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Bio-Orthogonal T Cell Targeting Strategy for Robustly Enhancing Cytotoxicity against Tumor Cells

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T cells can kill tumor cells by cell surface immunological recognition, but low affinity for tumor-associated antigens could lead to T cell off-target effects. Herein, a universal T cell targeting strategy based on bio-orthogonal chemistry and glycol-metabolic engineering is introduced to enhance recognition and cytotoxicity of T cells in tumor immunotherapy. Three kinds of bicycle [6.1.0] nonyne (BCN)-modified sugars are designed and synthesized, in which Ac_4ManN-BCN shows efficient incorporation into wide tumor cells with a BCN motif on surface glycans. Meanwhile, activated T cells are treated with Ac_4GalNAz to introduce azide (N_3) on the cell surface, initiating specific tumor targeting through a bio-orthogonal click reaction between N_3 and BCN. This artificial targeting strategy remarkably enhances recognition and migration of T cells to tumor cells, and increases the cytotoxicity 2 to 4 times for T cells against different kinds of tumor cells. Surprisingly, based on this strategy, the T cells even exhibit similar cytotoxicity with the chimeric antigen receptor T-cell against Raji cells in vitro at the effector: target cell ratios (E:T) of 1:1. Such a universal bio-orthogonal T cell-targeting strategy might further broaden applications of T cell therapy against tumors and provide a new strategy for T cell modification.

In recent years, adoptive T cell therapy (ACT) has been considered a potent and feasible cancer treatment modality in cancer immunotherapy.[1] However, due to the constant immune selective pressure and genetic instability,[2] tumor cells would give rise to variants displaying a multitude of evasion mechanisms, including downregulate antigen presentation to render T cells "blind" to tumor, or overexpression of decoy receptors to inhibit T cell activity.[3] To overcome these challenges, modification of T cell has rapidly evolved by harnessing artificial expression of chimeric antigen receptors (CARs) and T cell receptor (TCR),[3b,4] which recreate T cells with enhanced tumor-specific recognition and effective cytotoxicity, but unpredictable on-target, off-tumor toxicity is still the major safety concern for engineering T cell therapy.[5] Thus it is crucial to search novel targeting strategies for further clinic application of T cell immune therapeutics.[6]

Metabolic glycoengineering is an efficient way to introduce various chemical groups to cellular glycan through intrinsic biosynthetic pathways,[7] which enable the chemical modification of cells within their native environment.[8] Bertozzi and co-workers developed this technique to wide application range in combination with bio-orthogonal click chemistry, namely bio-orthogonal glycometabolic labeling.[9] The bio-orthogonal functional groups incorporated into cell surface glycans can be further labeled or anchored through bio-orthogonal click reaction.[10] Based on this technique, Kim and co-workers and Cheng and co-workers further designed a novel tumor-targeting strategy by introducing bio-orthogonal groups azide (-N_3) on tumor cell surface as an artificial target, which effectively increased the tumor-targeting and accumulation efficiency of nanoparticles through click reaction in vivo.[11] Thus, bio-orthogonal glycometabolic labeling can provide an artificial-targeting strategy that made tumor cells more uniform and highly specific.[11b,12] This strategy has been widely used in modulating the biodistribution and tumor-targeting efficacy of probe and therapeutic nanoparticles in vitro and in vivo,[13] but less used in improving the target recognition of immune cell therapy, for which enhanced tumor targeting is just the urgent issue to be solved.

