Journal for ImmunoTherapy of Cancer

Potency-optimized CD28-activating bispecific antibody for the targeted treatment of Nectin-4 positive cancers

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To cite: Kaur M, Rüger K, Chen EC, *et al*. Potency-optimized CD28-activating bispecific antibody for the targeted treatment of Nectin-4 positive cancers. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e011323. doi:10.1136/jitc-2024-011323

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-011323).

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Accepted 16 March 2025

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ABSTRACT

Background T-cell costimulation is crucial for an effective and sustained antitumor immune response, and inadequate expression of costimulatory ligands within tumors can impair T-cell function. Bispecific antibodies (bsAbs) targeting a tumor-associated antigen and the T-cell costimulatory receptor CD28 represent a novel class of immune-stimulatory therapeutics designed to enhance antitumor immune responses by selectively delivering T-cell costimulation directly to the tumor microenvironment. This approach holds the potential to improve the survival, proliferation, and cytotoxic function of antitumor T cells while minimizing the risk of systemic immune activation. Urothelial cancer (UC) is associated with significant morbidity and mortality worldwide, particularly in advanced disease settings. Nectin-4, a membrane protein highly expressed in UC with limited expression in healthy tissues, presents a compelling target for therapeutic intervention.

Methods Using our proprietary high-throughput antibody discovery pipeline, we identified a panel of novel antibodies with a range of affinities for CD28 and Nectin-4 and successfully engineered them as bsAbs. We tested the T-cell costimulatory function of these molecules *in vitro* using primary human T cells and human cancer cell lines. Based on these results, we selected a clinical candidate which we assessed in a syngeneic mouse tumor model system and investigated tolerability and pharmacokinetics (PK) in non-human primates (NHP).

Results Our *in vitro* studies demonstrated that these bsAbs effectively enhance T-cell activation and cytotoxicity against Nectin-4 positive tumor cells in the presence of T-cell receptor engagement. The bsAb panel exhibited a range of potencies, enabling the selection of a clinical candidate, termed RNDO-564, that maximized antitumor efficacy as well as the likelihood of a broad therapeutic window. Tumor-bearing syngeneic mouse models confirmed the *in vivo* efficacy of RNDO-564, demonstrating significant tumor regression both as a single agent and in combination with an immune checkpoint inhibitor. We observed favorable PK and tolerability profiles in NHP assessments.

Conclusions Our study reports the first CD28 bsAb targeting Nectin-4 and highlights the potential of CD28 × Nectin-4 bsAbs as a new immunotherapeutic modality. The findings support the clinical development of RNDO-564 in patients with locally advanced and metastatic UC and other Nectin-4 positive malignancies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ CD28 bispecific antibodies are an emerging class of immunotherapies with the potential to treat a wide range of cancers. However, little is known about the optimal design of CD28 engagers, including the ideal potency of these molecules for maximizing clinical success.

WHAT THIS STUDY ADDS

⇒ We describe the preclinical development of a CD28 × Nectin-4 bispecific antibody (bsAb) optimized for the treatment of advanced urothelial cancer (UC) that elicits costimulation of tumor-specific T cells at the site of the tumor. We detail our approach to empirically determine the optimal potency of CD28 engagement from a panel of molecules containing CD28 binding domains with different affinities. This is the first example of a CD28 bsAb targeting Nectin-4 and represents a new therapeutic strategy for UC.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study supports the clinical development of CD28 × Nectin-4 bsAbs for treatment of UC and other Nectin-4 positive cancers.

INTRODUCTION

Immune checkpoint therapy (ICT) has revolutionized cancer treatment; however, the limited response rates to single-agent ICT highlight the urgent need for additional therapeutic strategies.[1] In hematological malignancies, bispecific T-cell engaging antibodies (TCEs) have emerged as a promising therapeutic modality, evidenced by several recent Food and Drug Administration (FDA) approvals.[2] In contrast, TCEs have faced significant challenges in solid tumors, with limited efficacy or toxicity often implicated in

discontinuation of clinical use.[3 4] A key barrier to TCE efficacy in solid tumors is the complex and immunosuppressive tumor microenvironment which hinders both the antitumor activity and durability of these therapies.[5]

Recent reports suggest that an important characteristic of the suppressive solid tumor microenvironment is insufficient T-cell costimulation stemming from a lack of antigen-presenting cells expressing the CD28 ligands CD80 and CD86 as well as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-mediated sequestration of these proteins.[5–7] CD28 is a membrane-bound, homodimeric T-cell costimulatory receptor expressed by most T cells, and is integral to T-cell activation, proliferation, and the formation of a memory response.[8] Interaction with its ligands on antigen-presenting cells provides a necessary signal (termed Signal 2) that enhances T-cell receptor (TCR) signaling (termed Signal 1) and is required for full T-cell activation. Insufficient costimulation causes T cells to enter a state of anergy, characterized by hyporesponsiveness to cognate peptide major histocompatibility complex (MHC) resulting in reduced effector function and immunosuppression.[9 10] In the context of cancer therapy, augmenting CD28 signaling has the potential to amplify endogenous T-cell responses against tumor cells.[11] However, a key challenge lies in achieving selective activation of tumor-specific T cells to prevent systemic immune activation and associated toxicities, such as those seen in the phase I clinical trial of the CD28 super-agonist antibody TGN1412.[12 13]

A bispecific antibody (bsAb) approach that integrates CD28 engagement with the binding of a tumor-associated antigen (TAA) has the potential to direct costimulation specifically to the site of the tumor. This strategy has the benefit of enhancing the function of antitumor T cells while minimizing systemic immune activation. Several clinical trials are currently investigating this approach;[11] however, key questions remain, such as the optimal potency of CD28 activation in bsAb format and the potential for clinical development as monotherapies. Insights from TCE development have shown that optimizing potency through affinity tuning of anti-CD3 antibodies can improve therapeutic windows.[14 15] We hypothesize that similar tuning will be necessary to optimize the therapeutic window for CD28 bsAbs depending on the TAA, indication and potential combination partners. Therefore, there is a need for novel CD28-engaging antibodies with a range of potencies to explore optimal engagement strategies across various tumor targets and indications.

Nectin-4 is a cell adhesion molecule overexpressed in multiple malignancies, including urothelial, breast, skin, lung, and pancreatic cancers, with limited expression in healthy adult tissues.[16–18] Moreover, Nectin-4 has been implicated in promoting tumor cell proliferation, migration, and survival, making it a compelling target for therapeutic intervention.[18] In urothelial cancer (UC), a Nectin-4-directed antibody drug conjugate (ADC) is approved for use in locally advanced and metastatic disease (la/mUC) while additional recently approved therapies include several checkpoint inhibitors as well as FGFR inhibitors for a subset of patients.[19] Despite these advancements, options for la/mUC patients who become refractory to first-line therapies remain limited, with 5-year survival rates below 10% for those with metastatic disease.[20–23] Thus, there is an urgent need for novel therapeutic approaches and new modalities to improve patient outcomes.

In this study, we describe the identification and optimization of a CD28 × Nectin-4 clinical development candidate by evaluating a panel of bsAbs with a range of potencies. These molecules are designed to enhance immune-mediated tumor eradication while minimizing off-target effects by activating T cells only in the setting of both TCR/peptide-MHC engagement and Nectin-4-positive tumor cells. A detailed analysis of the binding properties, *in vitro* functional activity, and therapeutic potential of these molecules in preclinical models allowed us to select a clinical development candidate, termed RNDO-564, that is designed to support robust antitumor activity while preserving a wide therapeutic window. Our results suggest a promising new immune-activating therapeutic modality for the treatment of Nectin-4-positive cancers that is differentiated from the current standard of care and, to our knowledge, is the first time Nectin-4-targeted CD28 activation will be investigated as a treatment for UC.

RESULTS

Discovery of anti-CD28 antibodies

We used a next-generation sequencing (NGS)-based discovery approach to identify 764 unique monoclonal antibodies (mAbs) from antibody repertoires of humanized rodents immunized with CD28[24] (figure 1A). Based on high-throughput cell binding analysis to CD28-positive and -negative cell lines, we selected 27 mAbs for further characterization. Additional studies confirmed that 25 of the 27 candidates showed specific binding to human CD28 protein and a 30-fold range of binding strength to CD28-positive Jurkat cells, while none of the mAbs bound to mouse CD28 (figure 1B). We then evaluated the costimulatory activity of each CD28 mAb in combination with the anti-CD3 antibody OKT3 by measuring interleukin (IL)-2 production from human $CD4^+$ T cells incubated with each antibody co-treatment. All but one of our CD28 candidate mAbs enhanced IL-2 production above that of OKT3 stimulation alone, although generally not to the extent of the strong positive control CD28 mAb TGN1412. Sequence analysis of the CD28 mAbs revealed six clonotypes in the set of 27 antibodies based on CDR3 (complementarity determining region 3) similarity. To screen additional mAb candidates that could have more desirable features, such as varying affinities or better developability properties, we identified 269 additional members of these clonotypes from the full antibody repertoire data set. After another round of screening similar to that described for the initial set of 27 mAbs, we selected

