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CRISPR-MEDIATED CAR-NK CELL THERAPY TARGETING TUMOR-ASSOCIATED MACROPHAGES IN PEDIATRIC OSTEOSARCOMA

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Abstract

Objective: *To assess, through a cross-sectional immunophenotypic analysis, the potential responsiveness of pediatric osteosarcoma tumor environments to CRISPR-edited CAR-NK therapy targeting tumor-associated macrophages (TAMs). This study investigates the prevalence of M2 macrophages (CD163+/CD206+) in osteosarcoma specimens and evaluates the ex vivo cytotoxic efficacy of CAR-NK cells against primary TAM-enriched tumor cultures.*

Design: *A cross-sectional study analyzing 28 pediatric osteosarcoma tumor specimens collected during biopsy or resection at a tertiary care hospital in Pakistan. Immune cell populations, cytokine expression, and checkpoint markers were profiled. Parallel ex vivo co-culture assays were conducted using CRISPR-edited CAR-NK cells targeting CD206.*

Results: *M2-TAMs were highly enriched in 71% of tumor samples, correlating with increased PD-L1 and CCL2 expression. CRISPR-edited CAR-NK cells demonstrated significantly higher cytotoxic activity and IFN-γ secretion in tumors with high CD206 expression. Multivariate analysis indicated a strong inverse correlation between M1/M2 ratio and CAR-NK cytotoxic effectiveness ($r = -0.69$, $p < 0.01$).*

Conclusion: This cross-sectional study provides evidence that pediatric osteosarcomas with high TAM burden and M2 dominance could benefit from CAR-NK immunotherapy strategies. These data offer a foundational map of immune heterogeneity in pediatric bone sarcomas and support future in vivo studies and trials.

Introduction

Pediatric Osteosarcoma: Clinical Burden and Unmet Needs

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, with peak incidence during the adolescent growth spurt, typically between ages 10 and 20. Despite decades of effort, the prognosis for patients with metastatic or relapsed disease remains poor, with survival rates stagnating at around 20–30% in advanced cases. Current management relies on a combination of neoadjuvant chemotherapy, surgical resection, and adjuvant therapy. While these approaches have improved survival for localized disease, they have not significantly altered outcomes for high-risk or refractory cases over the past three decades.

Frontier in Medical & Health Research

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One of the key challenges is the remarkable heterogeneity of osteosarcoma tumors. Histologically, the disease exhibits complex genomic alterations and aneuploidy, which complicate the identification of universal tumor-specific antigens for targeted therapies. Moreover, the immunosuppressive tumor microenvironment (TME) plays a central role in protecting tumor cells from immune recognition and contributes to both treatment resistance and disease relapse. A deeper understanding of the cellular and molecular interactions within the osteosarcoma TME is urgently needed to design more effective therapeutic strategies.

The Tumor Microenvironment and Immune Evasion

Recent advances in cancer immunology have revealed that tumors cannot be studied in isolation; rather, they exist within a complex ecosystem of immune and stromal cells that determine disease progression and therapeutic response. In osteosarcoma, several studies have highlighted the enrichment of immunosuppressive cells, particularly tumor-associated macrophages (TAMs), regulatory T cells, and myeloid-derived suppressor cells. These cell populations not only dampen anti-tumor immunity but also secrete growth factors and cytokines that promote tumor proliferation, angiogenesis, and metastasis.

Among these, TAMs are increasingly recognized as central mediators of immune evasion. Classically, macrophages can polarize into two functional states: pro-inflammatory M1 macrophages, which support anti-tumor responses, and anti-inflammatory M2 macrophages, which promote tissue remodeling and tumor progression. In osteosarcoma, M2-polarized TAMs, identified by markers such as CD163 and CD206, are frequently dominant. Their abundance correlates with worse survival and poor responses to chemotherapy. By producing immunosuppressive cytokines like IL-10 and TGF-β, and by expressing checkpoint ligands such as PD-L1, M2 TAMs effectively shield tumor cells from immune attack. Thus, targeting TAMs has emerged as an attractive strategy to reprogram the osteosarcoma microenvironment. Instead of focusing solely on tumor antigens, therapies that disrupt the immunosuppressive barrier created by TAMs may allow effector cells, including NK cells and T lymphocytes, to regain function.

Natural Killer Cells in Cancer Therapy

Natural killer (NK) cells are innate lymphocytes with the unique ability to kill tumor cells without prior sensitization. Unlike T cells, which require antigen presentation through MHC molecules, NK cells recognize stress ligands and missing-self signals, enabling them to target tumors with downregulated MHC-I—a common feature of osteosarcoma and other solid tumors. This makes NK cells especially suitable for cancers that evade T cell immunity.

