provides carbons for the production of aspartate and glutamate, which in turn serve as carbon and nitrogen sources for de novo deoxyribonucleotide synthesis, thus influencing gene transcription (Schoors et al., 2015). Consequently, further studies will be required to determine whether this is also the case for pDCs. A second emerging question is whether pDC respiration is relevant for host defense against viruses. Here, using the LCMV systemic infection model, Wu et al. demonstrated that FAO contributes to viral clearance. However, genetic models will help to understand the importance of metabolic reprogramming in specific DC subsets in vivo. The work by Pearce and colleagues will pave the way for future studies investigating why pDCs need to take a deep breath against viral pathogens.

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Driving CARs into Sweet Roads: Targeting Glycosylated Antigens in Cancer

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Engineering T cells with chimeric antigen receptors (CARs) has demonstrated remarkable success in eradicating hematological malignancies. In this issue of *Immunity,* June and colleagues demonstrate the broad antitumor efficacy of a newly-designed CAR targeting the *O*-linked hypoglycosylated epitopes Tn and sialyl-Tn on cancer-associated MUC-1.

The emergence of cancer immunotherapy has led to a paradigm shift in the treatment of oncologic patients, mainly evidenced by the clinical success of immune checkpoint blockers. Monoclonal antibody (mAb)-based therapies targeting CTLA-4 and/or PD-1-PD-L1 pathways have yielded significant clinical benefits, including durable cancer regression and increased overall survival in patients with various malignancies by unleashing antitumor immune responses (Sharma and Allison, 2015). In parallel with this revolution, another exciting approach has been the use of genetically engineered T cells expressing synthetic receptors, called chimeric antigen receptor (CARs), to eliminate cancer cells by targeting mutation-derived neoantigens or aberrantly-expressed tumor-associated antigens (Newick et al., 2016). Although CAR technology has evolved significantly from the original composition of "first generation CARs" involving only an extracellular single-chain variable fragment (scFv) connected to CD3ζ signaling domains, to "second and third generation CARs" combining CD3ζ with one (41BB or CD28) or two (41BB and CD28) co-stimulatory domains, sustained efforts are being made to further improve these constructs in order to enhance their potency and prevent undesired "on target-off tumor" events (Newick et al., 2016).

CAR T cell therapy targeting CD19 (an antigen expressed on lymphoid tumors and dispensable in normal B cells), demonstrated impressive clinical responses in B cell malignancies, leading to its designation as a "breakthrough therapy" and approval by the US Food and Drug Administration for the treatment of

adult and pediatric relapsed and refractory acute lymphoblastic leukemia in July 2014 (Gill and June, 2015). This clinical success has fueled an intensive exploration of tumor-associated antigens- including carcinoembryonic antigen (CEA), the diganglioside GD2, mesothelin, and human epidermal growth factor receptor 2 (HER2)- that could enable adaptation of CAR-T cell therapies to solid tumors (Newick et al., 2016). This also includes MUC-1, a tumor-associated glycoprotein of the mucin family, which has been used as an immunotherapeutic target in both preventive and therapeutic settings (Kimura and Finn, 2013). Although retargeting of CAR T cells to tumor-associated MUC-1 has led to delayed tumor progression in breast cancer xenografts (Wilkie et al., 2008), limitations were encountered that were associated with MUC-1-imposed steric hindrance, antigenic heterogeneity and possible undesired effects, precluding the immediate translation of MUC-1-CAR T cells to clinical settings. In this issue of *Immunity*, Posey et al. (2016) demonstrate the broad antitumor activity and enhanced tumor selectivity of a newly-designed CAR-T cell targeting the *O*-linked hypoglycosylated epitopes Tn and sialyl-Tn (STn) on cancer-associated MUC-1.

