Characterization of JNJ-80948543 a novel CD79bxCD20xCD3 trispecific antibody for B-cell non-Hodgkin lymphoma

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Received: June 25, 2025.
Accepted: February 13, 2026.
Early view: February 26, 2026.

https://doi.org/10.3324/haematol.2025.288473

Abstract

Despite advances in targeted therapies, in the majority of patients, relapsed/refractory B-cell non-Hodgkin lymphoma (R/R B-NHL) remains incurable. Thus, there is a critical need to expand the treatment options for R/R B-NHL to improve patient outcomes. In this study, we characterized JNJ-80948543, a novel trispecific T-cell engager (TCE), designed to target CD79b$^+$ and/or CD20$^+$ lymphoma cells and bind to CD3 T cells with low affinity. By engaging two tumor antigens, JNJ-80948543 may enhance tumor binding through avidity effects, potentially improving eradication of heterogeneous cell populations and reducing the risk of antigen escape. Preclinical data confirmed potent T-cell-mediated cytotoxicity against CD79b$^+$ and/or CD20$^+$ cells, with increased potency upon dual antigen engagement, consistent with an avidity effect. To mitigate the cytokine release syndrome and T-cell exhaustion commonly associated with TCE, JNJ-80948543 was designed with a low-affinity CD3 arm. *In vitro*, JNJ-80948543 achieved effective cytotoxicity with lower cytokine release compared to a matched high-affinity CD3 trispecific, JNJ-80948556. Despite reduced cytokine secretion by JNJ-80948543, both antibodies demonstrated comparable antitumor activity in a xenograft mouse model. Collectively, the selectivity, potent cytotoxicity, tumor growth inhibition, and favorable cytokine profile of JNJ-80948543 supports its clinical development. Phase 1 clinical trials are ongoing to evaluate JNJ-80948543 as a monotherapy (clinicaltrials.gov identifier NCT05424822) and in combination with a co-stimulatory bispecific antibody (clinicaltrials.gov identifier NCT06139406) in patients with R/R B-NHL.

Introduction

B-cell non-Hodgkin lymphoma (B-NHL) is the most common hematologic malignancy worldwide. CD20 and CD79b are validated therapeutic targets in multiple B-cell malignancies due to restricted B-cell and B-NHL cell expression. Over the past decades, several CD79b- and CD20-targeting monoclonal antibodies (mAb), including rituximab (CD20 mAb) and the subsequent next-generation CD20-mAb, polatuzumab vedotin (CD79b-antibody drug conjugate [ADC]), have been developed and are clinically effective when used in combination with chemotherapy for treating different subtypes of B-NHL. However, despite advances in treatment, there remains a high incidence of relapsed and refractory disease. Further efforts are needed to develop more effective targeted therapies for patients with B-cell malignancies whose disease no longer responds to standard chemotherapy or immunotherapies. T-cell engaging (TCE) antibodies recognize CD3 on T cells and specific antigens on malignant cells. Several bispecific TCE, such as CD20xCD3

and CD19×CD3, have shown promising clinical response rates,[1] providing proof of concept that this therapeutic approach can be highly effective in treating B-cell malignancies. Notably, multiple CD20×CD3 bispecific antibodies (epcoritamab, glofitamab, and mosunetuzumab) have received regulatory approval, underscoring their clinical impact. However, their long-term efficacy in advanced cancers remains limited due to resistance mechanisms such as target antigen downregulation / loss or intratumor heterogeneity.[2,3]

CD79b is a critical component of the B-cell receptor (BCR), and is essential for its functionality, signal initiation, and signal transduction. CD79b mutations have been described as oncogenic drivers in diffuse large B-cell lymphoma (DLBCL), resulting in constitutive activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF κB) pathway.[4] Furthermore, a large proportion of B-NHL, including DLBCL and Burkitt lymphoma, are dependent on CD79b expression for survival, independently of its mutational status.[4-6] In addition, CD79b surface expression has also been shown to remain detectable upon tumor relapse / progression from previous standard-of-care treatment regimens.[7,8] Taken together, CD79b is an attractive target for a TCE approach, as the development of resistance to CD79b-targeted agents through antigen loss may be less likely to occur.[9]

CD20 is a transmembrane protein involved in B-cell activation and differentiation, and is present on all mature B cells and most B-NHL cells.[10] CD20 is organized in the plasma membrane as multimeric molecular complexes with other cell-surface and cytoplasmic proteins involved in BCR-activated calcium entry and contributes to signal transduction and B-cell proliferation.[11] Simultaneous targeting of B-cell lineage targets by combining CD20- and CD79b-targeted therapeutics with different mechanisms of action (ADC combined with antibody dependent cellular cytotoxicity [ADCC] / phagocytosis [ADCP] / complement dependent cytotoxicity [CDC] or TCE) have been explored in the clinical setting (clinicaltrials.gov identifiers NCT04231877, NCT04594798, NCT03671018, NCT04665765, NCT04479267, and NCT04182204) and showed some improved efficacy with a favorable safety profile in patients with relapsed or refractory (R/R) B-cell lymphoma.[12-15] However, no dual CD79b and CD20 targeted TCE approaches have been explored to date.[12-14,16]

Here, we describe a novel trispecific TCE antibody targeting CD79b, CD20, and CD3, JNJ-80948543, that facilitates T-cell mediated cytotoxicity of CD79b$^+$ and/or CD20$^+$ tumor cells both *in vitro* and *in vivo*. JNJ-80948543 was designed to induce proximity between T cells and B-cell NHL tumor cells, promoting immunological synapse formation and subsequent tumor cell lysis through perforin and granzyme release by cytotoxic T lymphocytes (CTL). JNJ-80948543 incorporates a low-affinity CD3 binding arm to mitigate cytokine release syndrome (CRS) and T-cell exhaustion, while JNJ-80948556 features a higher-affinity CD3 arm to enhance T-cell activation. Preclinical studies using cellular models with varying CD79b and CD20 expressions explored the impact of dual antigen engagement on cytolytic potential. *In vivo* xenograft models demonstrated potent antitumor activity for both constructs, with JNJ-80948543 showing reduced *in vitro* cytokine release compared to JNJ-80948556.

Methods

Generation of CD79b×CD20xCD3 trispecifics

JNJ-80948543 and JNJ-80948556 feature mutations in the fragment crystallizable (Fc) region to abolish interaction with Fc receptors. Heterodimerization was enhanced using the knobs-into-holes platform mutations. JNJ-80948543 contains a low-affinity and JNJ-80948556 a higher-affinity anti-CD3ε single-chain variable fragment (scFv) fused onto the N-terminus of the 'knob' Fc region and an anti-CD20 scFv attached to the C-terminus of the Fc region. The 'hole' chain comprises a high-affinity CD79b antigen-binding fragment and contains mutations to disrupt protein A binding of monomeric and homodimerized hole chains.

Cell lines and cell culture

All cell lines used were of human origin and obtained from either American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen. Cell lines were cultured in RPMI 1640 medium with GlutaMAX and with 10% fetal bovine serum without antibiotics at 37°C in a 5% carbon dioxide incubator.

T-cell-mediated cytotoxicity and T-cell activation using cell lines and healthy donor T cells

For the *in vitro* assays, tumor cell lines were plated with thawed purified frozen T cells at 5:1 or 1:1 effector to target ratio (E:T) and trispecific antibody (TsAb) or control antibodies and incubated at 37°C with 5% carbon dioxide for 48 or 72 hours (h). The supernatant was collected for cytokine analysis, and the cells were stained for analysis on a FACSLyric or a FACSymphony A1 cell analyzer (BD Biosciences). Cytotoxicity was assessed by quantifying viable carboxyfluorescein succinimidyl ester positive (CFSE$^+$) cancer cells per well. Viable cells in these assays were determined using Fixable Viability Dye eFluor™ 780. Cytotoxicity (%) was calculated as:

$$\text{Cytotoxicity} = \left\{ \frac{\text{Absolute} \star \text{viable cancer cells}}{\text{Average} \star \text{viable cancer cells in untreated wells}} \right\} \times 100$$

T-cell activation was measured by CD69 and CD25 expression on CD4$^+$ and/or CD8$^+$ T cells.

Cytokine measurement

Supernatants were collected from *in vitro* T-cell cytotox-

icity assays. Production of interferon-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, and tumor necrosis factor (TNF)-α was assayed by using the Meso Scale Discovery (MSD) human pro-inflammatory panel 1 kit as per the instructions. An additional single-plex analysis from the MSD for granulocyte-macrophage colony-stimulating factor (GM-CSF) was also performed according to the manufacturer's protocol.

CD79b antagonistic studies

The effect of JNJ-80948543 on CD79b downstream signaling was assessed by evaluating the inhibition of IL-10 secretion by MSD. The MSD assay was performed according to the manufacturer's protocol.

