



Original Articles

Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells



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ABSTRACT

We report here our clinical experience of a program of compassionate use of Celyvir – autologous marrow-derived mesenchymal stem cells (MSCs) carrying an oncolytic adenovirus – for treating children with advanced metastatic neuroblastoma. Children received weekly doses of Celyvir with no concomitant treatments. The tolerance was excellent, with very mild and self-limited viral-related symptoms. Patients could be distinguished based on their response to therapy: those who had a clinical response (either complete, partial or stabilization) and those who did not respond. We found differences between patients who responded versus those who did not when analyzing their respective MSCs, at the expression levels of adhesion molecules (CCR1, CXCR1 and CXCR4) and in migration capacities in transwell assays, and in immune-related molecules (IFN γ , HLA-DR). These results suggest interpatient differences in the homing and immune modulation capacities of the therapy administered. In addition, the pretherapy immune T cell status and the T effector response were markedly different between responders and non-responders. We conclude that multidoses of Celyvir have an excellent safety profile in children with metastatic neuroblastoma. Intrinsic patients' and MSCs' factors appear to be related to clinical outcome.

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Introduction

Oncolytic virotherapy is gaining interest in the clinic as a new weapon against cancer [1]. Various clinical trials have been conducted in adults and to a lesser extent also in pediatric patients [2]. Initial results have given information on the toxicities [3] and mechanism of action of this new strategy [4]. Different wild type or genetically engineered viral strains have been used, injected either systemically or intratumorally into patients with advanced or non-curable cancers. Oncolysis is not the only benefit that virotherapy can deliver; it is now well accepted that an important aspect of oncolytic viruses is the antitumor immune response they can initiate or reactivate within the patients, which translates into clinical re-

sponses [1]. The role of the immune system during virotherapies has a second edge though. The antiviral immune response of the patients is a major threat and limits the effects of oncolytic viruses [5,6]. The antiviral response may minimize the amount of viruses that eventually reach tumor sites upon systemic delivery, while increasing the doses may only cause higher toxicity and further sensitization toward the oncovirus.

We reported an initial clinical experience in the use of CELYVIR in 4 children with advanced neuroblastoma (NB) [7], the most frequent extracranial solid tumor in pediatric patients. Children with metastatic NB who relapse or develop refractory disease have a dismal prognosis; therefore, new strategies are needed in order to improve the outcome of these patients. CELYVIR is the acronym for autologous mesenchymal stem cells (MSCs) infected with ICOVIR-5, an oncolytic adenovirus [8] designed for systemic treatment of disseminated solid tumors. ICOVIR-5 contains several modifications that give it selective replication ability in cancer cells in which

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the Rb/E2F route is activated [9]. Our strategy consists of systemic infusions of CELYVIR aiming at enhancing the targeted delivery of the oncolytic adenoviruses to the metastases based in the natural tumor tropism of the MSCs. Here, we now report the complete program of compassionate use of this new antitumor medicine, after treating 12 additional children. We have gathered information that confirms the safety of this procedure, enabling numerous infusions per child, amounting to very high doses of virus with very low toxicities. We have also found some clinical responses in our cohort. Patients who responded to treatment showed interesting differences in immunity, before and during treatment, compared to children without response. In addition, their MSCs showed differences in the expression of cell adhesion molecules and immune-related genes when comparing both groups of patients.

Patients and methods

Patients

Twelve patients diagnosed with refractory neuroblastoma were enrolled in a program of compassionate use. The local Research Ethics Board and the Spanish Medicine Agency (AEMPS) approved each patient's treatment in an individualized basis, and informed consent was obtained from each participant. Table 1 shows patients' and infusions' characteristics. Toxicities were studied, recording clinical symptoms and signs of adverse effects, and by hematological and biochemical analysis done in blood samples prior to each infusion. Clinical responses were evaluated after the 6th dose with the level of serum enolase and with ^{123}I MIBG-scintigraphy, comparing the number of lesions pre- and post-therapy.

Cell culture

Bone marrow MSCs were obtained from the iliac crest of patients as previously described [7]. Briefly, mononuclear cells were obtained by Ficoll gradient centrifugation (400 g, 25 min, 20 °C) and cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and 1% penicillin–streptomycin (P/S, Gibco). The medium was replaced after 48 hours. Cells were maintained at 37 °C and 5% CO₂. MSC production complied with the principles of Good Manufacturing Practice (GMP) in an AEMPS-approved clean room.

For CELYVIR preparation, cell cultures were washed with PBS, trypsinized (TriPLE Express, Life Technologies, Carlsbad, CA) and MSCs resuspended in 25 mL of complete medium. MSCs received 30 Gy irradiation, fulfilling a request of AEMPS. 3×10^6 cells/mL cells were then infected with ICOVIR-5 [8] at 200 MOI during 120 min at 37 °C,

5% CO₂ in DMEM. Infected cells were then washed and resuspended in 50 mL of 0.9% saline supplemented with 2% human albumin.

Flow cytometry analysis

Flow cytometry analysis was performed with FACSCanto II and FACSDiva software v6.1.2 (BD Biosciences, Franklin Lakes, NJ). Data acquisition and analyses of cell adhesion and immune-related molecules were performed on the CELYVIR product, i.e., after MSCs were irradiated and infected with ICOVIR-5. The mean fluorescence intensity (MFI) was obtained for each adhesion molecule. Expression levels were normalized to those of their respective isotype control to allow comparisons. The list of molecules studied is available as Supplementary material.

Peripheral blood leukocyte subtypes were studied as previously described by our group [10]. Fresh tumor biopsies were mechanically disaggregated, filtered through a nylon mesh cell strainer, 100 μm (BD Bioscience), and cell suspension was processed for flow cytometry. Dead cells were excluded by 7-AAD staining.