Therefore, we herein report a bio-orthogonal universal-targeting strategy in enhancing recognition and avoiding off-target of T cell, in which complementary bio-orthogonal groups were separately incorporated in tumor cell and T cell through glycol-metabolic process to build implanted target reporter (**Scheme 1**). Motivated by this purpose, a serial of novel unnatural sugar were delicately designed and synthesized based on bio-orthogonal molecules:bicyclo [6.1.0] nonyne (BCN)-modified mannose, galactose, and glucose. BCN is a ring-strained alkyne that can react with both azides (N_3) and tetrazine (Tz) through metal-free click reactions, the reaction rates (k_2) of which were

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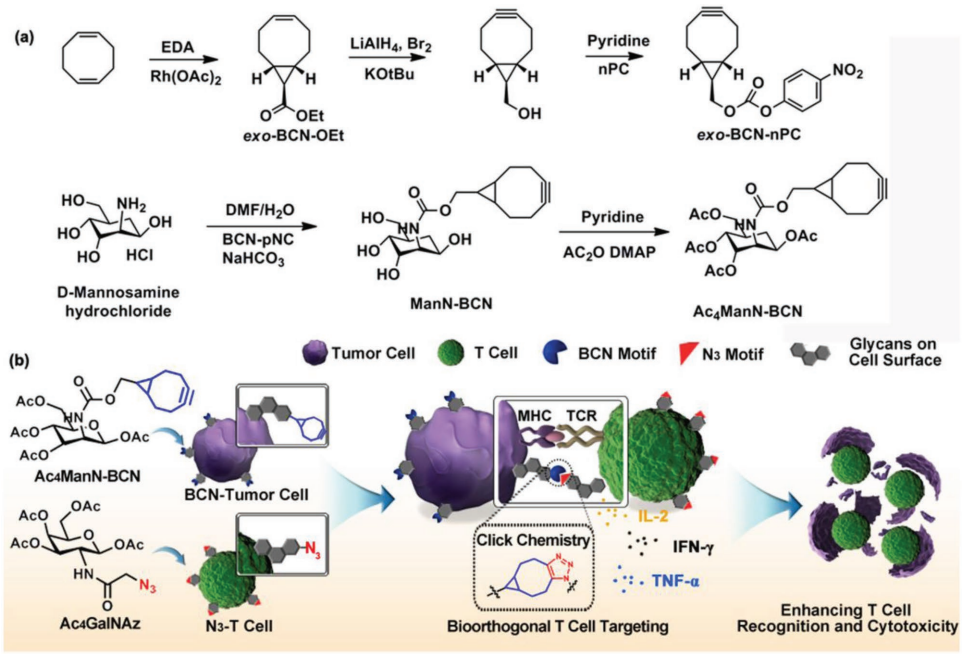

Scheme 1. a) The synthesis route of Ac_4ManN-BCN, one of BCN-modified nature sugars applied in subsequent experiments. b) The universal T cell targeting strategy mediated by bio-orthogonal glycol engineering for enhancing T cell recognition and cytotoxicity to tumor cell.

1.7 and 2.9 × 10^4 M^{-1} s^{-1} in phosphate buffer solution (PBS), respectively.[11c,14] Here we chose BCN as bio-orthogonal chemical reporter not only because of its fast reaction kinetics, but also because BCN showed higher hydrophilicity than dibenzocyclooctyne (DBCO) and better stability than trans-cyclooctene (TCO), which might decrease nonspecific binding to proteins and increase in vivo circulation.[14] These synthesized unnatural sugars were incorporated into tumor cells via metabolic labeling to introduce BCN reporters on cell surface (BCN-tumor cell), which can be used as artificial target motif of tumor cells. Simultaneously, commercialized Ac_4GalNAz was incorporated into activated T cells to introduce azide on cell surface (N_3-T Cell). After modification, tumor cells could fastly bond to T cells through bio-orthogonal click reaction, which further promote natural recognition T cell toward tumor cell, subsequently stimulate T cell and enhance its cytotoxicity. This strategy might provide a novel insight into cell recognition and interaction through bio-orthogonal glycometabolic labeling, and also broaden the applications of T cell immunotherapy for other tumor, even solid tumor, in vitro and in vivo.

