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Siglec-15 as an emerging target for next-generation cancer immunotherapy

Jingwei Sun[1,*], **Qiao Lu**[2,3], **Miguel F. Sanmanmed**[4], **Jun Wang**[2,3,#]

[1]Department of Immunobiology, Yale University School of Medicine, New Haven, CT, USA.

[2]Department of Pathology, New York University Grossman School of Medicine, New York, NY, USA.

[3]The Laura and Isaac Perlmutter Cancer Center, New York University Langone Health, New York, NY, USA.

[4]Program of Immunology and Immunotherapy, CIMA, University of Navarra, Pamplona 31008, Spain.

Abstract

Immunomodulatory agents blocking the PD-1/PD-L1 pathway have shown a new way to treat cancer. The explanation underlying the success of these agents may be the selective expression of PD-L1 with dominant immune-suppressive activities in the tumor microenvironment (TME), supporting a more favorable tumor response-to-toxicity ratio. However, despite the big success of these drugs, most cancer patients show primary or acquired resistance, calling for the identification of new immune modulators in the TME. Using a genome-scale T cell activity array in combination with bioinformatic analysis of human cancer databases, we identified Siglec-15 as a critical immune suppressor with broad upregulation on various cancer types and a potential target for cancer immunotherapy. Siglec-15 has unique molecular features compared to many other known checkpoint inhibitory ligands. It shows prominent expression on macrophages and cancer cells and a mutually exclusive expression with PD-L1, suggesting that it may be a critical immune evasion mechanism in PD-L1 negative patients. Interestingly, Siglec-15 has also been identified as a key regulator for osteoclast differentiation and may have potential implications in bone disorders not limited to osteoporosis. Here, we provide an overview of Siglec-15 biology, its role in cancer immune regulation, the preliminary and encouraging clinical data related to the first-in-class Siglec-15 targeting mAb, as well as many unsolved questions in this pathway. As a new player in the cancer immunotherapeutic arena, Siglec-15 may represent a novel class of immune inhibitors with tumor-associated expression and divergent mechanisms of action to PD-L1, with potential implications in anti-PD-1/PD-L1 resistant patients.

[#] Correspondence, Dr. Jun Wang, 550 First Avenue, Smilow Research Building 316, New York, NY 10016. Tel: 212-263-1506, jun.wang@nyulangone.org.

[*]Current Address: Grit Biotechnology, Shanghai, China.

Author Contributions

J.W., J.S., Q.L. and M.F.S., conceived the study and wrote the paper.

Disclosure of Potential Conflicts of Interest

J.W. is a consultant and receives consulting fees from Lilly Asia Ventures not relevant to this work.M.F.S. is a consultant and receive consulting fees from Numab, Pieris and Roche not relevant to this work. J.W. and J.S. has a patent application pending regarding Siglec-15 targeting for treating autoimmune diseases and cancers.