Histological studies

Tumor biopsy specimens from patient UPN5 were fixed in 10% neutral formalin. Paraffin-embedded sections were cut at 3 μm and stained for hematoxylin–eosin. Several antibodies were used for staining tumor infiltrating human leukocytes (see Supplementary Fig. S4). An automated staining system (Dako Autostainer, DakoCytomation, Denmark) was used in combination with a two-step peroxidase-labeled polymer system (Envision System, Dako, Denmark).

Cell migration assays

NB1691 cells (kindly provided by Dr. A. Davidoff of St. Jude's Children's Research Hospital) were seeded at 25,000 cells/cm² in the lower chamber of a transwell multiwell plate (BD Biosciences, Franklin Lakes, NJ), in 1% P/S DMEM, and 20,000 Celyvir-MSCs were seeded on the gelatine-coated upper chamber. After 24 h at 37 °C, 5% CO₂, transwells were washed and non-migrated cells were removed with a cotton swab previously dipped in PBS. Migrated cells were fixed in 10% formalin for 20 minutes and stained with 0.2% crystal violet in 10% formaldehyde for 15 minutes. Finally, transwells were washed twice with water and allowed to dry. Each sample was tested in triplicate.

For specific chemokine assays, 10 ng/mL of either CXCL12 or CCL5 was tested following the same scheme as for NB1691. Monoclonal antibodies against these two molecules (200 ng/mL) or monoclonal antibodies against their counterpart receptors (CXCR4 and CCR1,

Table 1
Patients' characteristics.

ID	Gender	Lines TH	Celyvir doses	Cells ($\times 10^6$)	Virus	PCR PB	Outcome
UPN5	F	3	70	2640	2.6E+14	+	SD
UPN6a	M	3	6	226	4.5E+12		CR
UPN6b		5	29	1013	2.3E+13	+	PR
UPN7	F	4	9	520	5.2E+13	ND	PD
UPN10	M	4	6	160	1.6E+13	ND	PD
UPN11	M	4	4	150	1.5E+13	ND	PD
UPN12	F	4	10	340	3.4E+13	ND	PD
UPN13	M	4	8	280	2.8E+13	ND	PR
UPN16	F	3	7	150	1.5E+13	+	PD
UPN18	F	4	14	300	3.0E+13	+	PD
UPN19	M	4	9	220	2.2E+13	+	PR
UPN20	F	3	6	290	2.9E+13	+	PD
UPN21	M	6	8	176	1.7E+13	+	PD

ID, identification; TH, therapy; PCR PB, detection of adenoviral genome in peripheral blood by PCR; UPN, unique patient number; SD, stable disease; PR, partial response; CR, complete response; PD, progressive disease; ND, not done.

respectively, at 100 ng/mL) were used as controls. Chemokines and antibodies were purchased from R&D Systems (Minneapolis, MN).

Gene expression profile

RNA from Celyvir-MSCs and from pre-irradiated MSCs was isolated with Absolutely RNA microprep kit (Agilent, Santa Clara, CA) and cDNA synthesized with SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA). qRT-PCR was performed with TaqMan Gene expression assays for the following genes: IL-6 Hs00985639_m1, IL-8 Hs00174103_m1, IL-10 Hs00961622_m1, CCL5 Hs00982282_m1, CXCL10 Hs01124251_g1, IFN γ Hs00989291_m1, TNF α Hs01113624_g1, GAPDH Hs02758991_g1, TGF β 1 Hs00998133_m1, TGF β 2 Hs00234244_m1, TGF β 3 Hs01086000_m1, VEGF-A Hs00900055_m1, IDO1 Hs00984148_m1, IDO2 Hs01589373_m1 and the Taqman Gex master mix (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Each sample was analyzed as independent duplicate for each set of primers and probes.

Statistics

The Shapiro–Wilk test was performed first to find whether values followed a normal distribution. Then, comparisons between quantitative variables were done using the Student's t-test (for samples with normal distribution) or the Wilcoxon's rank-sum test (Mann–Whitney statistic, for samples with non-normal distribution). Differences were considered significant with a p value below 5%. The STATA software 11.0 (StataCorp, College Station, TX) was used. Figures represent mean \pm standard error of the mean.

Results

CELYVIR therapy and follow-up

Twelve patients diagnosed of refractory neuroblastoma received multidoses of CELYVIR (systemic infusion of autologous MSCs infected with the oncolytic adenovirus ICOVIR-5) under a program of compassionate use. The children received weekly multidoses of CELYVIR (minimum 4, maximum 70; total 186) as sole treatment. Total cells (min. 150×10^6 , max. 2640×10^6) and total viral particles (min. 4.5×10^{12} , max. 2.64×10^{14}) varied among patients. Blood cell counts and serum biochemical parameters were in normal ranges following each infusion. Mild and auto limited viral-related toxicities were the only adverse effects detected. None of the 12 patients experienced grade 3+ toxicities. The most frequent toxicity observed was low-grade fever and flu-like symptoms in 10 patients. Clinical outcomes were progression (n = 8), stable disease (n = 1), partial response (n = 3) and complete response (n = 1). UPN6 received CELYVIR in 2 separate occasions. This patient reached a complete response after the first cycle; however, he had a brain plus extracranial (bone, skin) relapse 6 months later and received a second round of CELYVIR, achieving a partial response.

We compared different biomarkers in 2 groups of the above-described patients: those who had a positive clinical response to treatment (either complete, partial or stabilization; n = 5) and those who did not respond (n = 8).

Adenoviral replication was studied in peripheral blood (PB) samples using a PCR method [7]. We found adenoviral genomes in the PB of all patients studied (Table 1), without differences among patients that responded and those who did not. PCRs were performed 7 days after CELYVIR infusions, a time when positive results indicate *in vivo* secondary infection and replication.