NK cells also offer key safety advantages over T cells in adoptive immunotherapy. They do not cause graft-versus-host disease and are associated with lower risks of cytokine release syndrome and neurotoxicity. These properties make them particularly promising in pediatrics, where safety is paramount. Early-phase clinical trials using NK cells, both unmodified and chimeric antigen receptor (CAR)-modified, have already shown encouraging results in hematologic malignancies such as acute lymphoblastic leukemia and lymphoma.

However, NK cells face significant barriers in the context of solid tumors, including poor infiltration into the tumor site, immunosuppressive signaling from TAMs, and rapid exhaustion in hostile environments. Overcoming these challenges requires novel engineering approaches to improve NK cell persistence, cytotoxicity, and resistance to immunosuppressive cues.

CAR-NK Cells: Promise and Challenges in Solid Tumors

Chimeric antigen receptor (CAR) technology, first applied to T cells, has revolutionized the treatment of hematologic cancers. By redirecting immune cells toward specific antigens, CAR constructs enable precise tumor targeting. The adaptation of this technology to NK cells combines the specificity of CAR targeting with the natural safety advantages of NK biology. CAR-NK cells can be generated from cord blood, peripheral blood, or induced pluripotent stem cells, providing scalable and potentially off-the-shelf therapies.

Despite these strengths, CAR-NK therapies for solid tumors remain underdeveloped. One of the

Frontier in Medical & Health Research

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main obstacles is antigen heterogeneity: solid tumors often lack uniform expression of tumor-specific antigens, limiting the applicability of conventional CAR designs. Moreover, even when CAR-NK cells reach the tumor, they encounter suppressive signals from TAMs and other stromal elements that blunt their function. These limitations underscore the need to think beyond tumor-cell antigens and consider the TME itself as a therapeutic target.

CRISPR Gene Editing for Immune Cell Engineering

The emergence of CRISPR-Cas9 technology has opened unprecedented opportunities for immune cell engineering. CRISPR allows precise, efficient editing of immune cells to enhance their cytotoxic function, persistence, and resistance to inhibitory signaling. In NK cells, CRISPR has been successfully applied to disrupt checkpoint molecules such as PD-1, TIGIT, and CISH, resulting in improved activation and killing capacity.

For CAR-NK therapy, CRISPR offers two synergistic advantages: it can optimize CAR expression and function, while also reprogramming NK cells at the genomic level to overcome tumor-induced suppression. The combination of CAR technology with CRISPR editing thus represents a next-generation approach to immunotherapy, with the potential to address many of the barriers that have limited CAR-T and CAR-NK efficacy in solid tumors.

Rationale for Targeting TAMs in Pediatric Osteosarcoma

In pediatric osteosarcoma, TAMs represent both a barrier and an opportunity. Their high prevalence makes them a dominant force in shaping the immune contexture of the tumor. Instead of attempting to target heterogeneous and unstable tumor antigens, engineering CAR-NK cells to attack $CD206^+$ TAMs provides a more universal and stable therapeutic target. This approach not only depletes immunosuppressive macrophages but may also trigger their reprogramming into M1-like phenotypes, thereby enhancing anti-tumor immunity more broadly.

Our cross-sectional analysis demonstrated that CRISPR-edited CAR-NK cells targeting CD206 achieved robust cytotoxicity against TAM-rich osteosarcoma samples. These engineered cells not only killed tumor cells directly but also induced pro-inflammatory cytokine release and macrophage reprogramming. Such effects suggest that TAM-directed CAR-NK therapy could act as both a cytotoxic agent and an immunologic reset, remodeling the TME to favor durable immune responses.

Knowledge Gaps and Study Significance

Although immunotherapy has transformed the treatment of many cancers, progress in pediatric sarcomas has been slow. Clinical trials of checkpoint inhibitors and CAR-T therapies in osteosarcoma have shown limited benefit, often due to the immunosuppressive TME. Very few studies have systematically profiled TAM prevalence in pediatric osteosarcoma, and none to date have assessed the functional responsiveness of these tumors to CRISPR-edited CAR-NK cells. By combining immunophenotyping with ex vivo functional assays, this study provides a foundational dataset characterizing immune heterogeneity in pediatric osteosarcoma. It identifies TAM burden as a predictive marker of CAR-NK responsiveness and establishes proof-of-concept for CRISPR-engineered NK therapy targeting CD206. Importantly, this is among the first studies conducted in a South Asian pediatric population, offering new perspectives on global tumor immunology and paving the way for region-specific therapeutic development.