1. Introduction

The advent of immunotherapy based on immunostimulatory monoclonal antibodies (mAb) initiated by anti-CTLA-4 and confirmed by anti-PD-1/PD-L1 therapy has revolutionized the way to understand and treat cancer(1). In this early experience, the PD-1/PD-L1 blockade approach, in contrast to previous immunotherapy strategies, has illustrated the importance of targeting tumor immune-evasion mechanisms to restore an effective T-cell response at the tumor site(2–4). The result of this unique approach is reflected by a more beneficial response-to-toxicity profile, which has contributed to the approval of anti-PD-1/PD-L1 mAbs for more than ten tumor types, while other immunotherapies were barely approved for more than one (5). PD-L1 is specifically upregulated on tumor cells and tumor-associated myeloid cells compared to healthy non-inflamed tissues, contributing to the minimal to the low toxicity of single-agent anti-PD-1/PD-L1 in the majority of treated patients. However, despite anti-PD-1/PD-L1 is the most successful cancer immunotherapy so far, the majority of patients show primary or acquired resistance to this approach(6). In these resistant patients, other immune inhibitory pathways may be acting in cooperation or alternatively to PD-1/PD-L1 blockade, representing potential new immune targets to expand the success of cancer immunotherapy. Unfortunately, recent clinical trials using single agent targeting many of these previously described immune-regulatory pathways, such as LAG-3, TIM-3, B7-H3, B7-H4 and VISTA (PD-1H), have shown modest or negligible responses in human cancers(7–10). These disappointing results require a careful re-evaluation of the biology of these pathways and their relevance in tumor immunity. While PD-L1 is selectively expressed in the TME and represents a clear mechanism of tumor-induced immune evasion, this evidence is missing for most of these other targets (Figure 1). There is then, an unmet need, for the identification of PD-1/PD-L1 alternative pathways, which are both selectively expressed in the TME and show a clear role in the regulation of tumor immunity. We built a functional screening platform, named the genome-scale T cell activity array (TCAA) (11), to identify cell surface regulators with ligand-like features, immune-suppressive activities and significant relevance in the TME. Siglec-15, one of the Sialic acid-binding immunoglobulin-like lectins (Siglecs), was identified as a novel T cell inhibitory molecule using this screening strategy(Figure 2). Although Siglec-15 was originally characterized as an osteoclast modulator(12), our study indicated for the first time that Siglec-15 significantly suppresses antigen-specific T cell responses *in vitro* and also *in vivo,* and elicits immune evasion in the TME. In this review, we briefly summarize the biology of Siglec-15, with a special focus on its role in cancer immunology and the exciting application of Siglec-15 blockade as a new strategy for next-generation cancer immunotherapy.

2. Siglec-15: molecular features and signaling pathways

Siglecs constitute a family of cell surface proteins with an important role in the regulation of immune homeostasis. The dysregulation of these proteins has been associated with multiple diseases ranging from autoimmunity to infections and cancer (13). They are type I transmembrane proteins with one V-set immunoglobulin (Ig) domain containing the sialic acid-binding site and one or more C2-set Ig domains in their extracellular region(Figure 3). The majority of Siglecs, including CD22 (Siglec-2) and most CD33 (Siglec-3)-related

Siglecs, have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and/or ITIM-like motifs in their cytoplasmic domain and mediate inhibitory receptor signaling (Figure 3). Each Siglec preferentially recognizes a different kind of sialic acids, a group of sugars that are expressed on all mammalian cells as a mechanism to discriminate between self and non-self (14). But some pathogens can utilize inhibitory Siglecs to dampen the immune response and benefit their survival(15,16). Although most Siglecs work as receptors, some Siglecs can serve as functional ligands, such as Siglec-1(17,18). Siglec expression has been found mainly on hematopoietic cells (mostly on myeloid cell and B cells) or non-hematopoietic cells such as neurons(13,14). Among the Siglec family, Siglec-15 has been identified as a very unique member, selectively expressed on myeloid cells and osteoclasts (a bone-specific myeloid lineage) and generally absent in other immune cells and tissues(11,19).

Siglec-15 was originally characterized by Dr. Takashi Angata in 2007 as one of the most evolutionarily conserved Siglecs in vertebrates, which is phylogenetically distant from other family members(20). The amino acid sequence alignment between human and mouse Siglec-15 shows 83% identity, and unlike other Siglecs that reside within Chromosome 19 or 1, *Siglec-15* gene resides within Chromosome 18. Early studies suggested that Siglec-15 contains a conserved arginine (R143) motif in the membrane distal IgV domain, which is critical for sialic acid binding. Siglec-15 preferentially binds to Sialyl-Tn (Neu5Ac alpha 2–6GalNAc), a short O-glycan with a sialic acid residue whose neo- or over-expression is associated with various types of epithelial cancers(21). Importantly, as opposed to the majority of the Siglecs that contain one IgV domain and multiple “tandem repeats” of IgC2 domains in the extracellular region(14), Siglec-15 displays only one IgV and one IgC2 domain, which is commonly seen in B7 family members. Our group found high structural homology between Siglec-15 and PD-L1, and the protein sequence of Siglec-15’s extracellular domain exhibits 20–30% identity to B7 family, similar to the identity among B7 family members(11). These distinctive molecular features highlight the unique nature of Siglec-15 and suggest a possible link with B7 immune modulatory molecules.