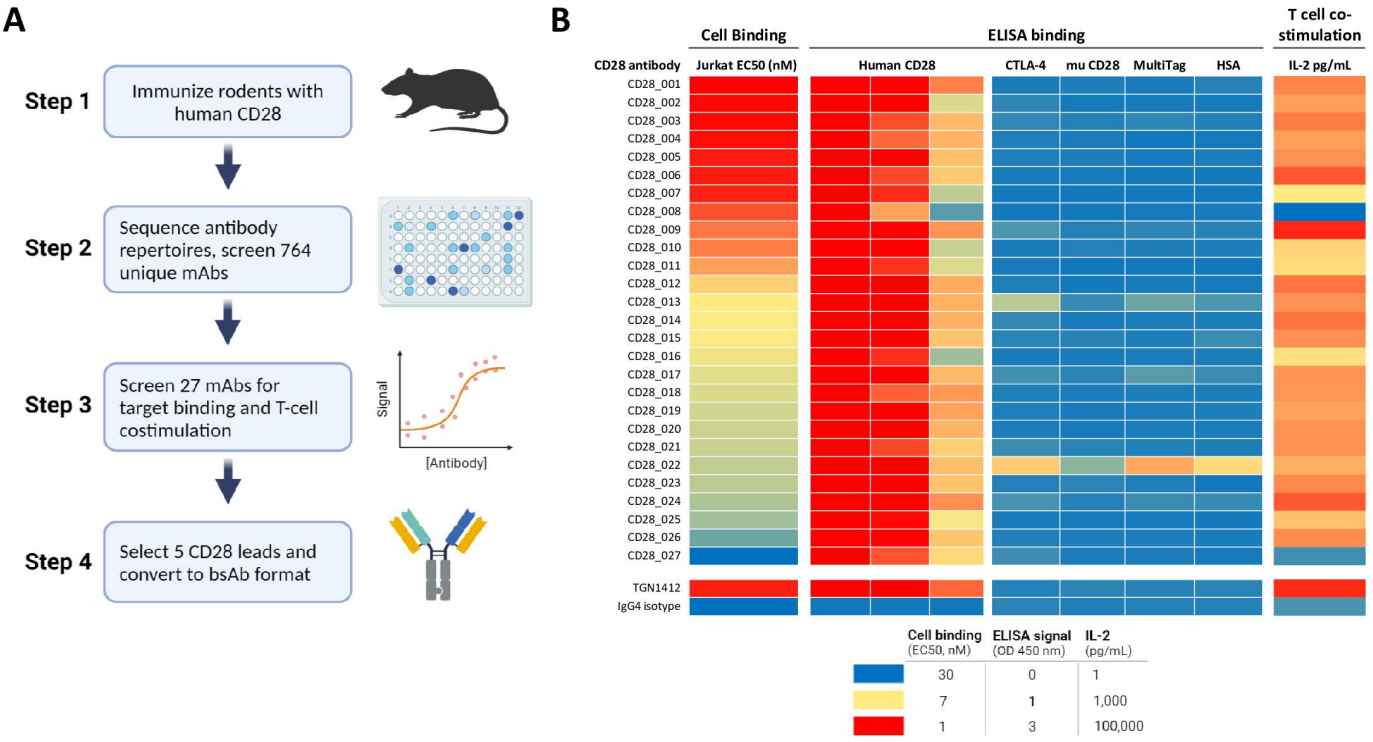


Figure 1 Discovery of a diverse panel of CD28 agonist antibodies. (A) Our CD28 antibody discovery approach consisted of four key steps: immunization of humanized rodents, antibody repertoire analysis and high-throughput screening of monoclonal antibodies (mAbs), detailed binding and functional analysis of a subset of antibodies and conversion of selected candidates to bsAb format. (B) Summary of step 3 of our CD28 antibody discovery approach. CD28-positive Jurkat cell binding dose response results for 27 anti-CD28 mAbs reported as EC50 values (column 2). Binding signal as measured by ELISA for each antibody tested at a concentration of 1.6 µg/mL, 0.16 µg/mL and 0.016 µg/mL to human CD28 (columns 3–5) and at 1.6 µg/mL to off-target proteins (columns 6–9). Human $CD4^+$ T-cell costimulation as measured by IL-2 production (column 10). IL-2 was measured in supernatants from 96-well plates containing 1×10^5 T cells in a 200 µL volume incubated overnight in the presence of 1 µg/mL plate-coated anti-CD3 antibody OKT3 and 16 µg/mL of each anti-CD28 mAb. bsAb, bispecific antibody; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; IL, interleukin; mu, mouse.

five CD28 mAbs (online supplemental figure S1), based on their specific CD28 binding, lack of sequence liabilities and range of target affinity (double to triple-digit nM affinities to recombinant human CD28 protein) and converted them into bsAb format.

Design and specificity of CD28 × Nectin-4 bsAbs

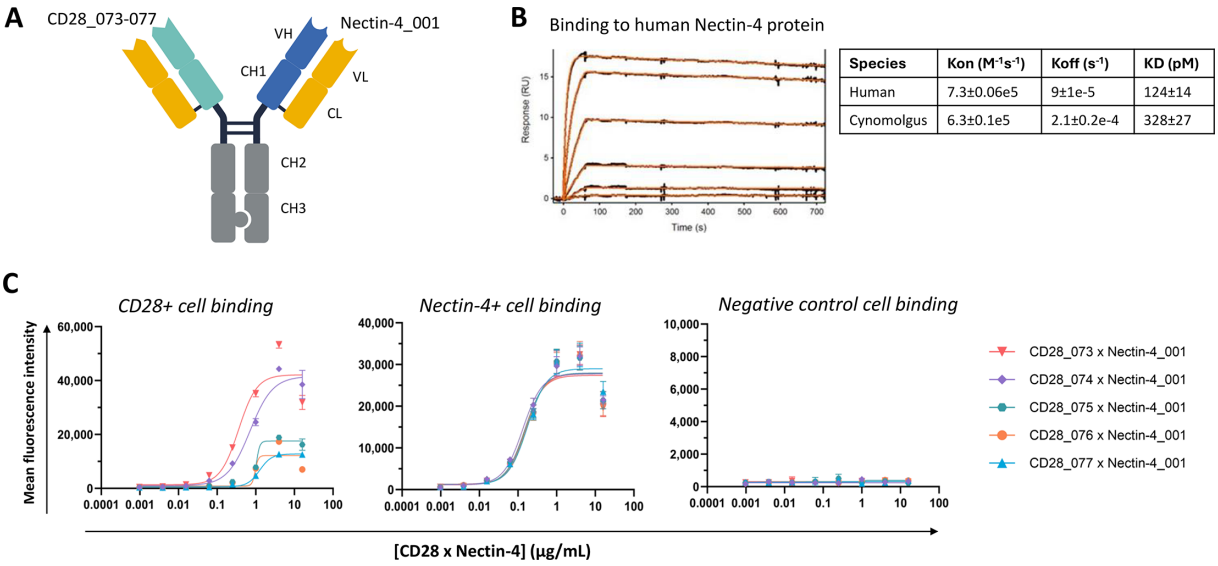
Following an antibody discovery strategy similar to that described for CD28, we identified the Nectin-4 binding antibody, Nectin-4_001, based on its high target affinity (124 pM and 328 pM to human and cynomolgus Nectin-4, respectively, figure 2B, online supplemental figure S2) and paired it with our panel of five CD28 antibodies for conversion to bsAb format (figure 2A). To prevent Fc receptor and complement binding of the bsAbs, we used a hinge-stabilized, silenced IgG4 Fc, with knobs-into-holes mutations to drive efficient formation of heterodimers.[25 26] We observed varying binding strength to Jurkat cells for the set of five CD28 × Nectin-4 bsAbs, as expected given the range of CD28 protein-binding affinities of these molecules (figure 2C). Binding to the Nectin-4 positive cell line T-47D was highly similar because the five bsAbs contain an identical Nectin-4-binding domain. None of the bsAbs showed binding to a target cell line negative for both CD28 and Nectin-4 (UM-UC-3).

Biophysical properties of CD28 × Nectin-4 bsAbs

The panel of CD28 × Nectin-4 bsAbs displayed robust transient expression yields and purity above 90% (table 1). We further polished the bsAbs to greater than 95% purity and analyzed the melting and aggregation temperatures (Tm and Tagg), finding values within the 90% threshold of approved mAbs.[27] We then assessed stability after four cycles of freeze-thaw and after thermal stress by incubation at 40°C for 1 month. All antibodies showed excellent stability with ≤2.0% change in monomer content.

CD28 × Nectin-4 bsAbs exhibit target-dependent T-cell costimulation

To determine whether the CD28 × Nectin-4 bsAbs can provide T-cell costimulation, we developed a co-culture assay using primary human T cells and human tumor cell lines ranging in Nectin-4 expression (online supplemental figure S3). TCR stimulus (Signal 1) in our *in vitro* assays was provided by a CD3 bispecific antibody (CD3 bsAb) targeting the TAA 5T4 (expressed by all tumor cell lines that we used). We first assessed tumor cell



Species	Kon ($M^{-1}s^{-1}$)	Koff (s^{-1})	KD (pM)
Human	7.3±0.06e5	9±1e-5	124±14
Cynomolgus	6.3±0.1e5	2.1±0.2e-4	328±27

Figure 2 CD28 × Nectin-4 bispecific antibody panel. (A) Schematic of bispecific antibody format. A human IgG4 Fc scaffold was used, including silencing mutations and knob-into-hole mutations to facilitate heterodimer formation. VH (variable heavy chain), VL (variable light chain), CH1/2/3 (heavy chain constant domain 1/2/3), CL (light chain constant domain). (B) Surface plasmon resonance analysis of human Nectin-4 binding by the bsAb CD28_075 × Nectin-4_001. The reported values are averages of triplicate measurements at two loading densities and a representative binding trace is shown. (C) Analysis of cell binding by flow cytometry of the panel of five CD28 × Nectin-4 bsAbs. Binding results to CD28-positive Jurkat cells, Nectin-4-positive T-47D cells and a CD28- and Nectin-4-negative cell line, UM-UC-3 are shown. The mean and SD of triplicate measurements are shown. bsAb, bispecific antibody.

cytotoxicity in the presence of a subefficacious concentration of CD3 bsAb combined with a titration of each CD28 × Nectin-4 bsAb across the panel of Nectin-4-positive and -negative cell lines (figure 3A). All the CD28 × Nectin-4 bsAbs showed dose-dependent enhanced cytotoxicity of the Nectin-4-positive cell lines. We next measured production of IL-2 following a similar assay design and observed increased IL-2 in the presence of Nectin-4-positive cells (figure 3B). Importantly, we did not detect cytotoxicity or cytokine production in the absence of Signal 1 (online supplemental figure S4A,B). We also assessed whether T-cell proliferation was enhanced by the CD28 × Nectin-4 bsAbs and found dose-dependent augmented proliferation of both $CD4^+$ and $CD8^+$ T cells only in the presence of Signal 1 (figure 3C, online supplemental figure S4C). CD28 antibodies that non-specifically activate T cells in the absence of Signal 1 pose a safety risk due to uncontrolled immune activation and are termed

Table 1 Biophysical properties of CD28 × Nectin-4 bispecific antibodies. All antibodies show >90% purity and yield between 24 and 42 milligrams (mgs) from a 200 mL transient transfection after a 1-step protein A purification. Melting (T_m) and aggregation (T_{agg}) temperatures were measured on 2-step purified proteins on the Uncle. Long-term thermal stability was tested by incubating samples for 4 weeks at 40°C. Stability was assessed by SEC-HPLC analysis of percent monomer content, decrease in percent monomer from the initial time point (T0) to 4 weeks (D28) at 40°C is reported. Stability after four freeze-thaw cycles (FT4) was analyzed by percent change in monomer compared with T0 by SEC-HPLC.

