Title: ***Characterization of the NK Regulatory Landscape in the Tumor Microenvironment of Pediatric Sarcomas***

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Significance & Hypothesis/Techniques

Pediatric sarcomas, including osteosarcoma (OS), Ewing sarcoma (EWS), and rhabdomyosarcoma (RMS), remain a major clinical challenge, especially in metastatic and relapsed disease, where survival has plateaued and no new effective therapies have been approved in recent decades. These tumors are typically characterized by an immunologically "cold" tumor microenvironment (TME), with limited immune infiltration and dominant immune evasion mechanisms. Natural killer (NK) cells, key effectors of innate immunity, are promising candidates for immunotherapy due to their potent antitumor activity; however, the mechanisms regulating NK-cell activity in pediatric sarcomas remain poorly defined. We hypothesize that (i) counteracting NK-cell suppression in the TME may enhance NK-cell infiltration, (ii) effective NK-cell infiltration may contribute to tumor control and prevention of metastatic progression, and (iii) remodeling the sarcoma TME may promote immune-mediated tumor destruction. The goal of this study is to characterize immune infiltration and regulation of NK-cell activity in pediatric sarcomas to identify actionable targets.

Innovation

This study provides an NK-focused characterization of the pediatric sarcoma TME by integrating protein-level analyses and transcriptomic validation across multiple sarcoma subtypes. This approach identifies immunoregulatory pathways and actionable immune checkpoints with direct translational relevance, including biomarker-driven patient selection in clinical trials.

Approach (Materials & Methods)

A total of 50 pediatric sarcoma samples were analyzed at La Paz University Hospital since 2023 (39 surgical specimens, 11 biopsies), including OS (n=16), OS metastases (n=9), EWS (n=11), RMS (n=4), and other subtypes (n=10). Fresh tumor samples (n=12) were processed for flow cytometric analysis of tumor-infiltrating lymphocytes (TILs). In parallel, tumor expression of ligands regulating NK-cell activity was assessed in patient samples, and semi-quantitative H-score analysis was performed in FFPE tissues. In silico transcriptomic analyses of OS and EWS cohorts were performed for validation.

Results

Pediatric sarcomas exhibited a predominantly immune-cold phenotype with scarce immune infiltration. NK cells consistently represented the predominant immune population among TILs with marked heterogeneity (Figure 1). Tumor cells displayed a conserved immune-evasive phenotype, with high expression of inhibitory ligands such as B7-H3 and CD155. Others, including FAS-L, HLA-I, CD112, PD-L1, and HLA-E, showed variable expression across sarcoma subtypes. In contrast, activating ligands such as MICA, MICB, and ULBP1–6 showed low expression in analyzed primary samples, suggesting insufficient activating signals to trigger robust NK-cell responses (figure 2). H-score analysis confirmed low and heterogeneous expression of activating ligands in tumor tissues, with ULBP3 showing the highest expression among NKG2D ligands. In silico analyses of independent OS and EWS cohorts demonstrated

that MICA and MICB were the most highly expressed NKG2D ligands at the RNA level, although overall expression remained variable (Figure 3).

Discussion & Impact

This study provides valuable insights into the immune landscape of pediatric sarcomas, particularly regarding NK-cell function and tumor immune evasion. The overexpression of B7-H3 highlights its potential as an immunotherapeutic target and supports development of B7-H3-specific CAR-NK strategies. Importantly, this work establishes a biomarker-driven framework for patient stratification and informs clinical translation, including H-score-guided selection in clinical trials and the design of future NK-based immunotherapies.

Figures:

Figure 1. Expression of Tumor Infiltrating Lymphocytes (TILs) in pediatric sarcoma primary samples

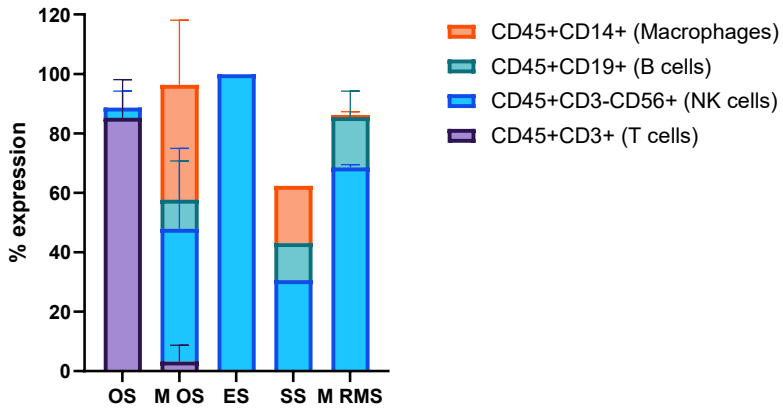

Figure 1. The presence of Macrophages (CD45+CD14+), B cells (CD45+CD19+), NK cells (CD45+CD3-CD56+) and T cells (CD45+CD3+) in the primary patient sarcoma cells samples. OS = Osteosarcoma (n=4), M OS = Metastatic osteosarcoma (n=4), ES = Ewing Sarcoma (n=1), SS = Synovial Sarcoma (n=1), M RMS = Metastatic Rhabdomyosarcoma (n=2).