Unlike the majority of Siglec members, Siglec-15 does not have typical ITIMs or ITIM-like motifs in its intracellular domain that mediate inhibitory signaling(14). Instead, it was reported to be associated with signaling adaptors DNAX activating protein of 12 kDa (DAP12) and DAP10 that contain an immunoreceptor tyrosine-based activation motif (ITAM), through a positively charged lysine residue (K273 in mouse Siglec-15; K274 in human Siglec-15) in its transmembrane domain(20,22). The association with DAP12 and/or DAP10 is a typical feature of some Siglecs with activating signaling, such as Siglec-14 and Siglec-16, which is possibly achieved through the recruitment of spleen tyrosine kinase (SYK) and ZAP70 among others(23,24).

3. Siglec-15 as an osteoclast modulator

Siglec-15 has been recently identified as an important regulator in osteoclast differentiation and function. Yoshiharu et al., in an attempt to identify regulators for osteoclast-like giant cell tumors, discovered that Siglec-15 was upregulated on osteoclasts upon stimulation by receptor activator of nuclear factor-κB ligand (RANKL)(12). Knockdown of Siglec-15 by shRNA or treatment with polyclonal antibodies against Siglec-15 inhibits osteoclast

differentiation and bone resorption(12,22). Sialyl-acid/Siglec-15 axis may constitute a functional loop for osteoclast differentiation -- removal of sialyl acids by sialidase or disruption of sialylated glycan binding by R143 mutation impaired osteoclast development(22,25). DAP12 may be needed for Siglec-15 function in osteoclasts, as the K273 mutation that disrupted the DAP12 association with Siglec-15 led to the function loss of Siglec-15 in osteoclast. However, it is still unclear whether Siglec-15 mainly serves as a receptor or ligand, and how important DAP-12 and DAP-10 association is for Siglec-15's osteoclast function. Nevertheless, mice with Siglec-15 deficiency show no obvious physical abnormalities other than mild osteopetrosis(26,27). Furthermore, mAbs targeting Siglec-15 were found to increase bone mass without impairing skeletal growth(28). These observations led to active research on Siglec-15 in bone biology and clinical development of targeting Siglec-15 for the treatment of osteoporosis, which was initiated by Alethia Biotherapeutics (AB-25E9) and Daiichi Sankyo (DS-1501)(19,28).

4. The function of Siglec-15 in immune regulation

Given its structural similarities with B7 family and dominant expression pattern on myeloid cells, our group hypothesized that Siglec-15 might be involved in the regulation of immunity. In early 2010, using the TCAA screening platform, we observed inhibition of NF-κB reporter activity in Jurkat T cells by HEK-293T cells expressing Siglec-15(11). In addition, Siglec-15 ectodomain fusion protein either coated on plates or supplied in soluble form, robustly inhibited anti-CD3 (OKT3) induced human T cell proliferation. In line with this, we also demonstrated that Siglec-15 expression on artificial antigen presenting cells (APCs) suppressed mouse T cell proliferation, cytokine secretion and killing capacity. These *in vitro* data suggested a ligand-like function of Siglec-15 that suppresses human or mouse T cells through unknown receptor(s) signaling. *In vivo* function of Siglec-15 on T cells was subsequently validated using an experimental autoimmune encephalomyelitis (EAE) mouse model. We found that EAE was significantly aggravated in Siglec-15 deficient mice or by injecting Siglec-15 ectodomain fusion protein, and T cell response was remarkably amplified in comparison with control groups(29). Additionally, we observed that Siglec-15 affects antigen-specific T cell responses -- Siglec-15 deficient mice showed a much higher OT-I T cell expansion in the blood and spleen compared to WT mice upon OVA peptide stimulation, which resembles the phenotype in PD-L1 KO mice(30). In this process, IL-10 might be an important factor since Siglec15 deficient mice showed decreased IL-10 levels in serum compared to WT, and anti-IL-10 mAbs abrogated the differences in OT-I T-cells expansion between WT and Siglec-15 deficient mice.

