

# Peripheral blood tumor-infiltrating lymphocytes in a neuroblastoma model

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## ABSTRACT

**Objective.** To detect tumor-infiltrating lymphocytes (TILs) in the peripheral blood (PB) of a preclinical neuroblastoma model.

**Materials and methods.** Two types of preclinical models – immunodeficient mice and immunocompetent mice – were generated by injecting a cell suspension of neuroblastoma cell line NB36769 with MYCN gene (TH-MYCN<sup>+</sup>) overexpression. Spleen, tumor, and peripheral blood were studied using flow cytometry to detect PD-1<sup>+</sup> T-cells. TCR- $\beta$  immunosequencing was performed in matched samples (tumor and peripheral blood).

**Results.** Most PB T-cells of immunodeficient mice were CD4 (control: 83.1%; tumor: 86.1%), with a small proportion of PD-1<sup>+</sup> T-cells (control: 0.4%; tumor: 0.3%). However, the percentage of PD-1<sup>+</sup> T-cells in the spleen was higher (control: 6.5%; tumor: 6.2%), and it was expressed in the CD4<sup>+</sup> subset only.

Regarding the TCR repertoire of immunocompetent mice, the proportion of the 10 most frequent sequences was significantly higher in tumors ( $11.09\% \pm 2.83\%$ ) than in the peripheral blood ( $1.59\% \pm 0.59\%$ ) ( $p=0.024$ ). These findings are suggestive of clonotype enrichment within the tumor. 9 out of the 10 most frequent tumor clones were identified in the matched peripheral blood sample in 2 mice, and 6 out of 10 in one mouse. In addition, TILs with shared sequences from different animals were found.

**Conclusions.** Our results in terms of immunophenotype and clonality suggest the presence of PB T-cells which could include TILs in a preclinical neuroblastoma model.

**KEY WORDS:** Neuroblastoma; Immunotherapy; Tumor-Infiltrating lymphocytes.

## LINFOCITOS INFILTRANTES DE TUMOR EN SANGRE PERIFÉRICA EN UN MODELO DE NEUROBLASTOMA

### RESUMEN

**Objetivo.** Comprobar la existencia de linfocitos T que incluyen linfocitos infiltrantes de tumor (TILs) en la sangre periférica (SP) de un modelo preclínico de neuroblastoma.

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**Material y métodos.** Utilizamos un modelo en ratones inmunodeficientes y otro en inmunocompetentes mediante inyección de suspensiones de la línea tumoral NB36769 con mutación de MYCN (TH-MYCN<sup>+</sup>). Se realizaron análisis por citometría de flujo (bazo, SP y tumor) y secuenciación del TCR- $\beta$  en el ADN de muestras pareadas de tumor y SP.

**Resultados.** En los ratones inmunodeficientes el componente principal en SP fue CD4: 83,1% (control) y 86,1% (tumor), siendo PD-1<sup>+</sup> el 0,4 y el 0,3%. En el bazo obtuvimos un mayor porcentaje de linfocitos T PD-1<sup>+</sup> que en SP, siendo similar en el control (6,5%) y en el ratón con tumor (6,2%), en subpoblación CD4<sup>+</sup> exclusivamente. En los ratones inmunocompetentes observamos que la proporción de los 10 clones más frecuentes en los tumores constituía el  $11,09\% \pm 2,83\%$  del repertorio del TCR, mientras en SP representaba el  $1,59\% \pm 0,59\%$  ( $p=0,024$ ). Estos resultados sugieren un enriquecimiento de clonotipos dentro del tumor. De los 10 clones más frecuentes en las muestras tumorales, localizamos 9 también en la SP en dos ratones y 6 en el tercero. Además, encontramos secuencias compartidas por TILs de animales diferentes.

**Conclusiones.** Nuestros resultados de inmunofenotipo y clonalidad apuntan a la existencia de linfocitos en SP que podrían contener TILs en un modelo experimental de neuroblastoma.