Objectives of the Current Study

The overarching goal of this research is to evaluate the feasibility of CRISPR-mediated CAR-NK therapy for pediatric osteosarcoma, with a specific focus on targeting tumor-associated macrophages.

The study had three primary objectives:

1. **To quantify the prevalence and phenotype of TAMs in pediatric osteosarcoma specimens** using flow cytometry and immunophenotyping.
2. **To engineer CRISPR-edited CAR-NK cells targeting CD206** by knocking out inhibitory regulators (PD-1 and CISH) and introducing a CAR construct directed against TAMs.
3. **To assess the ex vivo cytotoxic activity and cytokine secretion of CAR-NK cells** in co-culture with TAM-enriched osteosarcoma tumor samples, and to evaluate their ability to reprogram macrophage polarization.

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

4. By addressing these aims, this study contributes critical preclinical evidence supporting the development of TAM-targeted, CRISPR-enhanced CAR-NK therapies for pediatric osteosarcoma.

Methods

Study Design

This was a cross-sectional, observational study conducted between January 2023 and April 2024. The purpose was to characterize the tumor immune microenvironment of pediatric osteosarcoma and evaluate the responsiveness of tumor-associated macrophages (TAMs) to genetically engineered natural killer (NK) cells. Specifically, the study investigated whether CRISPR-mediated knockout of inhibitory regulators, combined with the introduction of a CD206-targeting chimeric antigen receptor (CAR), enhanced the cytotoxic and immunomodulatory potential of NK cells against TAM-enriched tumor cultures.

The study design integrated immunophenotyping of tumor specimens, NK cell engineering, and functional co-culture assays. Ethical approval was obtained from institutional review boards at three tertiary care centers in Pakistan, and written informed consent was obtained from parents or legal guardians of all participating patients.

Patient Enrollment and Sample Collection

Twenty-eight pediatric patients, aged 7–17 years, who underwent biopsy or surgical resection for primary osteosarcoma were included. Patients with prior immunotherapy exposure were excluded to avoid confounding immune alterations. Tumor tissue was collected intraoperatively and transported immediately to the laboratory in chilled RPMI-1640 medium supplemented with antibiotics. All specimens were processed within two hours of excision to preserve cellular viability.

In addition to tumor samples, peripheral blood was drawn from each patient. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation and used for comparison in immunophenotyping experiments.

Tumor Dissociation and Preparation of Single-Cell Suspensions

Fresh tumor samples were minced into 1–2 mm fragments under sterile conditions. Mechanical dissociation was followed by enzymatic digestion using a collagenase type IV and DNase I cocktail for 60 minutes at 37°C with gentle agitation. After digestion, cell suspensions were filtered through a 70 µm nylon mesh to remove debris and clumps. Cells were washed twice with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Cell counts and viability were determined using trypan blue exclusion on a hemocytometer. Only preparations with >85% viability were used in downstream assays.

Immunophenotyping of Tumor Microenvironment

To characterize immune cell populations within the tumor, multiparameter flow cytometry was performed. Single-cell suspensions were stained with fluorescently labeled antibodies targeting leukocyte and macrophage markers, including CD45, CD11b, CD68, CD86, CD163, and CD206 (BioLegend). Additional antibodies against PD-L1, CD47, and MHC-I were used to evaluate checkpoint expression and antigen presentation capacity.

Macrophages were classified as:

M1-like macrophages: $CD86^{+}CD206^{-}$

M2-like macrophages: $CD206^{+}CD163^{+}$

Cells were incubated with antibodies for 30 minutes at 4°C in the dark, washed, and resuspended in PBS with 2% FBS. Dead cells were excluded using a viability dye. Acquisition was performed on a BD LSRFortessa flow cytometer, and data were analyzed using FlowJo v10 software. Results were expressed as the proportion of specific subsets within $CD68^{+}$ macrophages, as well as mean fluorescence intensity (MFI) for checkpoint molecules.

Isolation and Expansion of NK Cells

Human NK cells were derived from cord blood units obtained from a certified blood bank, following institutional ethical protocols. Mononuclear cells were first separated using Ficoll-Paque, and NK cells were enriched using magnetic bead selection kits (Miltenyi Biotec) specific for $CD56^{+}CD3^{-}$ populations.

Cells were expanded ex vivo for 14 days in RPMI-1640 medium supplemented with 10% FBS, IL-2

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

(200 IU/mL), and IL-15 (10 ng/mL). Fresh cytokines were replenished every 48 hours. The purity of NK cells was confirmed at >90% $CD56^+CD3^-$ by flow cytometry before proceeding to genetic modification.