To construct BCN-modified mannose, galactose, and glucose, we firstly synthesized 4-nitrophenyl chloroformate activated bicycle [6.1.0] nonyne (nPC-BCN) according to the former literatures.[15] And then nPC-BCN was coupled with glycans (mannose, galactose, and glucose) to get N-BCN-carbonyl-d-mannosamine (ManN-BCN), N-BCN-carbonyl-d-galactosamine (GalN-BCN), and N-BCN-carbonyl-d-glucosamine (GluN-BCN), respectively. To increase cellular uptake, these unnatural glycans were peracetylated to form the tetra-O-acetyl compounds including tetraacetylated-N-BCN-carbonyl-d-mannosamine (Ac_4ManN-BCN), tetraacetylated-N-BCN-carbonyl-d-galactosamine (Ac_4GalN-BCN), and tetraacetylated-N-BCN-carbonyl-d-glucosamine (Ac_4GluN-BCN) (see Supporting Information and Scheme S1, Supporting Information).

Before investigating the metabolic labeling performance of these newly synthesized unnatural BCN-sugars, we evaluate their cytotoxicity to Raji cells in different concentration (10, 20, and 40 μM). Here we chose Raji cell because it is a stable cultured line of lymphoblastoid cells derived from B lymphoma, which presented sensitive response to T lymphocytes therapy. All of unnatural BCN-sugars showed no toxicity at 10 and 20 μM, but caused obvious lower cellular viability at 40 μM (Figure S1, Supporting Information). Then we studied whether these unnatural sugars could metabolically label cells with BCN groups in vitro. Raji cells were incubated with Ac_4ManN-BCN, Ac_4GalN-BCN, or Ac_4GluN-BCN at 20 μM for 2 d, respectively, and the potentially incorporated BCN groups on the cell glycoproteins were detected by incubation with N_3-conjugated fluorophore (N_3-Cy5.5) for 30 min (**Figure 1**). As shown in Figure 1, the control group treated with PBS shows negligible Cy5.5 signals on the cell surface; however, cells treated with unnatural Ac_4ManN-BCN presented strong fluorescence signals, while cells incubated with Ac_4GluN-BCN or Ac_4GalN-BCN only showed slightly increased fluorescence signal. Flow cytometry analyses of Raji cells following the same treatment consistently showed stronger Cy5.5 fluorescence intensity in Ac_4ManN-BCN group than in Ac_4GlcN-BCN and Ac_4GalN-BCN group, which might be due to that metabolic enzymes in different biosynthetic pathway showed different tolerance to each BCN-sugars. In addition, cells treated by Ac_4ManN-BCN were also stained by Tz-conjugated fluorophore (Tz-cy5), another bio-orthogonal group can react with BCN more quickly than N_3 group, and the flow cytometry results showed obvious fluorescence enhancement, further confirming that BCN groups have successfully incorporated on cell surface (Figure S2, Supporting Information). Ac_4ManN-BCN with different concentration (0, 10, or 20 μM) treated cells showed a dose-dependent