	Post 1-step purification		Post 2-step purification			
	Purity	Yield	Thermal stability		Long-term stability	Freeze-thaw
CD28 binder	Monomer	Transient expression (200 mL)	T_m	T_{agg}	Monomer decrease T0-D28	Monomer decrease T0-FT4
	%	mg	°C	°C	%	%
CD28_073 × Nectin-4_001	95	31	65	80	2	0
CD28_074 × Nectin-4_001	93	24	64	79	0	0
CD28_075 × Nectin-4_001	91	42	64	78	1	1
CD28_076 × Nectin-4_001	93	40	64	81	0	1
CD28_077 × Nectin-4_001	93	41	66	77	1	0

FT, Freeze-thaw; T_{agg}, aggregation temperature; T_m, melting temperature.

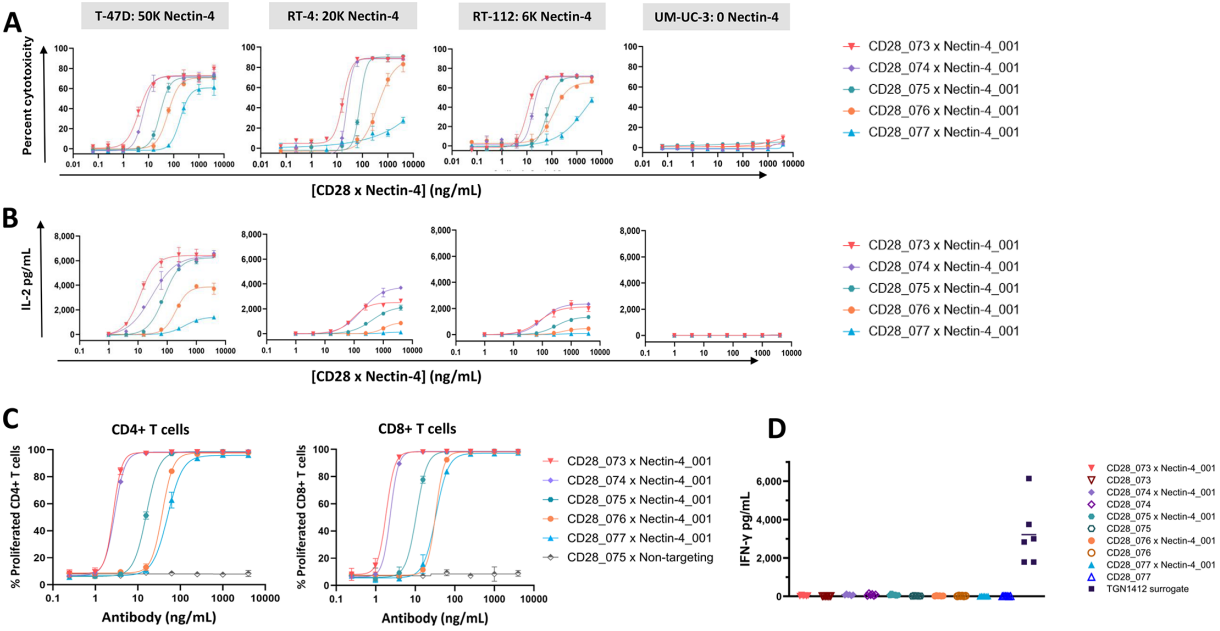


Figure 3 CD28 × Nectin-4 bsAbs exhibit target-dependent T-cell costimulation. (A) T cells were incubated with target cells at a 5:1 E:T ratio in a 200 µL assay volume. CD28 × Nectin-4 bsAbs were titrated with a limiting concentration of CD3 bsAb. To assess tumor cell cytotoxicity, the concentration of the CD3 bsAb was adjusted for each cell line to correspond to the EC10 concentrations as follows: T-47D at 5.5 pM, RT4, RT-112 and UM-UC-3 at 27.6 pM. Assays were incubated for three days, after which time cell viability was assessed. The mean and SD of triplicate values are shown. (B) In an IL-2 release assay, CD28 bsAbs were titrated with a fixed CD3 bsAb concentration of 103 pM, a level of Signal 1 optimal for analysis of cytokine release. Assay supernatants were collected after 24 hours of incubation and analyzed for production of IL-2. The mean and SD of duplicate values are shown. (C) T cells labeled with CellTrace Violet (CTV) were incubated with T-47D target cells at a 2:1 E:T ratio. Proliferation of $CD8^+$ and $CD4^+$ T cells by CTV dilution was measured after five days of incubation by flow cytometry. The mean and SD of triplicate measurements are plotted for panels A, B and C. (D) Assessment of the potential for super-agonist activity was measured by IFN-γ production in a solid-phase cytokine release assay containing $1{\times}10^5$ human peripheral blood mononuclear cells (PBMCs) incubated with immobilized CD28 × Nectin-4 bsAbs or the parental CD28 monoclonal antibodies in a 200 µL assay volume. Triplicate measurements of two representative PBMC donors are plotted. bsAb, bispecific antibody; E:T, effector to target; IFN, interferon; IL, interleukin.

super-agonists.[13] We assessed the potential for super-agonist activity in our CD28 × Nectin-4 bsAb panel by using an FDA-recommended assay where human peripheral blood mononuclear cells (PBMCs) are incubated with immobilized antibodies and tested for activation by cytokine release.[28 29] We compared our CD28 × Nectin-4 bsAbs as well as the CD28 parental mAbs to the quintessential CD28 super-agonist TGN1412 by measuring production of interferon (IFN)-γ. We did not observe super-agonist activity from the five CD28 × Nectin-4 bsAbs or parental CD28 mAbs, whereas the positive control TGN1412 showed robust IFN-γ production (figure 3D).

CD28 costimulation supports T-cell cytotoxic activity at a low E:T ratio

We next assessed the costimulatory activity of our CD28 × Nectin-4 molecules at a low effector to target (E:T) ratio of 1:4, as tumor-specific T cells are likely present at low numbers within the tumor microenvironment.[9 30] For comparison, we also tested a 5:1 E:T ratio, where the CD3 bsAb induced high levels of tumor cell cytotoxicity alone, with further potency enhancements after the addition of each of the CD28 × Nectin-4 bsAbs (figure 4A, online supplemental figure S5A). However, the CD3 bsAb alone at a 1:4 E:T ratio had no activity (figure 4B, online supplemental figure S5B). The addition of CD28 × Nectin-4 bsAbs restored activity to varying degrees correlating to the bsAb potency, suggesting the potential for activity in a setting with limited tumor-specific T cells.

CD28 × Nectin-4 costimulation differentially impacts cytokine production

We next investigated the impact of CD28 × Nectin-4 costimulation on production of cytokines associated with T-cell effector function and cytokine release syndrome (CRS)[31] (online supplemental figure S6). To measure the change in cytokine production from the addition of each CD28 × Nectin-4 bsAb, we calculated the fold enhancement of each co-treatment relative to the CD3 bsAb titration alone. IL-2 was the most enhanced cytokine in each co-treatment, with smaller increases in IFN-γ, IL-4, IL-1β and tumor necrosis factor (TNF)-α (figure 4C, online supplemental figure S7). Importantly, we did not detect substantial increases in the CRS-associated cytokine IL-6.