**PALABRAS CLAVE:** Neuroblastoma; Inmunoterapia; Linfocitos infiltrantes de tumor.

## INTRODUCTION

Neuroblastoma is the most frequent extracranial solid tumor in pediatric patients, with an annual incidence of 1.1 out of 100,000 children aged 0-14. It represents up to 7% of all malignant tumors in patients under 15<sup>(1)</sup>. In spite of the advances made in the treatment of this pathology in recent years, overall survival rates in patients with high risk tumors are under 40%, even when using intensive multimodal therapy<sup>(2,3)</sup>. Today, neuroblastoma accounts for up to 15% of all cancer deaths in pediatric patients<sup>(4)</sup>.

The advent of immunotherapy strategies such as adoptive cell therapy (ACT) for the treatment of solid tumors represents a new therapeutic option to improve results in

this population. Although there are numerous clinical trials on typically adult tumors such as melanoma, therapeutic potential in child tumors has been little explored<sup>(5)</sup>.

Tumor-infiltrating lymphocytes (TILs) are a heterogeneous population of  $\alpha\beta$  T-cells. Lymphoid cell infiltration in tumors has been associated with a more favorable prognosis in neuroblastoma patients<sup>(6)</sup>. Dr. Rosenberg's group demonstrated that peripheral blood (PB) PD1<sup>+</sup> T-cells of melanoma patients have anti-tumor T-lymphocytes, suggesting they are a circulating subpopulation of TILs<sup>(7-9)</sup>, with a CD3<sup>+</sup> immunophenotype, and CD4<sup>+</sup> and CD8<sup>+</sup> subpopulations expressing PD-1. Therefore, they can be detected using flow cytometry.

In the case of neuroblastoma – a difficult access retroperitoneal tumor –, invasive procedures are required to obtain tissue samples. The fact these cells might circulate in the PB could avoid such procedures and reduce morbidity in these patients.

In this work, a murine model was developed in order to detect PB circulating PD-1<sup>+</sup> lymphocytes that could be used as a source of such cells for ACT use.

## MATERIALS AND METHODS

- **Animals:** *NOD scid gamma* immunodeficient mice (*Mus musculus*), which do not have B- or T-lymphocytes, and 129/SvJ wild type (WT) immunocompetent mice were used. The animals were purchased at del National Cancer Institute (Frederick, Maryland, EEUU), and bred and maintained at the Servicio del Animalario del Centro de Investigaciones Energéticas, Medioambientales and Tecnológicas (CIEMAT) with registro 28079-21 A. All animal experiments were approved by the Spanish animal well-being body (OEBA) in CIEMAT and the Departamento Regional of Madrid of Medio Ambiente, with reference PROEX 186/15.
- **Cells:** 36769 neuroblastoma (36769NB) cell line was selected with amplification of the MYCN oncogene by a rat tyrosine hydroxylase promoter (TH-MYCN<sup>+</sup>) from 129/SvJ mice<sup>(10)</sup>. Such line was borrowed from Profesor Louis Chesler (The Royal Marsden Hospital, Londres). Tumor cells, also known as neurospheres, were suspension-cultured with DMEM:F12 (1:1) medium (Gibco™), supplemented with  $1 \times B27$  without vitamin A (Gibco™), 40 ng/ml mFGF (R&D Systems®), 20 ng/ml mEGF (R&D Systems®), and 1% penicillin-streptomycin (Gibco®) in an oven at 37°C and 5% CO<sub>2</sub>, and maintained at subconfluent levels with medium renovation every 3-4 days.
- **Neuroblastoma model in immunodeficient animals:**  $1 \times 10^6$  neurosphere suspensions were injected in 50  $\mu$ l of non-supplemented medium in the subcutaneous cell tissue of the right dorsal region using 25 G  $\times$  5/8" needles. On day 8 post-injection, suspensions of  $1 \times 10^5$