Xenograft studies

All experiments were carried out in accordance with The Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Johnson & Johnson, Beerse, Belgium.
Further details of the methods used can be found in the *Online Supplementary Methods*.

Results

Generation and characterization of specificity of JNJ-80948543

JNJ-80948543 is a novel TsAb binding to the epsilon subunit of the CD3 T-cell receptor complex (CD3ε; Uniprot ID P07766), CD79b (B-cell antigen receptor complex-associated protein β chain; Uniprot ID P40259) and CD20 (B lymphocyte antigen CD20; Uniprot ID P11836) tumor antigens (Figure 1A). JNJ-80948543 features mutations in the Fc region to abolish interaction with Fc receptors.
Each binding arm of JNJ-80948543 was evaluated for binding specificity in the Retrogenix® Cell Microarray Technology screen (Charles River Laboratories) and confirmed to be specific for the respective primary targets (*data not shown*). JNJ-80948543 was also shown to be specific for CD20 and CD79b in an *in vitro* functional assay (cytokine release) using a panel of 6 cancer cell lines that lack expression of CD79b and CD20, but that in transcriptomics are predicted to express >50% of the known cell surface proteins using a transcript per million cut-off of >5 (*Online Supplementary Figure S1*).

Expression of CD79b and CD20 in cancer cell lines and patient samples

The lineage markers, CD79b and CD20, are expressed in early to mature stages of normal B-cell development, and both antigens are undetectable in terminally differentiated plasma cells. CD79b and CD20 were highly expressed in several subtypes of B-NHL, including DLBCL, follicular lymphoma, and mantle cell lymphoma (*Online Supplementary Figure S2A-D*). In the majority of B-NHL samples, CD79b and CD20 were co-expressed, but within each B-NHL type there were up to 25% of cells that expressed either CD79b

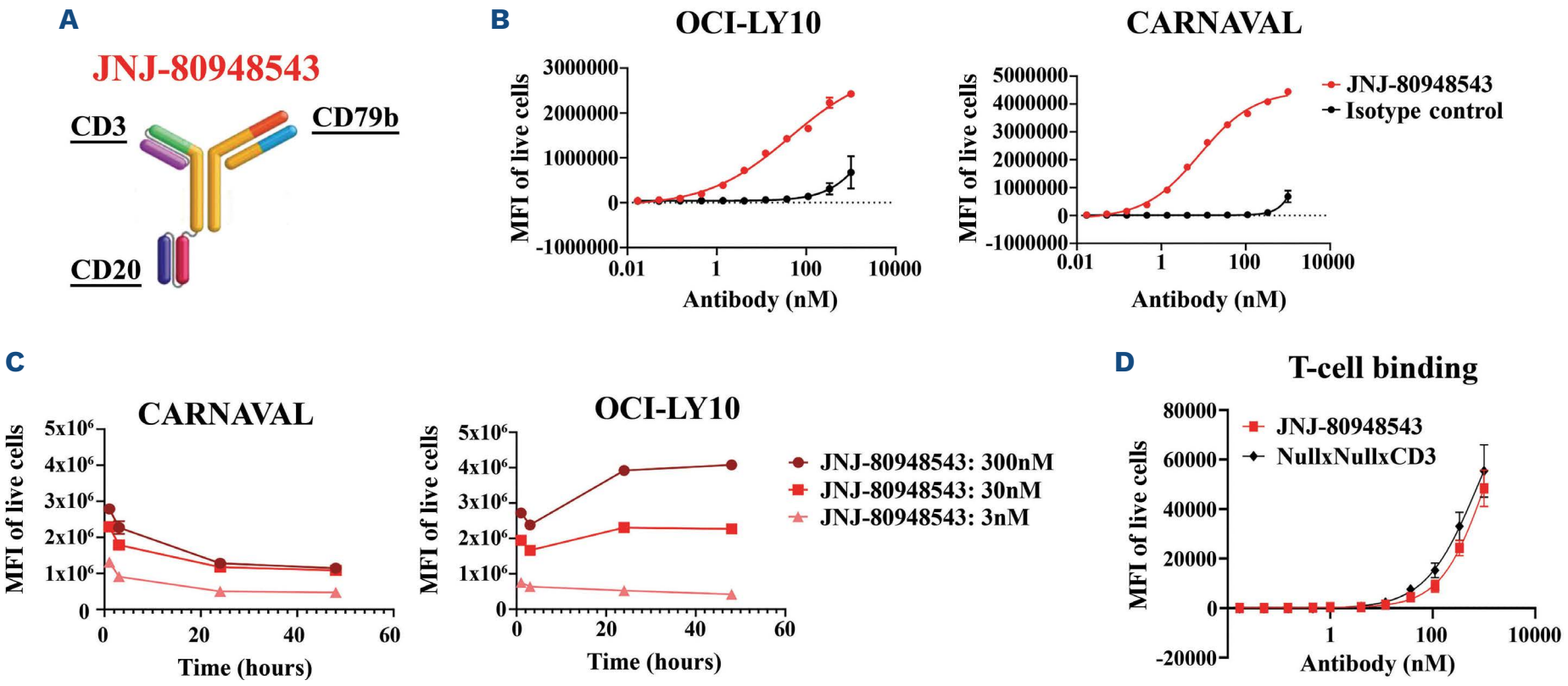

Figure 1. JNJ-80948543 profiling for binding to cancer cells and T cells. (A) JNJ-80948543 trispecific antibody schematic. (B) Dose-dependent binding of JNJ-80948543 to CARNAVAL and OCI-Ly10 B-cell non-Hodgkin lymphoma cells at 1 hour (h). Data from a single experiment with 3 independent replicates are shown as mean ± standard error of mean (SEM). (C) Binding of JNJ-80948543 over time (up to 48 h) to CARNAVAL and OCI-Ly10 B-NHL cells. Data from 2 independent experiments are shown as mean ± SEM (N=2 replicates). (D) Binding of JNJ-80948543 to primary T cells from 3 donors after 1-h incubation at 37°C. The experiment was performed once with 3 T-cell donors. Data are presented as mean ± SEM. MFI: median fluorescence intensity.

or CD20 (*Online Supplementary Figure S2B*). Using immunohistochemistry, CD79b and CD20 were also detected in formalin-fixed paraffin-embedded tissues obtained from patients with B-NHL at initial diagnosis or from those with R/R disease following treatment with R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone / prednisolone) (*Online Supplementary Figure S2D*). Additionally, analysis of receptor density in cell lines confirmed that CD79b and CD20 expression was largely restricted to B-NHL cells and was not observed in malignant cells from other lineages (*Online Supplementary Table S1*).

JNJ-80948543 binding profiles to B-cell non-Hodgkin lymphoma cell lines and CD3⁺ T cells

JNJ-80948543 acts as a bridge between tumor cells and T cells by binding CD79b and CD20 on target tumor cells and CD3 on T cells. To reduce the frequency and/or severity of CRS, T-cell anergy and T-cell exhaustion induced by TCE therapies, JNJ-80948543 was designed with a low-affinity CD3 binding arm (SPR, KD 221 nM). In lymphoma cell lines with varying CD79b and CD20 densities (*Online Supplementary Table S1*), JNJ-80948543 demonstrated stable binding to tumor cells over 48 h (Figure 1B, C). Higher concentrations of JNJ-80948543 were needed for binding to human T cells expressing endogenous CD3 compared with B-NHL cell lines, due to the low-affinity CD3 binding arm (Figure 1D, *Online Supplementary Figure S3A*).

JNJ-80948543 exhibits increased avidity and cytotoxic potency

JNJ-80948543 facilitates the formation of an immunological synapse by binding to both CD79b and CD20, initiating T-cell activation and enabling cytotoxicity of tumor cells by secretion of perforin and granzymes stored in the secretory vesicles of CTL. Expression of both targets on a tumor cell could potentially result in dual antigen-binding by JNJ-80948543, leading to increased avidity and cytotoxic potency. To address this question, TCE assays were performed using K562 target cells engineered to express CD79b, CD20, or both antigens (Figure 2A). JNJ-80948543 induced concentration-dependent cytotoxicity of target cells expressing either CD79b or CD20. The NullxCD20xCD3 antibody only induced concentration-dependent cytotoxicity of the K562_CD20 target cells and not the K562_CD79b cells. The activity of the NullxCD20xCD3 antibody with target cells expressing both antigens (K562_CD79b_CD20) was similar to that observed when incubated with K562_CD20 target cells. In contrast, when the assay was conducted with K562_CD79b_CD20 cells, JNJ-80948543 induced much greater cytotoxicity, which was approximately 1,000-fold greater than the potency observed with target cells expressing either antigen. These results are consistent with an avidity effect.