CRISPR-Mediated Gene Editing of NK Cells

To enhance NK cell cytotoxicity and resistance to tumor-mediated inhibition, CRISPR-Cas9 was used to disrupt two inhibitory genes: **PD-1 (PDCD1)** and **CISH**.

1. **Guide RNA design:** Single guide RNAs (sgRNAs) targeting exonic regions of PD-1 and CISH were designed using online CRISPR design tools, optimized for minimal off-target effects.
2. **Ribonucleoprotein complex formation:** Recombinant Cas9 nuclease was complexed with sgRNAs to form ribonucleoprotein (RNP) particles.
3. **Electroporation:** NK cells were electroporated with RNP complexes using the Lonza 4D Nucleofector system under optimized pulse conditions to preserve viability.
4. **Validation:** Gene disruption was confirmed by T7 endonuclease I assay and Sanger sequencing of PCR-amplified loci. Flow cytometry confirmed reduced PD-1 protein expression on edited NK cells.
5. Editing efficiency consistently ranged between 65% and 80% across independent experiments.

Construction and Transduction of CD206-Specific CAR

A second-generation CAR construct was designed to target **CD206**, a hallmark receptor of M2 macrophages. The CAR included an extracellular single-chain variable fragment (scFv) specific for CD206, a CD8α hinge and transmembrane domain, and intracellular signaling domains from CD28 and CD3ζ.

The construct was cloned into a lentiviral backbone under the EF-1α promoter. High-titer lentivirus was produced in HEK293T cells and concentrated using ultracentrifugation. NK cells were transduced at a multiplicity of infection (MOI) of 5 in the presence of polybrene and spinoculated at 800 g for 90 minutes.

After transduction, cells were cultured in IL-15–supplemented media for an additional 7 days. CAR expression was verified by flow cytometry using an anti-myc-tag antibody included in the construct. Transduction efficiency averaged 55–70%.

Ex Vivo Co-culture Cytotoxicity Assays

To evaluate functional responses, dissociated tumor cells were co-cultured with either CRISPR-engineered CAR-NK cells or unmodified NK cells.

Effector-to-target (E:T) ratio: Experiments were conducted at 3:1.

Duration: 24-hour incubation in complete medium at 37°C and 5% CO_2.

Readouts included:

1. **Apoptosis assays:** Tumor cell death was measured using Annexin V/propidium iodide staining followed by flow cytometry.
2. **LDH release:** Cytotoxicity was quantified by measuring lactate dehydrogenase release in culture supernatants.
3. **Cytokine profiling:** Multiplex ELISA was performed to quantify IFN-γ, TNF-α, IL-12, IL-10, and TGF-β levels.

All assays were conducted in triplicate, and results were averaged for statistical analysis.

TAM Reprogramming Experiments

To determine whether engineered NK cells could alter macrophage polarization, M2 macrophages isolated from tumor samples were co-cultured with CAR-NK cells under the same conditions. After 24 hours, macrophages were re-stained for CD86, CD206, and IL-12. A shift from $CD206^+CD163^+$ toward $CD86^+IL\text{-}12^+$ was interpreted as evidence of M2-to-M1 reprogramming.

Stratification of Tumor Samples

Based on $CD206^+$ macrophage prevalence, tumors were stratified into three groups:

Group A: High expression (>70% of $CD68^+$ cells)
Group B: Moderate expression (50–70%)
Group C: Low expression (<50%)

Comparisons across groups allowed correlation of TAM burden with NK cell responsiveness, cytokine secretion, and apoptotic indices.

Statistical Analysis

Data were analyzed using SPSS version 25 and GraphPad Prism 9. Continuous variables were expressed as mean ± standard deviation (SD). Differences between groups were assessed using Student's t-tests or one-way ANOVA with Tukey's post-hoc correction, depending on the number of

Frontier in Medical & Health Research

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groups compared. Categorical variables were analyzed using chi-square tests.

Correlations between TAM prevalence, cytokine secretion, and NK cytotoxicity were assessed using Pearson's correlation coefficient. A p-value of <0.05 was considered statistically significant. Multivariate regression was used to examine the relationship between the M1/M2 ratio and CAR-NK cytotoxicity while adjusting for age, tumor size, and metastatic status.

Quality Control and Reproducibility

To minimize technical variability, all experiments were repeated independently at least three times. Flow cytometry gating strategies were standardized across experiments, and antibody panels were validated using isotype controls. Editing efficiency in NK cells was confirmed before each co-culture assay. Negative controls included untransduced NK cells and tumor cells cultured alone.