We followed the absolute numbers of circulating lymphocytes before and after CELYVIR therapy and found changes in all patients. Patients who responded had significantly higher counts of

T lymphocytes pre-therapy (p = 0.0157, Wilcoxon's test). The difference was found both in CD4 and CD8 (Fig. 1). Absolute numbers of T lymphocytes remained higher, mainly in CD4 (not significantly for CD8), along therapy among the patients that showed a clinical response. Numbers of leukocytes of the innate immunity such as neutrophils (NT), monocytes (MO), natural killer (NK) cells, and of the adaptive response like B-lymphocytes were not significantly different when comparing responder and non responder patients. Starting from pre-therapy values, NK, B-lymphocyte and T-lymphocyte (CD4 and CD8) cell counts rose during treatment with CELYVIR. The increases above basal levels were not significantly different comparing patients that responded to CELYVIR versus those who did not. However, the variations over basal values were very mild among patients who responded to CELYVIR, while those who did not respond had marked increases in all populations of circulating lymphocytes (Supplementary Fig. S1).

We analyzed the kinetics of circulating CD4 and CD8 T lymphocyte subsets: naïve (N), central memory (CM), effector memory (EM) and effector memory 45RA (EMRA) (Fig. 2). Naïve CD4 and CD8 T lymphocyte numbers in children who responded were always above those of children with no response. Major changes in the numbers of these T cell subpopulations affected to the EM and EMRA subsets of children who did not respond to the therapy, both in CD4 and CD8 T lymphocytes, and also in CD8 CM. Compared to responder patients who presented an almost flat kinetic, non-responders showed a trend toward increasingly higher numbers of these subpopulations during therapy. These differences were not statistically significant. Finally, we also analyzed the subpopulation of circulating CD4⁺CD25⁺CD127^{low/negative} containing T regulatory (T_{reg}) lymphocytes [11] and did not find statistically significant differences between both groups of patients (Supplementary Fig. S2).

UPN5 was the patient who received the highest numbers of infusions and allowed us to perform an extensive follow-up (Supplementary Fig. S3). After diagnosis, she received 3 lines of therapy, with progression after each one. Weekly infusions of CELYVIR were started as the sole therapy, with stabilization of the disease in the first evaluation (after the first 2 months). Peripheral blood T lymphocyte counts steadily rose from low basal levels. From a clinical point of view, the improvement of her general condition during the first year or CELYVIR treatment was remarkable. She initiated therapy pale, tired, sad and anorexic. After four months, she no longer looked sick and gained 2.5 kg. Her Lansky performance status improved from 50 to 90. The clinical examination became normal (except the abdominal mass). CELYVIR was discontinued after 12 months and infusions of interleukin-2 (IL-2) were then administered, at doses of 6×10^6 U each, in order to *in vivo* stimulate the antitumor immune response. PB cell counts declined with this treatment, so IL-2 administration was discontinued after 2 months and CELYVIR infusions were then resumed. An initial increase of circulating T lymphocytes followed the reinfusion of CELYVIR and lasted for 3 months, after which the disease reactivated and progressed to an end-stage situation. During the last period of CELYVIR infusions, lymphocyte cell counts kept decreasing, coinciding with the progressive raise in biochemical markers of the tumor (see enolase and LDH curves in Supplementary Fig. S3). In UPN5 we obtained biopsies of the primary tumor before and after CELYVIR therapy. Immunohistochemistry studies in paraffin-embedded biopsies showed the presence of different populations of tumor infiltrating lymphocytes (TILs), pre and post CELYVIR therapy (Supplementary Fig. S4). Interestingly, Ki67 staining showed an increase in the activity of TILs within nodular structures in the tumor mass after CELYVIR therapy. Moreover, flow cytometry of tumor biopsies obtained at 2 different moments during CELYVIR therapy (stable disease and end-stage disease) showed notable changes in the CD4:CD8 ratio and naïve:central memory:effector immunophenotypes of TILs (Supplementary Fig. S5). The presence of adenovirus genome in the