To further corroborate the benefit of dual tumor antigen targeting, *in vitro* cytotoxicity of CD20⁺ CD79b⁺ B-NHL cell lines (WSU-DLCL2, OCI-Ly10, and CARNAVAL) was assessed in the presence of JNJ-80948543, CD79bxNullxCD3, or NullxCD20xCD3, and T cells from healthy donors as effector cells. CD79bxNullxCD3 or NullxCD20xCD3 both showed cytotoxic activity, but with lower potency compared to JNJ-80948543 (Figure 2B, *Online Supplementary Table S2*). T-cell activation and proliferation in co-culture of CD79b+/CD20⁺ cancer cells were higher in the presence of JNJ-80948543 than CD79bxNullxCD3, or NullxCD20xCD3 control antibodies (Figure 2C, *Online Supplementary Figure S3B*). Altogether, the data indicated that JNJ-80948543 may be effective in targeting tumor cells that express CD79b and/or CD20, and confirmed that dual tumor antigen-binding by JNJ-80948543 results in increased avidity and cytotoxic potency.

JNJ-80948543 induces *in vitro* T-cell activation and T-cell-mediated cytotoxicity of tumor cells independently of the level of CD79b and CD20

To further characterize the activity of JNJ-80948543, B-NHL cell lines with varying levels of CD20 and CD79b expression (*Online Supplementary Table S1*) were incubated with purified healthy human T cells in the presence of JNJ-80948543 for 48 or 72 h. JNJ-80948543 induced T-cell-mediated cytotoxicity of all CD79b⁺/CD20⁺ cell lines (Figure 3A, *Online Supplementary Table S3*) without eliciting a cytotoxic response to CD79b⁻/CD20⁻ cell lines K562 and SU-DHL1 (Figure 3A). As expected, the negative control antibodies (NullxNullxCD3) did not exhibit any cytotoxicity.

Recent immunoprofiling studies in DLBCL highlighted substantial differences in T-cell infiltration in cold and hot DLBCL tumors. Hot DLBCL tumors are characterized by high immune infiltration, whereas cold DLBCL tumors exhibit low immune cell presence, indicating an immune-depleted microenvironment.[17,18] To assess the impact of increased target burden on the cytotoxic potential of JNJ-80948543 *in vitro*, tumor cell viability in the presence of treatment and purified human pan CD3⁺ T cells was also assessed at a 1:1 E:T ratio, that might be more relevant in the context of cold DLBCL tumors, after either 48 or 72 h. JNJ-80948543 induced T-cell-mediated cytotoxicity but with lower maximum cytotoxicity and higher EC_{50} values compared to a 5:1 E:T ratio (Figure 3B, *Online Supplementary Table S3*).

In parallel, T-cell activation, measured by the level of CD25 expression on T cells, was assessed. JNJ-80948543 mediated T-cell activation only when incubated with CD79b⁺/CD20⁺ cell lines, but not in the presence of the CD79b⁻/CD20⁻ cell lines, demonstrating the specificity of T-cell activation (Figure 4A, *Online Supplementary Figure S4A*). Similarly, a negative control NullxNullxCD3 antibody did not induce significant T-cell activation in any of the cell lines.

To further characterize T-cell activation induced by JNJ-80948543, supernatants from the *in vitro* cytotoxicity assay were analyzed for cytokine levels using MSD. JNJ-80948543, engineered with a low-affinity CD3 arm, demonstrated T-cell

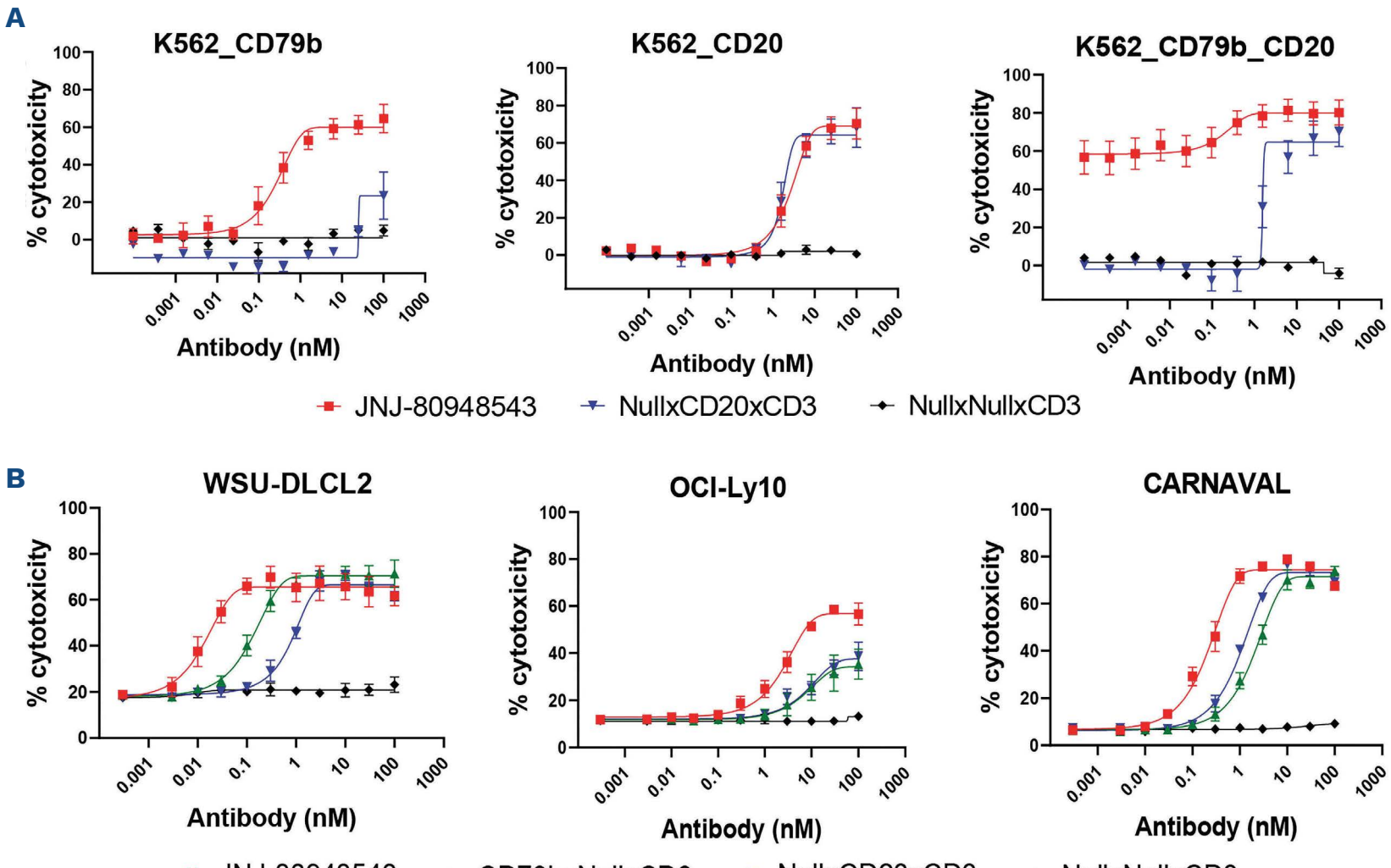

Figure 2. JNJ-80948543 mediates cytotoxicity of CD79b⁺ and CD20⁺ tumor cells and its activity is increased when binding to both tumor antigens. (A) Effect of JNJ-80948543 and NullxCD20xCD3 antibodies on cytotoxicity of K562 tumor cells expressing CD79b and/or CD20 in the presence of T cells from healthy donors at 5:1 effector to target ratio (E:T). T cells from 2 healthy donors were incubated with the indicated antibodies, and cell lines expressing CD79b and/or CD20. The percentage of cytotoxicity over a 6-day assay is shown as average mean ± standard error of mean (SEM). (B) Effect of JNJ-80948543, CD79bxNullxCD3 and NullxCD20xCD3 antibodies on cytotoxicity of WSU-DLCL2, OCI-Ly10, CARNAVAL tumor cells expressing CD79b and CD20 in the presence of T cells from healthy donors at a 5:1 E:T ratio for 72 hours (h). Data are shown as mean ± SEM (N=3 independent T-cell donors). Significance in activity between JNJ-80948543 and Null control antibodies was calculated using two-way ANOVA and Bonferroni multiple comparison test: WSU-DLCL2 cells JNJ-80948543 *versus* CD79bxNullxCD3 (P=0.0023), JNJ-80948543 *versus* NullxCD20xCD3 (P<0.0001), JNJ-80948543 *versus* NullxNullxCD3 (P<0.0001); OCI-Ly10 cells JNJ-80948543 *versus* CD79bxNullxCD3 (P<0.0001), JNJ-80948543 *versus* NullxCD20xCD3 (P<0.0001), JNJ-80948543 *versus* NullxNullxCD3 (P=0.0001); CARNAVAL cells JNJ-80948543 *versus* CD79bxNullxCD3 (P=0.0002), JNJ-80948543 *versus* NullxCD20xCD3 (P=0.0007), JNJ-80948543 *versus* NullxNullxCD3 (P<0.0001). (C) Effect of JNJ-80948543, CD79bxNullxCD3 and NullxCD20xCD3 antibodies on T-cell activation in cytotoxicity assays conducted in the presence of WSU-DLCL2, OCI-Ly10, CARNAVAL tumor cells and T cells from healthy donors at a 5:1 E:T ratio for 72 h. Data are shown as mean ± SEM (N=3 independent T-cell donors). Significance in activity between JNJ-80948543 and Null control antibodies was calculated using two-way ANOVA and Bonferroni multiple comparison test: WSU-DLCL2 cells JNJ-80948543 *versus* CD79bxNullxCD3 (P<0.0001), JNJ-80948543 *versus* NullxCD20xCD3 (P<0.0001), JNJ-80948543 *versus* NullxNullxCD3 (P<0.0001); OCI-Ly10 cells JNJ-80948543 *versus* CD79bxNullxCD3 (P<0.0001), JNJ-80948543 *versus* NullxCD20xCD3 (P=0.0011), JNJ-80948543 *versus* NullxNullxCD3 (P<0.0001); CARNAVAL cells JNJ-80948543 *versus* CD79bxNullxCD3 (P<0.0001), JNJ-80948543 *versus* NullxCD20xCD3 (P=0.0018), JNJ-80948543 *versus* NullxNullxCD3 (P<0.0001).