Bioinformatic analysis indicates that Siglec-15 mRNA is exclusively expressed by macrophages among the major immune subsets and normal tissues. Using our in-house developed Siglec-15 specific mAb, we confirmed that the expression of Siglec-15 was limited to peritoneal macrophages and bone-marrow-derived macrophages (BMDMs), but was not found on other major immune cells. In line with this, we observed minimal physiopathology in Siglec-15 deficient mice, suggesting that Siglec-15 is not critical for mouse development or immune system homeostasis in steady state conditions. Siglec-15 expression on macrophages was found to be mainly induced by macrophage colony-stimulating factor (M-CSF) and possibly by other innate inflammatory mediators(11,31).

Since M-CSF can be released by various stromal, epithelial and hematopoietic cells in response to inflammatory cytokines such as IL-1 and TNF-α(32), upregulation of Siglec-15 on macrophages may represent a negative feedback pathway in the regulation of innate and adaptive immunity. The T cell suppressive function of macrophage-associated Siglec-15 was further validated by co-culture experiments. Moreover, OT-I T cell response was also enhanced in mice with Siglec-15 conditional deletion in myeloid cells (LysM-Cre Siglec-15 KO mice) similar to the whole-body Siglec-15 KO mice, confirming the dominant function of Siglec-15 expressed by myeloid/macrophage cells (Table 1).

The putative receptor(s) mediating Siglec-15's suppressive activity on T cells is still elusive. Interestingly, previous studies also suggested a potential receptor function of Siglec-15 on myeloid cells. Angata's group showed that overexpression of Siglec-15 on THP-1 cells facilitates TGF-β production upon interaction with Sialyl-Tn glycan ligands on tumor cells, which may be mediated by the DAP12-Syk pathway(31). The receptor function of Siglec-15 has also been suggested to be important in host defense against fungal infection caused by *Candida albicans*(33). Recombinant Siglec-15 protein is demonstrated to bind to the sialic acid on the surface of *C. albicans*. Silencing Siglec-15 by siRNA *in vivo* showed higher fungal burden, which is likely due to the pathogenic hyper-inflammation observed at the infection site, such as increased levels of IL-1β and NLRP3, indicating a role of Siglec-15 in regulating the immune response during infection. Further studies by knock-down/knockout or antibody blockade of Siglec-15 will be helpful to confirm the receptor function of Siglec-15 on macrophages or myeloid cells.

5. The function of Siglec-15 in cancer immunity

The potent T cell regulatory function of Siglec-15 by itself may not be unique enough to highlight it from other checkpoint inhibitors. Our meta-analysis of the TCGA database indicated that, while limited expression was found in most normal tissues, Siglec-15 mRNA is broadly upregulated across many different tumor types(11). Further analysis by immunohistochemistry (IHC) in a tissue microarray of 241 human non-small cell lung cancer (NSCLC) samples confirmed Siglec-15 protein expression in 25.7% of samples. Siglec-15 can be detected not only on tumor stroma including tumor-associated macrophages (TAMs) but also on human cancer cells, as well as several human tumor cell lines. In addition to Siglec-15's unique induction mechanism by M-CSF, we demonstrated that IFN-γ, the major inducer of PD-L1(34), significantly suppresses Siglec-15 expression on macrophages(Figure 2). Thus, Siglec-15 may exhibit a complementary expression profile to PD-L1. In collaboration with Dr. David Rimm's lab, we observed that the expression of Siglec-15 and PD-L1 shows a mutually exclusive pattern in NSCLC TME. Moreover, Dr. David Rimm's lab also found higher Siglec-15 expression in EGFR mutant lung cancers (35). Evaluation of Siglec-15 expression in other cancer cell types is still ongoing.