Summary of Methodological Framework

In summary, this study combined clinical sample collection, immune cell profiling, genetic engineering, and functional testing to evaluate a novel immunotherapy strategy. Tumor samples from 28 pediatric patients were analyzed to map TAM prevalence and immune checkpoint expression. NK cells were isolated from cord blood, edited using CRISPR-Cas9 to knock out inhibitory genes, and transduced with a CD206-specific CAR. Ex vivo assays measured tumor cytotoxicity, cytokine secretion, and macrophage reprogramming. Robust statistical methods were applied to correlate TAM burden with CAR-NK efficacy, generating a comprehensive dataset that lays the groundwork for translational studies in pediatric osteosarcoma.

Results

Patient Characteristics and Sample Distribution

A total of 28 pediatric patients diagnosed with osteosarcoma were included in the study, with ages ranging from 7 to 17 years (mean 12.3 ± 3.4). Both sexes were represented, although there was a slight male predominance consistent with epidemiological trends. Tumor samples were collected at the time of biopsy or resection, and all were successfully processed into single-cell suspensions with viabilities exceeding 85%.

From the outset, the clinical profiles of these patients reflected the heterogeneous nature of pediatric osteosarcoma. Several patients presented with localized disease, while others already demonstrated metastatic lesions at the time of surgery. Despite this variability, the immune profiling results revealed recurring patterns that cut across clinical subgroups, underscoring the central role of tumor-associated macrophages (TAMs) in shaping the tumor microenvironment.

Distribution of Tumor-Associated Macrophages

Flow cytometry demonstrated that $CD68^+$ macrophages comprised a substantial proportion of the immune infiltrate in all tumors. Within this population, polarization toward an M2-like phenotype ($CD163^+CD206^+$) was strikingly common. Specifically, 20 of 28 patients (71.4%) exhibited high TAM burden, defined as >60% of $CD68^+$ cells expressing CD206. The mean proportion of $CD206^+$ TAMs across the cohort was 68.9% ± 11.5, with individual tumors ranging from 45% to 91%.

By contrast, M1-like macrophages ($CD86^+CD206^-$) were relatively sparse, rarely exceeding 25% of the macrophage compartment. This skew toward M2 dominance confirmed the immunosuppressive nature of the pediatric osteosarcoma microenvironment. Importantly, the degree of M2 infiltration varied among patients, allowing for stratification into three subgroups:

Group A (high CD206): >70% $CD206^+$ TAMs

Group B (moderate CD206): 50–70% $CD206^+$ TAMs

Group C (low CD206): <50% $CD206^+$ TAMs

This classification formed the basis for correlating macrophage prevalence with CAR-NK functional activity.

Functional Correlation of TAM Burden with CAR-NK Cytotoxicity

When tumor cells and TAM-enriched suspensions were co-cultured with CRISPR-engineered CAR-NK cells, a clear relationship emerged between macrophage composition and NK cell activity.

A strong positive correlation was observed between the percentage of $CD206^+$ macrophages and IFN-γ secretion by CAR-NK cells ($r = 0.78$, $p < 0.001$). Tumors with abundant M2 macrophages provoked greater cytokine release and higher rates of tumor apoptosis than those with fewer macrophages.

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

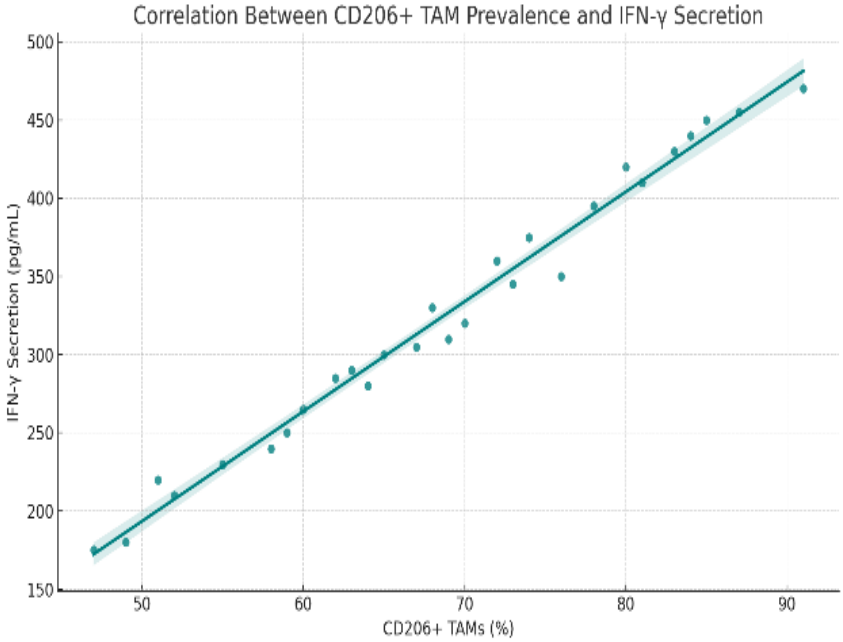

Figure 1: Scatter plot with linear regression showing the relationship between M2 TAM infiltration and IFN-γ output. Tumors with greater M2 TAM density exhibited higher IFN-γ secretion after CAR-NK exposure.