activation accompanied by low cytokine release across the cell lines tested (Figure 4B, *Online Supplementary Figure S4B, C*). JNJ-80948556 (*Online Supplementary Figure S5A*) is a matched CD79bxCD20xCD3 TsAb designed with a higher affinity CD3 (SPR, KD ~10-50 nM) compared to the CD3 affinity of JNJ-80948543 (SPR, KD 221 nM); this was included for comparison purposes. JNJ-80948556 (CARNAVAL, EC_{50}: 11 nM; OCI-Ly10 EC50: 55 nM) showed comparable cancer cell binding to JNJ-80948543 (CARNAVAL EC50: 8 nM; OCI-Ly10 EC50: 43.4 nM), with stable engagement over 48 h (Figure 1B, C, *Online Supplementary Figure S5C, D*). However, unlike JNJ-80948543 (EC_{50} > the highest tested concentration 1 µM) (Figure 1D), JNJ-80948556 demonstrated dose-dependent binding to T cells, with an EC_{50} of 104 nM (*Online*

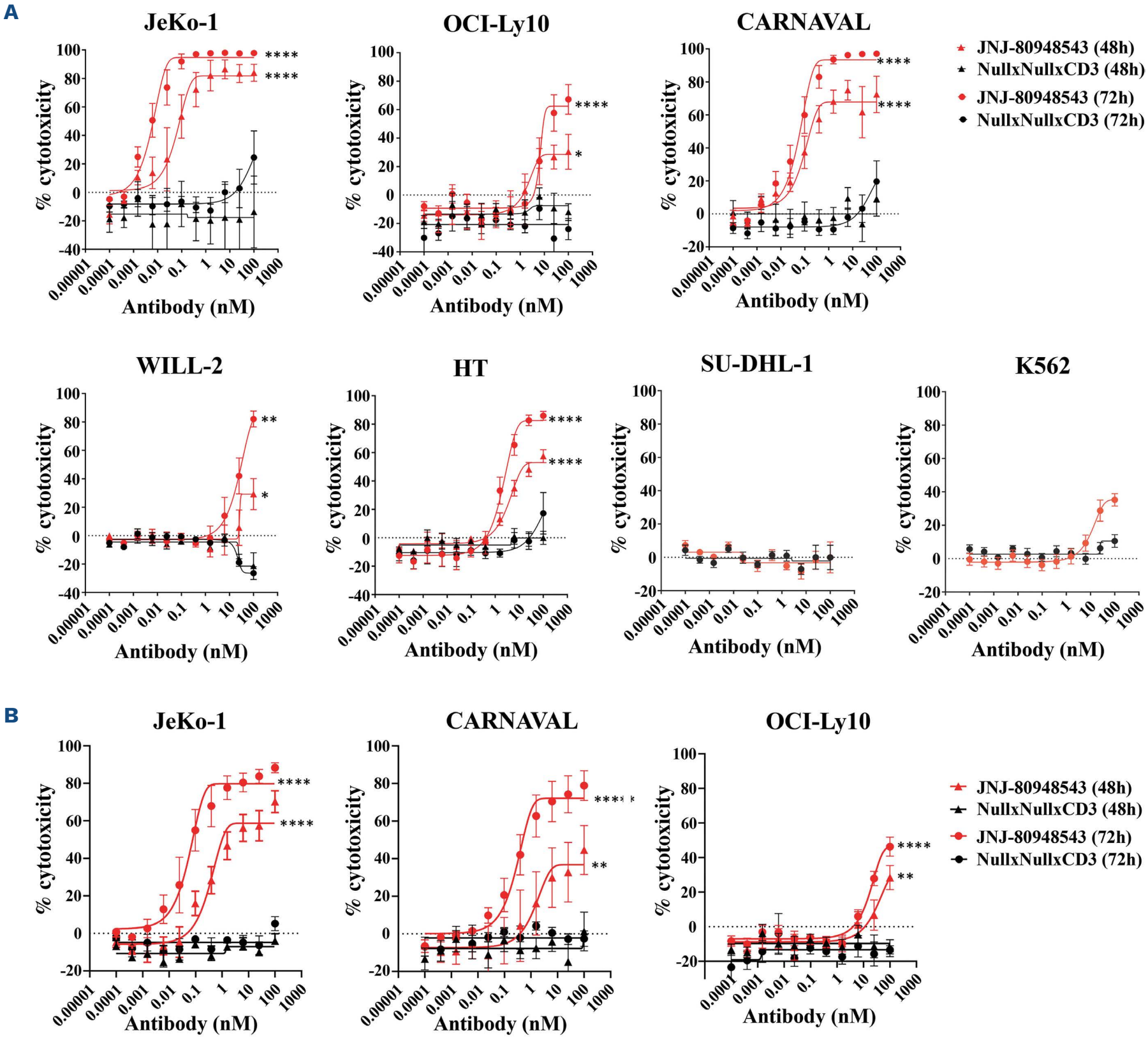

Figure 3. JNJ-80948543 mediates cytotoxicity of B-cell non-Hodgkin lymphoma cells with different CD79/CD20 expression levels. (A) Effect of JNJ-80948543 and NullxNullxCD3 antibodies on cytotoxicity of B-cell non-Hodgkin lymphoma cells expressing CD79b and/or CD20 in the presence of T cells from healthy donors (N=5-7) at a 5:1 effector to target ratio (E:T). Cytotoxicity of tumor cell targets was measured after 48 or 72 hours (h). All cell lines used were $CD79b^+$ and/or $CD20^+$ except SU DHL-1 and K562, which are $CD79b^-CD20^-$. Data from 6 independent experiments were averaged and mean ± standard error of mean (SEM) are shown. (B) Effect of JNJ-80948543 and NullxNullxCD3 antibodies on cytotoxicity of B-NHL cells expressing CD79b and/or CD20 in the presence of T cells from healthy donors (N=5-6) at a 1:1 E:T ratio. Cytotoxicity of tumor cell targets was measured after 48 or 72 h. All cell lines used were $CD79b^+$ and $CD20^+$. Data were averaged and mean ± SEM are shown. Significance in activity between JNJ-80948543 and NullxNullxCD3 control antibody per time point was calculated using two-way ANOVA and Bonferroni multiple comparison test: *P<0.05, **P<0.01, ****P<0.0001.