Changes in the cell surface glycome—the full collection of sugar structures expressed in cells and tissues—have largely been appreciated as hallmarks of malignant transformation, influencing diverse tumor-related processes, including tumor cell adhesion, migration, angiogenesis, and immune escape (Rabinovich and Croci, 2012). These altered glycan structures constitute the so-called tumor-associated carbohydrate antigens, and comprise not only the under- or overexpression of naturally-occurring glycans, but also the neo-expression of others. Although aberrant glycosylation contributes to all the described hallmarks of cancer, its translation to clinical therapies has stayed way behind other players implicated in oncogenic and metastatic processes. In this regard, one of the most common glycan aberrant features is the neo-expression of Thomsen-Friederich (TF)-related antigens, a series of truncated *O*-glycans including the Thomsen-noveau antigen (Tn: GalNAcα-1-*O*-Ser/Thr) and the sialyl-Tn antigen (STn), in which the C-6 of the GalNAc residue in Tn has been substituted with sialic acid. In most normal tissues, the Tn antigen solely appears as a precursor during the biosynthesis of mature *O*-glycans, being elongated by the concerted action of glycosyltransferases before *O*-linked glycoproteins are secreted or localized at the cell surface (Rabinovich and Croci, 2012). Although still under debate, the incomplete O-glycosylation found in several tumors arises as a result of several causes including altered expression or localization of glycosyltransferases, and/or mutations or epigenetic silencing of the molecular chaperone COSMC, required for stability of β(1,3)-galactosyltransferase (C1GalT1), an enzyme responsible of elongating the Tn antigen to form core 1 mature structures during *O*-glycan biosynthesis (Ju et al., 2008; Radhakrishnan et al., 2014).

Because of their preferential expression in cancer cells and their correlation with poor prognosis, both Tn and STn antigens have been proposed as tumor biomarkers and excellent candidates for immunotherapeutic strategies. Interestingly, although Tn and STn-based vaccination was well tolerated and revealed minimal toxicity in clinical trials, results were not as encouraging as expected (NCT00030823, Theratope NCT0046371), suggesting the need of more potent approaches to evoke robust anti-tumor immunity against these carbohydrate antigens. Here Posey et al. engineered T cells with CARs that selectively recognize Tn and STn antigens on MUC-1 to strike both hematological and solid tumors. This newly developed CAR-T cell utilizes the scFv portion of a previously developed antibody (5E5) that recognizes Tn and STn glycoepitopes within the MUC-1 protein backbone (Sørensen et al., 2006). The authors tested reactivity of the designed 5E5 CAR-T cells toward the corresponding Tn-glycopeptide on MUC-1 (MUC1-9Tn) or against a non-glycosylated MUC1 peptide and a glycoprotein expressing high levels of STn *O*-glycans (ovine submaxillary mucin; OSM) as controls. The results evidenced high binding affinity and selectivity of 5E5-CAR T cells to MUC1-9Tn, suggesting that the presence of the Tn and STn glycoepitopes and the MUC-1 protein framework are both required for full recognition. This selective recognition pattern was confirmed by the high amounts of cytokines (IL-2 and IFN-γ) produced by 5E5 CAR-T cells in response to MUC19-Tn stimulation in vitro. Immunohistochemical analysis revealed negative staining in most normal tissues analyzed when exposed to 5E5 Tn and STn-specific constructs. Of note, in those tissues showing weak staining (such as stomach, lung, pancreas, or kidney), confocal microscopy studies revealed localization of this glycoepitope within the Golgi apparatus, but its absence on the surface of normal cells. This result could be explained by the natural occurrence of Tn antigen as a precursor for *O*-glycan synthesis in this organelle. Cytotoxicity was assessed by bioluminescence assays, where 5E5 CAR-T cells exhibited potent killing activity against leukemic Jurkat cells in all the conditions analyzed. Importantly, the authors checked bystander effects by restoring C1GalT1 activity following transduction of cancer cells with a lentiviral vector encoding the chaperone COSMC. This effect abolished cytotoxicity induced by 5E5 CAR-T cells indicating selective reactivity of this chimeric construct against incompletely glycosylated MUC-1 (Figure 1).

Providing further evidence of the selective recognition of Tn- and STn-positive malignant tumors by 5E5 CAR T cells, Posey et al. evaluated their reactivity against a panel of cancer cell lines. Consistent with the natural diversity of the cellular glycome, only some cancer cells were killed by these engineered T cells, including the Capan-2 and Hs766T pancreatic cancer cell lines and MDA-MB-453 and MCF7 breast cancer cells. This effect tightly correlated with Tn and STn content in the *O*-glycoprofile of these cells, as shown by lectin staining and the expression of glycosyltransferases responsible for Tn-STn biosynthesis (high levels of ST6GalNAc1 along with low levels of C1GalT1 and COSMC).