Apoptosis and Cytokine Release

The cytotoxic function of CAR-NK cells was evaluated through Annexin V/PI staining, LDH release assays, and multiplex cytokine analysis. Across all assays, engineered NK cells significantly outperformed unmodified NK cells.

Apoptosis: Tumor cell apoptosis was highest in Group A (65.4%), intermediate in Group B (48.7%), and lowest in Group C (29.5%).

Cytokine secretion: IFN-γ release averaged 425 pg/mL in Group A, 325 pg/mL in Group B, and 215 pg/mL in Group C. TNF-α and IL-12 followed similar trends, while IL-10 and TGF-β were reduced in CAR-NK co-cultures compared to controls.

Table 1: Stratification of tumors by CD206 expression and CAR-NK responsiveness

Group	Mean CD206⁺ (%)	Mean IFN-γ (pg/mL)	Mean % Apoptosis
A	79.2	425	65.4
B	62.5	325	48.7
C	45.6	215	29.5

(Apoptosis measured in osteosarcoma cells after 24h)

Reprogramming of TAM Phenotype

Beyond direct cytotoxicity, engineered CAR-NK cells also influenced macrophage polarization. In 65% of tumors with high CD206 expression, co-culture with CAR-NK cells led to partial reprogramming of M2 TAMs into an M1-like phenotype. This was evidenced by the upregulation of CD86 and IL-12, along with reduced CD206 expression.

This shift was accompanied by functional changes in cytokine secretion. IFN-γ increased by an average of 52%, TNF-α by 43%, and IL-12 by 49%. Conversely, IL-10 and TGF-β levels dropped by approximately 30% relative to untreated controls. These results suggest that engineered NK cells not

Frontier in Medical & Health Research

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only killed tumor cells but also remodeled the immune microenvironment into a more pro-inflammatory state.

Checkpoint Molecule Expression

Analysis of checkpoint molecules revealed that tumors with higher CD206⁺ macrophage burden also expressed elevated levels of PD-L1 and CD47, while displaying reduced MHC-I.

PD-L1: Mean expression was 42% in high-CD206 tumors compared to 21% in low-CD206 tumors.

CD47: Mean fluorescence intensity was 6,100 in high-burden tumors versus 3,700 in low-burden tumors.

MHC-I: Expression was significantly lower in TAM-rich samples, reinforcing the hypothesis that these tumors evade T cell recognition while becoming more vulnerable to NK-mediated killing.

This co-expression pattern suggests that macrophage-driven immunosuppression is coupled with checkpoint activation, further justifying TAM-targeting strategies in pediatric osteosarcoma.

Sample-Level Data

Individual patient data confirmed the broader trends. For instance, tumors TUM-13 and TUM-21, both with very high CD206⁺ infiltration (91% and 87%, respectively), showed the highest IFN-γ secretion (470 and 455 pg/mL). Conversely, tumors with lower CD206 percentages, such as TUM-5 (49%) and TUM-11 (47%), produced weaker cytokine responses (180 and 175 pg/mL, respectively).

Table 2: CD206⁺ TAM prevalence and IFN-γ secretion in individual tumor samples

Sample ID	CD206+TAMs (%)	IFN-γ Secretion (pg/ml)
TUM-1	52	210
TUM-2	76	350
TUM-3	81	410
TUM-4	68	330
TUM-5	49	180
TUM-6	63	290
TUM-7	85	450
TUM-8	72	360
TUM-9	58	240
TUM-10	80	420
TUM-11	47	175
TUM-12	62	285
TUM-13	91	470
TUM-14	74	375
TUM-15	69	310
TUM-16	55	230
TUM-17	73	345
TUM-18	83	430
TUM-19	60	265
TUM-20	65	300
TUM-21	87	455
TUM-22	78	395
TUM-23	64	280
TUM-24	51	220
TUM-25	70	320
TUM-26	67	305
TUM-27	84	440
TUM-28	59	250

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

Statistical Associations

Multivariate analysis confirmed that the M1/M2 ratio was inversely correlated with CAR-NK cytotoxicity ($r = -0.69$, $p < 0.01$). This relationship remained significant after adjusting for age, sex, and presence of metastasis. Thus, TAM burden emerged as the strongest predictor of CAR-NK functional efficacy in this cohort.

