Changes in tumor infiltrating leukocytes in neuroblastoma treated with oncolytic virotherapy: insights from preclinical models and patients

Lidia Franco[1], Manuel Espinosa[2], Ana L Luís[2], África González-Murillo[1], Gustavo J Melen[1], Luís Madero[1], Javier García-Castro[3], Louis Chesler[4], Manuel Ramírez[1].
[1]Oncohematology, Hospital Universitario Niño Jesús, Madrid, Spain. [2]Pediatric Surgery, Hospital Universitario Niño Jesús, Spain. [3]Cellular Biotechnology Unit, Instituto de Salud Carlos III, Madrid, Spain. [4]Division of Clinical Studies, The Institute of Cancer Research, Sutton, Surrey, UK.

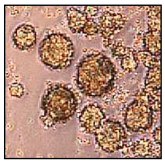
INTRODUCTION

Neuroblastoma (NB) is the most frequent solid extracranial childhood tumor. The prognosis of metastatic or relapsed NB is very poor, practically incurable. New therapeutic strategies for increasing cure rates are clearly needed. Our group has been developing a strategy for the treatment of refractory and metastatic childhood solid tumors, based on the administration of *Celyvir*: autologous mesenchymal cells that carry inside the oncolytic adenovirus ICOVIR-5 (EudraCT2008-000364-16; ClinicalTrials Identifier: NCT01844661).

The possibility that oncolytic virotherapy stimulates antitumor immune response opens new possibilities in the field of cancer immunotherapy. Preexisting lymphocytic infiltration in tumors (TILs) is associated with better prognosis in a variety of cancers. Recent studies also suggest that lymphocyte responses can identify those patients most likely to benefit from immune targeted therapies, opening the possibility that the effectiveness of immunotherapy can be improved through strategies that induce inflammation of the tumor. In our clinical experience we have found that the local and systemic administration of Celyvir caused changes within TILs, associated with a beneficial clinical response.

The main objective of this project is to characterize *Celyvir*-induced changes in TILs and tumor stroma using a murine NB model (which recapitulates the main genetic and clinical aspects of NB with amplified MYCN) and a murine *Celyvir* therapy similar to human.

Neuroblastoma model



There are many important issues about *Celyvir* therapy that do not have easy preclinical solution because of the lack of a good animal model. THMYCN transgenic mice have been used for the past 15 years in many studies of basic biology and preclinical research.

In this work NB neurospheres derived from TH-MYCN (129/SVJ) mice were cultured in DMEM:F12 (50%), 1xB27 (w/o VitA), 40ng/mL of mFGF and 20ng/mL of mEGF. Experiments were performed with 36769 TH-MYCN+ / ALK- cells.

Murine Celyvir

Mouse adenovirus type 1 (MAV-1) E1A protein contains three conserved regions (CR1, CR2 and CR3) that are comparable to the conserved regions found in Ad5 E1A, which interacts with members of the pRB family. MAV-1 *dl102* contains a CR2 deletion which confers oncolytic properties, similar to the Δ24 oncolytic Ad5 vector (Smith K et al, *Virology* 1996). Similar to human *Celyvir*, we infected adipocite derived mMSC with MAV-1 *dl102* oncolytic vector at a MOI of 200 during 90 min. After infection, cells were washed and prepared to treat mice, and m*Celyvir* was *I.V.* injected.

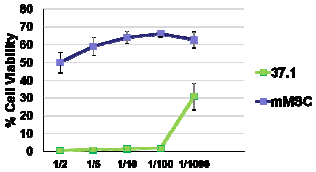


Figure 3. In vitro oncolytic properties of the MAV-1 *dl102* vector. 37.1 fibroblast-derived cell line is highly susceptible to *dl102* infection, it is used to production of virus stock, and here it is used as positive control. 37.1 and mMSC cells were infected in triplicate with serial dilutions of virus starting at ½ in DMEM w/o FBS. Cell viability was determined by flow cytometry after 7-8 days postinfection. We observed that mMSC used as vehicle had a mild susceptibility to MAV-1 *dl102* vector lysis compared with control cell line.

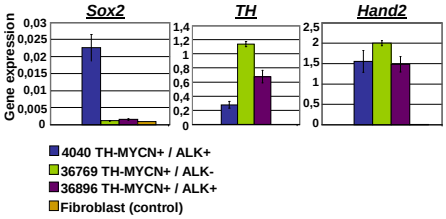
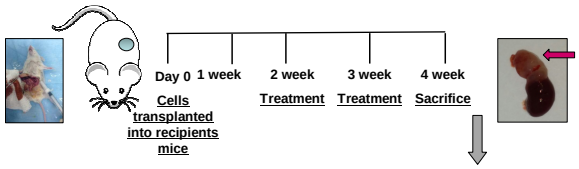


Figure 1. RT-Q-PCR were performed for *Sox2* (pluripotency), *Th* (tyrosine hydroxylase SN sympathetic neurons) and *Hand2 (neural crest derivatives expressed transcript 2)* genes. 4040 cell line expressed high levels of *Sox2* gene, but 36769 and 36869 cell lines expressed higher levels of neural linage genes, *TH* and *Hand2*, compared with 4040.

EXPERIMENTAL PROCEDURE



- Peripheral blood
- Spleen
- Bone marrow
- Tumor mass

Flow cytometry

qRT-PCR

Figure 2. At day 0, 10^6 tumor cells were orthotopically implanted on the adrenal gland of WT 129/SVJ mice (n=6). At week 2 and 3, mice were either treated with murine *Celyvir* (mMSC infected with MAV-1 *dl102*, n=3) or untreated (n=3). At week 4, mice were sacrified and tumors were analyzed by flow cytometry (leukocyte infiltration, Figure 5) or qRT–PCR (microenvironment, Figure 6).