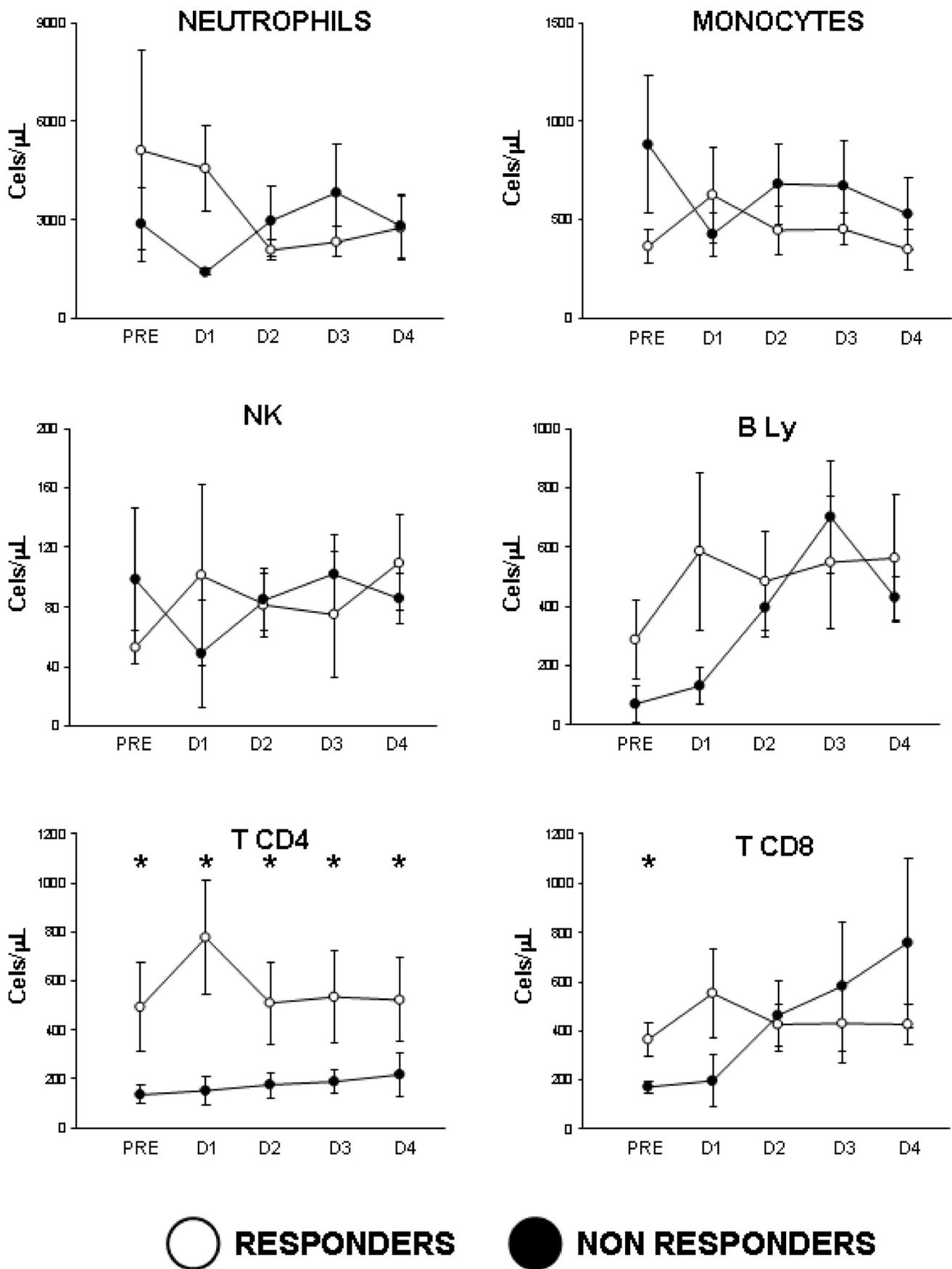


Fig. 1. Changes in circulating leukocyte subpopulation counts in patients treated with CELYVIR. Absolute peripheral blood cell counts of different leukocyte subpopulations from patients who responded (white circles) and who did not (black circles), pre-therapy and after the first 4 doses (D) of CELYVIR. Figures represent the mean \pm standard mean error. * $p < 0.05$.

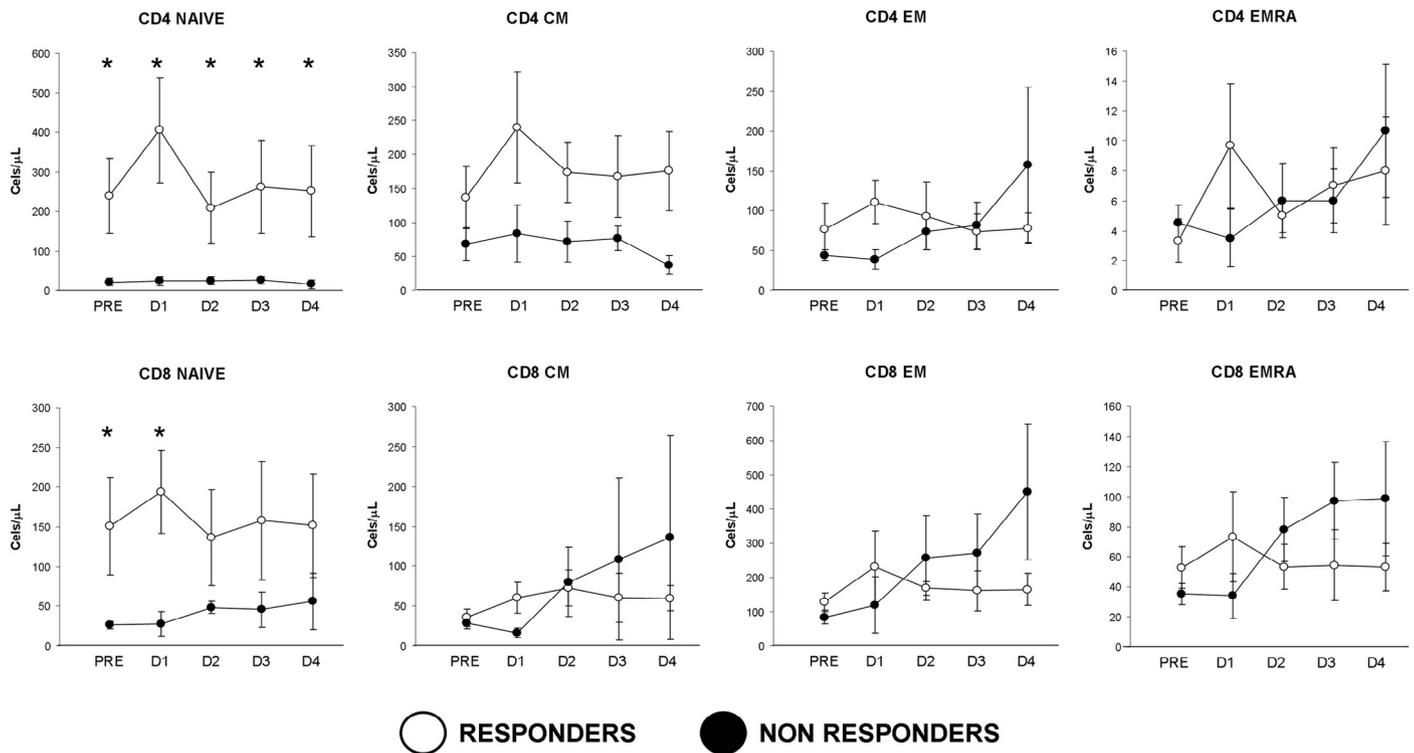


Fig. 2. Changes in circulating T lymphocyte subpopulation counts in patients treated with CELYVIR. Absolute peripheral blood cell counts of naïve (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻), and effector memory CD45RA (CD45RA⁺CCR7⁻), from patients who responded (white circles) and who did not (black circles), pre-therapy and after the first 4 doses (D) of CELYVIR. *p < 0.05.

tumor biopsies was studied by PCR with negative results in all samples.

