Molecular Mechanisms of OncoTherad Nano-Immunotherapy based on T-cell CX3CR1, Immune Checkpoints and Toll-like Receptor 4 Signaling Pathways: Clinical Importance in Bladder Cancer

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Background

Nearly half a century the immunotherapy with Bacillus Calmette-Guerin (BCG) has been the gold standard treatment for non-muscle invasive bladder cancer (NMIBC). However, many patients with high-risk disease experience recurrence, including those who progress and eventually become unresponsive to BCG. For decades, apart from radical cystectomy, few therapeutic options existed for this at-risk population. However, the advent of new immunotherapeutic agents has transformed the treatment of several types of tumors, including urothelial carcinoma. These immunotherapies have shown promising results in the treatment of bladder cancer. In this scenario, our research group developed the OncoTherad (MRB-CFI-1) immunotherapy, which has shown positive results in the treatment of cancer, especially NMIBC.

This study detailed and characterized the mechanisms of action of OncoTherad nano-immunotherapy based on modulation of Toll-like Receptor 4 (TLR4) signaling pathway, CX3C chemokine receptor 1 (CX3CR1, a marker of T-cell differentiation) and immune checkpoints in patients with *Bacillus Calmette-Guérin* (BCG)-unresponsive non-muscle invasive bladder cancer.

Methods

A single-center open-label (Paulinia Municipal Hospital, Brazil) and single-arm phase 1/2 study (Clinical Trial: RBR-6swqd2) was applied in 44 patients (30 male and 14 female) with BCG-unresponsive NMIBC (≥ 1 prior course of BCG therapy) submitted to OncoTherad immunotherapy for 24 months. Patient follow-ups were performed with systematic mapping biopsies of the bladder every 3 months for the first year and every 6 months thereafter for up to 2 years. Bladder biopsies of each patient (n=44) were divided into 2 groups: **Group 1 (initial biopsy, before OncoTherad treatment)**; **and Group 2 (bladder biopsy after OncoTherad treatment)**. Subsequently, the samples were submitted to immunohistochemistry analysis: TLR4-mediated IFN-γ production signaling pathway (TRIF, TBK1, IRF-3), $CX3CR1^{+}CD8^{+}$ T-cells, immune checkpoints (PD-1/PD-L1, CTLA-4) and Regulatory T cells (FOXP3).

Results

- After 24-months follow-up, pathological complete response rate was 72.7% (95% CI) and recurrence-free survival of 21.4 months;
- Bladder samples from patients submitted to OncoTherad treatment (Group 2) showed intensified TLR4, TRIF, TBK1, IRF-3 and IFN-γ immunoreactivities when compared ($p<0.01$) to initial biopsies (Group 1). Furthermore, as result of interferon signaling pathway (TRIF-dependent pathway) induction by OncoTherad, intensified CX3CR1 immunoreactivities ($p<0.01$) were found in the Group 2;
- In contrast, PD-1/PD-L1 immunoreactivities were decreased ($p<0.01$) in the Group 2 when compared to Group 1;
- No statistical differences were found between the two groups for FOXP3 and CTLA4.

Tabel 1. Primary and secondary endpoints: Results of a phase 1/ 2 clinical trial.

Endpoint	Results
Pathological Complete Response Rate (pCRR) Primary Endpoint	•100% pCRR at 3 and 6 months •97.7% pCRR at 9 months •86.4% pCRR at 12 months •77.3% pCRR at 18 months •72.7% pCRR at 24 months
Recurrence-Free Survival (RFS) Primary Endpoint	•Median is 21.4 months (at 24-month follow-up)
Duration of Response (DoR) Secondary Endpoint	•Median Duration of Response is 429 days (95% CI) (14.3 months)
Safety Secondary Endpoint	•61.4% treatment-related Grade 1-2 Adverse Events •11.4% treatment-related Grade 1-2 + Grade 3-4 Adverse Events • 27.2% no Adverse Events

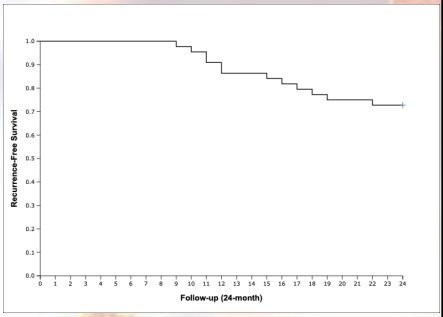

Figure 1. Kaplan-Meier curve for recurrence-free survival at 24-month follow-up with OncoTherad treatment.