Clinical development candidate selection

Our *in vitro* assessments demonstrated a range of activity in the panel of CD28 × Nectin-4 bsAbs. To identify an

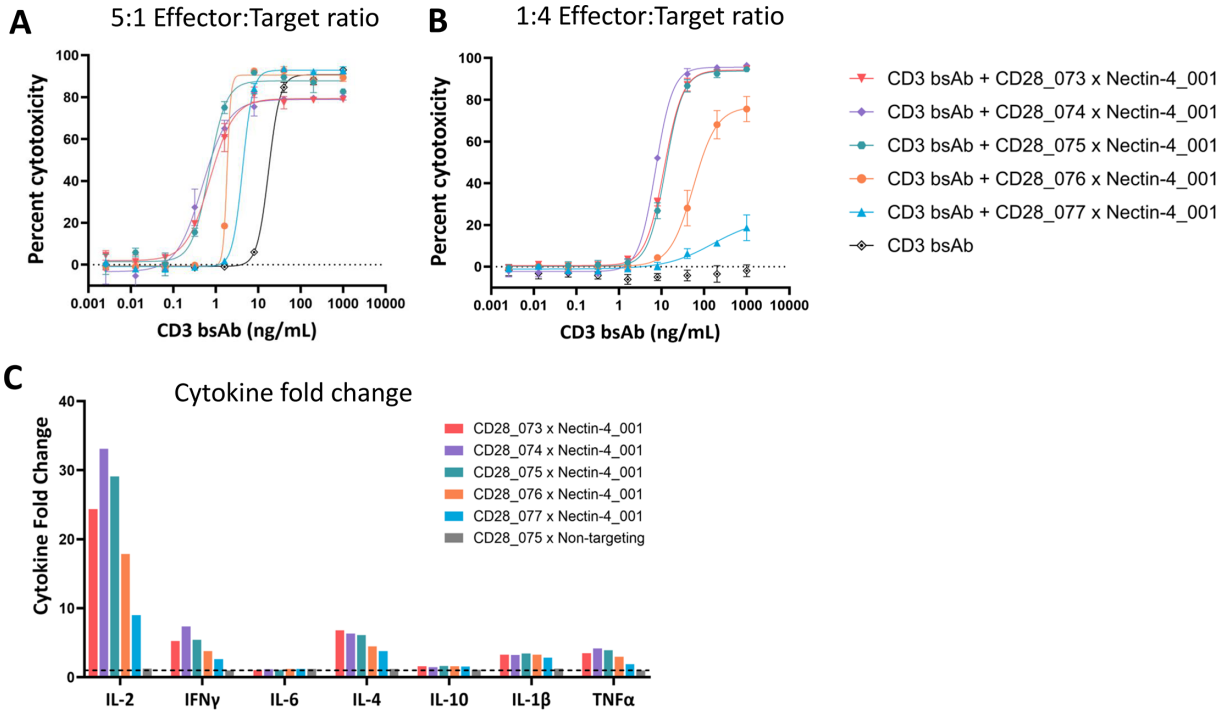


Figure 4 CD28 costimulation supports T-cell redirected cytotoxicity at low E:T ratios and differentially impacts cytokine production. 1×10^4 RT4 bladder cancer cells were incubated for three days with human T cells from one donor at either (A) a 5:1 or (B) 1:4 effector to target cell ratio and a titration of CD3 bsAb with or without 4 µg/mL of the indicated CD28 × Nectin-4 bsAb followed by assessment of tumor cell cytotoxicity. The mean and SD of triplicate values are shown. (C) Assessment of cytokine production was conducted by co-culturing 1×10^4 RT4 bladder cancer cells with freshly isolated human peripheral blood mononuclear cells (donor #9021) at a 5:1 effector-to-target (E:T) ratio and a titration of CD3 bsAb, with or without 4 µg/mL of each CD28 × Nectin-4 bsAb in a 200 µL volume. Assay supernatants were collected after 48 hours to assess production of the cytokines IL-2, IFN-γ, IL-6, IL-4, IL-10, IL-1β and TNF-α. Fold enhancement of production of each cytokine by the addition of each CD28 × Nectin-4 bsAb was calculated relative to the CD3 bsAb treatment alone by dividing the area under the curve for each co-treatment condition with that of the CD3 bsAb treatment alone (GraphPad Prism V.10). Data from a representative donor is shown. A dashed horizontal line indicates a fold change of 1. bsAb, bispecific antibody; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

optimal clinical candidate, we considered both potency and maximum activity. CD28_076× Nectin-4_001 and CD28_077× Nectin-4_001 had reduced maximum activity in some assays such as cytotoxicity at low E:T ratios (figure 4B, online supplemental figure S5B), suggesting a potential risk for insufficient clinical activity. In contrast, the three other bsAbs in the panel had similar maximum activity but a range of potencies (figure 3). CD28_075x Nectin-4_001 was the least potent of these three molecules, suggesting it may maintain efficacy while benefiting from a wider therapeutic window. Thus, we selected it for clinical development, renaming it to RNDO-564.

RNDO-564 enhances T-cell function in settings with mixed Nectin-4 positive and negative tumor cells

Recent studies report heterogeneous Nectin-4 expression in both primary and metastatic tumors in patients with la/mUC.[32 33] We assessed Nectin-4 expression on dissociated tumor cells (DTCs) from patients with UC (n=7), and found that the Nectin-4-positive fraction ranged from 9% to 51% (figure 5A). Thus, we were interested in determining the activity of RNDO-564 in a setting with mixed Nectin-4-positive and -negative tumor cells. We generated a Nectin-4-negative MCF-7 cell line (MCF-7^{KO}), then combined these cells and the Nectin-4-positive parental MCF-7 cells (MCF-7^{P}) at various ratios in a cytotoxicity assay containing RNDO-564 and a subefficacious concentration of CD3 bsAb. We assessed the viability of the $MCF7^{P}$ and $MCF7^{KO}$ populations, observing enhanced cytotoxicity against both $MCF7^{P}$ and $MCF7^{KO}$ target cells at each ratio tested (figure 5B). As the fraction of $MCF7^{KO}$ cells increased, maximum levels of cytotoxicity decreased, perhaps due to the reduction in target cells providing the costimulatory signal necessary to enhance T-cell function. However, activity was greatly elevated over that observed under the control condition containing 100% $MCF7^{KO}$ cells. We then assessed co-cultures of allogeneic T cells with bladder DTCs treated with RNDO-564 alone or in combination with CD3 bsAb (figure 5C). We observed increased T-cell activation and cytokine production from the combination treatment as well as smaller increases from each individual treatment. T-cell activation correlated to the fraction of Nectin-4-positive DTCs (online supplemental figure S8), in agreement with the pattern of cytotoxicity enhancement seen in our MCF7

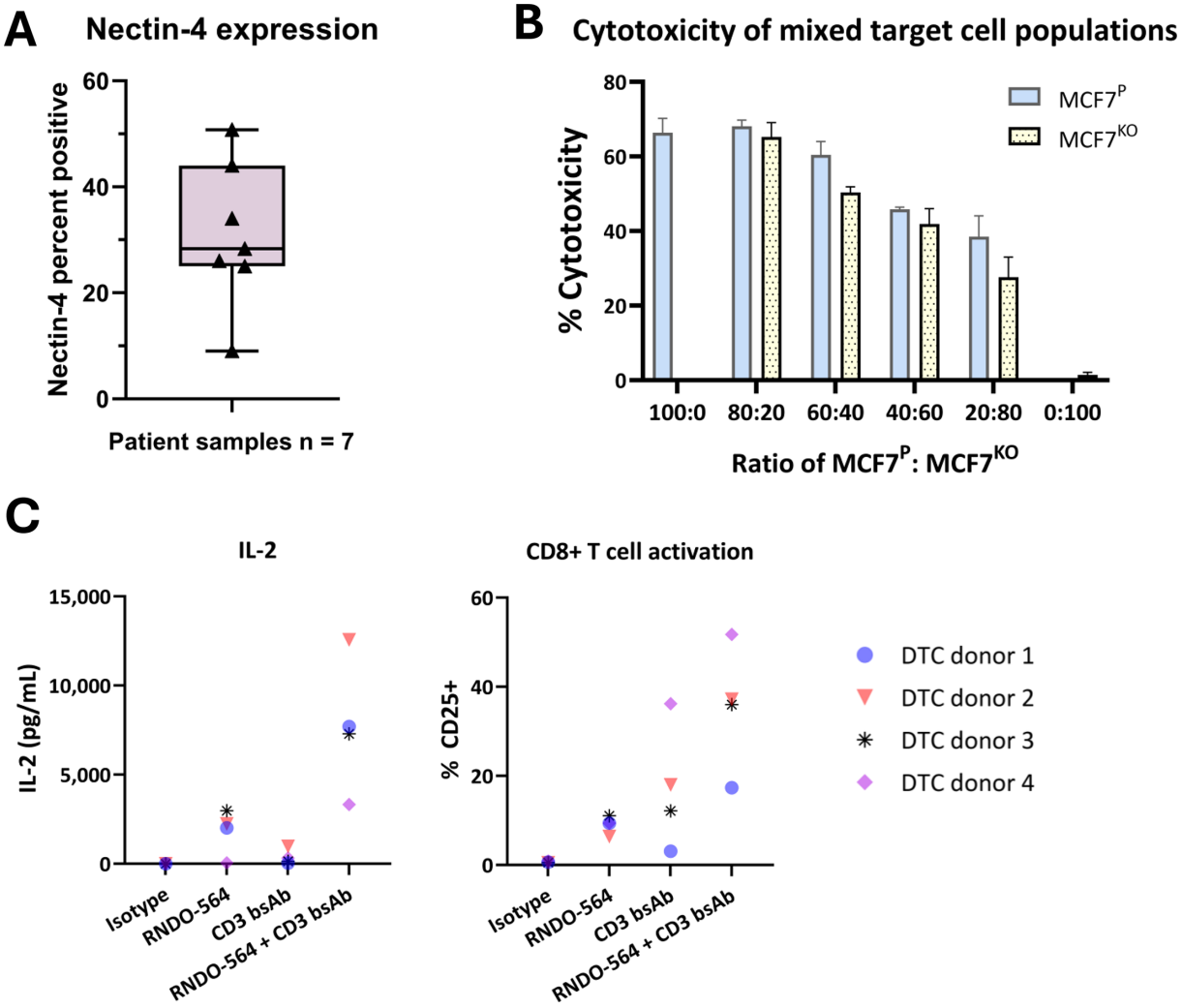


Figure 5 RNDO-564 enhances T-cell function in settings with mixed Nectin-4-positive and -negative target cells. (A) Nectin-4 expression was analyzed on seven patients with bladder cancer dissociated tumor cell samples (DTCs) by flow cytometry. Tumor cells were identified by EpCAM expression. (B) Parental MCF7 ($MCF7^{P}$) and Nectin-4 knock-out MCF7 ($MCF7^{KO}$) cells were labeled with CellTrace Violet and CFSE (carboxyfluorescein diacetate succinimidyl ester), to distinguish the two populations and mixed at varying ratios of $MCF7^{P}$: $MCF7^{KO}$ as indicated by the X-axis. Human T cells were added at a 1:1 E:T ratio, along with CD3 bsAb (5.5 pM) and RNDO-564 (1 µg/mL) and incubated for three days. Target cell cytotoxicity was assessed using flow cytometry by calculating the number of live labeled target cells per well compared with untreated controls. The mean and SD of triplicate values are shown. (C) Four of the DTC samples from panel A were co-cultured with allogeneic T cells at a 10:1 E:T ratio along with 64 µg/mL RNDO-564 with or without 4 ng/mL of CD3 bsAb. Assay supernatants were analyzed for cytokine production after 48 hours of incubation and T-cell activation was assessed by flow cytometry analysis of CD25 expression after 72 hours of incubation. bsAb, bispecific antibody; E:T, effector to target; IL, interleukin.

model system. The small increases from RNDO-564 monotherapy treatment may indicate alloreactivity between the T-cell donor and DTCs or activation of tumor-specific T cells within the DTC population. Taken together, these experiments demonstrate beneficial activity by RNDO-564 in a mixed Nectin-4-positive and -negative tumor cell setting with either tumor cell lines or UC patient DTCs.

