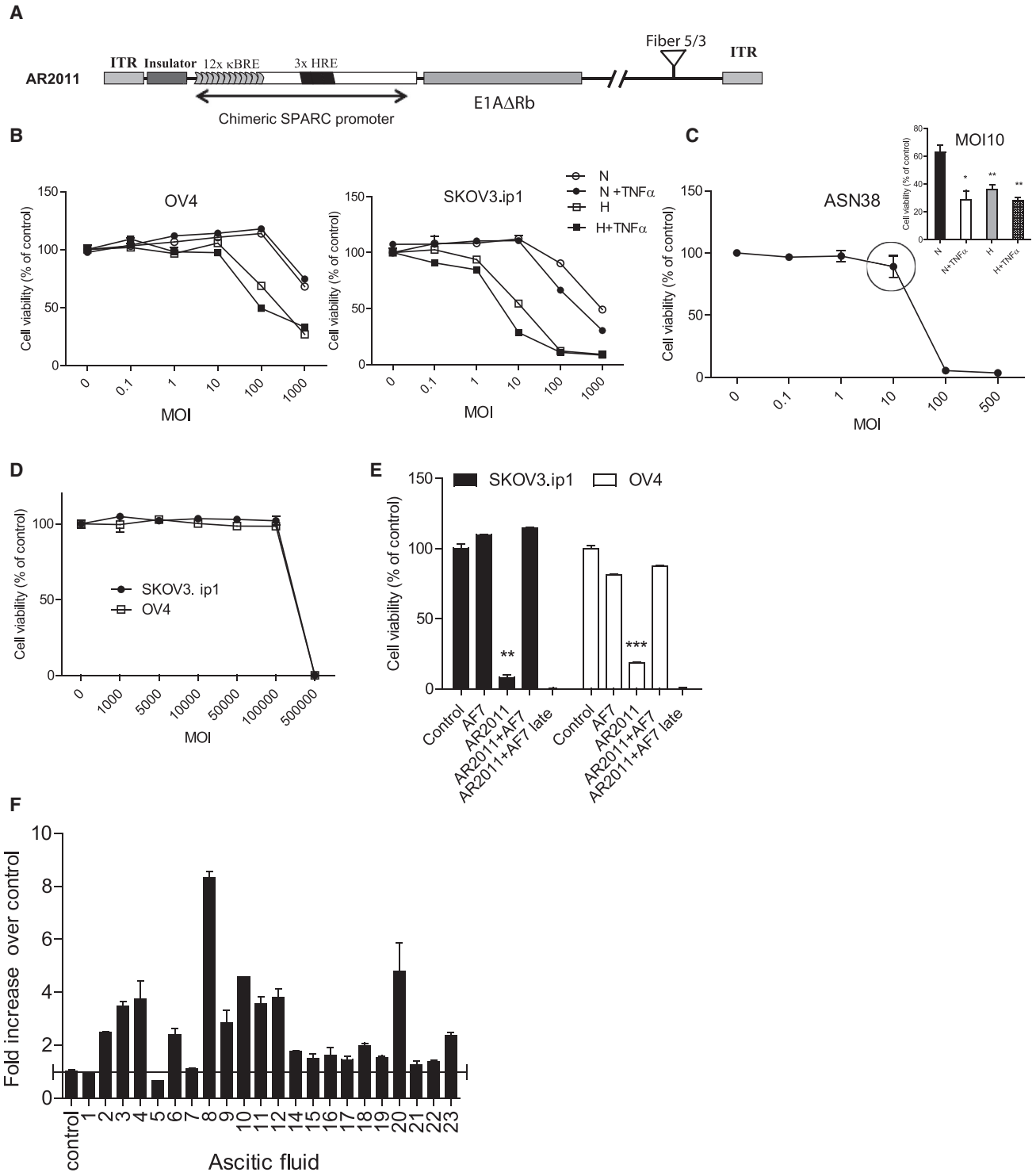

Figure 1. In Vitro Activity of AR2011 and AR2011-luc in the Presence of Ascitic Fluids

(A) Genomic organization of AR2011. (B) In vitro lytic activity of AR2011. Cells were infected with AR2011 at different MOIs and exposed to TNF-α in normoxia (N) or hypoxia (H). Cell viability was assessed by the MTS assay 6 days later. (C) In vitro lytic activity of AR2011 in sample ASN38 corresponding to malignant cells isolated from ovarian cancer ascitic AF38. Data in the inset show the viability with TNF-α in normoxia or hypoxia at an MOI of 10. (D) In vitro lytic activity of AR2011. Cells were transduced with

(legend continued on next page)

of biological activity of the virus was seen in 13 of 21 patients and 8 of 12 patients showed objective clinical benefit, as evaluated radiologically with the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.[16] Overall, studies with oncolytic Ads in recurrent ovarian cancer were very promising, but they also highlighted the need to construct more potent viruses expressing chimeric fibers to increase infection to avoid anti-adenoviral neutralizing antibodies.

Different studies described a significant inhibition of Ad-mediated gene transfer in the presence of ascitic fluid (AF) from patients with ovarian cancer.[17–19] Schulick et al.[20] showed that close to 60% of the studied population showed neutralizing antibodies against Ad type 5. Most anti-Ad antibodies recognize the fiber, penton, and hexon proteins of Ad.[17,21] There are two alternative, non-mutually exclusive ways to avoid neutralization, either by genetically engineering the immunogenic epitopes or by hiding the virus from the humoral response. One potential approach to hide viruses from the immune system is to build a shield. In this regard, mesenchymal stem cells (MSCs) were isolated from the bone marrow, adipose tissue, umbilical cord, and other tissues.[22] Different studies, including from our group, showed clear evidence that MSCs obtained from different sources can be used as carriers of oncolytic viruses to deliver the virus deeply inside the tumor mass.[23,24] MSCs might eventually hide the virus from immune attack and may reduce hepatic uptake, increasing viral availability.[25]

Menstrual blood stem cells (MenSCs) are an additional source of MSCs.[26] These adherent cells (also called "endometrial regenerative cells") could be maintained in tissue culture for more than 60 doublings, retained expression of mesenchymal markers without karyotypic abnormalities, and exhibited a remarkable totipotency.[26–28] MenSCs are easy to obtain in a non-invasive manner, and it was proposed that they are free of ethical dilemmas. MenScs, contrary to MSCs obtained from other sources, seem not to form teratomas.[26] MenSCs exhibited anti-tumoral and anti-neovascularization properties in a rat glioma model.[29] In a unique preliminary clinical trial, MenSCs were used allogeneically to treat 4 patients with multiple sclerosis intravenously and intrathecally; the investigators reported that there was no evidence of immunological reactions or treatment-associated adverse effects after more than 1 year.[30]

We previously described a stroma-targeted conditionally replicative oncolytic Ad named AdF512v1 that was pseudotyped with chimeric fiber 5/3, whose replication is driven by a 0.5-kb fragment of the SPARC promoter fragment that controls the expression of a mutated E1A unable to bind retinoblastoma protein (pRb) (E1AΔpRb).[31] AdF512v1 was extremely effective in the remission of established human ovarian cancer disseminated in the peritoneal cavity of nude mice.[31] Moreover, AdF512v1 was also able to replicate in fresh explants of patients' primary tumors and metastases refractory to chemotherapy[31]; AdF512v1 did not replicate in normal ovary tissue as the wild-type Ad did.[31]

In the present work, we generated a novel CRAd, AR2011, that includes a triple chimeric promoter[32] to drive the transcriptional activity of a mutated E1A. The present studies show that the in vitro lytic effect of AR2011 is blocked by soluble factors, such as antibodies present in AFs obtained from patients with ovarian cancer. This blockade can be overridden by the use of MenSCs loaded with the virus. Preclinical studies in nude mice demonstrate that MenSCs carrying AR2011 were able to inhibit, to a great extent, established human cell-derived peritoneal carcinomatosis.