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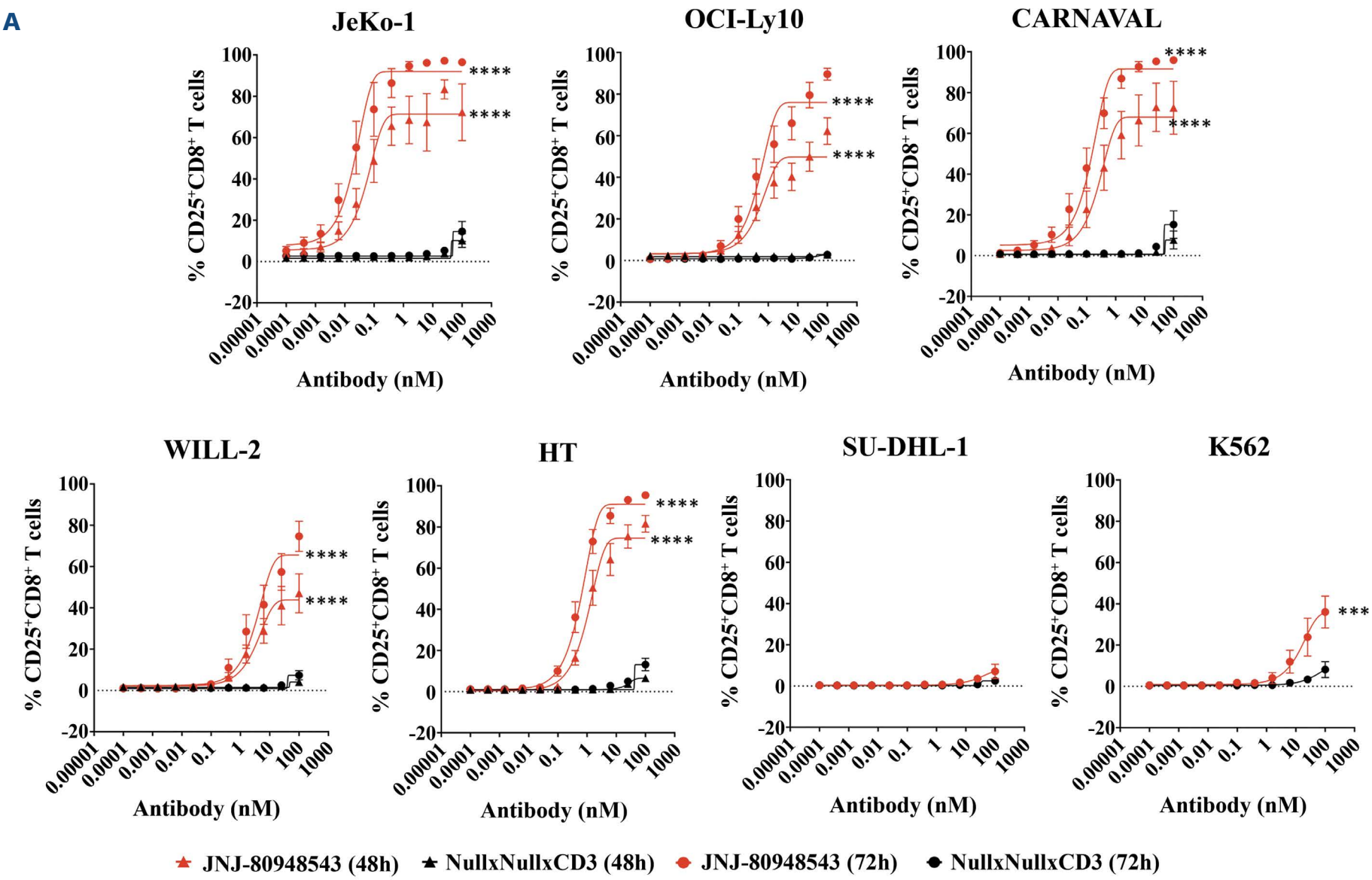

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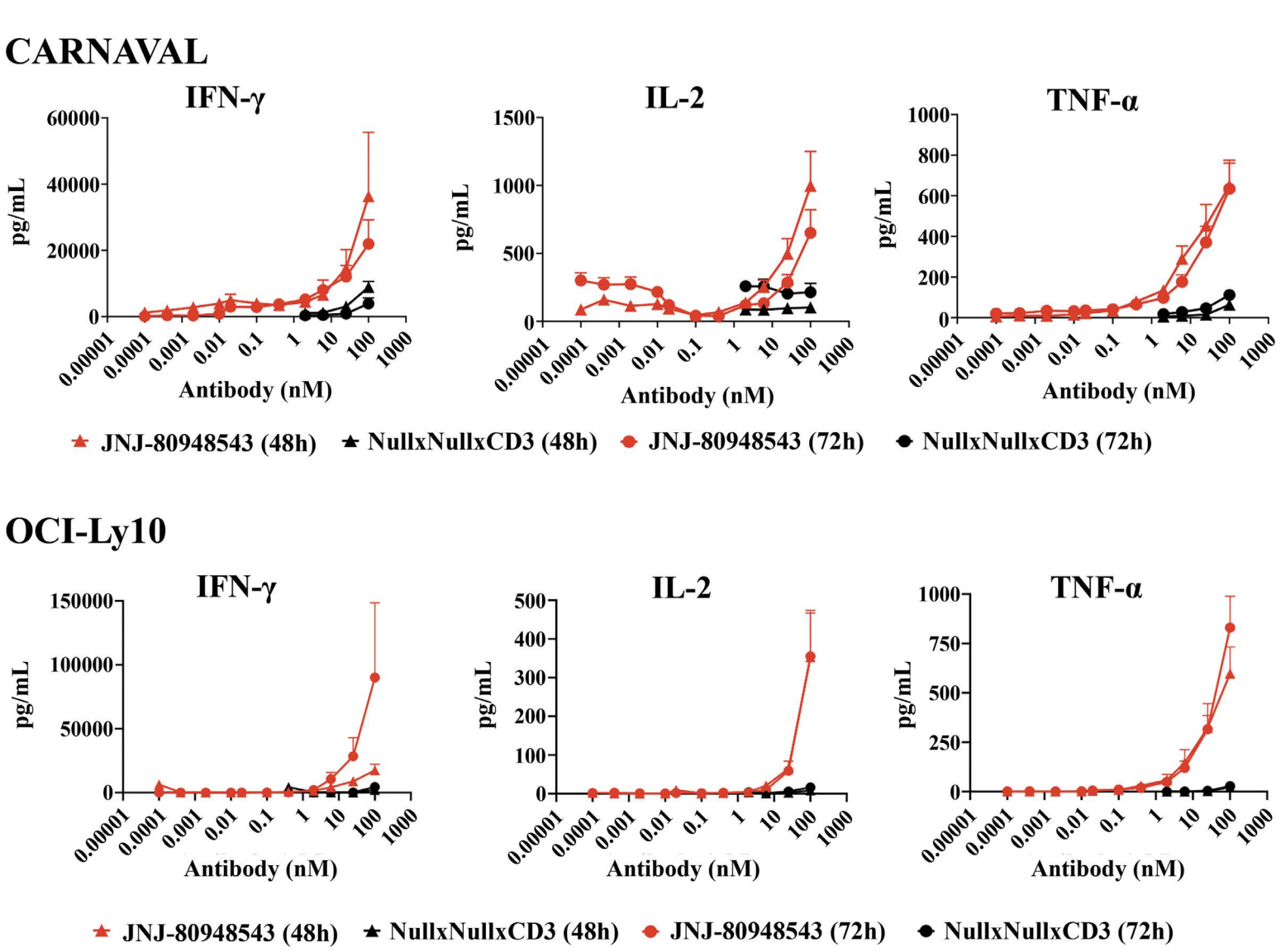

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Figure 4. JNJ-80948543 mediates T-cell activation in the presence of CD79b$^+$ CD20$^+$ B-cell non-Hodgkin lymphoma cells. (A) The percentage of CD8 T-cell activation was determined by flow cytometry (y axis) as percentage of CD25$^+$ cells. CFSE-labeled cell lines were combined with CD3$^+$ pan T cells at a 5:1 effector to target ratio (E:T) for either 48 or 72 hours (h) with increasing concentrations (x axis) of JNJ-80948543 or NullxNullxCD3. Values are averages of 5-6 individual T-cell donors. All cell lines are CD79b$^+$/CD20$^+$ except SU-DHL-1 and K562, which are CD79b$^-$/CD20$^-$. As SU-DHL-1 secretes IL-2 and thus induces CD25 on T cells in the co-culture system, for assessing % of CD25 on CD8 T cells gate has been set up relative to untreated wells with SU-DHL-1 and T cells. GraphPad Prism 9 was used to present data. Data from 6 independent experiments were pooled and represented as mean ± standard error of mean (SEM). Significance in activity between JNJ-80948543 and NullxNullxCD3 control antibody per time point was calculated using two-way ANOVA and Bonferroni multiple comparison test: ***P<0.001. ****P<0.0001. (B) T cells from 5 or 6 healthy donors were tested in T-cell redirection assays incubated with the indicated antibodies and CD79b$^+$CD20$^+$ CARNAVAL (top) and OCI-Ly10 (bottom) cells. The assay was conducted for 48 or 72 h at a 5:1 E:T ratio. Supernatant was analyzed for inflammatory cytokines using the Meso Scale Discovery (MSD) Proinflammatory kit (MSD K15049D). Representative graphs for interferon gamma (IFNγ), interleukin (IL)-2, and tumor necrosis factor alpha (TNFa) are shown. (Data for other cytokines are shown in *Online Supplementary Figure 4B, C.*)

Supplementary Figure S5B). These findings confirm that the CD3 binding arm is the only distinguishing feature between the two molecules. When comparing cytokine secretion with these two TsAb at 48 h in T-cell cytotoxicity assays with OCI-Ly10 and CARNAVAL cells (*Online Supplementary Figure S5E, F*), JNJ-80948543 induced lower cytokine levels than JNJ-80948556. This suggests that JNJ-80948543 can drive effective T-cell-mediated cytotoxicity with low cytokine secretion (*Online Supplementary Table S4*).