CD28 costimulation enhances the function of chronically stimulated T cells

Chronically stimulated T cells with reduced effector activity may be found in the tumor environment,[5] thus we were interested in determining if T-cell costimulation could reverse the loss of cytotoxic function associated with this state. To test this *in vitro*, we repeatedly activated T cells with beads conjugated to CD3 antibodies.[34] After serially activating the T cells, we profiled their exhaustion marker expression by flow cytometry, finding increases in PD-1, CD39, TIM-3, LAG3, CTLA-4 and TOX (online supplemental figure S9). We then assessed their function in a cytotoxicity assay with either CD3 bsAb or co-treatment with CD3 bsAb and RNDO-564 (figure 6A). Serially stimulated T cells treated with CD3 bsAb alone showed reduced tumor cell cytotoxicity, while the addition of RNDO-564 completely restored cytotoxic function. For comparison, we tested T-cell function prior to serial stimulation, observing complete cytotoxicity of tumor cells with or without costimulation.

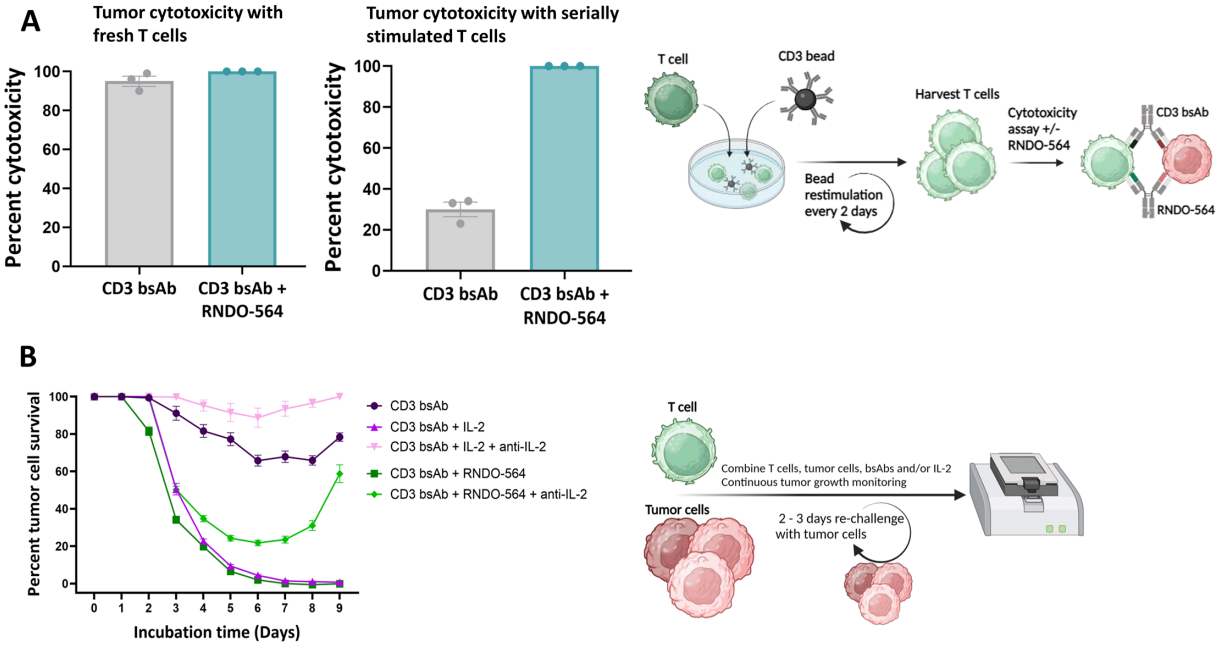


Figure 6 CD28 costimulation enhances the function of chronically stimulated T cells. (A) Human T cells were activated with anti-CD3 beads at a 1:1 ratio every two days. After the fourth stimulation, T cells were collected for use in a cytotoxicity assay with either CD3 bsAb (1 µg/mL) or a combination of CD3 bsAb and RNDO-564 (4 µg/mL) at a 2:1 E:T ratio with T-47D target cells. Target cell survival was measured using xCELLigence cell index values after six days of incubation. The mean of triplicate values is plotted with SD indicated by error bars. (B) 3×10^3 T47D cells were co-cultured with human T cells at 1:1 E:T ratio along with the indicated treatments of CD3 bsAb (1 µg/mL), RNDO-564 (4 µg/mL), IL-2 (10 U/mL) and anti-IL-2 antibody (4 µg/mL) in xCELLigence PET 96-well plates. T cells were re-stimulated by the addition of 3×10^3 T-47D target cells every 48–72 hours (on days 0, 3, 5 and 7) and percent target cell survival was measured using xCELLigence cell index values. Percent survival in each condition was calculated relative to the average of untreated control wells. Each condition was tested in four to eight replicates and the mean and SD of all replicates are plotted. bsAb, bispecific antibody; E:T, effector to target; IL, interleukin.

Given that CD28 costimulation supports high levels of IL-2 production, we next investigated the impact of this cytokine on tumor cell cytotoxicity in a serial stimulation assay where T cells were repeatedly challenged with tumor cells in the presence of CD3 bsAb (figure 6B). We monitored tumor cell viability to assess T-cell cytotoxic function when treated with either CD3 bsAb and RNDO-564 (dual treatment) or CD3 bsAb + RNDO-564 + an IL-2 antagonist antibody (dual treatment + αIL-2). For comparison, we also tested treatment with CD3 bsAb alone and CD3 bsAb + IL-2 with and without the addition of IL-2 antagonism. Interestingly, dual treatment + αIL-2 initially showed similar levels of tumor cell cytotoxicity as dual treatment; however, by the third challenge tumor cell cytotoxicity plateaued and tumor growth control was lost. We also observed limited tumor cell cytotoxicity in the presence of monotherapy treatment with CD3 bsAb alone. In contrast, treatment with CD3 bsAb + IL-2 showed tumor cell cytotoxicity equivalent to the dual treatment condition. These results highlight the important role of IL-2 in supporting sustained T-cell function and indicate that CD28 costimulation enhances T-cell function through additional mechanisms independent of IL-2. CD28 costimulation is important for the formation of a T-cell memory response[8] and so we investigated the memory phenotype following treatment with a dose titration of RNDO-564 or a non-targeting CD28 bsAb negative control in the presence of Signal 1. RNDO-564 preferentially supported a central memory phenotype in $CD8^+$ and $CD4^+$ T cells (online supplemental figure S10).

RNDO-564 is effective as a single agent and in combination with ICT in tumor-bearing syngeneic mouse models

To evaluate the *in vivo* activity of RNDO-564, transgenic mice expressing human CD28 extracellular domain were subcutaneously implanted with a mouse tumor cell line (MC38) expressing human Nectin-4 (figure 7A, online supplemental figure S11). MC38 cells express retroviral antigens which are recognized as foreign by mouse T cells, thus providing Signal 1.[35] Therefore, we hypothesized that T-cell costimulation with RNDO-564 would elicit monotherapy activity by enhancing the endogenous immune response. After tumors reached an average volume of 100 mm^3, twice weekly treatments were initiated at 10, 1, and 0.1 mg/kg. We observed significant tumor regression in all treatment groups, with complete responses in all members of the 10 mg/kg group by day 45 (figure 7B). We next tested the combination of RNDO-564 with an anti-mouse PD-1 (programmed cell death protein 1) antibody, both dosed at 1 mg/kg, selected based on their moderate activity as monotherapy treatments. All animals in the combination group showed stable disease or complete response, whereas in both monotherapy groups 25% of animals did not respond