RESULTS

AFs Obtained from Patients with Ovarian Cancer Exhibited a Dual Effect on Viral Activity

AR2011 is a 5/3 pseudotyped oncolytic Ad that combines the 0.5 kb secreted protein acidic and rich in cysteine (SPARC) promoter fragment with hypoxia-response elements (HREs) and nuclear factor κB (NF-κB) response elements to drive E1AΔpRb expression (Figure 1A). AR2011 exhibited a clear lytic effect on SKOV3.ip1 and OV4 human ovarian cancer cells in vitro, which was exacerbated under hypoxia and upon the addition of tumor necrosis factor α (TNF-α) (Figure 1B). AR2011 also exerted a lytic effect on malignant cells isolated from different ovarian cancer ascites (Figure 1C; A.L.A., A.N.C., M. Gangemi, C.M. Malnero, I.R. Bermúdez, A.S., N.C., O.L.P., and M.V.L., unpublished data).

We collected 31 samples of peritoneal ascites from patients with advanced ovarian cancer and confirmed the presence of malignant cells in all of the samples by using cytokeratin staining (Figure S1A). As a first attempt to establish the clinical barriers that an oncolytic virus might face in tackling advanced ovarian cancer, we sought to determine the effect that AFs might have on AR2011 lytic capacity. Initially, we assessed the lytic capacity of AR2011 in the presence of serial dilutions of the different AFs using human ovarian cancer cells SKOV3.ip1 as a target. Table 1 shows the large variability in half maximal inhibitory concentrations (IC_{50}) of the different AFs on AR2011 activity; indeed, IC_{50} values ranged from 1:1 dilution up to 1:1,370 dilution (Table 1). Using an ELISA test, we confirmed the presence of preexisting anti-Ad antibodies in all of the AFs assessed (Table 1); AF antibodies recognized the fiber, hexon, and penton viral proteins (Figure S1B). Interestingly, we observed no correlation between antibody titers and the inhibition of AR2011 lytic activity by the AFs, indicating that additional soluble factors might hamper viral activity. The lytic activity observed at an MOI of 1,000 in the absence of AFs, could be reached only at an MOI of 500,000 in the presence of AFs (compare Figure 1D with 1B). To note, we were unable to see any

AR2011 in the presence of a mix of AFs 7, 9, 10, 11, 18, 20, 27, and 28 as described in Materials and Methods. Viability was assessed at day 6 by the MTS assay. (E) In vitro lytic effect of AR2011 in the presence of AF added simultaneously with the virus or 4 hr after infection. For further details, see the Materials and Methods. (F) Adenoviral promoter activity assessed in the presence of different AFs using luciferase as a reporter gene (AR2011-luc). Control corresponds to medium without AF. Error bars represent the mean ± SEM (n = 3). **p < 0.01, ***p < 0.001. AF, ascitic fluid; ITR, inverted terminal repeat.

Table 1. IC_{50} and ELISA Titer of Ascitic Fluid Samples

Sample	IC_{50}	Titer by ELISA
AF1	197	ND
AF2	267	ND
AF3	578	6,667
AF4	476	1,428
AF5	25	803
AF6	287	419
AF7	50	667
AF8	64	6,667
AF9	18	667
AF10	15	667
AF11	7	2,222
AF12	68	935
AF14	61	889
AF15	442	2,222
AF16	20	1,052
AF17	35	550
AF18	397	535
AF19	373	506
AF20	1,370	1,907
AF21	332	895
AF22	5	386
AF24	3	ND
AF27	3	1,034
AF28	1	288
AF34	41	564
AF35	99	1,848
AF36	108	ND
AF39	28	425
AF40	10	761
AF43	9	1,168
AF44	7	1,205

ND, not determined.

inhibitory effect of the AFs when the ascites were added 4 hr after AR2011 infection (Figures 1E and S1C; data not shown).

In previous studies, we showed that soluble factors produced by either malignant or stromal cells can augment viral activity most likely by enhancing the activity of the tumor-specific promoters (TSPs) that drive viral replication.[33] Since AFs are rich in such soluble factors, we assessed whether AFs would be able to enhance the activity of our triple chimeric promoter. For this purpose, we constructed a non-replicative version of AR2011 (AR2011-luc), where the triple chimeric promoter drove the transcriptional activity of luciferase as a reporter gene. We observed that 19 of 22 AFs were able to augment luciferase levels, indicating that soluble factors produced by the AFs could enhance viral activity by acting at the promoter level (Figure 1F). Interestingly, this boost seems to be specific to the AR2011 promoter, since the effect was not observed with F512[31] and a SV40 promoter (data not shown).

AF Blockade of Viral Lytic Activity Can Be Overriden Using Menstrual MSCs as Carriers of AR2011

The previous data demonstrated that peritoneal-derived AFs obtained from patients with ovarian cancer have a dual effect on viral lytic activity, including a stimulatory effect driven by soluble factors that enhance viral TSP activity and an inhibitory effect driven mainly by neutralizing antibodies. To overcome the blockade exerted by AFs, we sought to use menstrual blood MSCs as a shield to hide AR2011 and avoid neutralization. We confirmed the mesenchymal nature of the MenSCs by showing the expression of CD73, CD90, and CD105 using qRT-PCR (Figure 2A) and flow cytometry analysis (Figure 2B) and the lack of expression of the hematopoietic markers CD45, CD34, CD11b, CD79A, and human leukocyte antigen-antigen D related (HLA-DR) (Figure 2B). MenSCs could be differentiated to adipogenic, osteogenic, and chondrogenic linages, confirming the multipotent differentiation capacity of these cells (Figure 2C).

Next, we sought to determine the window of time necessary for MenSCs to reach the tumor mass before they are killed by the viral cargo. MenSCs were effectively killed by AR2011 at an MOI of 10,000 at day 3 after infection of cells with the virus (Figure 3A). Thus, infecting MenSCs with an MOI of 10,000 will provide a 2-day window before MenSCs are killed by their cargo. For comparison, SKOV3 ovarian cancer cells were almost completely killed by AR2011 after 3-day incubation at a lower MOI of 1,000 (Figure 3A).