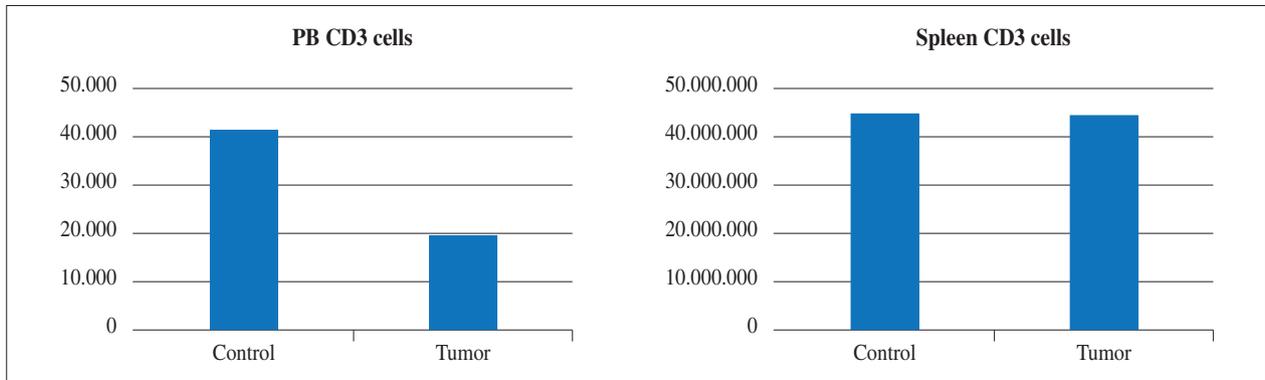
TILs from neuroblastomas generated through an orthotopic transplantation model in 129/SvJ immunocompetent mice with the same line (NB36769) were inoculated in the same region.

- **Neuroblastoma model in immunocompetent animals:**  $4 \times 10^5$  neurosphere suspensions were implanted in 30  $\mu$ l of non-supplemented medium through an orthotopic injection in the left adrenal gland as described by our group<sup>(11)</sup>. Sacrifice was performed 4 weeks following implantation. PB samples were achieved through exsanguination, and tumor samples were achieved through excision. PB samples were preserved with 50  $\mu$ l of EDTA 0.5 M, at room temperature. Red blood cell lysis solution (Quiclysis™, Cytognos) was applied at room temperature, in the dark, for 10 minutes. Cells were washed with PBS pH 7.2 (Gibco™) twice. Splensens were processed through mechanical disaggregation on a 40  $\mu$ m filter in conical tubes with 30 ml of PBS for cell suspension. Tumors were processed through mechanical disaggregation and enzyme digestion with RPMI-1640 medium (Gibco™), supplemented with 1 mg/ml collagenase D (Roche) and 2500U DNAase (Pulmozyme®, Roche), at 37°C, for 45 minutes. Enzyme digestion was FBS inactivated, and cell suspensions were filtered using a 40  $\mu$ m filter. The resulting pellet was washed twice and re-suspended in 30 ml of PBS. Cell suspension aliquots were dry frozen for DNA isolation, the rest of them being re-suspended in the PBS for flow cytometry (FACSCanto™ II, Becton Dickinson).
- **Flow cytometry:**  $1 \times 10^5$  cells from the samples processed with CD45.2, CD3, CD4, CD8, PD1, and 7-AAD (Biolegend®) monoclonal antibodies were incubated in 100  $\mu$ l of PBS for 20 minutes at 4°C, in the dark.
- **Clonality:** DNA of frozen unicellular tumor suspensions from six 129/SvJ transplanted mice and DNA of PB matched samples from three 129/SvJ transplanted mice were purified with the QIAamp DNeasy kit for blood and tissues (Qiagen N.V.)<sup>(12)</sup>. Immunosequencing of the TCR- $\beta$  region was carried out by Adaptive Biotechnologies Corp. (Seattle, WA, USA) in purified DNA, and data were analyzed using the ImmunoSEQ® analyzer. Results were expressed as a percentage of the total T-cell patterns originated by the 10 most frequent sequences<sup>(13)</sup>.