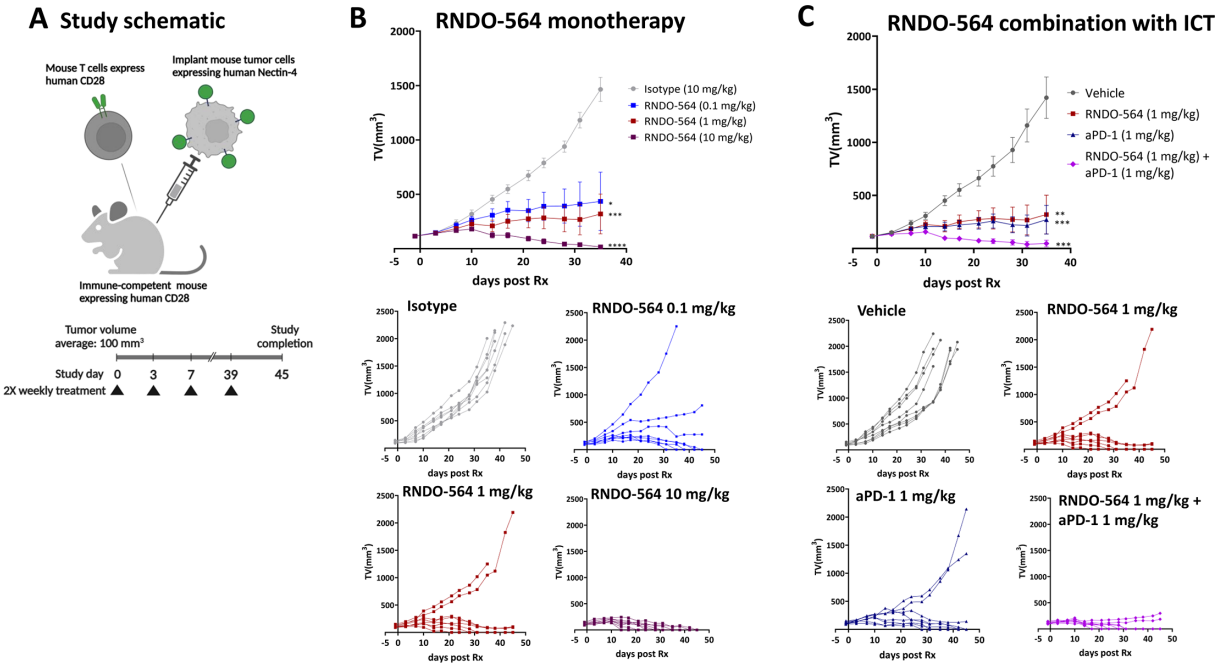


Figure 7 RNDO-564 has single-agent activity in an immune-primed syngeneic tumor model. (A) Study schematic. (B and C) Immuno-competent mice expressing human CD28 in place of mouse CD28 were subcutaneously implanted with MC38-hNectin-4 tumor cells. When the tumors reached 100 mm^3 the animals were randomized to treatment groups (eight/group) and RNDO-564 (0.1, 1 or 10 mg/kg) or an isotype control (10 mg/kg) was injected intraperitoneally, two times a week for 39 days. (C) Additional groups with combination treatments were tested. Anti-PD-1 antibody at 1 mg/kg was tested alone or as a combination with 1 mg/kg of RNDO-564 and compared with a vehicle control. Tumor volumes were monitored over time and depicted as average±SEM. P values were calculated by two-way analysis of variance using Dunnett's correction and shown for day 35 for each treatment group relative to isotype control (A) or vehicle (B) (ns – not significant, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001). Individual animal curves are shown below each figure. aPD-1, anti-programmed cell death protein 1; ICT, immune checkpoint therapy.

to treatment (figure 7C). These results provide evidence for complementary activity between PD-1 inhibition and CD28 agonism consistent with literature reports.[36–38]

Pharmacokinetics and tolerability of intravenous RNDO-564 in non-human primates

As RNDO-564 cross-reacts with cynomolgus monkey CD28 and Nectin-4, this species represents a relevant model for pharmacokinetics (PK) and tolerability. Cynomolgus monkeys were injected with an intravenous infusion of either 1 or 10 mg/kg RNDO-564 followed by observation and intravenous sampling. The mean half-life was 6.2 days, consistent with expectations of a standard IgG (online supplemental figure S12A).[39] Analysis of immune cell composition and production of T-cell activation markers did not reveal any RNDO-564 dependent significant changes in any immune cell subset or increase in T-cell activation (data not shown). We also measured serum levels of the cytokines IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ and TNF-α, observing no significant changes in cytokines across any of the individuals (online supplemental figure S12B). These results suggest RNDO-564 is well-tolerated and exhibits normal PK.

Extended developability assessment of RNDO-564

The favorable developability characteristics observed during assessment of our bsAb panel (table 1) translated to desirable manufacturability properties for RNDO-564. A CHO stable cell line expressing RNDO-564 produces 5.2 g/L of desired product in a bioreactor at ≥95% purity (by size exclusion high performance liquid chromatography (SEC-HPLC)). A standard mAb purification process with affinity capture, low pH viral inactivation, intermediate anion exchange, and a final mixed mode polishing step can be successfully applied to deliver pure bsAb with high yields. RNDO-564 shows negligible change in purity under stress conditions, including elevated temperature, oxidative stress, low pH and freeze-thaw. For example, after incubation at 40°C for 4 weeks RNDO-564 formulated at 50 mg/mL showed a ≤1% change in monomer content by SEC-HPLC (online supplemental table S1).

DISCUSSION

CD28 agonist therapeutics were largely abandoned following the acute toxicity observed in the 2006 TeGenero phase one trial evaluating the experimental mAb TGN1412. However, subsequent studies of TGN1412 established preclinical assays that reduce the target risk by identifying CD28 super-agonists early in discovery. Furthermore, advances in bsAb technology enable tumor-antigen binding-dependent CD28 costimulation, further minimizing the risk of systemic immune activation. These

developments resulted in renewed interest in CD28-targeted therapies[40–42] and multiple candidates are in early-phase clinical trials. Despite this progress, several key questions in the CD28 bsAb engager field remain. In this study, we describe the development of a panel of novel antibodies that specifically activate human CD28 only in the presence of TCR engagement. In bsAb format, these antibodies exhibit Nectin-4-binding and Signal 1-dependent T-cell costimulation. CD28-mediated costimulation resulted in enhanced effector T-cell functions, including cytokine production, proliferation, and tumor cell cytotoxicity. The dual requirements of Signal 1 and Nectin-4-binding ensure that CD28 activation will occur in the relevant context of the tumor microenvironment but not in healthy tissues. The properties of this panel of CD28 engagers allow for targeting a variety of different TAAs in bsAb format as there is low risk of immune activation in healthy tissues expressing the TAA but lacking Signal 1. This contrasts with CD3-targeted T-cell engagers, where the expression of a TAA in healthy tissues limits its utility due to toxicity risk and requires complex engineering strategies to drive tumor-specific activity.[3 43] We designed our CD28 panel to include a wide range of T-cell costimulatory potencies, permitting empirical testing for the optimal strength of this signal. Lessons from CD3 engaging bsAbs revealed that the most potent molecules do not always yield the best clinical outcomes, with success often limited by a narrow therapeutic window.[14] Similarly, we hypothesize that CD28 engagement may require potency modification depending on the indication, clinical strategy and TAA being targeted. In selecting our clinical development candidate from the diverse panel of CD28 × Nectin-4 bsAbs, we aimed to ensure efficacy while gaining the benefits of a reduced-potency molecule. Some of the lowest potency bsAbs in our panel demonstrated reduced activity *in vitro* in challenging conditions like low E:T ratios, potentially posing a risk of inadequate clinical activity in a monotherapy setting. Our clinical development candidate, RNDO-564, achieved equivalent maximum tumor cell cytotoxic activity compared with the most potent molecules in our panel, but at higher doses. As a moderate-potency molecule, we hypothesize that RNDO-564 will have a larger therapeutic window in the clinic as well as more precise dosing control and reduced sink effect from CD28 expression on peripheral T cells compared with higher potency bsAbs. In characterizing our CD28 × Nectin-4 bsAbs we observed enhancement of IL-2 production in the presence of Signal 1. This is consistent with previous studies showing that CD28 regulates IL-2 production through activation of multiple transcription factors.[8] However, the comparison between enhancement of IL-2 and six additional cytokines highlights the degree of preferential upregulation of this cytokine and has not been previously described for other CD28 engagers. The minimal increases in cytokines most associated with CRS, IL-6 and IL-1β, suggest that CD28 bsAbs are unlikely to present the same toxicity limitations as CD3 engagers. Early clinical trial data further support this, reporting only grade 1–2 CRS and no high-grade events attributable to CD28 bsAb treatment.[44–46] The first effective immunotherapy for cancer treatment, Proleukin (recombinant IL-2) has shown durable therapeutic benefit, although significant toxicity has limited its clinical use.[47] Tumor-localized production of IL-2 through CD28 engagement could support expansion and function of effector T cells and avoid the toxicities associated with systemic exposure to IL-2. In our *in vitro* serial stimulation assay, we aimed to explore the contribution of IL-2 to sustained T-cell activity by using an IL-2 blocking antibody as well as IL-2 supplementation. Our results reveal that supplementation with IL-2 mimics the enhanced tumor cell cytotoxicity provided by CD28 costimulation, but blocking IL-2 does not fully reverse the benefit of CD28 costimulation. This result is consistent with the known mechanism of action of CD28, which modulates multiple T-cell pathways beyond IL-2 synthesis.[8] Further studies are needed to dissect the contributions of these additional signaling pathways to enhanced T-cell function in this assay setting. However, a caveat to interpreting these studies is that this *in vitro* system does not recapitulate the complexity of the tumor microenvironment, and the T-cell states present therein. Our *in vivo* results support the therapeutic potential of RNDO-564 either as a monotherapy or in combination with ICT. Treatment with RNDO-564 led to complete tumor regression in syngeneic mouse models, highlighting the ability of this molecule to potentiate T-cell-mediated cytotoxicity and tumor clearance in a model where endogenous Signal 1 is present. This is the first publication demonstrating complete response from CD28 bsAb monotherapy, as other reports have shown partial monotherapy activity from CD28 bsAb treatments.[37 40 41] UC is considered an immune-primed tumor where Signal 1 is likely present, as evidenced by the approvals of multiple checkpoint inhibitors. One limitation of our studies, however, is that the comparability of endogenous Signal 1 between patients with UC and our syngeneic tumor model is unknown. Future *in vivo* studies investigating co-treatment of our CD28 bsAbs with CD3 TCEs are also warranted to evaluate the therapeutic potential of this combination approach, which has been shown to be effective by other groups.[38 40–42] The success of ICT and CD3 TCEs have demonstrated the significant potential of T-cell activating immunotherapies in oncology, while highlighting the need for more efficacious approaches capable of producing deep and durable responses. Recent studies have emphasized the critical role of CD28 signaling in antitumor immune responses, revealing that insufficient costimulation within the tumor microenvironment may contribute to immune suppression and sustained CD28 engagement is required for response to ICT.[6 36 48] Our study demonstrates for the first time that a potency-optimized bsAb targeting CD28 and Nectin-4 offers a promising therapeutic strategy for Nectin-4-positive malignancies such as la/mUC. This is further supported by our investigation of non-human primates (NHP) tolerability, where we found

no evidence of systemic toxicity or adverse immune activation. The immune-primed nature of la/mUC makes it an ideal indication for the clinical testing of RNDO-564 as a monotherapy, as well as in combination with other approved agents in this indication, such as ICT. As the field matures in its understanding of CD28 bsAb therapies, future research may explore combination strategies with other immunomodulatory agents to enhance therapeutic efficacy and overcome potential resistance mechanisms. Approaches currently under investigation include combinations of CD28 bsAbs with ICT and CD3 TCEs, but combination with other agents may also be warranted to improve responses.[11 38 42 44–46] Expanding the clinical investigation of CD28 bsAbs to include a broader range of TAAs and indications will further increase the positive impact of this therapeutic modality.