To confirm that MenSCs can be used to vehiculize AR2011, we co-plated MenSCs preloaded with AR2011 (MenSC-AR) with SKVO3.ip1 or OV4 cells and assessed cells viability after 6 days. Co-plating MenSC-AR with ovarian cancer cells at a 1:1 ratio led to the complete elimination of ovarian cancer cells (Figure 3B). Contrary to the blockade exerted by AFs on AR2011 lytic activity (see Table 1), AFs were almost completely unable to block the lytic effect of MenSC-AR on ovarian cancer cells (Figure 3B). A strong lytic effect of MenSC-AR on ovarian cancer cells was observed even at a ratio of 10:1 (ovarian cancer cells/MenSC-AR) (Figure 3B), although the lytic effect of MenSC-AR at this cell ratio was almost completely blocked in the presence of the AF (Figure 3B). As a control, we observed that MenSC-AR cells were completely killed by their cargo under these experimental conditions (Figure 3B). The capacity of MenSC-AR to kill ovarian cancer cells was further confirmed in tridimensional spheroids; indeed, both AR2011 and MenSC-AR were able to eliminate around 60% of SKOV.3ip cells growing in three-dimensional spheroids (Figure 3C). Under three-dimensional conditions, AFs completely blocked the AR2011 lytic effect on ovarian cancer cells but exerted only a partial inhibitory effect on the lytic capacity of MenSC-AR (Figure 3C).

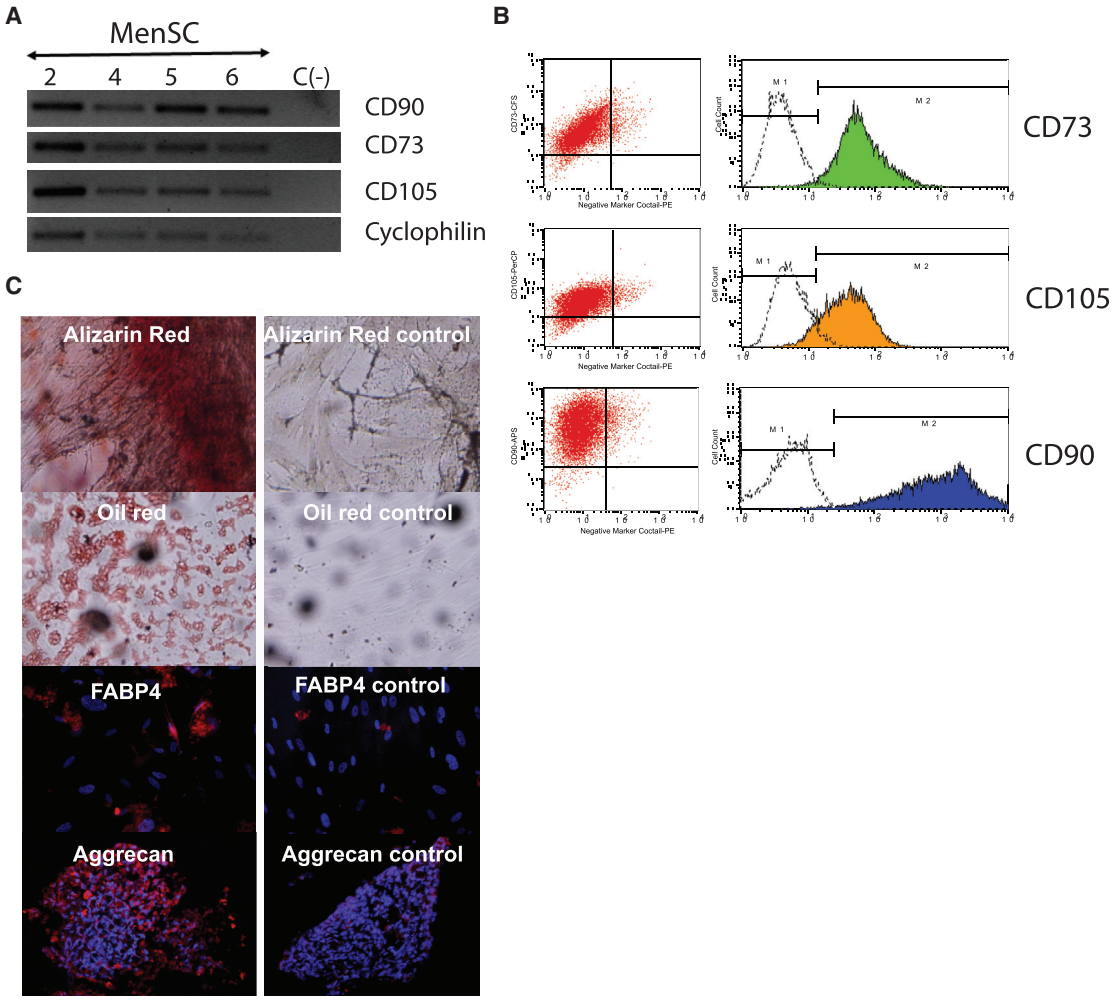

Figure 2. Phenotypic Characterization of MenSCs

(A) RT-PCR of mesenchymal markers corresponding to MenSC samples obtained from two different donors. C(–) indicates the negative control of the PCR; cyclophilin was used as the housekeeping gene. (B) Cell surface expression of CD73, CD105, and CD90 in MenSCs corresponding to one sample of six showing the same phenotype. Cells demonstrate positive expression of CD73, CD105, and CD90 as well as negative expression of all markers included in the negative marker cocktail of the kit used. Quadrants were set based on isotype controls. The histogram on the right shows the cells with positive expression for the corresponding antibodies (filled histogram) over the negative isotype cocktail (open histogram). (C) In vitro induction of MenSC differentiation. Alizarin red staining was used as a marker of osteogenic differentiation, oil red staining and FABP4 expression were used as markers of adipogenic differentiation, and aggrecan expression was used as a marker of differentiation to chondrocytes (for further details, see the Materials and Methods).

MenSCs Exhibit Tropism toward Ovarian Cancer Cells and Tumors

The previous data confirmed that MenSCs can be useful to overcome the inhibitory effect of AFs on viral lytic activity. Next, we sought to determine the chemotactic ability of MenSCs. By using transwell systems, we confirmed that MenSCs were able to migrate in vitro toward conditioned media produced by malignant cells (Figure 4A); interestingly, MenSCs were also chemoattracted by conditioned media produced by the MenSCs themselves and by ovarian cancer-derived AFs (Figure 4A). To identify the soluble factors that might mediate MenSC chemoattraction by AFs, we performed antibody arrays searching for chemotactic chemokines and cytokines. Cytokine and chemokine levels were highly heterogeneous among the different AFs. However, a comparison among the different AFs showed that they shared high levels of expression of certain chemokines such as interleukin (IL)-8 and cytokines such as IL-6, among several other chemokines and cytokines (Figure 4B; Table S1) that can chemoattract MenSCs.

To confirm the tropism of MenSCs toward the tumor mass, we injected $DiIC_{18}(7)$ (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) (DiR)-stained MenSCs i.p. in nude mice harboring 10-day-old peritoneal carcinomatosis following SKOV3.ip1-luc administration. 48 hr later, we observed the co-localization of luciferase expressed by the ovarian cancer cells and DiR, indicating that MenSCs reached the tumor nodules in less than 48 hr (Figure 4C).