## RESULTS

### Immunodeficient murine model

*NOD scid gamma* mice do not have T-lymphocytes so they are suitable for analyzing exogenous T-lymphocyte



**Figure 1.** PB and spleen lymphocyte component following TIL injection (total cells/ml) in immunodeficient mice.

behavior. In our case, the only source of T-lymphocytes were TILs previously obtained from neuroblastomas developed in immunocompetent mice (see “Methods”). The control mouse had a higher number of total PB T-lymphocytes than the tumor mouse (41,175 cells/ml, 2.7% vs. 20,000 cells/ml, 3.2%). After analyzing spleen lymphocyte infiltration, the percentage of T-lymphocytes was similar in both mice (control: 44,804,375 cells/ml, 20.9% vs. tumor: 44,446,500 cells/ml, 20.4%) (Fig. 1).

The main component of PB T-lymphocyte subpopulations was CD4<sup>+</sup>: 83.1% in the control mouse, and 86.1% in the tumor mouse. PD-1<sup>+</sup> accounted for 0.4% and 0.3%, respectively. No CD8<sup>+</sup> lymphocytes were identified in the control mouse, and CD8<sup>+</sup> percentage was minimal (0.1%) in the tumor mouse, with no CD8<sup>+</sup>PD-1<sup>+</sup> lymphocytes observed. In the spleen, the percentage of PD-1<sup>+</sup> T-lymphocytes was higher than in the PB, with similar numbers in the control mouse (6.5%) and in the tumor mouse (6.2%). In both cases, PD-1<sup>+</sup> T subpopulation was made up of CD4<sup>+</sup>, with no CD8<sup>+</sup>PD-1<sup>+</sup> lymphocytes (Fig. 2).

### Immunocompetent murine model

Flow cytometry demonstrated a similar PB T-lymphocyte distribution in the immunocompetent model, with CD4<sup>+</sup>PD1<sup>+</sup> lymphocytes being predominant. In addition, TIL and PB TCR repertoire was studied in order to detect clonal T-lymphocytes in both anatomical sites. 6 mice with neuroblastoma were analyzed using massive sequencing of TCR-β's CDR3 region. In 3 of them, tumors only were analyzed.

PB T-lymphocyte levels, analyzed through CD3, were 1,175,667 ± 773,224 cells/ml (32.23 ± 7.48%), whereas tumor T-lymphocyte levels were 261,500 ± 71,409 cells/ml (0.1 ± 1.7E-17%) (Fig. 3).

The 10 most frequent clones in the matched samples (tumor-PB) of 3 mice were analyzed, with a proportion of 11.09% ± 2.83% of the TCR repertoire in tumors, and a proportion of 1.59% ± 0.59% in the PB (p=0.024). These results are suggestive of clonotype enrichment within the tumor (Fig. 4).

**Table 1.** Sequences shared by tumor samples.

Amino acid	Sum (productive frequency)	Present in
CASSQDRGSYEQYF	0,614106	3
CASSPGQGAGEQYF	3	3
CASSQNQAPLF	0,195684	2
CASSQGQSSYEQYF	0,834896	2
CASSQDWGDEQYF	0,356441	2
CASSPRTGDYAEQFF	1	2
CASGDLGGSATLYF	2	2