MATERIALS AND METHODS

Antibody discovery

An NGS-based discovery approach identified unique candidate antibodies to CD28 and Nectin-4 from immunized rodents, similar to published methods.[24] The Nectin-4 discovery campaign used the cell lines HT-1376 and HCT-116 for positive and negative expression of Nectin-4, respectively, during screening.

ELISA

Methods were performed as described.[49] Recombinant CD28 (all species), Nectin-4 (all species), and CTLA-4 were sourced from Acro Biosystems. MultiTag protein was purchased from GenScript and HSA from MilliporeSigma.

Cell lines and culture

RT-112 cells were obtained from DSMZ, all other cell lines were obtained from the American Type Culture Collection (ATCC). Cells were cultured according to supplier guidelines and maintained in humidified incubators at 37°C, 5% CO2. For media conditions, see online supplemental methods. The MCF-7^{KO} cell line was engineered by introducing a single base pair deletion in exon 1 of the Nectin-4 gene and validated by sequencing. Human PBMCs and T cells were isolated from leukopaks using negative isolation (STEMCELL Technologies).

Protein production

Antibodies were produced in ExpiCHO cells (Thermo Fisher), following the manufacturer's protocol. Clarified supernatants were purified by protein A affinity capture followed by CHT Type 1 polishing.

Biophysical characterization

SEC-HPLC: Analytical SEC was run on a Shimadzu LC-40 HPLC system, using a TSKgel SuperSW3000 column (4.6 mm I.D. × 30 cm) in mobile phase composed of 0.1 M Sodium Phosphate, 0.3 M Sodium Chloride, pH6.7. Freeze-thaw stability: Four freeze-thaw cycles consisting of freezing at −80ºC for 1 hour and thawing at 25°C were conducted. Samples were analyzed by SEC-HPLC. T_m and T_{agg} characterization: T_m and T_{agg} were measured in duplicate on the UNcle (Unchained Labs). Temperature ramp was 25°–95°C, with a ramp rate of 0.5°C/min. T_m was determined in the UNcle software, using the inflection point of the barycentric fluorescence signal mean. T_{agg} was determined by SLS at 266 nm. Thermal stability: BsAbs were aliquoted at 5 mg/mL and incubated at 40°C for four weeks. All samples were analyzed by SEC-HPLC and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Surface plasmon resonance

Binding interactions were characterized at 25°C. C1 sensor (Cytiva) chip was coated with Streptavidin (Acro Biosystems) at 20 µg/mL. Two concentrations of Avi-tagged Nectin-4 antigen (Acro Biosystems) were injected to create low-density surfaces around 2 and 15 resonance units (RU). RNDO-564 was tested in a threefold concentration series from 160 nM to 660 pM. Binding constants were calculated with a global fitting from both density surfaces using a 1:1 interaction model. See online supplemental methods.

In vitro cytotoxicity assays

Target cells were co-cultured with human T cells at assay-dependent E:T ratios and antibody treatments. Plates were incubated for three days, and cell viability was determined using CellTiter-Glo V.2.0 (Promega) reagent. For cytotoxicity of co-cultured MCF7^{P} and MCF7^{KO}, cells were labeled with carboxyfluorescein diacetate succinimidyl ester and CellTrace Violet (CTV), respectively, (Thermo Fisher), then plated at different ratios and PanT cells were added at a 1:1 E:T ratio along with CD3 and CD28 bsAbs. Cytotoxicity was analyzed on a volumetric flow cytometer (Attune NxT, Thermo Fisher).

Repeat stimulation assay

Bead-based assays used CD3 beads (Gibco) with restimulation 48–72 hours at a 1:1 ratio. T cells were harvested after four stimulations and cytotoxicity assays were performed on an xCELLigence RTCA instrument (Agilent Technologies) with T-47D target cells at a 2:1 E:T ratio and incubated for six days. Target cell-based serial stimulation assays co-cultured T cells with target cells at a 1:1 E:T ratio in RTCA E-plates (Agilent Technologies). Cells were treated with antibodies, IL-2 (Miltenyi Biotech), and anti-IL-2 (R&D Systems) as indicated. T cells were re-stimulated with target cells every two to three days. Tumor cell viability was recorded throughout the assay on an xCELLigence RTCA instrument.

Cytokine analysis

Target cells were co-cultured with T cells at assay-dependent E:T ratios and treated with CD3 and CD28 bsAbs. Supernatants were collected as indicated and cytokines were detected by Meso Scale Discovery technology (Meso Scale Diagnostics). See online supplemental methods.

T-cell proliferation

T cells labeled with 1 µM CTV were co-cultured with target cells at a 2:1 E:T ratio and antibody treatments for five days. T cells were collected and stained with Zombie NIR Fixable Viability Kit (BioLegend) and fluorophore-conjugated antibodies to CD3, CD4 and CD8. Proliferation was determined by assessment of CTV dilution.

Solid phase T-cell activation

Antibodies were incubated in 96-well plates for two hours at 37°C, then wells were washed with phosphate-buffered saline. 1.5×10^5 human PBMCs were added in 200 µL media and incubated for 24 hours with the immobilized antibodies.

Nectin-4 expression and T-cell activation on dissociated bladder tumor cells

Bladder cancer dissociated tumor cells (Discovery Life Sciences) were stained with Zombie NIR Fixable Viability Kit, washed and resuspended in fluorescence-activated cell sorting (FACS) buffer with TruStain FcX (BioLegend) according to manufacturer protocol. Cells were stained with anti-Human Nectin-4 (R&D Systems) and anti-human EpCAM (BD Biosciences) for one hour on ice in the dark then washed twice in ice-cold FACS buffer and resuspended in FACS buffer for analysis. DTCs were co-cultured with allogeneic T cells at an E:T of 10:1 and RNDO-564±CD3 bsAb for 72 hours. Supernatants were collected at 48 hours for IL-2 measurement by electrochemiluminescence (Meso scale discovery) and cells were processed for flow cytometry at 72 hours (see online supplemental methods).

In vivo studies

Mice expressing human CD28 were implanted subcutaneously with 2×10^6 MC38 cells expressing human Nectin-4 (Biocytogen). When the tumors reached an average of 100 mm^3, mice were randomized into treatment groups of eight animals. RNDO-564, alone or in combination with mouse anti-PD-1 (RMP1-14, Bio X Cell), was administered twice weekly intraperitoneally for four weeks and tumor volumes were measured by calipers twice weekly until the tumors reached study endpoint or humane endpoint of 2,000 mm^3.

NHP studies

Two mixed-gender cohorts of cynomolgus monkeys (Macaca fascicularis) were given a single dose of 1 or 10 mg/kg RNDO-564 as a 30 min intravenous injection (Charles River Laboratories). Animals were monitored for mortality, clinical observations, body weights, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis). Blood was collected for immune surface phenotyping, cytokine analysis and bioanalysis. Serum concentration of RNDO-564 was evaluated by a dual antigen binding assay using MSD technology. Non-compartmental analysis (NCA) was used to generate PK parameters using Phoenix V.64 WinNonlin V.8.3 software (Certara).

Acknowledgements We would like to acknowledge Rong Deng for the estimation of half-life and PK parameters for the cynomolgus monkey study, and Gizelle Taleno for assistance collecting IL-2 production data from the T cell and bladder cancer DTC co-culture experiment. Some of the data included in this manuscript have been presented at the 2024 meeting of the Society for Immunotherapy of Cancer annual meeting.[50] The graphical abstract and illustrations in Figures 1A, 2A 6, and 7A were created with Biorender.com.

Contributors MK, KR and SCC collected data, and contributed to assay design, data interpretation and manuscript preparation. ECC collected data and contributed to assay design and data interpretation. USR contributed to assay design, data interpretation and manuscript preparation. LMD, SMA, RC, IS and SC collected data. SFA, KEH and NDT contributed to data interpretation and manuscript preparation. MR contributed to data interpretation. SCC is the guarantor.

Funding This study was funded by Rondo Therapeutics Inc.

Competing interests All authors are employees and stockholders of Rondo Therapeutics Inc.

Patient consent for publication Not applicable.

Ethics approval Rodent care and immunizations were performed by Antibody Solutions (Sunnyvale, CA, USA) in accordance with Institutional Animal Care and Use Committee (IACUC) (number IP-01) and in accordance with NIH office of laboratory Animal Welfare, PHS# D18-01024. The mouse tumor growth inhibition study was approved by IACUC of Biocytogen Boston following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), AUP number 2018N001. The cynomolgus monkey PK and tolerability study was approved by IACUC of Charles River laboratories, an AAALAC approved facility, NIH assurance ID number: D16-00594 (A4112-01). Human T cells were collected in accordance with all ethical, regulatory and scientific guidelines (Stemcell Technologies).