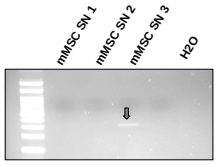


Figure 4. MAV-1 *dl102* virus was shown to replicate and to have a potent anti-tumor activity in a panel of murine tumor cell lines. We confirmed the replication of the virus in mMSC by convencional PCR.

RESULTS

mCelyvir-induced changes in tumor leukocites infiltration

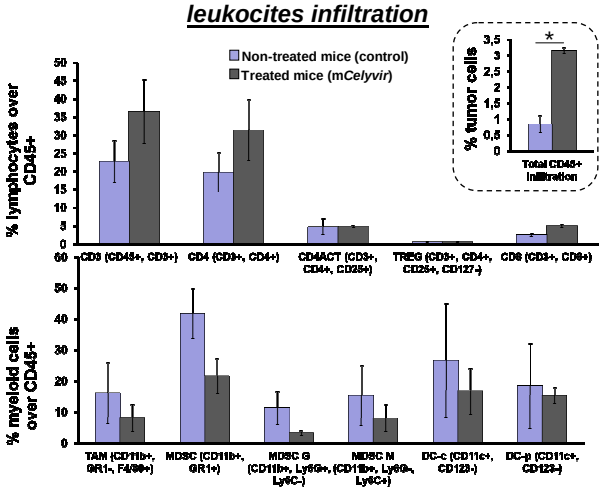


Figure 5. Changes in the distribution of different tumor infiltration leukocyte subpopulations in NB treated with Celyvir.

Our results (mean ± mean standard error) showed that m*Celyvir*-treated mice have higher percentage of TILs compared to non-treated mice (*p<0,05), especially of $CD3^+CD4^+$ lymphocytes, which suggest that m*Celyvir* treatment stimulates the recruitment of these immune cells into the tumor microenvirnment. On the other hand, levels of myeloid subpopulations tended to be lower in m*Celyvir*-treated mice compared to non-treated mice. Myeloid activation is classically associated with immune suppression in the tumor microenvironment, so these results might suggest that immune response is being activated in the tumor microenvironment due to the treatment with m*Celyvir*.

mCelyvir-induced changes in expression of environment signaling molecules

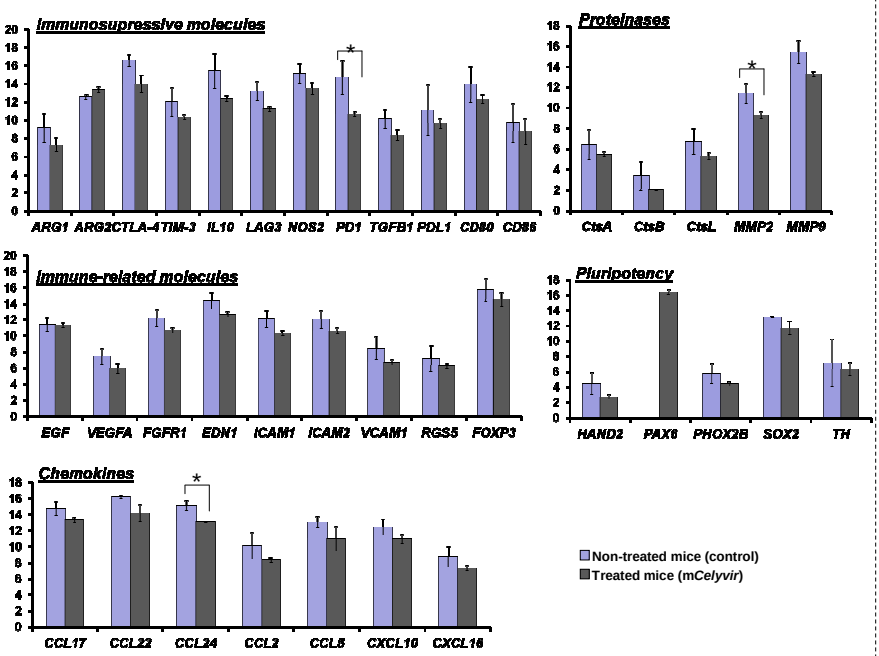


Figure 6. Changes in the tumor microenvironment in NB treated with *Celyvir*. Gene expression assays were performed for 38 genes related to immunosupressive molecules, proteinases, cell trafficking molecules, pluripotency and chemokines. All these molecules have an important role in cancer immunosurveillance and tumor progression.

We found that *PD1*, *MMP2* and *CCL24* showed statically significant differences between treated and non-treated mice (*p<0,05), being these levels lower in m*Celyvir*-treated mice. In other genes (*CTLA-4, ARG2, IL10, LAG3, CCL17, EDN1, ICAM1* and *MMP9*) we also found a trend to decrease after m*Celyvir* treatment, but differences were not statically significant due to low number of mice analyzed.

It is important to note that *CTLA-4* and *PD1* expression were diminished in treated mice, suggesting an increased T-cell activation after treatment with m*Celyvir* associated to changes within the tumor microenvironment.

CONCLUSIONS

1.Systemic administrations of Celyvir induced changes at local tumor sites. These changes affected both the infiltration by immunecompetent cells (lymphoid and myeloid cells, effectors and regulators) and the tumor-tolerant microenvironment.
2.The immunocompetent animal neuroblastoma model and the conditionally-replicative MAV-1 *dl102* should allow us to dissect crucial aspects of the mechanism of action of *Celyvir*, helping in optimizing this strategy in the clinic setting.

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