JNJ-80948543 mediates CD79b$^+$CD20$^+$ tumor cell cytotoxicity in human whole blood

To evaluate the impact of JNJ-80948543 in a more physiologically relevant setting, T-cell cytotoxicity assays were conducted using healthy human whole blood as a source of T-cell effectors and in co-culture with fluorescently labeled CD79b$^+$CD20$^+$ CARNAVAL or OCI-Ly10 cells. Both non-malignant B cells from the whole blood and added tumor cells were evaluated for cytolysis. T-cell activation and serum cytokine levels were also evaluated. JNJ-80948543 elicited concentration-dependent cytotoxicity of CD20$^+$CD79b$^+$ CARNAVAL and OCI-Ly10 cells after 48 and 72 h (Figure 5A, B) with concomitant T-cell activation (Figure 5C, D), but with low cytokine secretion (*Online Supplementary Figure S6A, B*). JNJ-80948543 also elicited concentration-dependent cytotoxicity of non-malignant autologous B cells in the presence of CARNAVAL and OCI-Ly10 cells after 48 and 72 h (*Online Supplementary Figure S6C, D*). At 72 h, the median EC_{50} of JNJ-80948543-induced T-cell-mediated autologous B-cell depletion was 0.459 and 0.563 nM in the presence of CARNAVAL and OCI-Ly10 cells, respectively.

In addition, in an autologous assay set up without added cancer cells, JNJ-80948543 induced T-cell-mediated non-malignant autologous B-cell depletion (Figure 5E), with a median EC_{50} of 1.19 nM. T cells were also activated in a concentration-dependent manner in the presence of JNJ-80948543 after 48 or 72 h of incubation, as measured by the frequency of CD25$^+$ on CD8$^+$ T cells (Figure 5F). The level of cytokine release varied across donors. In the concentration range tested, no plateau was reached for the majority of the cytokines, and cytokine release was observed mostly at the highest tested concentrations of JNJ-80948543, in line with its lower-affinity CD3 binding arm (*Online Supplementary Figure S6E*).

CD79b antagonistic activity of JNJ-80948543

Phosphorylation of CD79a and CD79b initiates BCR signaling. One of the prominent downstream signaling pathways engaged after BCR stimulation is the classical NF-κB pathway, which is frequently activated in ABC-DLBCL due to oncogenic mutations in CD79a/b.[4] NF-κB signaling regulates the expression of multiple cytokines, including IL-10. The ability of JNJ-80948543 to inhibit the secretion of IL-10 was assessed by OCI-Ly10 and HBL-1 ABC DLBCL cells harboring either CD79a ITAM or CD79b mutations. JNJ-80948543 elicited a concentration-dependent inhibition of IL-10 secretion by OCI-Ly10 and HBL-1 cells (Figure 5G, H). To provide evidence that this effect on IL-10 secretion was solely dependent on CD79b binding, matched CD79bxNullxCD3, NullxCD20xCD3 and NullxNullxCD3 control antibodies were included along JNJ-80948543. NullxCD20xCD3 and NullxNullxCD3 antibodies did not show any effect on IL-10 inhibition, while CD79bxNullxCD3 and JNJ-80948543 had overlapping activities in both cell lines (Figure 5G, H). These results demonstrate that JNJ-80948543 inhibits IL-10 secretion and potentially affects BCR signaling. Further experiments are necessary to comprehensively validate its impact on NF-κB signaling and its broader effects on the BCR pathway.

JNJ-80948543-induced T-cell-mediated tumor growth inhibition of B-cell lymphoma xenografts *in vivo*

In vivo efficacy of JNJ-80948543 was evaluated in 2 independent DLBCL models: CARNAVAL (prevention tumor model) and OCI-Ly10 (established tumor model). In the CARNAVAL prevention model (Figure 6A, *Online Supplementary Figure S7A*), twice a week intraperitoneal (IP) treatment with JNJ-80948543 or vehicle (Dulbecco's phosphate-buffered saline [DPBS]) was administered after subcutaneous (SC) injection of tumor cells. At day 22, 1 or 5 mg/kg JNJ-80948543 prevented tumor growth in the majority of mice, resulting in 95% and 100% tumor growth inhibition (TGI), respectively. In the OCI-Ly10 established model (Figure 6B, *Online Supplementary Figure S7B*), twice a week treatment starting at day 14 with 3 or 10 mg/kg JNJ-80948543 or vehicle was ad-

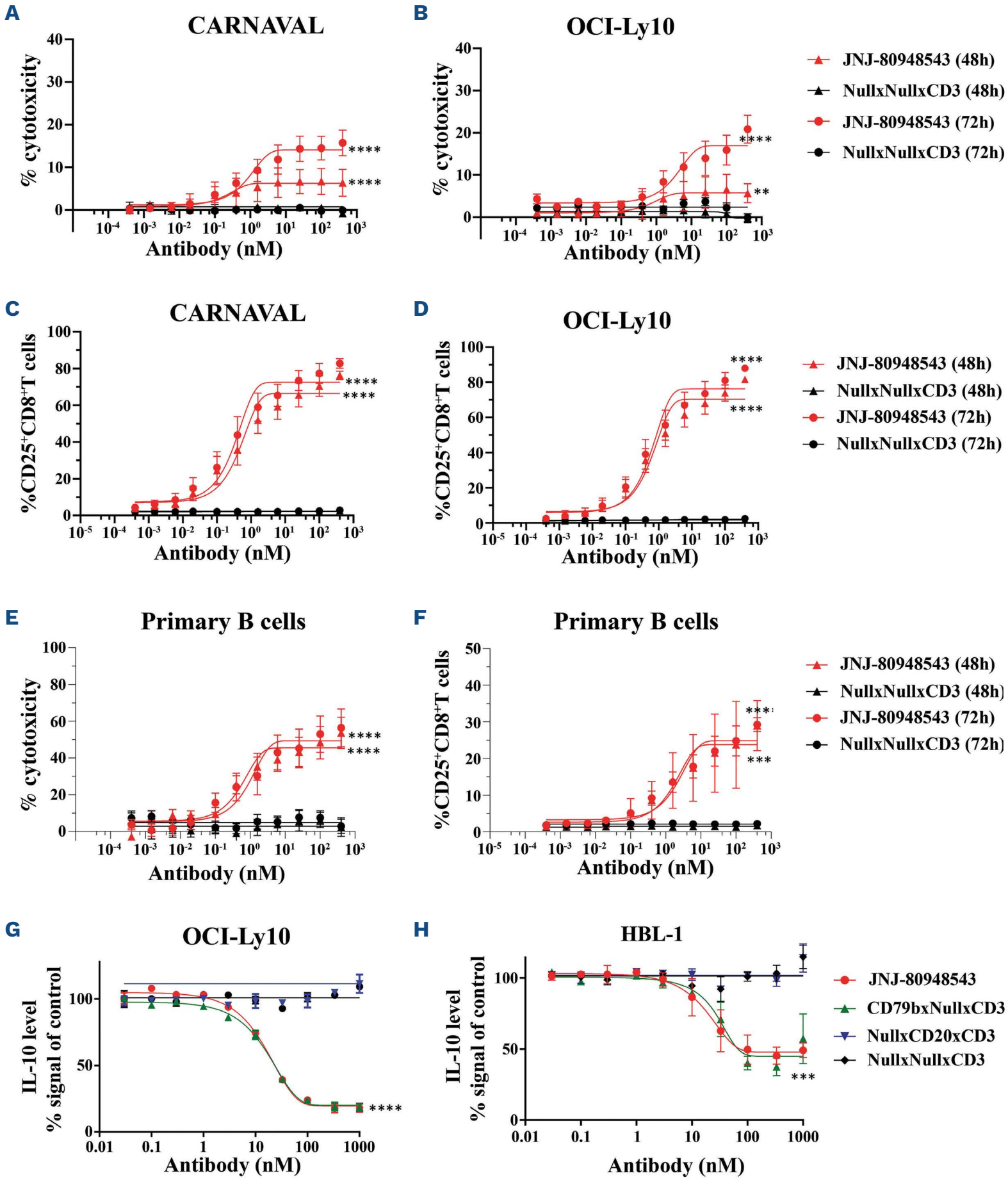

Figure 5. JNJ-80948543 mediates B-cell non-Hodgkin lymphoma cell cytotoxicity and T-cell activation in whole blood setting. JNJ-80948543 mediates CD79b antagonistic activity. (A-D) Whole blood from 6 healthy donors was tested in T-cell redirection assays with the indicated antibodies and CD79b$^+$CD20$^+$ target cell lines: CARNAVAL (A and C) or OCI-Ly10 (B and D). The percentage of cytotoxicity of cancer cells (A and B) or T-cell activation (C and D) was determined after 48 or 72 hours (h). The assay was conducted at an effector to target ratio (E:T) ratio of 1:1 with respect to added tumor cells. (E and F) The percentage of cytotoxicity of primary B cells (E) or T-cell activation (F) was assessed in the whole blood assay without added cancer cells. The E:T ratio for primary B cells ranged from 5:1 to 10:1. Data were averaged and mean ± standard error of mean (SEM) are shown. (G) JNJ-80948543, CD79bxNullxCD3, NullxCD20xCD3 and NullxNullxCD3 antibodies were added at a range of concentrations to OCI-Ly10 cells for 24 h to assess the effect on IL-10 secretion, as surrogate of nuclear factor kappa light chain enhancer of activated B cells (NF κB) signaling inhibition downstream of CD79b. Interleukin (IL)-10 levels were normalized to untreated control cells and expressed as a percentage. (H) JNJ-80948543, CD79bxNullxCD3, NullxCD20xCD3 and NullxNullxCD3 antibodies were added at a range of concentrations to HBL-1 cells for 24 h to assess the effect on IL-10 secretion, as surrogate of NF κB signaling inhibition downstream of CD79b. IL-10 levels were normalized to untreated control cells and expressed as a percentage. Significance in activity between JNJ-80948543 and NullxNullxCD3 control antibody per time point was calculated using two-way ANOVA and Bonferroni multiple comparison test: $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