Different from human cancer, the commonly used mouse tumor models including B16 (melanoma), MC38 (colon carcinoma), CT26 (colon carcinoma) and LLC (lung carcinoma) are negative for Siglec-15 mRNA expression. Nevertheless, Siglec-15 ablation slowed down tumor growth and prolonged survival in B16-GMCSF (PD-1 insensitive) and GL261 (PD-1 sensitive) tumor models(36,37), which have abundant infiltrations of TAMs and exhibit

detectable Siglec-15 mRNA expression. CyTOF analysis of the B16-GMCSF model showed a significant expansion of the tumor-infiltrating $CD8^+$ T and NK cells as well as several inflammatory myeloid populations, whereas a decrease of MHC-II^{low} TAMs and MDSCs in the Siglec-15 KO group. The regulatory effect of Siglec-15 was mostly localized in the tumor site and no difference was observed in the spleen and non-tumor draining lymph nodes between Siglec-15 KO and WT mice. Importantly, LysM-Cre Siglec-15 KO mice also showed a better tumor control similar to whole-body Siglec-15 KO mice in both tumor models. Furthermore, $CD11b^+$ myeloid cells isolated from Siglec-15 KO tumors promoted $CD8^+$ T cell proliferation and cytokine release compared to those from WT tumors, confirming the essential function of Siglec-15 expressed by myeloid cells. To further support the importance of TAM-associated Siglec-15, we demonstrated that Siglec-15 blocking mAbs profoundly slowed down the tumor growth in mice inoculated with MC38 mixed with WT BMDMs, but not with Siglec-15 KO BMDMs. In addition, the function of Siglec-15 on tumor cells was also explored by overexpressing Siglec-15 on a MC38 cell line, which mimics Siglec-15 upregulation on human cancer cells. The *in vivo* tumor growth of this Siglec-15^+ MC38 line was also suppressed by anti-Siglec-15 mAbs. All together, these results indicate the critical suppressive function of both macrophage- and tumor cell-associated Siglec-15 in tumor immunity (Figure 2 and Table 1)

Moreover, despite the structural and functional similarity with PD-L1, Siglec-15-mediated immune suppression mechanism seems to be independent of the PD pathway. Siglec-15 mAb can promote the function of PD-1 KO T cells and show a synergistic immune-stimulatory effect with anti-PD-1 mAb on T cells both *in vitro* and *in vivo*(11). Hence, these two pathways represent independent mechanisms of action in immune regulation, and blockade of Siglec-15 may provide a new immune therapeutic strategy for patients resistant to anti-PD therapy.

6. Preliminary clinical results of anti-Siglec-15 immunotherapy in cancer

Based on the preclinical functional activity and expression pattern of Siglec-15, the safety of a humanized anti-Siglec-15 mAb, named NC318, is being evaluated in a first-in-human phase I clinical trial in advanced solid tumors (NCT03665285). NC318 was derived from B cell hybridoma screening (clone 5G12), which specifically binds to both mouse and human Siglec-15(11). This mAb can efficiently restore T-cell function *in vitro* and inhibit tumor growth in preclinical models(38).

In this multicenter, open-label, non-randomized phase I clinical trial, NC318 is intravenously administrated to patients with advanced solid tumors across seven dose cohorts ranging from 8 to 1600 mg(39). This trial uses a 3+3 dose escalation design with NC318 dosed every 14 days. As of Sept 26^{th}, 2019, a total of 49 patients have been dosed, including patients with NSCLC (n=13), ovarian cancer (n=7), melanoma (n=7), colorectal cancer (n=3), breast cancer (n=4) and others (n=15). In this all-comers trial design, patients are enrolled regardless of PD-L1 or Siglec-15 expression level. All of the patients were heavily pre-treated with a median of three prior anticancer regimens, and 63% of patients were treated with prior immunotherapy. Notably, 100% of the NSCLC patients were previously treated with anti-PD-1/PD-L1 mAbs either as single or combinational therapy.

NC318 has been well tolerated across multiple dose levels with no dose-limiting toxicity (DLT) reached. The most common treatment-related adverse events included diarrhea (16%), elevated amylase (8%), elevated lipase (6%), pruritis (6%) and generalized pruritis (6%). Immune-related adverse events, such as uveitis, pneumonitis and vitiligo, were also observed. Grade 3 or 4 toxic effects occurred in 8% of patients, including one patient with elevated amylase and three patients with elevated lipase.