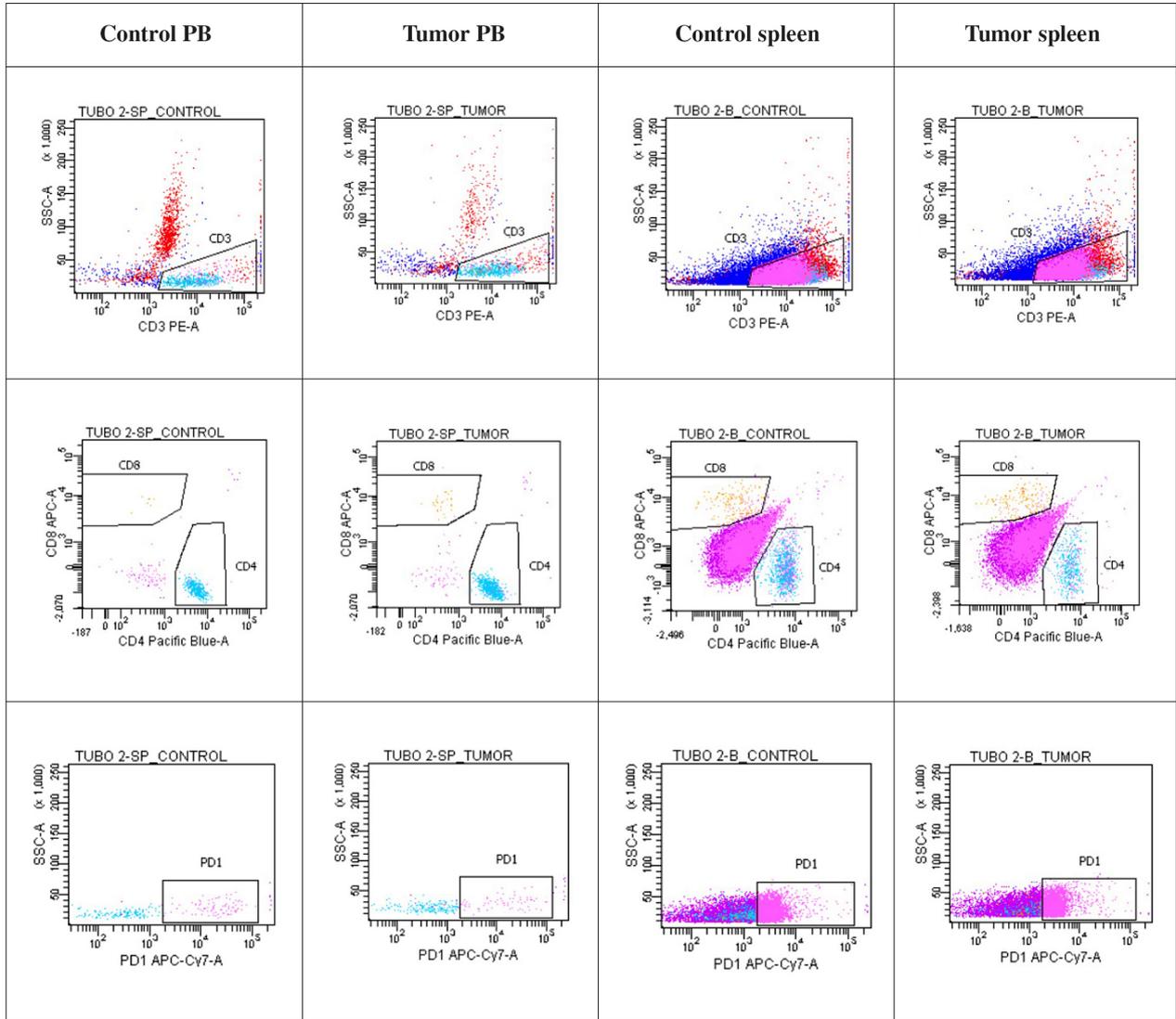
*Sequences shared by tumor samples that could represent TIL specific clonotypes against neuroblastoma.*

After studying T-cell clonal distribution in the tumors of the 6 mice, no common sequences for all of them were found. However, there were two sequences shared by three mice and five sequences shared by two mice, accounting for 7.64% of the TCR repertoire in all samples. These sequences could correspond to specific tumor clones against neuroblastoma (Table 1).

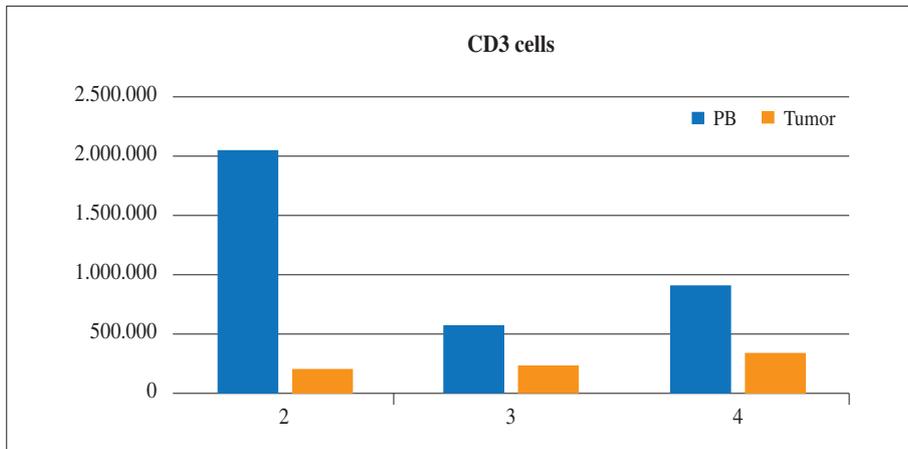
Finally, the analysis of the matched samples demonstrated an average 132 ± 79 shared sequences out of 242 ± 68 total sequences in the tumors, and 60,306 ± 25,765 sequences in the PB. Of the 10 most frequent clones in tumor samples, 9 were also found in the PB in two mice, and 6 in a third mice (Fig. 5).