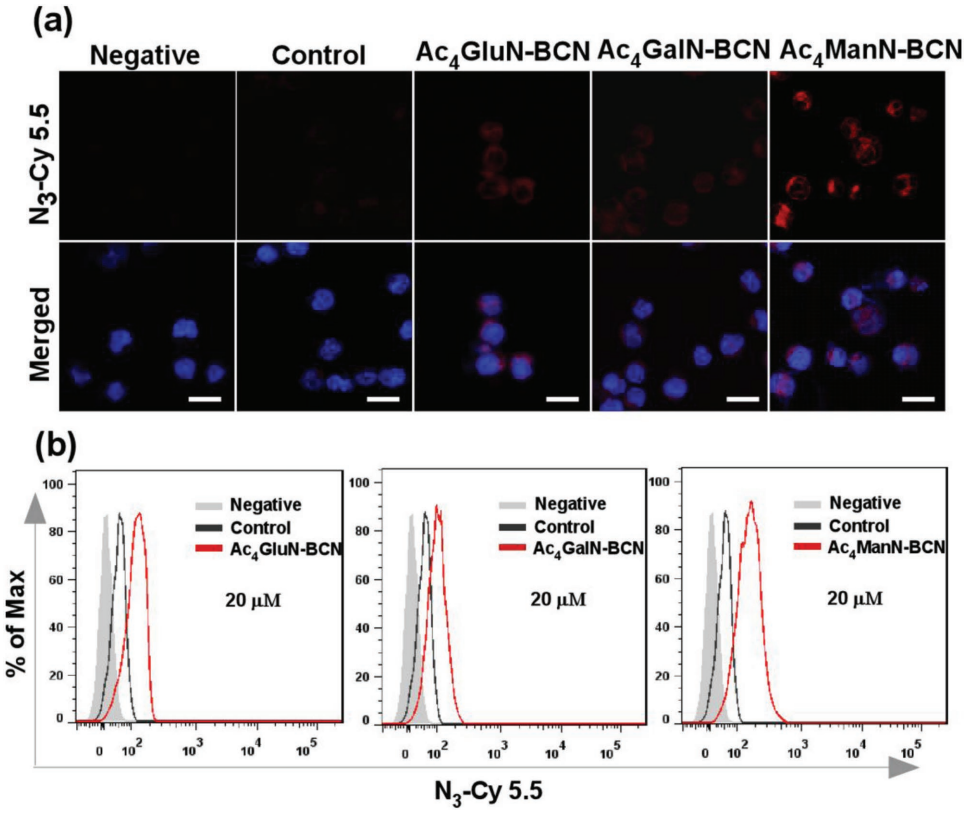

Figure 1. The metabolic labeling performance of these newly synthesized unnatural BCN-sugars including Ac_4ManN-BCN, Ac_4GalN-BCN, and Ac_4GluN-BCN. a) Confocal imaging and b) flow cytometry analyses of Raji cell with or without BCN-sugars after staining by N_3-conjugated fluorophore (N_3-Cy5.5) for 30 min (the bar in image is 10 μm). Negative group is BCN-sugars treated Raji cell but without stained by N_3-Cy5.5 for 30 min. Control group is unlabeled Raji cell stained by N_3-Cy5.5 for 30 min.

increase of fluorescence signal, with 82.5% N_3-Cy5.5 positive cells compared with untreated cells at 20 μM (**Figure 2**a). To further verify the universal metabolic labeling ability of Ac_4ManN-BCN in different cell lines, it was incubated with human lung carcinoma cell line (A549 cells), human rhabdomyoma cell line (RD cells), human hepatocarcinoma cell line (HepG2 cells), human breast cancer cell line (MCF-7 cells), respectively. Compared to Raji cells, these cells had higher tolerance to Ac_4ManN-BCN, not presenting any cytotoxicity even when the treated concentration was at 50 μM (Figure S3, Supporting Information). After modification by Ac_4ManN-BCN, the tested cells were stained by N_3-Cy5.5 and tested by laser scanning confocal microscopy (LSCM) and flow cytometry (FCM) analysis (Figure S4, Supporting Information). As expected, cells treated with Ac_4ManN-BCN showed significantly higher fluorescence than control group. These results collectively demonstrated that Ac_4ManN-BCN can metabolically label cells as a proper substrate for biosynthetic enzymes in sialic acid pathway, and act as an optimal choice for introducing BCN on cell surface to be an artificial target motif of tumor cells.

In order to introduce bio-orthogonal glycoengineering mediated targeting strategy to immunotherapy, T cells were labeled with different concentration of commercial Ac_4GlaNAz (0, 25, 50, or 100 μM) for introducing N_3 on cell surface. T cells used in this project were extracted from human peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers, and the percentage of $CD3^+$ positive T cells finally reached ≈99% under this culture conditions. T cells pretreated with Ac_4GlaNAz were stained by DBCO-bearing fluorophore (DBCO-Fluor 488) through click reaction and analyzed by confocal imaging and flow cytometry. The data showed that T cells were efficiently labeled by Ac_4GlaNAz in a dose-dependent manner. Above 85% of DBCO-Fluor 488 positive cells were monitored in T cells treated by 50 μM of Ac_4GlaNAz, but 100 μM of Ac_4GlaNAz showed no significant increased cell fluorescent intensity, suggesting that the dose of 50 μM was the optimum concentration for the next experiments (Figure 2b and Figure S5, Supporting Information).

