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Glycans Pave the Way for Immunotherapy in Triple-Negative Breast Cancer

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The clinical efficacy of therapies targeting the PD-1/PD-L1 pathway is still limited. In this issue of *Cancer Cell*, Li and colleagues identify a PD-L1 glycosylation-based mechanism in triple-negative breast cancer that fosters immunosuppression by enhancing interactions with PD-1. Targeting glycosylated PD-L1 with a drug-conjugated antibody opens new avenues for treatment.

Immune checkpoint inhibition represents a major breakthrough in the treatment of cancer. In particular, programmed death receptor-1 (PD-1) and its ligand PD-L1 are recognized as powerful targets to enhance tumor-directed T cell functions. PD-1 is an inhibitory receptor expressed on the surface of activated T cells, B cells, NK cells, monocytes, and dendritic cells, responsible for maintaining immune tolerance, blunting exuberant inflammation, and preventing autoimmune diseases. Engagement of PD-1 by its specific ligand PD-L1 conveys inhibitory signals that ultimately lead to T cell exhaustion and immunosuppression (Topalian et al., 2015). This inhibitory mechanism is co-opted by cancer cells, mainly through expression of PD-L1, to evade immune attack. Immunotherapy based on monoclonal antibodies targeting the PD-1/PD-L1 pathway has changed the treatment landscape for advanced melanoma, Hodgkin lymphoma, non-small-cell lung cancer, renal cell cancer, gastric cancer, and head and neck squamous cell carcinoma (Topalian et al., 2015). Moreover, PD-1 blockade has recently been shown to be active across a range of solid tumors with mismatch-repair deficiency (Le et al., 2017). However, only a small proportion of breast cancer patients respond to anti-PD-1/PD-L1 therapy (Katz and Alsharedi, 2017).

Triple-negative breast cancer (TNBC), clinically defined as breast tumors lacking expression of estrogen receptor, progesterone receptor, and the receptor tyrosine kinase ERBB2, is the molecular subtype with the worst prognosis and survival rates (Perou et al., 2000). At present, chemotherapy is the standard of care in the adjuvant, neoadjuvant, and metastatic settings. Among breast cancer types, TNBC exhibits the highest frequency of PD-L1-positive cells, highest mutational index, and most prominent immune infiltrate (Katz and Alsharedi, 2017). Although these parameters constitute key hallmarks of the so-called "cancer immunogram" and are positive predictors of immunotherapy responses (Blank et al., 2016), clinical studies in TNBC patients revealed low response rates to immune checkpoint blockade (Katz and Alsharedi, 2017). Thus, development of more effective immunotherapeutic modalities and validation of new biomarkers of treatment response are urgently needed.

In this issue of *Cancer Cell*, Li et al. identify a mechanism, based on PD-L1 glycosylation, that fosters immunosuppression in TNBC microenvironments (Li et al., 2018). The authors found that *N*-glycosylation of PD-L1 on TNBC cells is essential for its interaction with PD-1 receptor, enabling transmission of inhibitory signals and favoring T cell exhaustion. Remarkably, targeting glycosylated PD-L1 was highly effective in eradicating TNBC tumors (Figure 1).

In previous studies, the authors showed that glycogen synthase kinase 3β (GSK3β), an active protein kinase, induces phosphorylation-dependent proteasome degradation of non-glycosylated PD-L1. However, glycosylation driven by epidermal growth factor (EGF) signaling