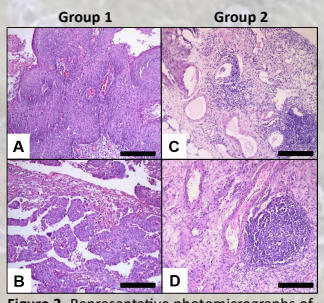

Figure 2. Representative photomicrographs of histopathological analyses from (A-B) Group 1 (initial biopsy) and (C-D) Group 2 (after OncoTherad treatment). pTa high-grade (A-B). Interstitial cystitis (C). Follicular cystitis (D) Bars = 50 µm.

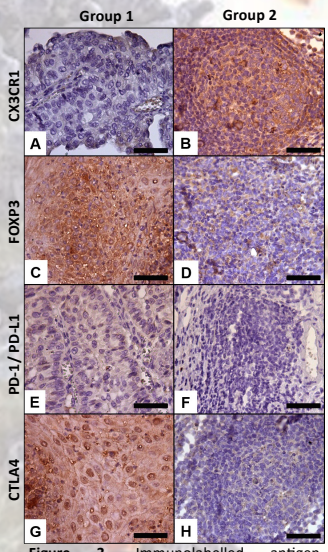

Figure 3. Immunolabelled antigen intensities for CX3CR1 (A-B), FOXP3 (C-D), PD-1/PD-L1 (E-F), and CTLA4 (G-H) in the bladder samples from Group 1 (initial biopsy) and Group 2 (after OncoTherad treatment). Bars = 50 µm.

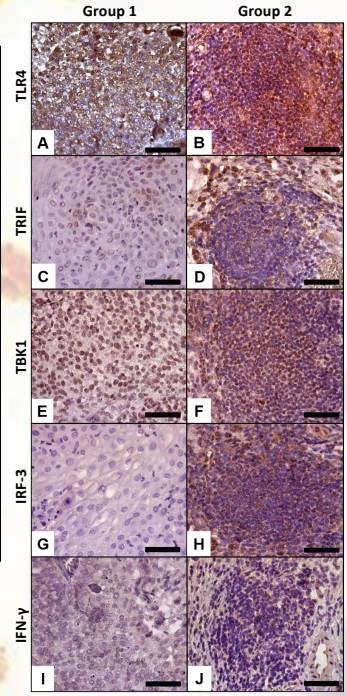

Figure 4. Immunolabelled antigen intensities for TLR-4 (A-B), TRIF (C-D), TBK1 (E-F), IRF-3 (G-H) and IFN-γ (I-J) in the bladder samples from Group 1 (initial biopsy) and Group 2 (after OncoTherad treatment). Bars = 50 µm.

Tabel 2. Total immunoreactivity (%) for TLR4-mediated IFN-γ production signaling pathway, $CX3CR1^{+}CD8^{+}$ T-cells, immune checkpoints and regulatory T cells antigens in the Groups 1 and 2.

	Groups	
Antigens	**Group 1**	**Group 2**
TLR4	80.33 ± 5.0 a	88.83 ± 6.6 b
TRIF	80.96 ± 6.9 a	91.24 ± 4.0 b
TBK-1	83.92 ± 5.8 a	91.01 ± 3.4 b
IRF-3	82.36 ± 6.2 a	90.32 ± 4.5 b
IFN-γ	63.32 ± 3.2 a	82.99 ± 3.7 b
CX3CR1	70.00 ± 15.7 a	89.20 ± 3.3 b
FOXP3	83.28 ± 6.1 a	80.86 ± 11.8 a
PD-1/ PD-L1	86.78 ± 7.3 a	76.43 ± 4.7 b
CTLA4	80.10 ± 15.0 a	84.98 ± 2.8 a

Values equivalent to the means of the percentage of positive urothelial cells cells for each specific antigen. ANOVA Kruskal-Wallis, Student-Newman-Keuls test. On the same line, values followed by different letters indicate the difference between the groups ($p < 0.01$).

Conclusions

OncoTherad nano-immunotherapy led to activation of TLR4-mediated innate immune system, resulting in increased interferon signaling pathway, which was fundamental in the activation of antitumor $CD8^{+}$ T-cells and decrease of PD-1/ PD-L1 expression in the bladder microenvironment. These important findings are relevant concerning the treatment of patients with NMIBC presenting high-risk of progression that are BCG-unresponsive.

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