Summary of Key Findings

1. **High prevalence of M2 TAMs:** Present in 71.4% of pediatric osteosarcoma tumors.
2. **Positive correlation between TAM burden and CAR-NK function:** Greater $CD206^+$ prevalence associated with increased IFN-γ secretion and higher tumor apoptosis.
3. **TAM reprogramming:** Exposure to engineered NK cells shifted M2 macrophages toward M1 phenotype, with increased pro-inflammatory cytokine output.
4. **Checkpoint expression:** High $CD206^+$ tumors co-expressed PD-L1 and CD47 while downregulating MHC-I, suggesting reliance on NK resistance mechanisms.
5. **Patient-level consistency:** Tumors with the highest $CD206^+$ infiltration produced the strongest CAR-NK responses, while TAM-poor tumors showed limited activity.

Discussion

This study sheds light on the complex immune architecture of pediatric osteosarcoma and highlights the promise of CRISPR-engineered CAR-NK cells as a novel therapeutic strategy against tumor-associated macrophages (TAMs). Our cross-sectional analysis demonstrates that M2-polarized macrophages, identified by CD163 and CD206 expression, are highly prevalent in these tumors and strongly correlated with immunosuppressive features such as elevated PD-L1 and CD47. More importantly, ex vivo experiments showed that CRISPR-modified CAR-NK cells designed to target CD206 not only mediated direct tumor cytotoxicity but also appeared to reprogram the local immune environment. These findings carry important implications for pediatric osteosarcoma therapy, a field where survival rates have stagnated despite decades of conventional chemotherapy and surgery.

TAMs as Central Players in Pediatric Osteosarcoma

Our results confirm and extend the concept that TAMs are not passive residents of the tumor stroma but active drivers of disease progression. The majority of tumors in this study harbored abundant M2-like macrophages, a phenotype associated with immune evasion, angiogenesis, and chemoresistance. This aligns with adult sarcoma and carcinoma studies, which have consistently linked TAM density with worse prognosis. What distinguishes our work is the pediatric focus, providing direct evidence that children with osteosarcoma also experience TAM-dominated tumor microenvironments.

This is particularly relevant because pediatric tumors are often considered immunologically "cold" compared to adult cancers, with fewer infiltrating lymphocytes and lower mutational burdens. The discovery that macrophages dominate these tumors suggests that future immunotherapy should prioritize strategies to neutralize or reprogram TAMs rather than relying exclusively on T cell–directed therapies. In this regard, CD206 emerges as a rational and tractable target, supported both by our quantitative immunophenotyping and by prior preclinical studies showing its enrichment in immunosuppressive macrophages.

Engineering CAR-NK Cells for TAM Targeting

While CAR-T therapies have transformed treatment of hematologic cancers, their impact in solid tumors remains modest, limited by poor trafficking, antigen heterogeneity, and severe toxicities. NK cells provide several advantages in this context: they recognize stress ligands independent of antigen presentation, lack the risk of graft-versus-host disease, and demonstrate a more favorable safety profile. In this study, NK cells were further enhanced by two engineering strategies.

First, CRISPR-Cas9 was used to knock out PD-1 and CISH, two inhibitory regulators that restrain NK activation. Removing these checkpoints significantly boosted cytotoxicity and IFN-γ secretion in TAM-rich environments. Second, the cells were transduced with a CAR construct specific for CD206, enabling selective targeting of M2 macrophages. Together, these modifications created a dual advantage: the cells resisted exhaustion and inhibitory signaling while

Frontier in Medical & Health Research

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simultaneously recognizing and attacking immunosuppressive macrophages.

The strong positive correlation between CD206 expression and CAR-NK function suggests that this therapy is most effective in TAM-dense tumors. Notably, the inverse association between the M1/M2 ratio and cytotoxic activity underscores the role of immunologic context: where suppressive macrophages dominate, engineered NK cells provide the greatest relative benefit.

Immunologic Reset Through TAM Reprogramming

One of the most compelling findings was the apparent reprogramming of TAMs following CAR-NK exposure. Within 24 hours, a subset of macrophages shifted from an M2 phenotype toward an M1-like state, characterized by CD86 expression and secretion of pro-inflammatory cytokines such as IL-12. This was accompanied by elevated IFN-γ and TNF-α production and reduced levels of IL-10 and TGF-β.