### DISCUSSION

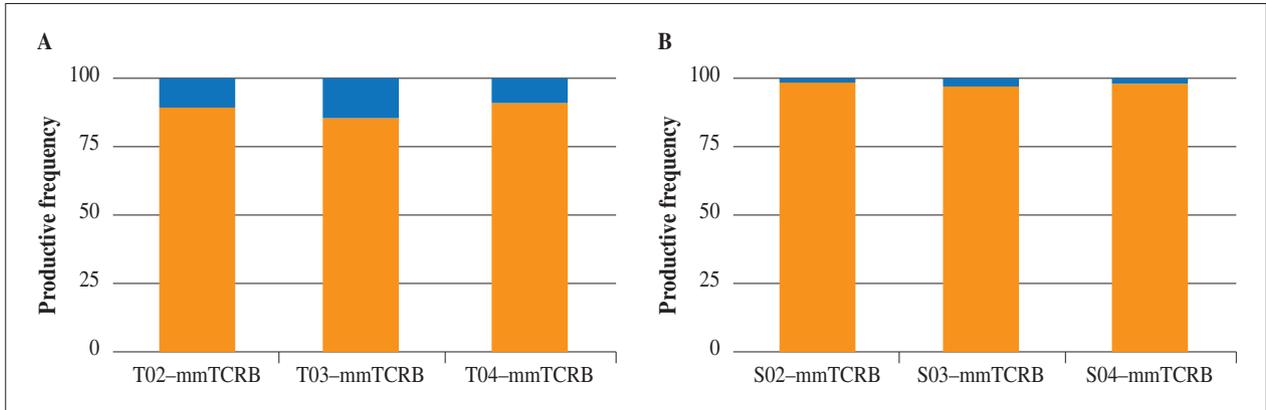
Today, TILs have demonstrated to be a useful therapeutic tool in the treatment of tumors such as melanoma<sup>(14)</sup>. According to a recent study, an anti-tumor immune response specific to each patient can be detected by identifying PD1<sup>+</sup> T-cells in the PB<sup>(7)</sup>. However, in the case of child tumors, the phenotypical and functional character-