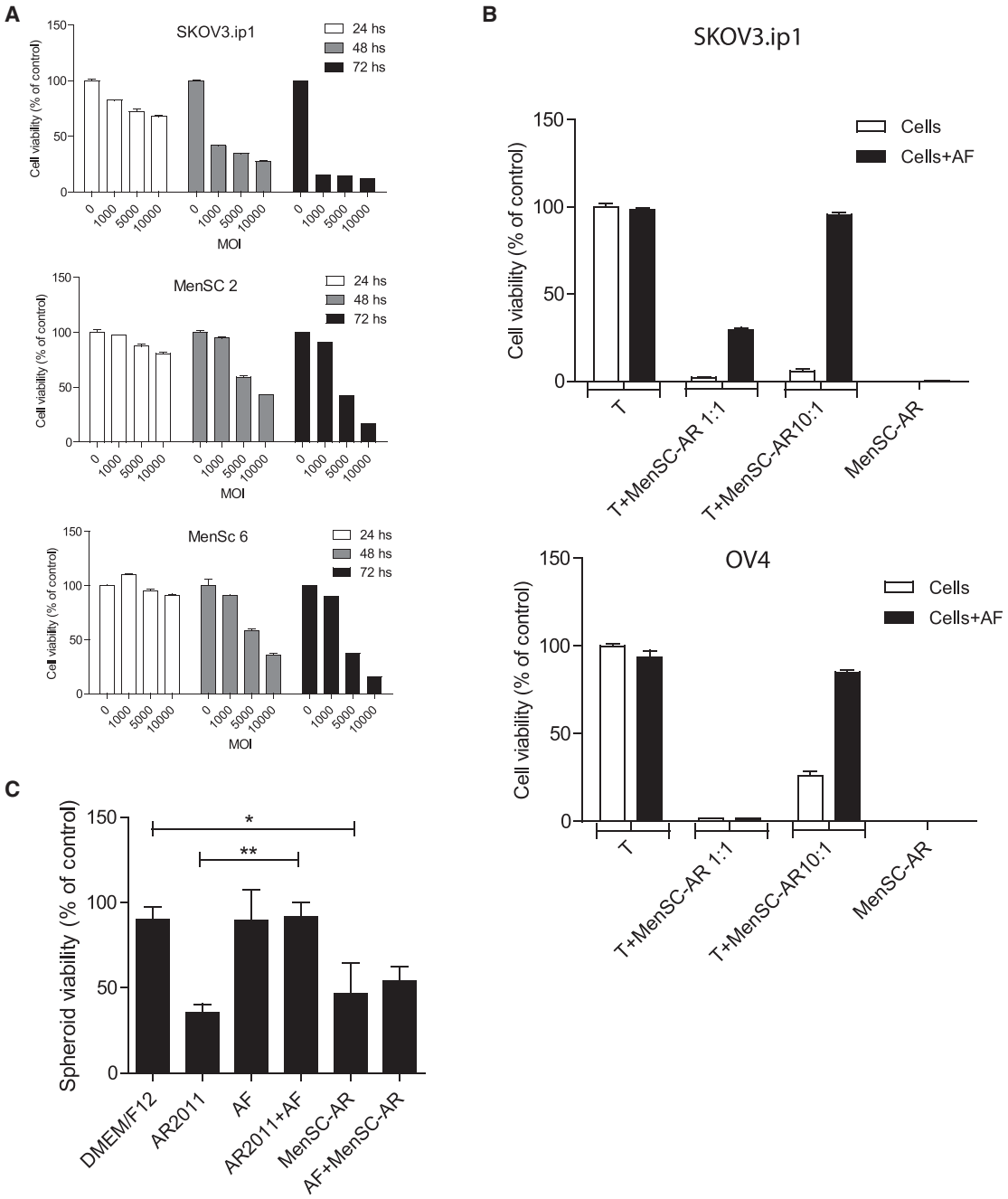

Figure 3. In Vitro Effects of MenSCs Loaded with AR2011

(A) In vitro lytic effect of AR2011 on ovarian cancer cells and two MenSC samples from the same donor. Cells were incubated with the virus and viability was assessed at the indicated time points. (B) In vitro viability at 6 days of a mix of ovarian cancer cells and MenSC-AR in the presence or absence of AFs. We show as a control the effects on each single cell type. (C) In vitro lytic activity of AR2011 and MenSC-AR on spheroids made of SKOV3.ip1 cells. Spheroid cell viability was assessed with the MTS assay. Error bars represent the mean ± SEM (n = 3). *p < 0.05, **p < 0.01.

The positive immunohistochemical staining of excised ovary tumors for the mesenchymal marker CD105 confirmed that MenSCs reached and penetrated the tumor nodules deep inside the tumor mass (Figure 4D). We assessed the presence of viral particles intratumorally 5 days after MenSC-AR administration, confirming that MenSC-AR reached the tumor mass and released the virus; interestingly, MenSC-AR cells were also observed in the spleen, although viral levels were 2.6-fold higher in the tumor than in spleen (Figure 4E).