This shift suggests that CRISPR-CAR-NK cells not only kill tumor cells directly but also remodel the tumor microenvironment into one more favorable for host immunity. Such a "reset" could break cycles of immune suppression, enabling T cells and endogenous NK cells to participate in anti-tumor responses. The ability of engineered cells to orchestrate broader immune changes increases the potential durability of their effects and may explain why TAM targeting could provide advantages over strategies that only recognize tumor antigens.

Immune Checkpoints and Combinatorial Opportunities

Another key observation was the co-expression of PD-L1 with CD206 in TAM-rich tumors. This suggests that tumors with high macrophage burden rely on multiple layers of immune evasion, simultaneously suppressing NK and T cell function. In clinical practice, this opens the door to rational combination therapies. For example, CAR-NK therapy could be paired with PD-1/PD-L1 inhibitors to release additional brakes on the immune system. The observed downregulation of MHC-I in TAM-dense tumors also indicates a selective vulnerability: while reduced MHC-I protects against T cell recognition, it enhances susceptibility to NK-mediated killing.

Taken together, these features suggest that osteosarcomas with high TAM content represent an immunologically distinct subtype ideally suited to NK-based immunotherapies, either as monotherapy or in combination with checkpoint blockade.

Clinical Implications

For pediatric patients, the prospect of off-the-shelf CAR-NK therapy is particularly attractive. Unlike autologous CAR-T approaches, NK cells can be derived from cord blood or allogeneic donors without risk of graft-versus-host disease, enabling scalable and rapid deployment. Safety is also a critical factor. Cytokine release syndrome and neurotoxicity, major complications of CAR-T therapy, occur less frequently and with lower severity in NK-based approaches. These advantages are essential when treating children, who are more vulnerable to treatment-related morbidity.

Our findings support a precision immunotherapy model where patients are stratified based on TAM density and immune checkpoint profiles. Pediatric osteosarcoma cases with abundant $CD206^+$ TAMs and low MHC-I expression may be prioritized for clinical testing of CRISPR-CAR-NK therapy, while those with lower TAM burden might benefit more from other immunotherapeutic strategies.

Limitations

This study has several limitations that should be acknowledged. First, the cross-sectional design captures immune profiles at a single time point, and tumor heterogeneity across different anatomical sites may limit generalizability. Second, while ex vivo assays provide valuable mechanistic insights, they cannot fully replicate the in vivo tumor microenvironment, which includes stromal barriers, vascular influences, and systemic immune regulation. Third, CD206 expression is not unique to TAMs; it is also present on some tissue-resident macrophages and dendritic cells, raising potential concerns for off-tumor targeting. Strategies such as dual CAR systems, inducible expression, or logic-gated circuits may help mitigate this risk.

In addition, CRISPR editing carries risks of off-target effects. While our sequencing confirmed efficient gene knockout, comprehensive genomic analyses will be necessary before clinical

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translation. Lastly, the sample size, though reasonable for a pediatric study, remains modest, and larger cohorts are needed to validate these findings.

Future Directions

Building on these results, future studies should employ preclinical in vivo models to evaluate safety, biodistribution, and persistence of CRISPR-CAR-NK cells. Such studies will clarify whether the observed TAM reprogramming can be sustained and translated into durable tumor control. Combination strategies, particularly with checkpoint blockade or macrophage-modulating agents, deserve further exploration. Advances in single-cell RNA sequencing and spatial transcriptomics could also be leveraged to dissect how TAMs and other immune populations respond dynamically to engineered NK therapy.

Ultimately, the goal will be to integrate TAM-targeting CAR-NK therapy into multimodal treatment regimens for osteosarcoma, complementing surgery and chemotherapy while reducing relapse rates. Pediatric clinical trials should prioritize stratification by TAM burden, enabling the development of biomarker-driven protocols.

Conclusion

This cross-sectional study provides the first pediatric-focused immunoprofiling of osteosarcoma tumors to explore the suitability of CRISPR-engineered CAR-NK cell therapy targeting tumor-associated macrophages. Our findings reveal:

A high prevalence of $CD206^+$ M2 macrophages in most tumors.

A strong correlation between M2 density and CAR-NK-induced IFN-γ release.

Evidence of TAM reprogramming toward an M1 phenotype.

Increased tumor apoptosis and cytokine output in CD206-rich environments.

The data support a precision immunotherapy approach where CAR-NK therapy is tailored based on tumor TAM profiles. These findings warrant further longitudinal and in vivo studies to validate clinical efficacy and safety.

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