To evaluate the effect on cell recognition for bio-orthogonal reaction over cell surface, the interaction between Ac_4GlaNAz treated T cells (N_3-T cell) and Ac_4ManN-BCN treated Raji cells (BCN-Raji cell) was monitored by confocal microscopy. As shown in Figure S6 in the Supporting Information, the cluster of N_3-T cells and BCN-Raji cells boosted gradually from 10 to 20 min, but only less cell aggregation in control group, indicating bio-orthogonal reaction over cell surface can initiate the strong affinity between two types of cells, further enhancing the recognition and activation of T cells. In addition, the dynamical process of N_3-T cells binding to BCN-Raji cells was also investigated using time lapse confocal imaging (**Figure 3**a). Interestingly, when N_3-T cells or BCN-Raji cells randomly moved around to each other, they rapidly bound together

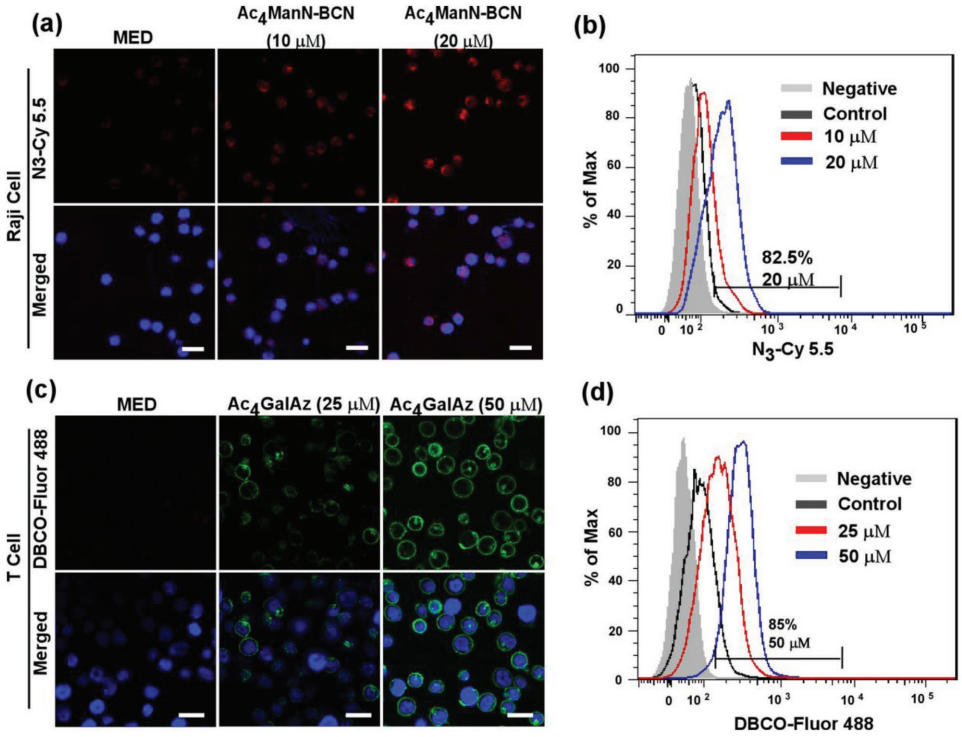

Figure 2. The metabolic labeling performance of Ac_4ManN-BCN and commercialized Ac_4GalNAz in B lymphoma cells and active T cells, respectively. a) Confocal imaging and b) flow cytometry analyses of Raji cell with or without treatment by Ac_4ManN-BCN after incubation with N_3-Cy5.5 for 30 min. c) Confocal imaging and d) flow cytometry analyses of T cell with or without Ac_4GalNAz after incubation with DBCO-Fluor 488 for 30 min. Negative group is BCN-sugars treated Raji cell but without incubating with N_3-Cy5.5 or DBCO-Fluor 488 for 30 min. Med or Control group is unlabeled Raji cell or T cell incubated with corresponding fluorophore for 30 min. The bar in image is 10 μm.