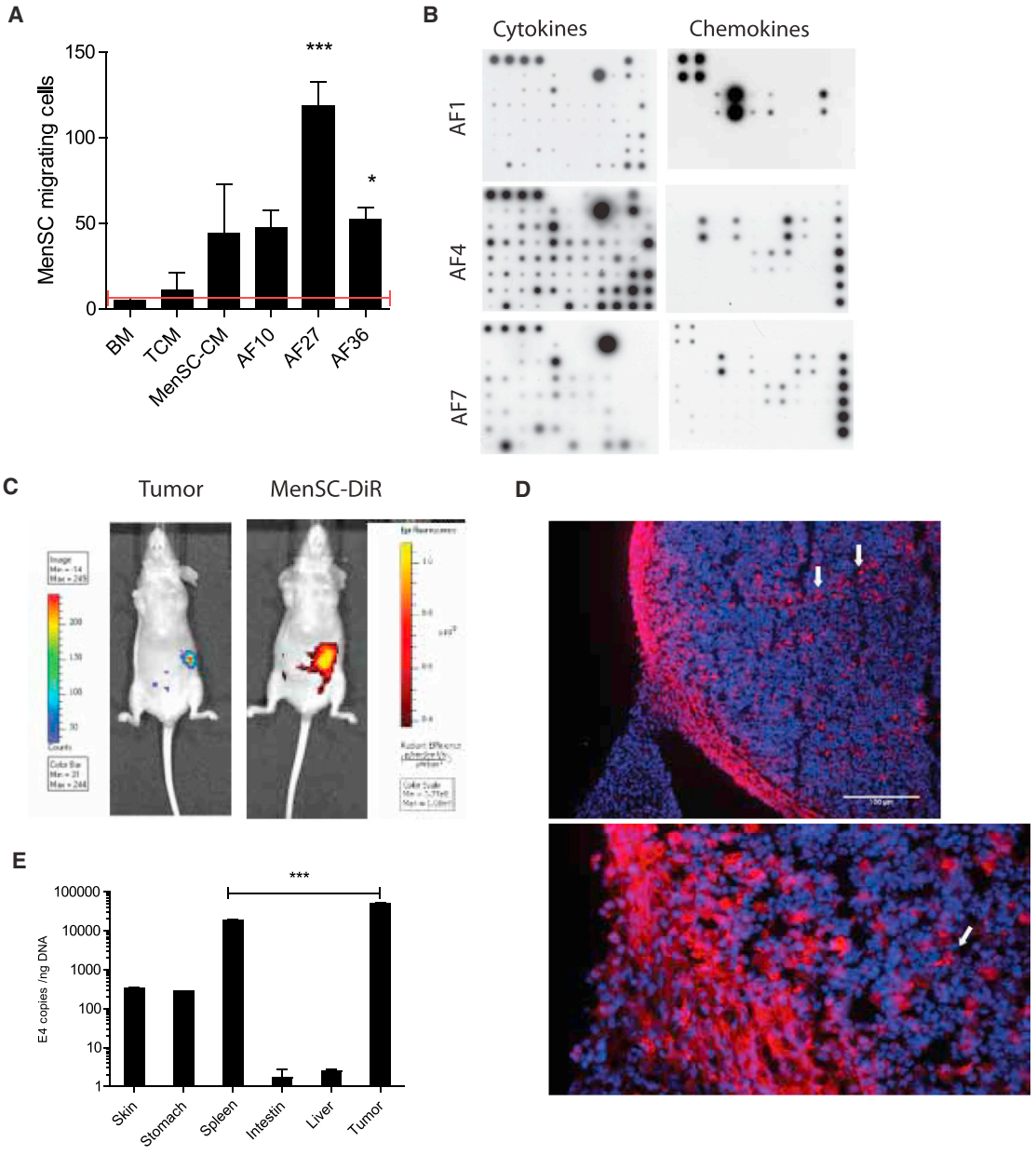

Figure 4. In Vitro and In Vivo Migratory Ability of MenSCs

(A) In vitro chemotactic migration of MenSCs. All media were diluted 1:1 with BM. Data correspond to the total migrating cells. (B) Cytokine and chemokine antibody arrays of AF samples. For further details, see the Materials and Methods. (C) In vivo migratory ability of MenSCs showing the co-localization at day 2 of luminescence emission by SKOV3-Luc tumors and fluorescence emission by DiR-labeled MenSCs. (D) Immunohistochemical detection of CD105 inside SKOV3-Luc tumors at day 2 after MenSC injection. Arrows show CD105 expression by MenSCs inside the tumor. (E) MenSC-AR biodistribution of two mice 5 days after MenSC-AR administration. Viral E4 levels were used as a surrogate marker of the MenSC-AR. One-way ANOVA was performed. Error bars represent the mean ± SD. *$p < 0.05$, ***$p < 0.001$. BM, basal medium, TCM, tumor conditioned medium; MenSC-CM, MenSC conditioned medium.

MenSCs Can Vehiculize Oncolytic Ad to Target i.p. Disseminated Ovarian Cancer

We next aimed to demonstrate whether MenSCs preloaded with AR2011 can also exert a therapeutic effect. For this purpose, mice harboring 13-day-old i.p. disseminated SKOV3.ip1-luc tumors were treated i.p. with three injections of saline, 2×10^6 MenSCs, 2×10^6 MenSC-AR cells, or 5×10^{10} viral particles (vp) of AR2011. Tumor growth was followed by bioluminescent imaging. We observed a steady increase in tumor size in control animals, whereas tumor growth was strongly inhibited in mice treated with either AR2011 or

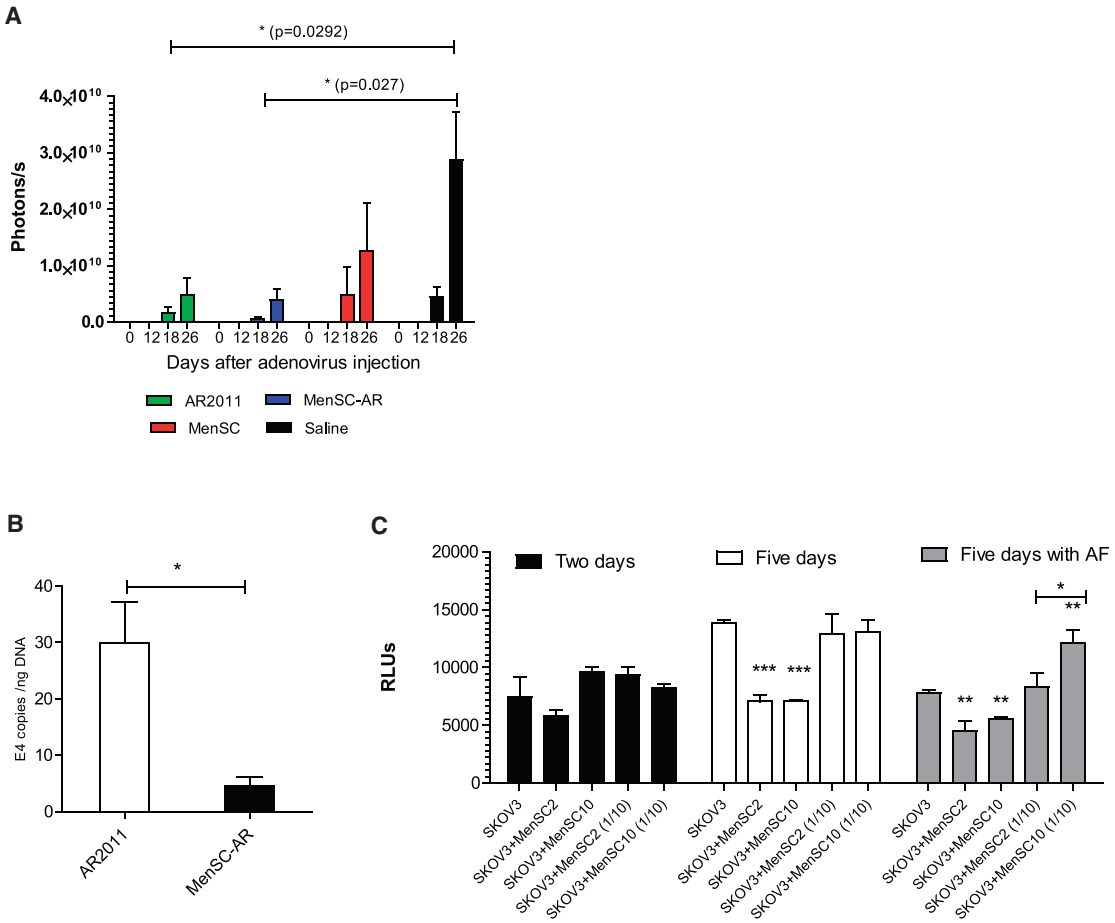

Figure 5. In Vivo Tumor Growth

(A) Evaluation of the antitumor effect of the different treatments. Mice were injected with 3×10^6 SKOV3.ip1-luc cells and treatment (AR2011, MenSC-AR, or MenSC alone) was initiated after 13 days. Tumor growth was quantified as photon emission per experimental time point. Error bars represent the mean ± SEM. The numbers of mice were as follows: AR2011 (n = 5), MenSC-AR (n = 5), MenSC (n = 3), and saline (n = 5). *$p < 0.05$. For more details, see the Materials and Methods. (B) Amount of viral particles at the end of the experiment. Tumors corresponding to each one of the mice were assessed for viral E4 levels. (C) Ovarian cancer cells were incubated 2–5 days in the presence or absence of MenSCs at different ratios and with or without AF. The Student's t test was performed. Error bars represent the mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

MenSC-AR (Figure 5A). The number of mice that remained alive at 64 days was slightly higher in the AR2011-treated group (n = 2 of 5) than in the MenSC-AR group (n = 1 of 5). The levels of viral E4 in the tumor masses at the end of the experiments were six times higher in mice treated with the virus alone than in the group treated with MenSC-AR (Figure 5B). Of note was the non-statistically significant inhibition observed in mice treated with MenSCs alone, suggesting that these cells might also contribute to tumor growth inhibition. Therefore, we performed additional in vitro experiments to determine whether MenSCs can exert an inhibitory effect on human ovarian cancer cell growth. Indeed, we observed that co-plating of SKOV3.ip1-luc cells with MenSCs led to the growth inhibition of cancer cells after 5 days of co-plating; this growth inhibitory effect was ablated when MenSCs were co-plated with malignant cells at a 1:10 ratio, which resembles the in vivo ratio inside the tumor nodules (Figure 5C). The addition of AF showed conflicting results, since it inhibited tumor cell growth at 5 days but its effects on the mix of ovarian cancer cells and MenSCs differed depending on the cell ratio (Figure 5C).