Adhesion molecule profile of MSCs and clinical outcome

MSCs exhibit tropism for damaged tissues as well as the tumor microenvironment and many different receptors have been implicated in the homing of MSCs. We studied expression levels of several adhesion molecules on the surface of the CELYVIR product and found that irradiated and infected MSCs of patients that had a positive clinical response expressed significantly ($p < 0.05$) higher levels of CXCR1 and CCR1 than MSCs of patients with no response (Fig. 3). In addition, the levels of CXCR4 tended to be higher, almost statistically significant ($p < 0.1$), in the group of patients that responded after receiving CELYVIR (Fig. 3). Supplementary Table S1 shows the results of all molecules studied.

MSCs migrated toward chemotactic stimuli and neuroblastoma cells

Since chemokine receptors expressed by MSCs were associated to clinical outcome, we studied whether the results on expression levels translated into functional differences. We first studied whether CELYVIR products used for the patients were able to migrate toward chemotactic stimuli. We performed 2 sets of experiments using transwell assays. We first induced chemotaxis by adding single chemokines (CCL5 – ligand for CCR1 – or SDF1 – ligand for CXCR4) to the lower chamber of standard transwell systems. Results showed that the MSCs used in CELYVIR responded to each chemotactic stimulus with migration values significantly higher than those in basal conditions (Fig. 4A and 4B). Chemotaxis was inhibited when blocking either the chemoattractant or the chemokine receptor with

specific antibodies. In the second set of experiments, neuroblastoma cells were placed in the lower chamber and used as chemotactic stimuli. The MSCs used in the production of CELYVIR responded by significantly increasing their migration toward tumor cells (Fig. 4C). We finally compared chemotactic responses of CELYVIR products of patients that had a positive clinical response versus those of patients with no response, in the above mentioned transwell assays. Although the migration values were higher in MSCs from patients who had clinical responses, the differences were not statistically significant (Fig. 4D and 4E).

Effects of irradiation on MSC chemotactic capacities

The preparation of CELYVIR implies that MSCs receive lethal irradiation and are infected by an oncolytic virus. Irradiating MSC was a requirement of AEMPS because of the controversial reports on the capacity of MSCs for favoring tumor progression. It is not known how this manipulation may affect the capacity of MSCs in CELYVIR products for migrating and homing into the metastatic nodules before delivering their oncolytic cargos. We have studied the expression levels of cell adhesion molecules and the chemotactic capacity of MSCs before and after receiving a dose of 30 Gy, as part of the process of CELYVIR production. Most of the cell adhesion molecules studied upregulated their expression levels upon irradiation (not shown). However, this effect did not translate into higher migration capacity in the transwell assays. In fact, irradiated MSCs had significantly lower migration values toward chemokines or neuroblastoma cells compared to non-irradiated MSCs (Supplementary Fig. S6). These results were not different among MSCs from children that responded to CELYVIR or those who did not, reflecting a universal negative effect of high-dose irradiation in MSC migration capacity.

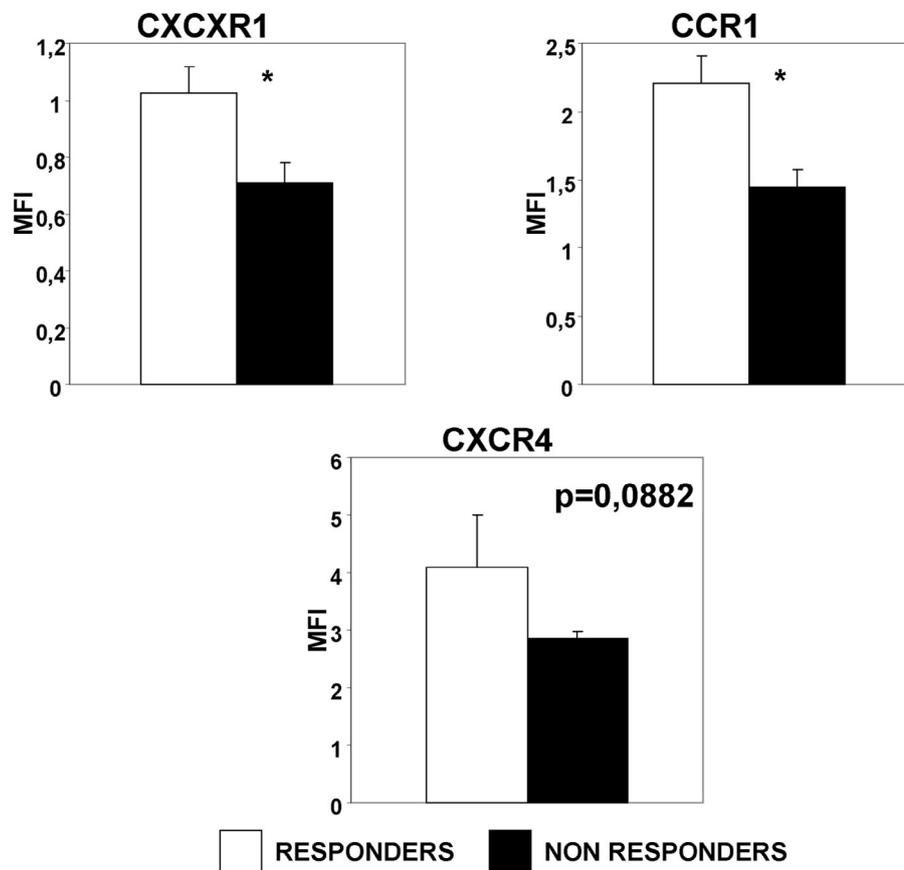


Fig. 3. Mesenchymal stem cells in the CELYVIR products of patients who responded expressed higher levels of chemokine receptors. Relative expression levels of CCR1, CXCR1 and CXCR4 were calculated by the mean fluorescence intensity by flow cytometry and normalized to the corresponding isotype. Patients who responded, white bars; non-responder patients, black bars. * $p < 0.05$.