and attracted more cells aggregating into big cell clusters from 0 to 20 min (Figure 3b and Video S1, Supporting Information), whereas unlabeled T cells and Raji cells exhibited a relative random motion and less aggregation in such short time (Figure 3b and Video S2, Supporting Information). To confirm effect on cell migration and invasion of bio-orthogonal reaction

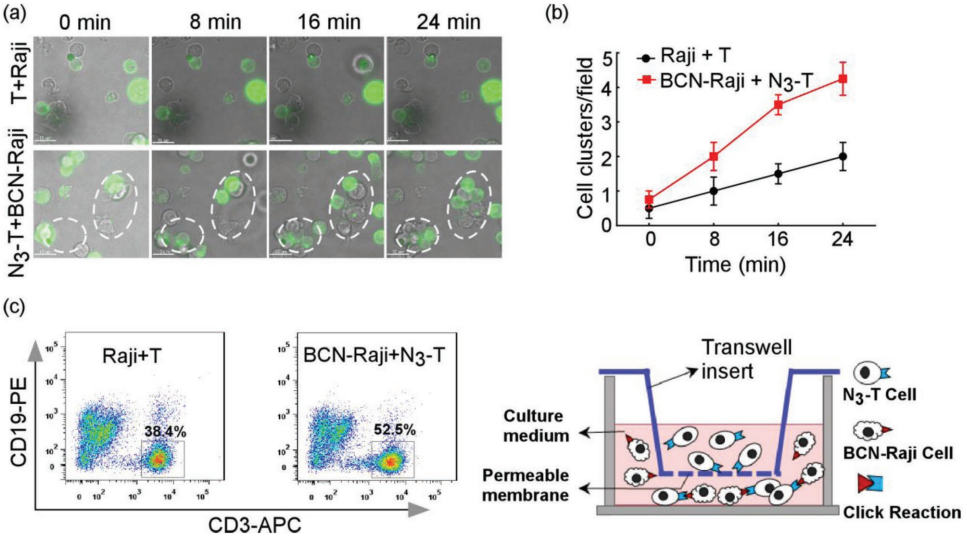

Figure 3. The effect of bio-orthogonal click reaction over cell surface on cell recognition and migration. a) Time lapse confocal imaging of coculture of T cell and Raji cells with or without treatment by Ac_4ManN-BCN (20 μM) or Ac_4GalNAz (50 μM), respectively, at 0, 8, 16, and 24 min. The bar in image is 15 μm. Cell with green fluorescence is T cell stained by anti-CD3. Cell without fluorescence is Raji cell. b) Count the cell cluster in the coculture of T cell and Raji cells with or without treatment by Ac_4ManN-BCN (20 μM) or Ac_4GalNAz (50 μM), respectively, at 0, 8, 16, and 24 min. c) Transwell results of the migration between T cell and Raji cells or between N_3-T cell and BCN-Raji cells, respectively. Anti-CD3 (APC) and anti-CD19 (PE) separately stained T cells and Raji cells.

over cell surface, N_3-T cells and BCN-Raji cells were examined in transwell chambers and analyzed by flow cytometry assay (Figure 3c). Compared to untreated T cells, the migration rate of N_3-T cells increased by 36.7% after 30 min coculture, demonstrating that rapid bio-orthogonal reaction over cell surface might also promote the adhesion and migration of T cell to target cell.