DISCUSSION

Here, we show for the first time (to our knowledge) that menstrual blood MSCs can be used to deliver oncolytic Ads, avoiding the blockade that AF-derived factors (i.e., antibodies) exert on viral lytic activity. MenSCs were used in the present study to deliver a novel tumor stroma-targeted, microenvironment-responsive CRAd named AR2011, which includes a triple chimeric TSP that drives the expression of a mutant E1A unable to bind to pRb. The blockade of AR2011 activity by ovarian cancer ascites was overridden when the CRAd was loaded inside MenSCs.

Almost all patients that have an ovarian cancer recurrence develop peritoneal ascites that affect both their quality of life and response

to therapy. Ovarian cancer ascites are a reservoir of pro-inflammatory soluble factors and Ad neutralizing antibodies.[17–19] Increased levels of anti-Ad antibodies were detected in ascites of patients with ovarian cancer treated i.p. with a non-replicative Ad.[18,34] We found Ad neutralizing antibodies in all 31 ascite samples obtained from patients with ovarian cancer at advanced stages of the disease. The antibody titer differed from one patient to another and did not correlate with the levels of blockade exerted on viral lytic activity. This lack of correlation could be due either to the presence of alternative blockade compounds or to the fact that soluble factors present in the AFs also exerted a stimulatory effect on viral activity (as shown here). Indeed, 27 of 31 AFs that blocked viral lysis were able to stimulate the activity of the triple chimeric TSP that drove AR2011 replication. In previous studies, we showed that soluble factors produced by malignant and stromal cells can stimulate viral replication.[31,33] Few of the factors that we identified in the AFs, such as IL-8, IL-6, TNF-α, and transforming growth factor β (TGF-β), were shown to enhance the transcriptional levels of SPARC[35–37]; TNF-α and VEGF can stimulate NF-κB-responsive elements, and IL-6 can also stimulate VEGF production through hypoxia-inducible factor (HIF)-1 motifs.[38,39] Therefore, AFs can both stimulate TSP activity and exert a blockade on CRAd activity.

Mesenchymal stem (stromal) cells, mainly obtained from bone marrow, have been used in few works for virus delivery as a strategy to reduce hepatic uptake of the virus and limit virus neutralization by preexisting serum antibodies.[40] The death of MSCs by their own viral cargo, once they reach the tumor area, might also avoid the immunosuppressive status that MSCs can establish at the tumor site.[41] Bone marrow-derived MSCs loaded with oncolytic Ad were therapeutically effective in a preclinical model of i.p. disseminated ovarian cancer with reduced hepatotoxicity.[25,42] Systemically administered MSCs preloaded with an oncolytic Ad significantly inhibited tumor growth in mice harboring established A375N melanomas, overcoming the natural resistance of the tumor to intratumor administration of the CRAd.[23] Among MSCs obtained from different sources, menstrual stem cells exhibited properties that make them likely candidates for future clinical trials. MenSCs are being non-invasively obtained from menstrual blood and they are therefore devoid of ethical dilemmas; they are easily grown and very stable in culture, can be expanded at a large scale, and appear to be non-tumorigenic in mice.[5,26–30] MenSCs were also shown to retain expression of mesenchymal markers without karyotypic abnormalities.[26] Moreover, MenSC were allogeneically used without evidence of immunological reactions or treatment-associated adverse effects, making these cells a likely candidate for therapeutic approaches in postmenopausal women.[30]

We demonstrated in the present study that the use of MenSCs as a carrier of AR2011 overrides the blockade that AFs exert on viral lytic activity on cells growing in 2D monolayers as in three-dimensional spheroids. In addition, we identified soluble chemokines and cytokines present in AFs that were described to be chemotactic for MSCs.[43,44] Thus, ovarian cancer AFs play two opposite roles: on one hand, they act as a barrier for viruses to encounter the tumor cells; on the other hand, secreted soluble factors present in AFs can be of great benefit to recruit MenSCs to the vicinity of the tumor mass and also stimulate the activity of the viral cargo.

In these initial preclinical studies with CRAd-loaded MenSCs, we observed that the therapeutic efficacy of MenSC-AR on a peritoneal carcinomatosis was comparable to that observed for AR2011 alone. However, it is important to stress that MenSCs were able to override the blockade effect on viral lytic activity exerted by AFs in vitro; thus, MSCs can act as a shield to avoid viral neutralization by circulating antibodies. Moreover, the lower levels of viral E4 found in the tumor mass at the end of the in vivo experiments suggest that a similar therapeutic effect can be reached with lower viral doses loaded in the MenSCs, reducing potential toxicity. In addition, soluble factors can chemoattract MenSCs that might aid in systemic administration of viruses. Also of note, the absence of CRAd in the liver indicates that the MenSCs can act as a shield to avoid viral uptake. Despite the fact that the in vivo studies did not show an improved therapeutic effect, there are different aspects of this study that suggest that MenSCs can improve oncolytic Ad performance in a clinical scenario for systemic viral treatment in particular.

In the biodistribution studies, we also observed that the CRAd vehiculized by the MenSC locates mainly in the tumor, while the liver shows only neglectable levels. To our surprise, we observed unusual levels of viral particles in the spleen. Whether these are CRAd particles trapped in the spleen or the result of CRAd infection of splenocytes warrants further studies.

Our in vivo studies showed that MenSCs exhibited non-statistically significant antitumor activity. MenSCs inhibited ovarian cancer cell growth in vitro when co-plated at a 1:1 ratio, although this effect was completely absent at a 1:10 ratio (MenSCs/cancer cells), which closely resembles the ratio we observed in vivo (see Figure 4D). On the other hand, the fact that the amount of virus detected in the tumor mass at the end of the experiment was six times larger in the group treated with AR2011 alone indicates that, indeed, both the MenSCs and CRAd contributed to achieve the same levels of tumor growth inhibition. A recent study showed that MenSCs were able to inhibit the in vitro and in vivo growth of ovarian cancer cells through a complex mechanism that involves cell cycle arrest, induction of apoptosis, disruption of mitochondria membrane potential, and decreased pro-angiogenic ability.[45] Although the in vivo data in studies by Bu et al.[45] were obtained from subcutaneous tumors made of a 1:2 ratio of ovarian cancer cells and MenSCs, their results confirm that MenSCs might exert direct antitumor effects most likely through the secretion of soluble factors. The question of whether MSCs exert a direct pro- or anti-tumorigenic effect is a matter of debate and might be related to the experimental models.[46,47]

In summary, we show here that the major advantages of using MenSCs as carriers of oncolytic virus include their capacity to avoid the blockade by antibodies present in the AFs and that they can be

recruited by soluble factors present in the AFs that can also serve as viral enhancers; MenSCs also showed highly reduced affinity for the liver as well as an intrinsic antitumor activity. Thus, MenSCs, either autologous or allogenic, could represent a useful tool for enhancing viral oncolysis that can be combined with mainstay and novel targeted therapies.