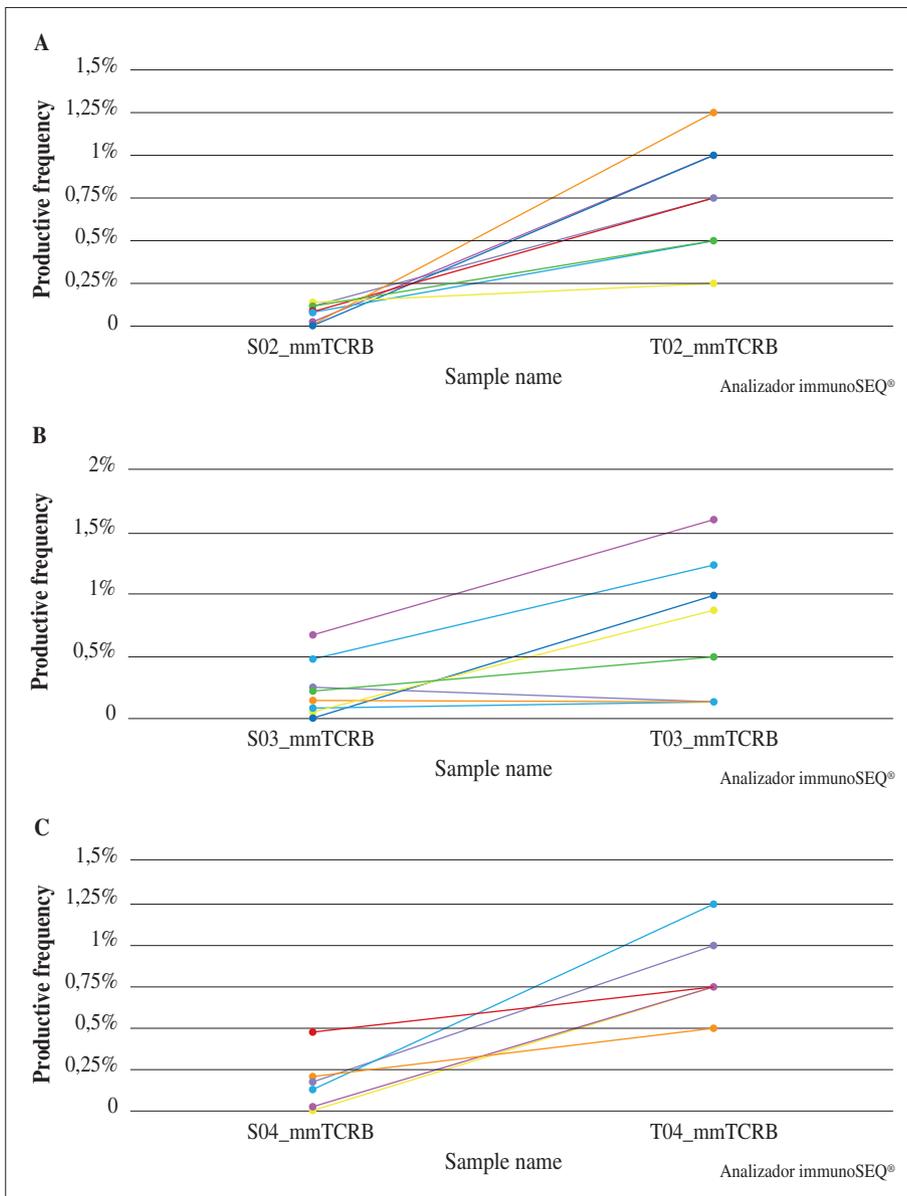
**Figure 2.** PB and spleen T-lymphocyte subpopulations following TIL injection in immunodeficient mice measured by flow cytometry.



**Figure 3.** PB and tumor T-lymphocyte levels in immunocompetent mice.



**Figure 4.** PB and tumor distribution of the 10 most frequent T-lymphocyte clones (blue) through TCR- $\beta$  sequencing. A) Mouse 02, mouse 03, and mouse 04 tumors. B) Mouse 02, mouse 03, and mouse 04 PB.



**Figure 5.** Graphic relationship of the TCR sequences shared by PB and tumor. A) Mouse 02. B) Mouse 03. C) Mouse 04.

istics of these immune populations are barely described in the literature, and the presence of PB circulating PD1<sup>+</sup> TILs in neuroblastoma has not been studied yet<sup>(7,15-19)</sup>. But if such presence was demonstrated, this would make surgical procedures for the extraction of ACT material unnecessary<sup>(7,15-20)</sup>.