To evaluate the cytotoxicity of N_3-T cells against BCN-Raji cells, we employed Raji cell stably transfected with firefly luciferase (Luci-Raji) as test cell line because its fluorescence intensity is proportional to the survival rate of Raji cells, which can be visualized in luminescence imaging assay. Different concentration of Ac_4ManN-BCN (10 or 20 μM) treated Luci-Raji cells (BCN-Luci-Raji cells) were incubated with Ac_4GlaNAz (50 μM) treated T cells (N_3-T cells) at 1:1 effector-to-target cell (E/T) ratio. As shown in **Figure 4**a,b, luminescence intensity of BCN-Luci-Raji cell was significantly decreased after incubated with N_3-T cell, nearly decreasing by 60% as Raji cells treated with 20 μM of Ac_4ManN-BCN. Subsequently, the N_3-T cells mediated cytotoxicity against BCN-Raji cells was further determined by lactate dehydrogenase (LDH) assay. The results in Figure 4d showed LDH released from BCN-Raji cells in a concentration-dependent manner after cocultured with N_3-T cells at 1:1 E/T ratio. Especially for Raji cells treated by 20 μM of Ac_4ManN-BCN, N_3-T cells mediated cytotoxicity increased by 2.7 times compared to untreated T cells. Similar to LDH release assay, Calcein-AM/PI staining assay also proved the enhanced cytotoxicity of N_3-T cells against BCN-Raji cells compared to the unlabeled T cell group at 1:1 E/T ratio (Figure 4c and Figure S7, Supporting Information), demonstrating bio-orthogonal reaction over cell surface not only promote intercellular recognition but also enhance T cell cytotoxicity against tumor cell. Additional LDH assay was tested to evaluate CAR-T cell mediated cytotoxicity as a comparison at 1:1 E/T ratio. To our surprise, N_3-T cells and CAR-T cells exhibited near cytotoxicity against—with or without Ac_4ManN-BCN (20 μM) treated—Raji cells, respectively (Figure 4e), indicating that such bio-orthogonal T cell targeting strategy could be a potentially effective method to enhance T cell treatment against tumor.

We also investigated the universal cytotoxicity mediated by N_3-T cells against BCN-modified tumor cells (including A549 cell, RD cell, HepG2 cell, and MCF-7 cells) at 1:1 E/T ratio. As shown in Figure S8 in the Supporting Information, N_3-T cells exhibited 2–4 times increased killing effects than untreated T cell to all of BCN-modified tumor cells, especially for BCN-HepG2 cells, yielding nearly 4 times higher LDH release than control group after coculture with N_3-T cells. These results strongly proved that bio-orthogonal glycoengineering mediated tumor targeting can universally enhance sensitivity and cytotoxicity of T cell to tumor cells.