Immune molecule profile of MSCs and clinical outcome

We determined the levels of immune related genes expressed by the infused MSCs using RTqPCR assays (Supplementary Table S2). Expression levels were normalized to those of MSCs from healthy donors. We found that IFN γ was expressed at significantly lower levels by the CELYVIR product of children with clinical responses (Fig. 5A), while IDO, IL6, IL8 and VEGF α levels showed a decrease in these patients which almost reached statistical significance ($p < 0.1$). We also studied the expression levels of several immune-related molecules on the surface of MSCs of patients treated with CELYVIR by flow cytometry (Supplementary Table S3). We found that irradiated and infected MSCs of patients that had a positive clinical response expressed significantly higher levels of HLA-DR than MSCs of patients with no response ($p < 0.05$) (Fig. 5B).

Discussion

Intratumorally or systemically administered oncolytic viruses for humans with advanced cancers have been increasingly explored during years [12]. We used MSCs as carriers for systemic administration of an oncolytic adenovirus as a first-in-humans therapy in a small cohort of children with refractory NB, with initial promising results [7]. We here update our previously reported experience with CELYVIR to 12 additional children with this aggressive type of cancer, confirming the excellent toxicity profile of the therapy. After infusing a significant number of doses, amounting to high numbers of cells and viral particles in each patient, we only documented auto limited mild or minor viral-related toxicities (fever,

chills, and discomfort). It is important to underscore this fact because the cohort of patients had previously received at least 3 lines of chemotherapies, with the concomitant toxicities known in heavily treated cancer patients. It appears that the combination of mesenchymal cells and oncolytic adenovirus is a safe treatment that can be administered in an intensive schedule and for which a maximum tolerated dose has not been achieved in our protocol. The highest single dose administered in our cohort was 5 million cells/kg. Since we found clinical responses with doses below the highest one, we routinely set 2 million cells/kg as target for single doses. As shown in Table 1, patient UPN5 received a total of 70 doses, adding up more than 2 billion cells and 2.6×10^{14} viral particles. These numbers of viral particles have not been previously administered in humans with so mild toxicity. It is not currently known either the optimum dosage or the dosing schedule of oncolytic viruses in the clinic, but our experience indicates that the use of mesenchymal cells may be a strategy for increasing the amount of oncolytic virus administered to patients avoiding or minimizing dose limiting toxicities.

In vivo detection of the virus genome days after CELYVIR infusion was an indirect confirmation of the replicative capacity of the infused adenovirus. The presence of ICOVIR-5 was documented by PCR in all patients. By the time the samples were drawn, the detection of the viral DNA should correspond to the *in vivo* secondary infection of tumor cells and suggested that CELYVIR carried ICOVIR-5 to areas with metastases.

We had some clinical responses among the children treated with CELYVIR. This confirmed our first report [7] and allowed us to search for markers that may help in improving our capacity for identifying patients who may benefit from this therapy. We reasoned that