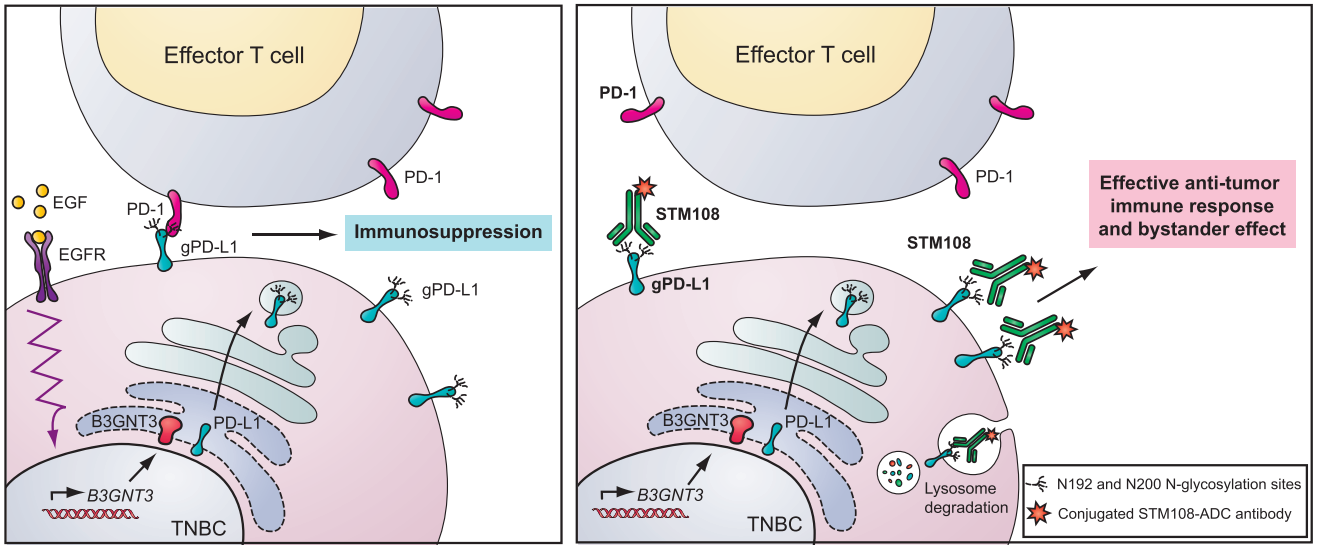

Figure 1. PD-L1 Glycosylation Controls Tumor-Derived Immunosuppression in Triple-Negative Breast Cancer (TNBC)
Left: In TNBC, PD-L1 glycosylation is required for PD-L1/PD-1 interactions, leading to sustained immunosuppression. EGF/EGFR signaling upregulates the expression of the B3GNT3 glycosyltransferase that controls PD-L1 *N*-glycosylation, an event that is essential for its interaction with PD-1 and subsequent suppression of T cell effector function. Right: The antibody drug conjugate STM108-ADC binds to the N192 and N200 glycosylation sites of PD-L1, inducing its internalization and lysosome degradation. Inhibition of PD-1/PD-L1 signaling associated with the bystander effect of STM108-ADC leads to potent anti-tumor activity in a TNBC mouse model.

stabilizes PD-L1 protein via GSK3β inactivation (Li et al., 2016). Now the authors went further to demonstrate the role of PD-L1 glycosylation as a critical determinant of tumor-driven immunosuppression and a therapeutic target in TNBC.

Regulated glycosylation controls critical cellular processes, including cell signaling, activation, proliferation, and survival (Cerliani et al., 2017). Programmed remodeling of glycosyltransferases and glycosidases, controlling the synthesis of *N*- and *O*-glycans, can modify cell-surface receptors and/or their ligands, modulating their stimulatory or inhibitory function. Illustrating this concept, complex *N*-glycans can control receptor signaling and endocytosis in mammary carcinoma cells, leading to changes in cytokine responses, epithelial-mesenchymal transition, and promotion of tumor metastasis (Partridge et al., 2004). Moreover, changes in glycosylation may also control tumor angiogenesis by allowing the binding of galectin-1, an endogenous glycan-binding protein, to endothelial cells (Croci et al., 2014).