MATERIALS AND METHODS

Cell Lines and Cell Culture

HEK293 cells were purchased from Microbix; sources of 911 cells, the ovarian adenocarcinoma cell lines SKOV3.ip1 and SKOV3.ip1-luc, and OV-4 cells were described previously.[31] All cell lines were grown in DMEM/F12 medium supplemented with 15% fetal bovine serum (FBS) (Natocor), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and were maintained in a 37°C atmosphere containing 5% CO_2.

Isolation of Ovarian Cancer Cells from AFs

AFs were obtained from patients with ovarian cancer at the Hospital Municipal de Oncología Marie Curie. Institutional review board approval was obtained from the Hospital Municipal de Oncología Marie Curie and the Leloir Institute. Declaration of Helsinki protocols were followed and patients gave written informed consent. The time between sample harvesting and processing was kept to an absolute minimum (<2 hr). Samples were centrifuged at 1,500 rpm for 10 min to clear ascites from the cells. Cleared ascites were fractioned and stored at −80°C until use. Cells were seeded in a 100-mm culture dish and incubated overnight in DMEM/F12 medium supplemented with 15% FBS, brought to confluence, and stored in liquid nitrogen until use.

Isolation of Menstrual Mesenchymal Stem Cells

Human MenSCs were isolated from the menstrual blood of healthy donors using a menstrual cup (Maggacup Argentina) during the second day of menstruation. The best yield of cells was obtained when 2 mL menstrual blood was mixed with 2 mL PBS and placed in a 100-mm tissue culture dish with DMEM/F12 medium supplemented with 15% FBS (Natocor), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mL amphotericin B (Gibco) at 37°C and 5% CO_2. After 24 hr, non-adherent cells were removed by washing with PBS, while adherent cells were cultured until they reached 80%–90% confluence. Cells were trypsinized, subcultured, and used for experiments between passages 2 and 15.

Human Cytokine and Chemokine Antibody Array

AAH.CYT 5.8 and AAH.CHE 1.8 (RayBiotech) membrane-based antibody arrays for screening and comparing expression levels of many cytokines and chemokines in a single assay were used with AFs from patients. The arrays were used according to the manufacturer's instructions. Semi-quantification was performed using CellProfiler software, considering IL-8 as 100% of expression.

Construction and Production of Ads

AR2011 is a recombinant Ad derived from AdF512v1,[31] in which we cloned a triple chimeric promoter[32] upstream of the E1ΔRb gene. For this purpose, the triple chimeric promoter was amplified by PCR using the plasmid pS-2Kb512HRE-E1A as a template.[32] A *Not*I/*Hind*III PCR fragment of 1,125 bp including the insulator and the entire triple chimeric promoter was cloned in the *Not*I/*Hind*III sites of the shuttle vector pS-I-F512-E1ARB[33] and named pS-2Kb512HRE-E1Arb. This new pshuttle was digested with *Pme*I and used for homologous DNA recombination with pVK500C 5/3.[48] A positive clone was selected, sequenced, and amplified by transforming DH5α ElectroMAX cells (Invitrogen), followed by DNA Maxiprep (QIAGEN). The resulting plasmid pVK-2Kb512HRE-E1ARB 5/3 was linearized with *Pac*I, purified by ethanol precipitation, and transfected in 911 cells using LTX Lipofectamine (Invitrogen). The rescued Ad (AR2011) was used to infect 293 cells and to produce the stocks.[33]

In Vitro Cytotoxicity Assays

Lytic Effect of AR2011

1×10^4 MenSCs or ovarian cancer cells were seeded in 24-well tissue culture plates and infected with the virus at indicated titers in 200 μL DMEM/F12 containing 2% FBS.[33] After 4 hr post-infection, 800 μL fresh medium containing 15% FBS in the presence or absence of 5 ng/mL TNF-α (Peprotech) was added, followed by 6 days of incubation in normoxia or hypoxia (0.1% O_2). After 6 days, cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega).

In the experiments using AFs, we infected tumor cells with different MOIs in the presence of 500 μL/well of undiluted AF; 48 hr later, the medium was replaced with fresh DMEM/F12 containing 15% FBS and viability was measured 4 days later using the MTS assay (day 6 from infection). In the experiments described in Figure 3B, MenSCs were infected with AR2011 at an MOI of 10,000. The next day, MenSC-AR cells were trypsinized and mixed with tumor cells at the indicated ratio in the presence or absence of undiluted AF. Cell viability was measured after 6 days, as described.

In the experiments described in Figure 1E, tumor cells were incubated in the presence of 100 μL undiluted AF or DMEM/F12 plus 100 μL AR2011 at a final MOI of 250. 4 hr later, 800 μL DMEM/F12 containing 15% FBS was added. In the case of "AF late," the 800 μL contained 100 μL undiluted AF and 700 μL DMEM/F12 including 15% FBS. Cell viability was assessed 6 days later with the MTS assay.

Three-Dimensional Spheroids

Spheroids made of 10^4 SKOV3.ip1-luc cells[49] were grown for 3 days, followed by the addition of AR2011 (MOI of 10,000), MenSC-AR (2,000 per spheroid infected at an MOI of 10,000 a day before), or medium. Studies were performed in the presence or absence of AF (100%) for 3 days, followed by washing and the addition of DMEM/F12 15% FBS. Six days later, cell viability was assessed with the MTT assay.

Table 2. Primers Used in This Work

Primer	Primer Type	Sequence 5′-3′
Cyclophilin	forward	GAAGAGTGCGATCAAGAACCCATGAC
	reverse	GTCTCTCCTCCTTCTCCTCCTATC TTTACTT
CD90	forward	ATCCCAGAACCATGAACCTGGCC
	reverse	CGTACTGGATGGGTGAACTGCTGG
CD105	forward	CACATCCTGAGGGTCCTGCC
	reverse	GCATGTTGTGGTTGGCGTCG
CD73	forward	AGTCCACTGGAGAGTTCCTGCA
	reverse	TGAGAGGGTCATAACTGGGCAC
E4	forward	ACAAGCTCCTCCCGCGTTAG
	reverse	ACTACGTCCGGCGTTCCAT

Assessment of Antibody Titers

SKOV3.ip1 cells were plated into 96-well plates (5,000/well) and allowed to grow overnight before infection. A 5-fold dilution series of ascites was prepared in DMEM/F12 and 50 μL was added over 50 μL DMEM/F12 and 2% FBS containing AR2011 to obtain an MOI of 500. After 4-hr incubation, we added 100 μL DMEM/F12 and 15% FBS. At day 6, cell viability was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega). We established the IC_{50} as the ascite dilution that inhibited 50% of the lytic activity of AR2011.

To assess the MOI that can reach and infect ovarian cancer cells in the presence of AF, we seeded ovarian cancer cells into 24-well plates (10,000/well) and allowed them to grow overnight before infection. Cells were infected with AR2011 at 10^3 to 5×10^5 MOI in the presence of 200 μL AF. After 4-hr incubation, 800 μL DMEM/F12 was added and viability was measured 6 days later as described.