Figure 2. Expression of NK inhibitory and activating ligands in pediatric sarcoma primary samples

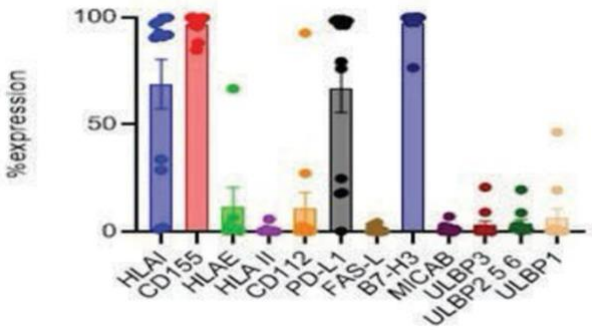

Figure 2. The expression levels of inhibitory and activating NK ligands on the primary patient sarcoma cells generated in the laboratory (n=12)

Figure 3. RNA expression of NKG2D ligands in Osteosarcoma and Ewing Sarcoma primary samples

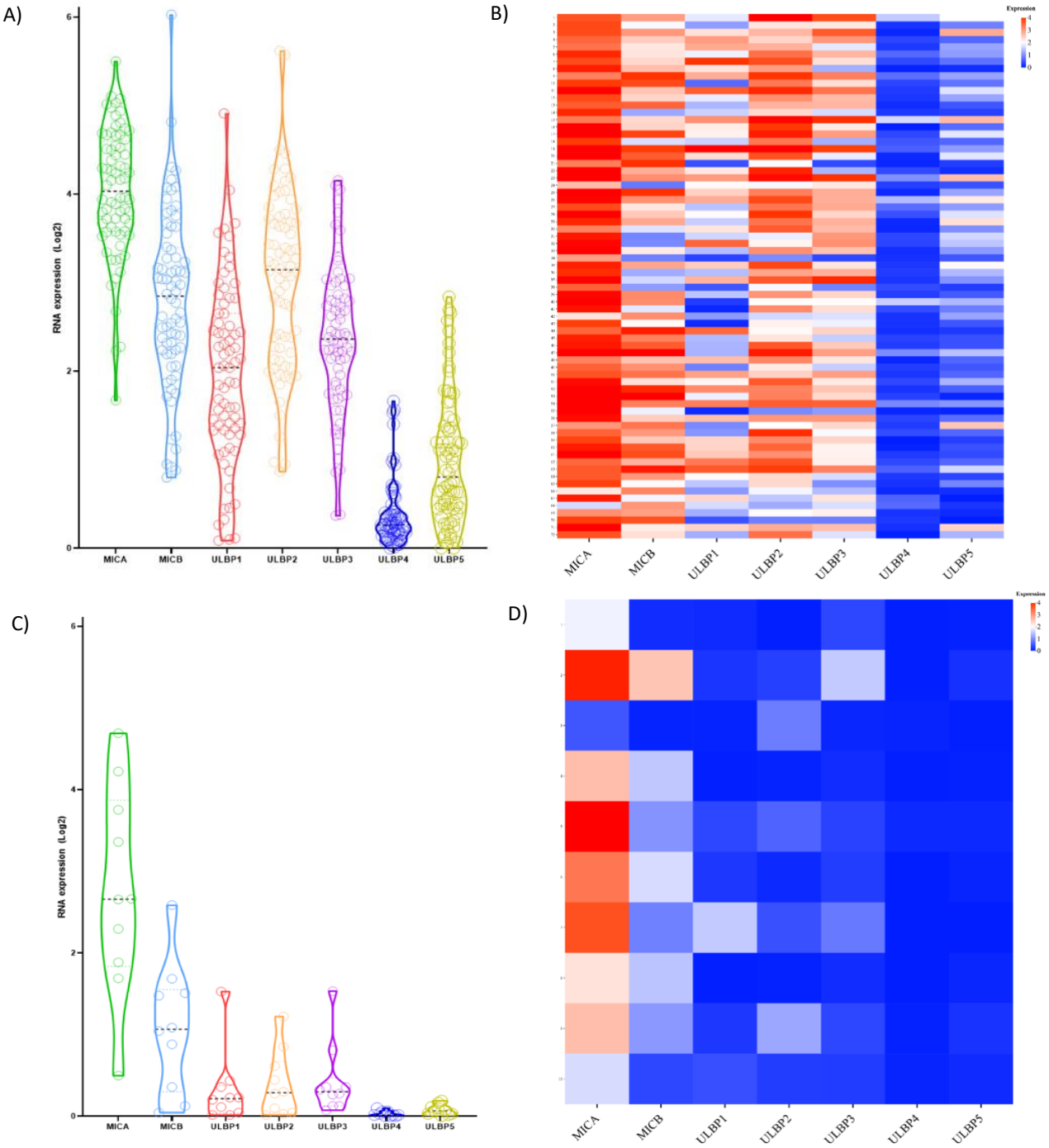

Figure 3. RNA expression (Log2) of NKG2D ligands in Osteosarcoma and Ewing sarcoma primary samples. **A)** and **C)** Violin plots representing RNA expression (Log2) in Osteosarcoma (A) and Ewing Sarcoma (C) samples. The median is represented by dashed line. **B)** and **D)** Heatmap representing RNA expression (Log2) in osteosarcoma (B) and Ewing Sarcoma (D) samples.

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