Provenance and peer review Not commissioned; externally peer-reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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REFERENCES

1 Sharma P, Goswami S, Raychaudhuri D, *et al*. Immune checkpoint therapy-current perspectives and future directions. *Cell* 2023;186:1652–69.
2 Surowka M, Klein C. A pivotal decade for bispecific antibodies? *MAbs* 2024;16:2321635.
3 Middelburg J, Kemper K, Engelberts P, *et al*. Overcoming Challenges for CD3-Bispecific Antibody Therapy in Solid Tumors. *Cancers (Basel)* 2021;13:287.
4 Baeuerle PA, Wesche H. T-cell-engaging antibodies for the treatment of solid tumors: challenges and opportunities. *Curr Opin Oncol* 2022;34:552–8.
5 Mani N, Andrews D, Obeng RC. Modulation of T cell function and survival by the tumor microenvironment. *Front Cell Dev Biol* 2023;11:1191774.
6 Prokhnevska N, Cardenas MA, Valanparambil RM, *et al*. $CD8^+$ T cell activation in cancer comprises an initial activation phase in lymph

nodes followed by effector differentiation within the tumor. *Immunity* 2023;56:107–24.
7 Spranger S, Dai D, Horton B, *et al*. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* 2017;31:711–23.
8 Esensten JH, Helou YA, Chopra G, *et al*. CD28 Costimulation: From Mechanism to Therapy. *Immunity* 2016;44:973–88.
9 Oliveira G, Wu CJ. Dynamics and specificities of T cells in cancer immunotherapy. *Nat Rev Cancer* 2023;23:295–316.
10 Schwartz RH. T cell anergy. *Annu Rev Immunol* 2003;21:305–34.
11 Lotze MT, Olejniczak SH, Skokos D. CD28 co-stimulation: novel insights and applications in cancer immunotherapy. *Nat Rev Immunol* 2024;24:878–95.
12 Stebbings R, Findlay L, Edwards C, *et al*. "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics. *J Immunol* 2007;179:3325–31.
13 Suntharalingam G, Perry MR, Ward S, *et al*. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 2006;355:1018–28.
14 Poussin M, Sereno A, Wu X, *et al*. Dichotomous impact of affinity on the function of T cell engaging bispecific antibodies. *J Immunother Cancer* 2021;9:e002444.
15 Vafa O, Trinklein ND. Perspective: Designing T-Cell Engagers With Better Therapeutic Windows. *Front Oncol* 2020;10:446.
16 Challita-Eid PM, Satpayev D, Yang P, *et al*. Enfortumab Vedotin Antibody-Drug Conjugate Targeting Nectin-4 Is a Highly Potent Therapeutic Agent in Multiple Preclinical Cancer Models. *Cancer Res* 2016;76:3003–13.
17 Takano A, Ishikawa N, Nishino R, *et al*. Identification of nectin-4 oncoprotein as a diagnostic and therapeutic target for lung cancer. *Cancer Res* 2009;69:6694–703.
18 Nishiwada S, Sho M, Yasuda S, *et al*. Nectin-4 expression contributes to tumor proliferation, angiogenesis and patient prognosis in human pancreatic cancer. *J Exp Clin Cancer Res* 2015;34:30.
19 Galsky MD, Balar AV, Black PC, *et al*. Society for Immunotherapy of Cancer (SITC) clinical practice guideline on immunotherapy for the treatment of urothelial cancer. *J Immunother Cancer* 2021;9:e002552.
20 Rddad Y. After sacituzumab pulled in bladder cancer what now? Oncology News Central; 2024.
21 van der Heijden MS, Sonpavde G, Powles T, *et al*. Nivolumab plus Gemcitabine-Cisplatin in Advanced Urothelial Carcinoma. *N Engl J Med* 2023;389:1778–89.
22 Powles T, Valderrama BP, Gupta S, *et al*. Enfortumab Vedotin and Pembrolizumab in Untreated Advanced Urothelial Cancer. *N Engl J Med* 2024;390:875–88.
23 Surveillance Research Program. SEER*Explorer: an interactive website for seer cancer statistics. National Cancer Institute; 2024. Available: https://seer.cancer.gov/statistics-network/explorer/
24 Harris KE, Aldred SF, Davison LM, *et al*. Sequence-Based Discovery Demonstrates That Fixed Light Chain Human Transgenic Rats Produce a Diverse Repertoire of Antigen-Specific Antibodies. *Front Immunol* 2018;9:889.
25 Ridgway JBB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody C_H3 domains for heavy chain heterodimerization. *Protein Eng Des Sel* 1996;9:617–21.
26 Merchant AM, Zhu Z, Yuan JQ, *et al*. An efficient route to human bispecific IgG. *Nat Biotechnol* 1998;16:677–81.
27 Jain T, Boland T, Vásquez M. Identifying developability risks for clinical progression of antibodies using high-throughput in vitro and in silico approaches. *MAbs* 2023;15:2200540.
28 Stebbings R, Eastwood D, Poole S, *et al*. After TGN1412: recent developments in cytokine release assays. *J Immunotoxicol* 2013;10:75–82.
29 Findlay L, Eastwood D, Stebbings R, *et al*. Improved in vitro methods to predict the in vivo toxicity in man of therapeutic monoclonal antibodies including TGN1412. *J Immunol Methods* 2010;352:1–12.
30 Meier SL, Satpathy AT, Wells DK. Bystander T cells in cancer immunology and therapy. *Nat Cancer* 2022;3:143–55.
31 Morris EC, Neelapu SS, Giavridis T, *et al*. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. *Nat Rev Immunol* 2022;22:85–96.
32 Klümper N, Ralser DJ, Ellinger J, *et al*. Membranous NECTIN-4 Expression Frequently Decreases during Metastatic Spread of Urothelial Carcinoma and Is Associated with Enfortumab Vedotin Resistance. *Clin Cancer Res* 2023;29:1496–505.
33 Chu CE, Sjöström M, Egusa EA, *et al*. Heterogeneity in *NECTIN4* Expression Across Molecular Subtypes of Urothelial Cancer Mediates Sensitivity to Enfortumab Vedotin. *Clin Cancer Res* 2021;27:5123–30.
34 Jenkins E, Whitehead T, Fellermeyer M, *et al*. The current state and future of T-cell exhaustion research. *Oxf Open Immunol* 2023;4:iqad006.
35 Yadav M, Jhunjhunwala S, Phung QT, *et al*. Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature New Biol* 2014;515:572–6.
36 Kamphorst AO, Wieland A, Nasti T, *et al*. Rescue of exhausted CD8 T cells by PD-1–targeted therapies is CD28-dependent. *Science* 2017;355:1423–7.
37 Waite JC, Wang B, Haber L, *et al*. Tumor-targeted CD28 bispecific antibodies enhance the antitumor efficacy of PD-1 immunotherapy. *Sci Transl Med* 2020;12:eaba2325.
38 Moore GL, Zeng VG, Diaz JE, *et al*. A B7-H3-Targeted CD28 Bispecific Antibody Enhances the Activity of Anti-PD-1 and CD3 T-cell Engager Immunotherapies. *Mol Cancer Ther* 2025;24:331–44.
39 Betts A, Keunecke A, van Steeg TJ, *et al*. Linear pharmacokinetic parameters for monoclonal antibodies are similar within a species and across different pharmacological targets: A comparison between human, cynomolgus monkey and hFcRn Tg32 transgenic mouse using a population-modeling approach. *MAbs* 2018;10:751–64.
40 Skokos D, Waite JC, Haber L, *et al*. A class of costimulatory CD28-bispecific antibodies that enhance the antitumor activity of CD3-bispecific antibodies. *Sci Transl Med* 2020;12:eaaw7888.
41 Wei J, Montalvo-Ortiz W, Yu L, *et al*. CD22-targeted CD28 bispecific antibody enhances antitumor efficacy of odronextamab in refractory diffuse large B cell lymphoma models. *Sci Transl Med* 2022;14.
42 Sam J, Hofer T, Kuettel C, *et al*. CD19-CD28: an affinity-optimized CD28 agonist for combination with glofitamab (CD20-TCB) as off-the-shelf immunotherapy. *Blood* 2024;143:2152–65.
43 Klein C, Brinkmann U, Reichert JM, *et al*. The present and future of bispecific antibodies for cancer therapy. *Nat Rev Drug Discov* 2024;23:301–19.
44 Segal NH, Girda E, Sohal D, *et al*. A phase 1/2 study of REGN7075 in combination with cemiplimab (cemi) in patients (pts) with advanced solid tumors: Efficacy and safety results. *JCO* 2024;42:2503.
45 Stein MN, Zhang J, Kelly WK, *et al*. Preliminary results from a phase 1/2 study of co-stimulatory bispecific PSMAxCD28 antibody REGN5678 in patients (pts) with metastatic castration-resistant prostate cancer (mCRPC). *JCO* 2023;41:154.
46 Winer IS, O'Cearbhaill RE, Bouberhan S, *et al*. 127P REGN5668 (MUC16xCD28 bispecific antibody) with cemiplimab (anti-PD-1 antibody) in recurrent ovarian cancer: Phase I dose-escalation study. *Immuno-Oncology and Technology* 2023;20:100599.
47 Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. *J Immunol* 2014;192:5451–8.
48 Humblin E, Korpas I, Lu J, *et al*. Sustained cd28 costimulation is required for self-renewal and differentiation of TCF-1 + PD-1 + CD8 T cells. 2023.
49 Chen EC, Gilchuk P, Zost SJ, *et al*. Systematic analysis of human antibody response to ebolavirus glycoprotein shows high prevalence of neutralizing public clonotypes. *Cell Rep* 2023;42:112370.
50 Clarke S, Chen EC, Trinklein N. 939 A novel CD28 x Nectin-4 costimulatory bispecific antibody targeting metastatic bladder cancer. *J Immunother Cancer* 2024;1058.