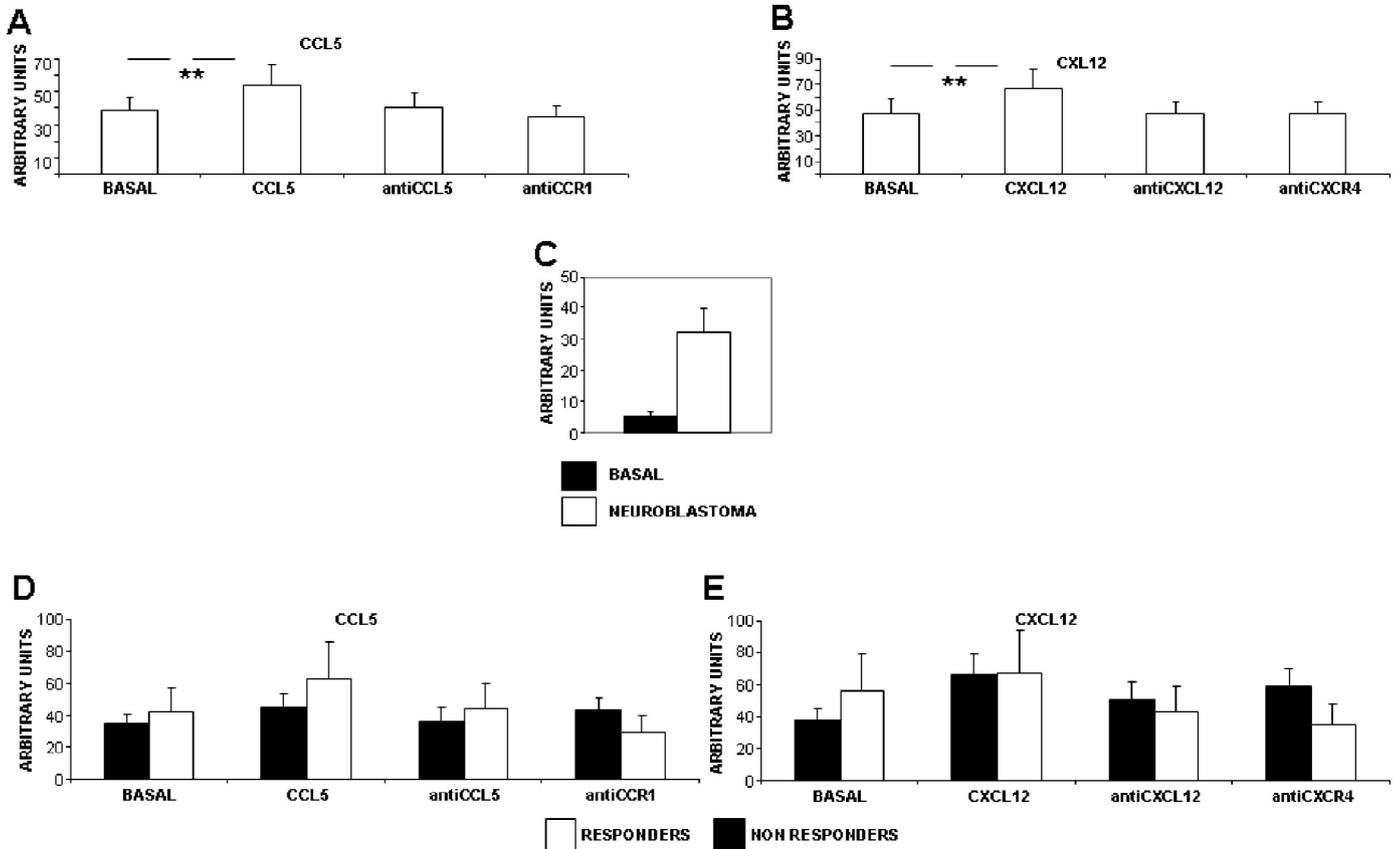


Fig. 4. Mesenchymal stem cells in the CELYVIR products responded to chemotactic stimuli. Results of migration of the CELYVIR product in transwell assays. The lower chambers of the transwell systems were prepared with CCL5 (A), CXCL12 (B), either alone or plus the corresponding blocking antibodies; or a neuroblastoma cell line (C). (D and E) Results of migration of the CELYVIR product in transwell assays from patients who responded (white bars) and patients who did not (black bars). The lower chambers of the transwell systems were prepared with CCL5 (D) or CXCL12 (E), or the corresponding blocking antibodies. **p < 0.01.

several factors may influence the achievement of a response in our strategy: the capacity of carrier cells for targeting metastatic sites, the oncolytic death of tumor cells *in vivo* and the role of the immune system in the success of oncolytic virotherapies. Even though sample size limited the power of statistic comparisons in this cohort of patients, we found patterns and tendencies that are interesting to be analyzed.

The migration and homing capacities of carrier MSCs in CELYVIR are likely linked to the profile of cell adhesion molecules they express and may enable them to migrate toward the sites of metastasis. We hypothesized that an optimum profile should result in better clinical outcomes. We performed an extensive study of cell adhesion molecules expressed by the MSCs used to treat our patients and compared the level of expression of responders versus non responders.

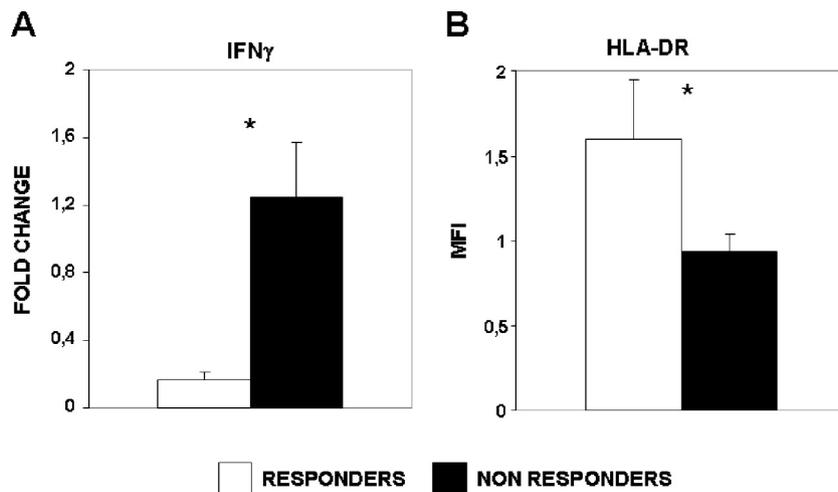


Fig. 5. Mesenchymal stem cells in the CELYVIR products from patients who responded expressed higher levels of immune molecules. (A) Relative expression levels of the IFN γ mRNA determined by RTqPCR. (B) The expression level of HLA-DR was calculated by the mean fluorescence intensity by flow cytometry and normalized to the corresponding isotype. Patients who responded, white bars; non-responder patients, black bars. *p < 0.05.

Of all the molecules studied, 2 chemokine receptors, CCR1 and CXCR1, were expressed at significantly different levels comparing both groups of patients. Other authors have shown that CXCR1 enhances the ability of human MSC to migrate toward gliomas [13] and both CXCR1 and CCR1 increase the migration of MSCs into sites of degenerated tissue of myocardium [14,15]. In every case, the levels of the chemokine receptors in MSC were higher among the responders, suggesting a better capacity for migrating toward sites where the corresponding chemokine is produced. The migration capacity of the cells was corroborated in transwell assays, either with single stimuli or with a NB cell line as source of chemotactic stimuli, demonstrating the capacity of the MSCs in CELYVIR for responding toward the ligands of these receptors. For CXCR4, the differences were almost statistically significant, an interesting finding since NB is known to metastasize to the bone marrow, and the axis CXCR4–CXCL12 is involved in the homing of hematopoietic stem cells [16] and MSCs [17] into the bone marrow upon transplantation, and also in the homing of MSCs into tumors [18]. The *in vivo* source of the corresponding chemokines in our patients was not studied; however, it has been reported that NB cells produce IL-8 (ligand of CXCR1) [19]. An alternative source of chemokines is the tumor microenvironment, which favor immune cell recruitment and angiogenesis [20]. In addition, many of the ligands for these receptors are associated to inflammation, a process related to cancer. Alternatively, the organs where NB cells naturally metastasize may be the source of the chemokine, as it has been commented previously for CXCL12. These molecules may also promote the growth and survival of neuroectodermal tumor cells [21].

MSC lethal irradiation, a requirement of our regulatory agency, may diminish the potential capacity of MSCs as carrier cells in virotherapy. Irradiation induces senescence [22], apoptosis [23] and autophagy [24] in MSC. We studied the impact of lethal irradiation on the migration capacity of CELYVIR and found a negative effect. Our results suggest that irradiation may jeopardize their *in vivo* performance and the clinical benefits that might be associated to their migratory ability upon infusion.