This work studies whether neuroblastoma patients present PB circulating TILs. To do so, a murine model was used for simulation purposes. In order to identify such cells, an immunophenotypic criterion such as PD-1 expression in circulating T-lymphocytes was applied. Dr. Rosenberg's group demonstrated that PB PD-1<sup>+</sup> T-cells in melanoma have anti-tumor T-lymphocytes, suggesting they are a circulating TIL subpopulation<sup>(7-9)</sup>. In a first trial, the circulation of these cells in immunodeficient mice was studied, with CD4<sup>+</sup>PD1<sup>+</sup> being the main component in the PB, and barely no CD8<sup>+</sup>PD1<sup>+</sup> lymphocytes. Later, the presence of these cells in immunocompetent mice with neuroblastoma generated through NB36769 cell line injection was confirmed, with flow cytometry demonstrating a similar distribution in the PB, CD4<sup>+</sup>PD1<sup>+</sup> lymphocytes being predominant. This is the first work analyzing the presence of these cells in the PB in a preclinical neuroblastoma model. Among the very few examples available in the literature, Waki K et al.'s work is worth mentioning as this study on non-microcytic lung cancer (NMLC1) also showed predominance of PB CD4<sup>+</sup>PD1<sup>+</sup> T-lymphocytes with an effector-memory phenotype (CD45RA<sup>+</sup>CCR7<sup>-</sup>). In addition, melanoma studies have confirmed that CD8<sup>+</sup>PD1<sup>+</sup> TIL infiltration has a significant presence in tumors and a minor one in the PB<sup>(17,21)</sup>. These data are consistent with the results achieved in our experiments, where the CD4<sup>+</sup>PD1<sup>+</sup> subpopulation always exceeded the CD8<sup>+</sup>PD1<sup>+</sup> subpopulation, both in the tumor-implanted immunodeficient model (TILs were the only source of circulating T-lymphocytes) and in the neuroblastoma immunocompetent model.

The other criterion used to detect circulating TILs is the similarity of gene (and amino acid) sequences in TCR-β's hypervariable complementarity-determining region 3 (CDR3)<sup>(13,22-29)</sup> by means of new generation sequencing techniques (NGS)<sup>(22,24)</sup>, which allow for specific T-cell detection and tracking<sup>(25)</sup>. In the case of neuroblastoma, there are few publications analyzing TILs' TCR repertoire. In a study analyzing TILs' CDR3 region in 6 neuroblastoma patients, half of them had significant T-lymphocyte clonal expansion not observed in the blood, although this work was carried out using old sequencing techniques<sup>(28)</sup>. We also found oligoclonality in TILs with respect to PB. In the analysis of the 10 most frequent clones in tumors, clonotype enrichment was noted. Moreover, consistent with the literature, no sequences shared by all tumors analyzed were found. However, there were two sequences shared by three tumors and five sequences shared by two tumors. The presence of similar clone-specific sequences in the T-lymphocytes of different animals sharing MHC anti-

gens suggests that these T-lymphocyte subpopulations have expanded in response to an antigen shared by various individuals and presented by MHC molecules. Therefore, they could correspond to tumor specific clones against neuroblastoma. The relevance of our results regarding these findings lies in the *in vivo* demonstration of the potential response to common antigens expressed by the neuroblastoma, which could be useful for tracking T-cells accountable for this response, and also for ACT use. Analyzing the intra-tumor TCR repertoire in neuroblastoma patients using NGS techniques could allow for a better understanding of TIL tumor infiltration's diversity, reactivity level, and antigen specificity.

Dr. Rosenberg's group recently demonstrated an anti-tumor immune response specific to each patient can be detected by identifying PD1<sup>+</sup> T-cells in the PB<sup>(7)</sup>. Our flow cytometry results corroborate the presence of subpopulations potentially containing anti-tumor T-lymphocytes (PD1<sup>+</sup>) in the PB of neuroblastoma mice. In addition, similarly to Gros et al.'s work, tumor infiltration immunosequencing allowed us to detect shared clonotypes between matched samples of tumor and PB, which reinforces the hypothesis that T-cells involved in anti-tumor immune response circulate in the body also in the case of neuroblastoma.

Our work has a number of limitations. First, even though murine cancer models have been used for the study of anti-tumor immune response and the development of immunotherapy strategies in preclinical experiments for decades, there are few data available on TCR repertoire, which complicates comparison<sup>(25)</sup>. Regarding immunophenotypic characteristics, CD39 and CD103 expression were not analyzed. It should be noted that these markers in co-expression identify CD8 TILs with an anti-tumor capacity in various human tumors<sup>(30)</sup>. Finally, the number of preclinical cases was limited as a result of the high costs involved, which reduces statistical power when it comes to demonstrating results. However, the trends observed in our experiments encourage us to pursue this line of research given the potential benefits it could provide for pediatric patients with neuroblastoma and other solid tumors.

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