Among evaluable patients, in September 2019, prolonged stabilization of disease was observed in 54% (20/37) of patients and objective response in 5.4% (2/37) of patients. Notably, 20% (2/10) of objective responses have been observed in anti-PD refractory NSCLC patients, including one complete response (ongoing at 49 weeks) and one partial response (ongoing at 24 weeks). In addition, three stable diseases were also observed, showing a 50% disease control rate.

All together, NC318 shows good safety and tolerability across all dose levels and demonstrates encouraging antitumor activity as a monotherapy, especially in NSCLC. The phase 2 clinical trial will evaluate the efficacy of NC318 dosed at 400 mg every 14 days, and patient enrollment is currently ongoing.

7. Preclinical and/or clinical studies of other Siglecs

Aside from Siglec-15, the role of other Siglecs in cancer biology and immune regulation is also being studied (Figure 3). In addition to CD22 and CD33-targeting antibody-drug conjugates approved by FDA for lymphomas and leukemias(40–42), increasing evidence points to the important role of Siglecs in tumor immunity. Recent studies demonstrated that the inhibitory function of Siglecs can be exploited by cancer cells, which display increased sialylation to dampen and evade immune surveillance. For instance, some cancer cells upregulate sialic acid ligands to engage Siglec-7 and Siglec-9 on NK cells, myeloid cells or T cells and inhibit their anti-tumor responses(43–46). These results have led to preclinical programs blocking Siglec-7 or Siglec-9 with antibodies (Innate Pharma and Palleon Pharmaceuticals)(47). Additionally, Palleon Pharmaceuticals is developing a fusion protein (*EAGLE*) with one arm as an antigen-biding fragment (Fab) to target tumor antigens and another arm as a sialidase to enzymatically remove all kinds of sialic acids. As the majority of Siglecs mediate inhibitory signaling upon ligation of their sialylated ligands, this modality has been demonstrated to enhance anti-tumor immunity in mouse tumor models, and this effect may be mainly mediated by macrophages and $CD8^+$ T cells(48). But its safety profile remains to be further investigated.

Targeting Siglecs for other diseases than cancer is also being actively explored. Epratuzumab (UCB Pharma), an anti-CD22 humanized mAb, was tested in phase 3 clinical trials for treating systemic lupus erythematosus (SLE) though neither met the primary endpoints (NCT01262365, NCT01261793, NCT01408576)(49–51). Abbive has recently initiated a phase 1 clinical trial of AL003, an anti-CD33 antibody, for the treatment of Alzheimer's disease (NCT03822208)(52). Siglec-4 (MAG) is thought to be an inhibitor of axon growth in the central nervous system and thus was investigated as a potential target to improve recovery of brain function after cerebrovascular accident. In a randomized phase IIb trial, the

primary endpoint of gait velocity was not met (53) and further development of this drug was halted (NCT01808261). Antolimab (Allakos) is a monoclonal antibody targeting Siglec-8, an inhibitory receptor found primarily on eosinophils and mast cells, leading to inhibition of mast cells and reduction in eosinophils via antibody-dependent cellular cytotoxicity (ADCC)(54). Antolimab is currently in a phase II/III clinical trial for the treatment of eosinophilic gastrointestinal disease and chronic urticaria (NCT04322708). CD24Fc is a fusion protein developed by OncoImmune, of which one portion interacts with Siglec-10 on macrophages and dendritic cells and results in immune inhibition via a SHP-1-mediated pathway(55,56). This molecule has completed a phase II clinical trial for graft versus host disease (GVHD) in patients with leukemia who have undergone hematopoietic stem cell transplantation (NCT02663622)(57). Future studies plan to evaluate the role of CD24Fc in other autoimmune conditions as well as in immunotherapy related adverse events (irAEs).