The success of CAR-T cell therapy relies on the correct activation, functionality, and metabolism of T cells, as evidenced by first generation CAR-T cells which became repeatedly anergic upon recognition of tumor antigens (Newick et al., 2016). Here the authors showed robust cytotoxic activity of 5E5 CAR-T cells in vivo in a xenograft model of T cell leukemia, by inoculating fluorescent Jurkat

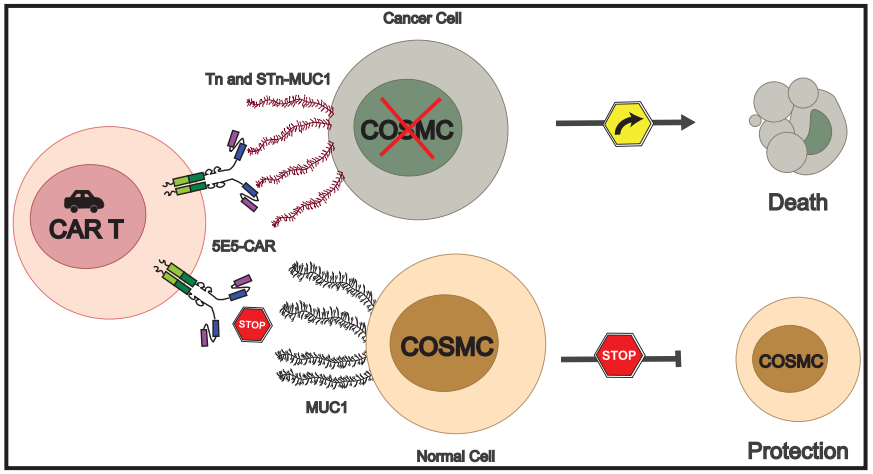

Figure 1. Selective Recognition of the Tumor-Associated Hypoglycosylated Forms Tn-STn on MUC-1 by T Cells Engineered to Express the Chimeric Antigen Receptor 5E5

In the absence of the molecular chaperone COSMC (required for stability of the C1GalT1 glycosyltransferase), exposure of the Tn-STn glycoepitope on MUC-1 is favored, leading to selective recognition of tumor cells by 5E5 CAR-T cells. In contrast, sustained expression of COSMC inhibits exposure of these hypoglycosylated epitopes, preventing cytotoxicity induced by engineered 5E5 CAR-T cells.

leukemic T cells into immunocompromised NSG mice. Moreover, they showed lack of tumor control by CD19 CAR-T cells, as a proof of antigen specificity, since Jurkat tumor cells do not express the B cell restricted antigen CD19. Furthermore, because bystander effects and possible damage to normal tissue are important concerns of these treatments (Gill and June, 2015), the authors addressed this possibility in an in vivo experiment by inoculating NSG mice with Jurkat tumor cells transduced with lentiviral vectors expressing CD19 in which COSMC activity was restored. Remarkably, CD19-expressing, COSMC-reconstituted Jurkat cells were eliminated in vivo by CD19 CAR-T cells, but not by MUC1-Tn-STn-specific 5E5 CAR-T cells, implying a strong specificity of 5E5 CAR constructs toward truncated *O*-glycoantigens (Figure 1). Finally, although CAR-T cell treatment showed impressive results in leukemia and lymphoma patients, clinical effectiveness was still hard to achieve in solid tumors (Newick et al., 2016). Here, the authors showed, in a pancreatic tumor model (which does not fully recapitulate the histological characteristics of a dense, fibrotic human pancreatic tumor) the ability of 5E5 CAR-T cells to control growth of a solid tumor, without apparent damage to normal bystander tissue.

Although much remains to be learned regarding signaling potency, mechanisms of action, trafficking pattern, and safety profiles of CAR-T cells recognizing Tn and STn antigens, the work by Posey et al. constitutes a proof of concept that aberrant tumor glycosylation could be specifically targeted by CAR-T cell therapy to control both hematological and solid malignancies (Figure 1), warranting further design and implementation of rational clinical trials. In addition, future studies should be aimed at examining the impact of complementary strategies that combine the potency and selectivity of 5E5 CAR-T cells together with immune checkpoint blockers, specific chemokines, and vessel-normalizing agents that could guarantee interruption of local immune-inhibitory signals and efficient T cell recruitment to sites of tumor growth.

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