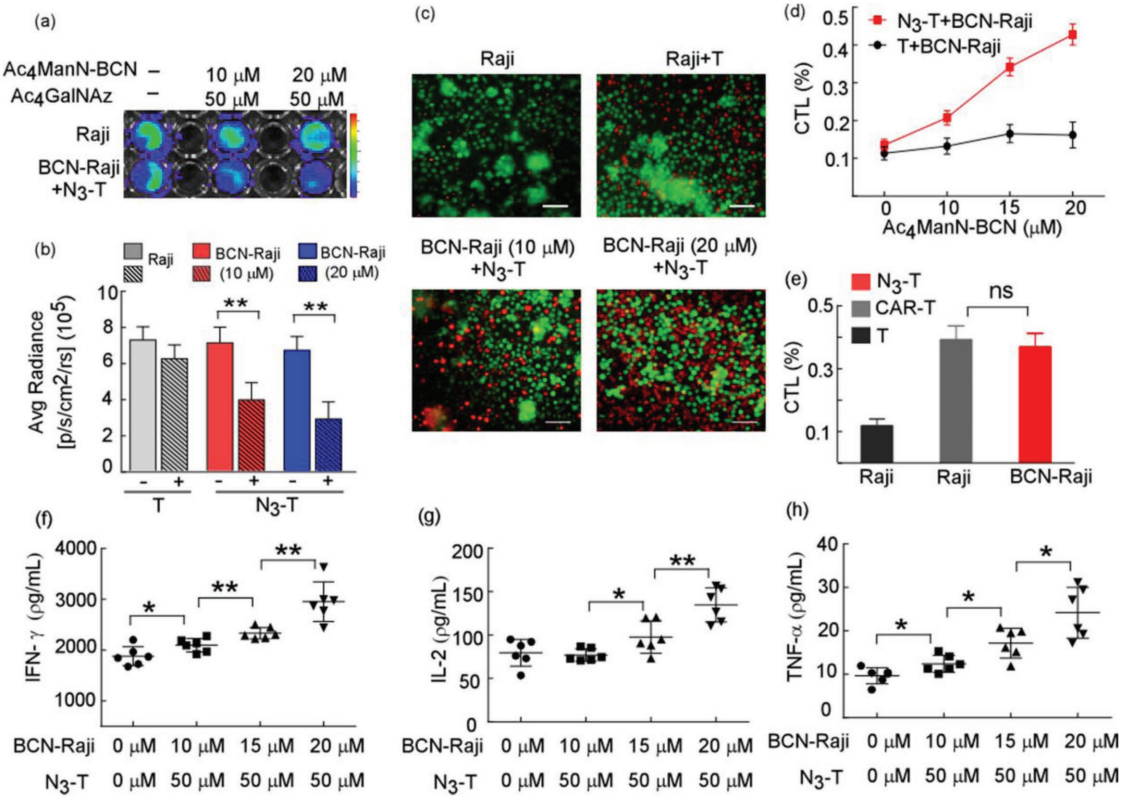

Figure 4. The cytotoxicity and cytokines secretion of N_3-labeled T cell (N_3-T cell) against BCN-labeled Raji cell (BCN-Raji). a,b) Luminescence imaging assay and quantitative analysis, and c) Calcein-AM/PI staining assay of cytotoxicity of Ac_4GalNAz (0 and 50 μM) treated T cell against Ac_4ManN-BCN (0, 10, and 20 μM) treated Raji cell. The bar in image is 100 μm. d) LDH release for Ac_4GalNAz (0 and 50 μM) treated T cell cocultured with Ac_4ManN-BCN (0, 10, 15, and 20 μM) treated Raji cell. e) LDH release for Ac_4GalNAz (50 μM) treated T cell against Ac_4ManN-BCN (20 μM) treated Raji cell, and CAR-T cell against Raji cell. f–h) Cytokines secretion for Ac_4GalNAz (0 and 50 μM) treated T cell cocultured with Ac_4ManN-BCN (0, 10, 15, and 20 μM) treated Raji cell.

For further confirming apoptosis regulated by T cells, we analyzed cytokine production of T cell or N_3-T cells separately in response to Raji cells or BCN-Raji cells (Figure 4f–h). As shown in Figure 4f–h, N_3-T cells can effectively recognize BCN-Raji cells and produce effector cytokines including interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). With the increase of the concentration of Ac_4ManN-BCN treated Raji cells, the antitumor cytokines secretion was significantly enhanced in N_3-T cells, indicating that bio-orthogonal reaction over cell surface can effectively improve the recognition and activation of T cells toward tumor cells, which might be the important reason that cause the enhanced tumor toxicity for T cells after modification.

In conclusion, the BCN modified unnatural sugars, especially for Ac_4ManN-BCN, can be efficiently and nondestructively incorporated into wild tumor cell surface glycans. The BCN motif on cell surface exhibited an excellent bio-orthogonal targeting tag with rapid reaction rate, which might enlarge the application of biodiagnostic and therapeutic approaches. Furthermore, in conjunction with the highly efficient and specific bio-orthogonal reaction, Ac_4GalNAz treated T cells presented remarkable high-efficiency targeting and enhanced cytotoxicity against BCN-labeled tumor cells, even showing equal cytotoxicity with CAR-T cells against BCN-Raji cell. It suggested that T cell with robust metabolic modification can effectively kill tumor cells and will provide a rapid and valid therapeutic method of tumor. Such universal bio-orthogonal T cell targeting strategy might pave a way to improve T cell therapies for cell targeting and tumor infiltrating, and provide a brand-new strategy for cell immunotherapy and solid tumor therapy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

bio-orthogonal glycometabolic labeling, click chemistry, cytotoxicity, T cell targeting, T cell therapy

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