ELISA

Analysis of immunoglobulin G (IgG) antibodies was performed by sandwich ELISA. An ELISA 96-well plate was coated with 0.3 μg/100 μL of Ad in saline solution per well (0.58 μg total protein/μL stock of AR2011) for 2 hr at 37°C. The plate was then washed and blocked with 5% skim milk in 0.05% PBS-Tween at room temperature. After washing, dilutions of AF were added and incubated for 2 hr at room temperature. Anti-Ad antibodies were detected with goat anti-human IgG-horseradish peroxidase (HRP) conjugate (Abcam); absorbance was obtained at 450 nm after adding the HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma). Titer was defined as the dilution producing an absorbance at 450 nm that was three times greater than the background.[17]

Luciferase Assays

SKOV3.ip1 cells were plated into 24-well plates (50,000/well) 1 day before infection at MOI of 500 of AR2011-luc in 200 μL volume. After 4-hr incubation, a 1:5 dilution of AF was added. 48 hr later, the luciferase assay was assessed as described.[33]

Chemotactic Assays

Serum-starved MenSCs diluted in 0.2% BSA DMEM/F12 were seeded (1,200/well) in 8-μm pore membranes of AP48-well Boyden chambers (Neuro Probe) in AF (1:1 dilution with 0.2% BSA DMEM/F12) or control chemoattractant. After 4 hr, membranes were fixed for 5 min in 4% paraformaldehyde (PFA) and stained with 1 μg/mL Hoechst. Non-migrated cells located on the upper surface were scraped and migrated cells were registered under fluorescent microscopy at ×20 magnification. Migrated MenSCs were automatically counted in each image using a CellProfiler homemade pipeline.[50] Each treatment was assayed twice in triplicate.

PCR

Total RNA was extracted from cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA synthesis was performed using Superscript II Reverse Transcriptase and Oligo dT (Invitrogen) according to the manufacturer's instructions using 5 μg total RNA. Specific primer pairs were designed to span introns and avoid eventual DNA contamination detection (Table 2). RT-PCR reactions were conducted in a 25-μL reaction mixture containing 2 μL cDNA, 1× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM of each dinucleotide triphosphate (dNTP), 0.05 μM of each forward and reverse primer, and 1 U Platinum Taq Polymerase (Invitrogen). Amplification conditions were as follows: denaturing at 95°C for 5 min, 40 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Cyclophilin was used as the internal amplification control. PCR reactions were carried out using the Multigene Thermal Cycle (Labnet International).

Adenoviral genome copies based on the E4 gene were quantified by real-time PCR using a standard curve as previously described.[31] DNA from tumor or tissue was purified with the DNeasy Tissue kit (QIAGEN). The primers used for amplification of the E4 were forward E4F and reverse E4R (Table 2).[31]

Flow Cytometry

Cells (2.5×10^5) were analyzed with the Human Mesenchymal Stem Cell Verification Kit (R&D Systems) and a FACSCalibur flow cytometer (Becton Dickinson) according to the manufacturers' instructions. In all cases, 10,000 events were measured in each analysis.

Cell Immunofluorescence Assays

Cancer cells grown on a glass coverslip were washed with PBS and fixed with 4% paraformaldehyde for 30 min. After permeabilization with 0.5% Triton X-100 for 10 min, the samples were blocked with 10% FBS/PBS and 0.5% Triton X-100 for 1 hr. Samples were exposed to the primary antibody (1:100 mouse anti-pan-cytokeratin [AE1/AE3]; Abcam) for 1–2 hr in PBS/2.5% FBS, followed by anti-mouse Cy2-conjugated secondary antibody (Jackson ImmunoResearch) for 1 hr. Cells were washed four times with PBS and mounted on glass slides.

To detect MenSCs in tumors, we used an anti-human Endoglin/CD105 polyclonal antibody. Tumor samples were fixed in 10%

neutral-buffered formalin before they were embedded in paraffin and cut into 8-μm sections. The 8-μm-thick tumor sections were deparaffinized in xylene and rehydrated in graded ethanol. For antigen unmasking, sections were immersed in citrate buffer (pH 6) and boiled twice in a microwave oven. Endogenous peroxidase activity was blocked by soaking the sections in 3% hydrogen peroxide in methanol for 15 min. Non-specific binding sites were blocked by incubating the sections in normal donkey serum (10% in PBS). Excess serum was then removed and the tissue sections were incubated overnight with anti-Endoglin/CD105 polyclonal antibody (R&D Systems) at 1:50 dilution. After the slides were washed twice in PBS for 10 min, the sections were incubated with Cy2 donkey anti-goat antibody (Jackson ImmunoResearch) at 1:400 dilution for 45 min; they were then washed with PBS and counterstained with DAPI (blue). Finally, the sections were dehydrated and mounted. All images were obtained by using an Olympus BX60 microscope.

MenSC Differentiation

MenSC differentiation to adipocytes, chondrocytes, and osteocytes was performed with the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems) according to the manufacturer's instructions.

In Vivo Studies

None of the mice showed signs of wasting or other visible indications of toxicity and all animals in the study received food and water ad libitum.

DiR Staining of MenSCs

5- to 6-week-old athymic N:NIH(S)-nu female mice were injected i.p. with 3×10^6 SKOV3.ip1-luc cells/200 μL. 10 days later, MenSCs were stained during 1 hr with DiR (Invitrogen); afterward, mice were injected with 3.3×10^6 cells once i.p. After 48 hr, mice were observed in the IVIS Imaging System (Xenogen) using bioluminescence and fluorescence to detect tumor cells and MenSCs, respectively.

Studies with Virus-Loaded MenSCs

Biodistribution Assays. 5- to 6-week-old athymic N:NIH(S)-nu female mice were injected i.p. with 3×10^6 SKOV3.ip1-luc cells/200 μL. 13 days later, mice were injected once with 2×10^6 MenSC-AR cells (MOI of 10,000). 5 days later, mice were euthanized and the skin, liver, spleen, stomach, intestine, and tumor were harvested to quantify E4 levels as a readout of viral particles.

Therapeutic Efficacy. 5- to 6-week-old athymic N:NIH(S)-nu female mice were injected i.p. with 3×10^6 SKOV3.ip1-luc cells/200 μL. Mice harboring 13-day-old i.p. tumors were treated i.p. with three injections (days 0, 4, and 7) of the following: saline (T1), 2×10^6 MenSCs (T2), 2×10^6 MenSC-AR cells (T3), and 5×10^{10} vp AR2011 (T4). Tumor growth was evaluated by bioluminescent imaging using an IVIS Imaging System (Xenogen) at days 12, 18, and 26 after treatments. By days 40–64, mice were euthanized according to laboratory animal guidelines and tumor samples were collected to perform Ad quantification.

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee of the Fundación Instituto Leloir (protocol no. 69OP). The Fundación Instituto Leloir has an approved Animal Welfare Assurance as a foreign institution with the Office of Laboratory Animal Welfare (no. A5168-01).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at http://dx.doi.org/10.1016/j.omto.2017.06.002.

AUTHOR CONTRIBUTIONS

Conceptualization, M.V.L. and O.L.P.; Methodology, M.V.L.; Investigation, A.L.A., A.N.C., L.N.G., C.R., L.S., and M.V.L.; Writing – Original Draft, M.V.L.; Writing – Review & Editing, M.V.L. and O.L.P.; Funding Acquisition, M.V.L. and O.L.P.; Resources, N.C. and A.S..; Supervision, M.V.L.

CONFLICTS OF INTEREST

The rights for the commercial use of AR2011 have been licensed by Fundación Instituto Leloir and CONICET to Unleash Immuno Oncolytics.

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