ministered to mice with tumor volumes averaging 108 mm^3. On day 34 treatment ended, and complete responses were observed in 8 of 10 and 10 of 10 mice treated with 3 or 10 mg/kg JNJ-80948543 by day 38, respectively (Figure 6B). At day 38, treatment with 3 or 10 mg/kg JNJ-80948543 resulted in 92% or 98% tumor regression (TR), respectively, as compared with the vehicle. A parallel evaluation of JNJ-80948556 in the established OCI-Ly10 model at identical dose levels (3

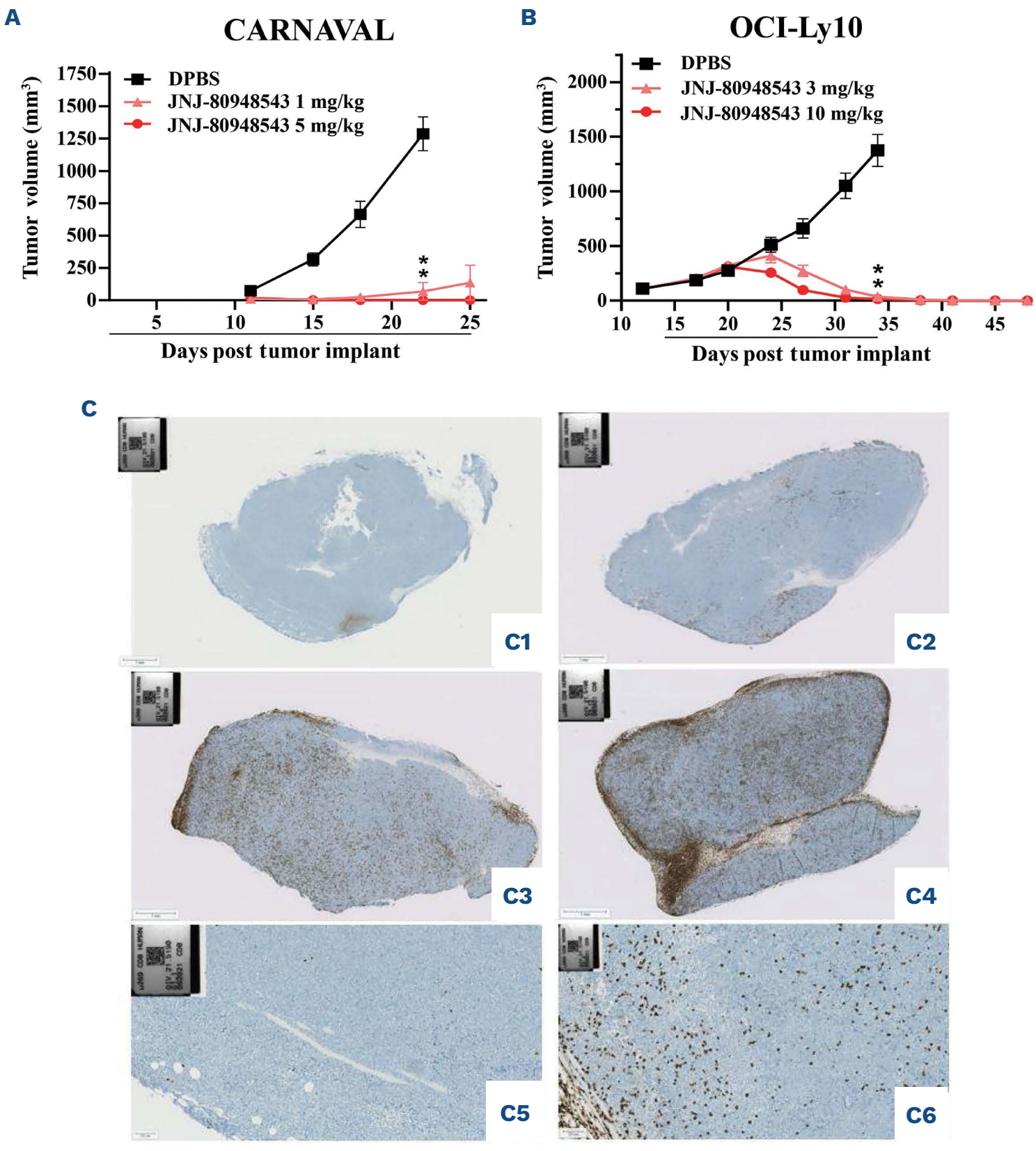

Figure 6. Antitumor efficacy of JNJ-80948543 on CARNAVAL and OCI-Ly10 xenografts and its impact on xenograft T-cell infiltration. (A and B) T-cell-humanized NSG mice injected subcutaneously with (A) CARNAVAL tumors or (B) OCI-Ly10 tumors were dosed intraperitoneally with JNJ-80948543 at 1 and 5 mg/kg for CARNAVAL or at 3 and 10 mg/kg for OCI-Ly10. Dosing time frame is denoted by bar below the x axis. Tumor volume was measured twice weekly and results presented as the mean tumor volume ± standard error of mean (SEM) for each group (N=10/group). Data are shown while at least 2/3 of animals remained in a group. Statistical significance as compared with the DPBS control evaluated using a mixed model for repeated measures: *$P \leq 0.05$. (C) Effect of JNJ-80948543 on $CD8^+$ T-cell infiltration in SC OCI-Ly10 DLBCL tumors grown in T-cell-humanized mice. Tumor cells were implanted on day 0, T cells were injected on day 20, and doses were administered on days 21, 24, 27, and 31. Tumor samples were collected for analysis at 4, 24, 72, 96, and 168 hours (h) post 4th dose (3 tumors / treatment group). Representative immunohistochemistry micrographs are shown after staining for human CD8. (C1) Tumor treated with Dulbecco's phosphate buffered saline (DPBS) (vehicle) 4 h post 4th dose; (C2, C3, C4) tumors treated with JNJ-80948543 at 3 mg/kg at 4, 72, and 168 h post 4th dose, respectively; (C1-C4) scanned at 40× magnification. (C5 and C6) Higher magnifications of tumors treated with or JNJ-80948543 at 3 mg/kg at 4 h post dose, respectively. Magnification bars represent 1 mm (C1-C4) and 100 μm (C5 and C6).

or 10 mg/kg) demonstrated *in vivo* efficacy comparable to JNJ-80948543 (*Online Supplementary Figure S7C*).

JNJ-80948543 mediates T-cell tumor infiltration

The effects of JNJ-80948543 treatment on T-cell tumor infiltration were assessed in the OCI-Ly10 DLBCL established SC tumor model. After four doses, a trend of TGI was observed in mice treated with 1.0 or 3.0 mg/kg JNJ-80948543 compared with vehicle-treated mice (*Online Supplementary Figure S7D*). After the fourth dose was administered, tumor samples were harvested at 4, 24, 72, 96, and 168 h to assess human $CD8^+$ tumor-infiltrating T cells (Figure 6C). A marked increase in tumor-infiltrating $CD8^+$ T cells was observed with 3.0 mg/kg JNJ-80948543 at 4 h compared with vehicle-treated tumors. Higher levels of $CD8^+$ T cells were observed at 72 and 168 h in tumors treated with 3 mg/kg JNJ-80948543. Similar results were observed in tumors treated with 1 mg/kg JNJ-80948543 (*data not shown*). These results demonstrate that treatment with JNJ-80948543 resulted in tumor infiltration of $CD8^+$ T cells.

While NSG mouse models enable engraftment of human lymphoma cells and assessment of T-cell-mediated cytotoxicity, this does not capture the complexity of immune interactions present in patients. As a result, immune modulation and cytokine dynamics could not be fully evaluated in this setting. Future clinical studies will be essential to confirm these observations and assess the broader immunological effects of JNJ-80948543.