Li and colleagues first studied whether glycosylation is critical for the co-stimulatory or co-inhibitory function of immune receptors. Strikingly, after enzymatic removal of *N*-linked oligosaccharides, only co-inhibitory but not co-stimulatory ligand-receptor pairs exhibited significant loss of binding. Although further studies are warranted to elucidate the molecular basis of these differential effects, glycosylation-dependent regulation of inhibitory receptors could be capitalized on to develop glycan-targeted therapies. Because PD-L1 exhibited the most significant loss in receptor binding after *N*-glycan removal, the authors focused on PD-L1 glycosylation for further validation. By re-expressing glycosylated or non-glycosylated PD-L1 constructs in PD-L1-silenced tumor cells and treating them with *N*- or *O*-glycosylation inhibitors, the authors confirmed that *N*- but not *O*-glycosylation is required for PD-L1/PD-1 interactions and suppression of T cell activity. Accordingly, TNBC cells expressing non-glycosylated PD-L1 were more sensitive to T-cell-mediated cytotoxicity and grew significantly more slowly than tumor cells expressing glycosylated PD-L1 when inoculated in immunocompetent, but not in immunodeficient, mice. These results emphasize the central role of immune-mediated mechanisms underlying tumor rejection mediated by unglycosylated PD-L1.

To further understand the mechanisms underlying PD-L1 glycosylation, the authors analyzed expression of EGFR and glycosyltransferases involved in *N*-glycan biosynthesis using the TCGA database with a focus on TNBC. They identified the β-1,3-N-acetylglucosaminyltransferase (B3GNT3), an enzyme involved in the biosynthesis of poly-N-acetyllactosamine (LacNAc), as a key determinant factor of PD-L1 glycosylation regulated by EGF. This glycosyltransferase catalyzes the incorporation of LacNAc residues, including those present on N192 and N200 sites of PD-L1 protein. Strikingly, when either B3GNT3 or EGFR was ectopically expressed in non-TNBC cell lines, PD-L1/PD-1 interaction was enhanced. These findings were substantiated by elegant experiments showing inhibition of tumor growth in mice receiving 4T1 cells (TNBC model) devoid of B3GNT3 and/or PD-L1. At the molecular level, EGF controlled PD-L1 glycosylation by upregulating expression of B3GNT3 via the TCF4 transcription factor downstream of the EGF-GSK3β-β-catenin pathway.

To explore the therapeutic potential of these findings, Li and colleagues focused on the development of specific neutralizing monoclonal antibody-targeting glycosylated PD-L1. Among 3,000 hybridomas screened, the authors isolated one particular antibody clone, STM108, that can specifically recognize poly-LacNAc residues incorporated by B3GNT3 on

N192 and N200 glycosylation sites of the PD-L1 protein. Importantly, STM108 profoundly affected glycosylation-dependent PD-1/PD-L1 interactions, enhanced T cell effector function, and elicited potent anti-tumor activity. Given that glycosylated PD-L1 is mostly expressed on tumor cells, the authors went further and generated an antibody drug conjugate (STM108-ADC) based on the addition of the antimitotic drug monomethylauristatin E to STM108. *In vivo* experiments confirmed the promising preclinical activity, bystander effects, and good safety profile of this new conjugated monoclonal antibody in mice inoculated with either 4T1 cells expressing human glycosylated PD-L1 (4T1-hgPD-L1) or a combination of 4T1-hgPD-L1 with wild-type tumor cells. Analysis of the mechanisms of action of STM108 antibody at the cellular level revealed induction of PD-L1 internalization to lysosomes and subsequent protein complex degradation (Figure 1). This endocytic process was shown to be caveolae dependent, was absent in B3GNT3 knockout tumor cells, and involved PD-L1 N192 and N200 glycosylation sites.

Although much remains to be learned, this study underscores the relevance of glycosylation in promoting the function of an immune inhibitory receptor. Moreover, it highlights a potential strategy to improve the efficacy of checkpoint blockade therapy by targeting PD-L1 glycosylation. From a translational perspective, the biological activity of STM108 antibody remains to be evaluated in TNBC patient-derived xenografts in humanized mice, as well as its safety profile in phase I clinical trials. Finally, several questions remain to be addressed: Is PD-L1 glycosylation a possible biomarker of therapy response? How does chemotherapy affect PD-L1 glycosylation? How does glycosylation impact other immune checkpoint receptors? The results presented by Li and colleagues provide proof of concept that rationalized drug cancer development should be based on understanding the molecular basis of receptor stabilization, signaling, and function. The potent anti-tumor activity of a drug-conjugated monoclonal antibody against glycosylated PD-L1 paves the way for the next generation of immuno-oncology drugs targeting post-translational protein modifications.

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