The immune system has a recognized role in the outcome of virotherapies [25]. Our results indicated that the status of the patient's immune system at the moment of initiating therapy might be crucial for achieving a clinical benefit. More specifically, the numbers of T lymphocytes might need to be above a threshold, since patients who did not respond in our cohort had significantly less circulating T cells than patients who did respond. We did not find a comparable result for other leukocytes, such as B cells (adaptive immunity) or NK cells, neutrophils and monocytes (innate immunity) underscoring the value of T lymphocytes in the antitumor immune attack after virotherapy. Therefore, although an immune response seems universal among these patients, only those with a presumably diverse enough TCR repertoire (highly enough T cell numbers) may develop a clinically significant antitumor immune response. In accordance with this, it is interesting that the numbers of naïve T cells pre-therapy were significantly higher in the group of patients who responded. We also found data suggesting an immune response upon administration of virus, since the numbers of circulating lymphocytes raised from basal levels in all patients. The increase was more important among patients who did not respond to CELYVIR. The kinetic of memory subsets in CD4 and CD8 T lymphocytes during therapy among the non-responders strongly suggested an antiviral immune response. The CCR7^{neg} memory T cell subpopulations are enriched in cells with effector molecules (IFN- γ and perforin), with homing preference into inflamed peripheral tissues, while the CD45RA⁺CCR7⁻ population is defined as a terminally (or late) differentiated population of effector T cells [26]. In several viral infections in humans, the numbers of helper and cytotoxic T lymphocytes with effector phenotypes increased along the expansion and resolution phases of the disease [27]; therefore, the

increase of EM and EMRA subsets during therapy may reflect the immune response to the continuous viral antigenic presentation. On the contrary, changes in circulating immune cell numbers were very mild in the group of responder patients, which might suggest a less active antiadenoviral response. In the only patient with available tumor biopsies, the clinical response was associated to activation of TILs, as shown by the Ki67 immunohistochemistry results of primary samples. The trials with a GM-CSF armed vaccinia oncovirus [28] and with a GM-CSF armed herpes oncovirus [29] in humans have detected infiltration of tumors by immune cells, both at injected and non-injected lesions. In accordance with the results in humans, studies in mice [30,31] found that localized intratumoral therapy with oncolytic viruses induced inflammatory responses, which activated lymphocytic infiltration and antitumor effect without virus spread in distant tumors (not injected with the virus). The authors hypothesized that high levels of viral replication were not needed to reach the effect, and the inflammation coincided with tumor infiltration with T CD4 and CD8 TILs. We have observed similar results using ICOVIR-5 in an immunocompetent murine model [32]. These results demonstrate that localized therapy with oncolytic virus can activate an immune inflammatory infiltration in tumors. It is not yet known whether the effect of virotherapy is direct on the lymphocytes or may also act through downmodulating the tolerant tumor microenvironment.

MSCs have a well known role in human antigen presenting cells [33–36] and effector and regulatory leukocytes of the innate and adaptive responses [37–40]. In our strategy, MSCs may not only act as carrier cells but might as well modulate the immune responses taking place after CELYVIR infusions, i.e., the antiadenoviral and the antitumor immune responses. We studied several molecules that may have impact in the role of MSCs in immune responses. From our gene expression assays, it appeared that clinical responses were achieved when the MSCs presented a lower pro-inflammatory profile after adenoviral infection (significantly lower expression of IFN γ , and a trend toward lower levels of IL6, IL8, IDO and VEGF α). In addition, expression of HLA class II by the same MSCs correlated with a better clinical outcome. It is not easy to draw a clear conclusion with these results and the fact that inflammation may favor the benefit of virotherapies. It may be argued that our studies were not done with the MSCs *in situ*, and it is known that MSCs modulate their gene and protein expression profile depending on the environment in which they are placed [41]. Another factor is the fact that MSCs were lethally irradiated in our strategy, a manipulation that deeply affected the function of these cells, although it has been reported that irradiated MSCs do not lose their immunomodulatory abilities [42]. In any case it is tempting to speculate that a lower antiadenoviral response would be a requisite for obtaining clinical benefits in our strategy, based on two observations. First, autologous MSCs from responder patients showed a less inflammatory gene expression profile after ICOVIR5 infection, compared to MSCs from non-responders, indicating a lower cellular response against adenovirus. Second, non-responder patients increased their peripheral blood cell counts and the proportions of terminally differentiated effector T lymphocytes during CELYVIR treatment in contrast to responders. We observed similar effects using a canine CELYVIR treatment in dogs with spontaneous tumors (Cejalvo et al., manuscript in preparation). Taken together these data would suggest an inverse correlation between clinical benefits and antiadenoviral immune system activation. In this scene, MSCs from patients who responded to adenovirus with a non-proinflammatory or tolerogenic profile might hide the virus from the recognition and attack of the innate and adaptive immune systems before and after CELYVIR deliver their load at the metastasis, favouring better conditions for the *in situ* oncolytic effect. Once the MSCs have eventually disappeared from the scene, the increased oncolysis that would ensue should increment the chances for the initiation or reactivation of

an antitumor immune response. In addition, these data would suggest that adenoviral immunodominance is lower among responder patients. The immunodominance hypothesis proposes that a strong immune response against dominant viral antigens prevents the development of an efficacious immune response against tumor antigens [43]. If so, a thorough understanding of why some patients present a lower adenoviral immunodominance would be essential to understand the therapeutic effects of CELYVIR.

In conclusion, our results confirm that the use of mesenchymal progenitor cells carrying an oncolytic adenovirus is a safe procedure in treating children with advanced NB, which can be administered in a multidose protocol with very high quantities of virus and an excellent tolerance. Our work has limitations due to the small sample size, the lack of direct data on the homing of CELYVIR and of the antiadenoviral and antitumor activities after therapy, all of them inherent to any trial of this type. Nevertheless, based on our results we suggest that the presence of MSCs in the medicine product may have an impact in the response to the therapy beyond their role as cell carriers. Several characteristics of the MSCs, related to their migratory capacities and response to the viral infection, may help us in designing an optimum version of this new antitumor strategy. Taking into account the patients' outcome and our experimental results, we envision that the development of an improved version of CELYVIR could bring into play a MSC source specifically selected on the basis of the characteristics we have already seen that are probably keys in a putative responder-like phenotype (e.g. immunophenotype, migration ability, non-irradiated).

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Conflict of interest

The authors declare there are no conflicts to disclose.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.11.036.

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