Discussion

In this study, we describe a novel TsAb TCE targeting CD79b, CD20, and CD3, JNJ-80948543, that binds to T cells and $CD79b^+$ and/or $CD20^+$ B-NHL, enabling potent and specific T-cell-mediated cytotoxicity *in vitro* and *in vivo*. JNJ-80948543 effectively eliminated tumor cells expressing varying levels of CD79b or CD20 *in vitro*.

Dual targeting of CD79b and CD20 addresses key limitations of current therapies. While rituximab-based regimens have improved outcomes in patients with DLBCL, approximately 20-40%[19] will recur and require novel options. Several different treatment modalities have been approved in the last decade or are currently being tested in the clinic for B-NHL, including monoclonal enhanced effector and bispecific TCE antibodies, ADC, and chimeric antigen receptor T (CAR-T) cells, primarily targeting single antigens (CD19, CD20, and CD79b). However, no dual CD79b and CD20 targeted TCE approaches have been explored to date.

Clinical combination studies underscore the potential benefit of dual tumor antigen targeting. Polatuzumab vedotin (CD79b-ADC) in combination with bendamustine and rituximab showed improvement in complete response and progression-free survival (PFS) as compared with bendamustine and rituximab in patients with R/R DLBCL after failure of two or more lines of therapy.[13] These results led to the accelerated approval of Pola-BR by the US Food and Drug Administration in 2019.[12] Additionally, the combination of polatuzumab vedotin and rituximab plus cyclophosphamide, doxorubicin, and prednisone (Pola-R-CHP) showed significant improvement in PFS in previously untreated DLBCL patients compared with R-CHOP,[14] leading to the approval of the Pola-R-CHP regimen in many countries (2022/2023). In addition, a phase Ib/II trial of mosunetuzumab (CD20x-CD3 TCE) plus polatuzumab vedotin in R/R aggressive LBCL demonstrated a favorable safety profile with highly durable responses.[16] This concept aligns with emerging strategies in combining CD20×CD3 bispecific antibodies with other agents. For example, epcoritamab plus R-CHOP achieved an objective response rate (ORR) of 100% and CR of 87% in previously untreated LBCL patients (EPOCORE-NHL 2). Similarly, glofitamab combined with polatuzumab vedotin and rituximab (Pola-R-CHP) demonstrated high complete response (CR) rates in high-risk LBCL patients (clinicaltrials.gov identifier NCT06047080), and a chemotherapy-light regimen of glofitamab, rituximab, and polatuzumab (R-Pola-Glo) is being evaluated in elderly/unfit patients (clinicaltrials.gov identifier NCT05798156). In the R/R setting, mosunetuzumab plus polatuzumab vedotin significantly improved PFS compared to rituximab, gemcitabine and oxaliplatin in the phase III SUNMO trial (clinicaltrials.gov identifier NCT05171647). This concept aligns with emerging strategies in cellular therapies, where dual-target CAR-T constructs are under clinical evaluation. CD19/CD20 CAR-T therapies have demonstrated ORR of up to 91% and CR rates of 73% in R/R B-NHL with manageable CRS and low immune effector cell-associated neurotoxicity syndrome (ICANS) incidence.[20] Similarly, CD19/CD22 CAR-T approaches report ORR of 83.7% and CR of 78% across multiple trials,[21] with a recent phase II study showing 100% ORR and 67.7% CR in LBCL (clinicaltrials.gov identifier NCT06081478). Furthermore, tri-specific CAR-T constructs targeting CD19/CD20/CD22 (clinicaltrials.gov identifier NCT07168486) are under investigation to further mitigate antigen escape. These developments highlight the therapeutic rationale for multi-antigen targeting across modalities, reinforcing the potential of JNJ-80948543 to deliver improved efficacy and durability in B-NHL.

Treatment relapse after B-cell lineage targeted therapies have been linked to antigen loss. Profiling of B-NHL samples showed that up to 25% of cells expressed either CD79b or CD20. Previous studies reported that loss of CD20 expression ranging from 7.9-60%, attributed to reduced transcription or acquisition of truncating mutations, have been reported as mechanisms for resistance to anti-CD20 therapies including rituximab and mosunetuzumab.[2,22-28] Unlike CD20, antigen loss of CD79b does not appear to be a major driver of resistance in either pre-clinical or clinical studies. For example, CD79b antigen loss as a mechanism for resistance to polatuzumab vedotin in DLBCL has not been widely observed;[13] this could be because it requires BCR signal transduction. It was shown

that loss of CD79b signaling led to reduced survival in ABC and GCB DLBCL, and Burkitt lymphoma.[4-6] Furthermore, ablation or nonsense mutations of CD79a or CD79b were observed in B-lymphocyte deficient mice and resulted in the developmental arrest of B cells at the pre-B-cell stage.[29] Taken together, these studies indicate that CD79b is an attractive target for a TCE approach, as the development of resistance to CD79b-targeted agents through antigen loss may be less likely to occur. Therefore, as characterized in this study, the dual antigen-targeting properties of JNJ-80948543 could potentially prevent occurrences of R/R disease in patients with B-NHL. Dual tumor-antigen targeting with a TCE TsAb has only recently been exploited.[30,31] This can potentially maximize tumor eradication in the presence of a heterogeneous cell population[32-34] and prevent treatment resistance by tumor antigen escape, resulting in sustained response to treatment.

Cytokine release syndrome and ICANS are commonly observed with CAR-T cell therapy and CD3 bispecific TCE antibodies.[35,36] Although CRS can be managed in the clinic by stepping up dosing schedules, administering corticosteroid pre-medications and the pharmacological inhibition of IL-6R or TNFa, improving the design of next generation CD3 TCE to reduce the incidence of high-grade CRS is warranted to improve safety and limit the need for hospitalization. Initial TCE were often biased towards high-affinity CD3 binders,[37] which elicited potent tumor cell cytotoxicity. However, they led to a high level of cytokine release that required patients to be treated in hospital with longer periods of steroids. Direct comparison of JNJ-80948543 and JNJ-80948556 *in vitro* showed that CD79bxCD20xCD3 TsAb with higher affinity CD3 resulted in higher cytokine secretion.

JNJ-80948543 was engineered with a low-affinity CD3 binding arm to reduce T-cell-mediated toxicity, aiming to improve safety and position the TCE as a preferred combination partner in future lymphoma studies. In this preclinical study, JNJ-80948543 induced cytotoxicity of CD79b$^+$ and/or CD20$^+$ tumor cells with corresponding T-cell activation, while eliciting lower cytokine secretion compared to the higher-affinity TsAb, JNJ-80948556. Despite this difference in cytokine release, both molecules demonstrated comparable efficacy in a xenograft model. Based on these findings, JNJ-80948543 was prioritized for clinical evaluation. Moreover, these results support the concept that JNJ-80948543 has the potential to uncouple tumor cell cytotoxicity from excessive cytokine release, potentially offering lymphoma patients a curative therapy with an improved benefit-risk profile. Further comparisons with other clinically relevant immunotherapies would help contextualize its therapeutic potential.

B-cell aplasia (BCA) is a recognized on-target effect of B-cell-directed therapies, the extent and duration of which vary according to antigen and modality. Agents targeting CD19, which is broadly expressed from early B-cell stages to plasma cells, tend to cause more profound and prolonged B-cell depletion compared to those targeting CD20 or CD79b. CAR-T therapies generally induce more profound and sustained BCA compared to TCE, reflecting differences in mechanism and persistence. For JNJ-80948543, while *in vitro* studies indicate an impact on healthy B cells, its clinical effect on BCA is still under evaluation in ongoing trials.

In conclusion, JNJ-80948543 exhibited potent and specific elimination of CD79b and/or CD20-expressing cells with a favorable cytokine profile. Phase I clinical trials are ongoing to evaluate JNJ-80948543 as a monotherapy (clinicaltrials.gov NCT05424822) and as combination therapy with a co-stimulatory molecule (clinicaltrials.gov NCT06139406) in patients with R/R B-NHL.

Disclosures

All the co-authors are current employees or have been employees in the past 2 years of Johnson & Johnson, and may own stock/stock options in the company.

Contributions

AK, DY, NV, IC, RA, TP, LJ, TS, MB, NN, TH, EL, LF, CS, NM, BW, JC, MF, CH, SW, HH and UP performed research; AK, DY, TP, EL, LF, SrSr, SiSu, AM, ND, JK, CH, RA, MPD, YE and UP discussed and analyzed data; SaSi contributed to perform research, and discuss and analyze the data; AK and UP wrote the manuscript. All authors edited the manuscript and approved the final version for publication.

Acknowledgments

This study was funded by Johnson & Johnson. Medical writing support was provided by Paul Cao (Johnson & Johnson) and was funded by Johnson & Johnson.

Data-sharing statement

The data supporting the findings of this study are available within the article or in the Online Supplementary Appendix.

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