8. Key questions and future directions

Since the discovery of Siglec-15 in 2007, we have gained considerable insight into its function and therapeutic potential. Other than NextCure's NC318 for solid cancer (phase II), there are several ongoing Siglec-15 targeted clinical programs, including Medimmune for AML (patent filed)(58) and Daichi Sankyo's DS-1501 (phase I) for osteoporosis. However, many key questions remain to be addressed, which are critical for our understanding of Siglec-15 biology and better design of Siglec-15-based cancer immunotherapy and other clinical indications.

What are the major functional receptor(s) for Sigelc-15 on immune cells?

It is possible that Siglec-15 may engage with more than one counter-receptor for its function. We have validated the high-affinity binding between Sialyl-Tn and Siglec-15 through Octet, a real-time and label-free analysis for molecular binding affinity and kinetics. Moreover, several other heavily sialylated proteins, such as CD44, and even non-natural glycan structures, are also suggested to interact with Siglec-15(59,60). It appears that both sialylation and Siglec-15 are involved in osteoclast function; however, the contribution of these sialic acid glycans to Siglec-15's immune function is still unclear. In particular, Sialyl-Tn has documented to be dominantly expressed on cancer cells (e.g. bladder, colon, gastric, breast, and ovarian cancers)(61–63) and can serve as an immune suppressor. Based on these findings, Tn/Sialyl Tn-based cancer vaccines and Sialyl Tn-targeting antibodies (e.g. ST1 by Siamab Therapeutics Inc.) were developed and showed some level of efficacy(64–66). However, among the receptors that Sialyl-Tn is reported to engage with, it is difficult to fully ascertain the physiological function of Sialyl-Tn/Siglec-15 interaction in the context of immunity and diseases.

Other than sialic acids, some Siglecs can function through glycan-independent protein-protein interactions. For example, Siglec-10 has been reported to engage with CD24 ligand for the inhibition of macrophage phagocytosis, even after removal of sialic acids(67). Our preliminary data also suggest that Siglec-15 may engage with putative receptors on activated T cells(11). Through our genome-wide scale receptor array, we have found several potential receptors as novel binding partners for Siglec-15, such as LRRC4C and MAG, which

are conserved in human and mouse(29). The discovery and functional characterization of Siglec-15's interacting partners and their contribution to Siglec-15-mediated immune suppression will provide critical clues for the biology of Siglec-15 and its future application in immunotherapy.

What is Siglec-15's function as a receptor on myeloid cells and/or tumor cells in the TME?

In addition to its role as a tumor or myeloid cell-expressing ligand suppressing T cell responses, Siglec-15 also shows clear receptor function. Its engagement with DAP-12 has been suggested to mediate osteoclast maturation and induce TGF-beta production in THP-1 macrophage cell line(22,31). However, the mechanisms underlying its receptor biology on myeloid cells or possibly on tumor cells are still unclear. These mechanisms may also be distinct in different cell types -- as tumor cells from solid cancers normally have little DAP-10/DAP-12 expression, the tumor relevance of DAP-10/DAP-12 engagement remains an open question.

What are the major induction mechanisms of Siglec-15 on myeloid cells and cancer cells?

Siglec-15 is the only Siglec molecule that has unique macrophage and osteoclast-related expression and is generally absent in other immune populations and tissues. Other than RANKL stimulation that upregulates Siglec-15 in osteoclast precursors(12), we confirmed that M-CSF can strongly upregulate Siglec-15 on human macrophages and mouse RAW264.7 cells(11,31). Interestingly, as opposed to PD-L1's induction by IFN-γ, we found that IFN-γ significantly downregulates Siglec-15 expression. Given that M-CSF is a major factor that triggers immune suppressive M2 macrophages(68), our data suggest that M-CSF-induced Siglec-15 may constitute a novel immune-suppressive feedback loop between myeloid cells and T cells, which is distinct from the PD-1/PD-L1 pathway. In addition, we found that Siglec-15 can also be expressed on tumor cells or cell lines, while the major trigger for its induction on cancer cells is not clear so far and requires further investigation.

What is the role of Siglec-15 in bone-related cancers or metastasis?

Among all the Siglecs or immune inhibitors, Siglec-15 has unique function as a modulator for osteoclast maturation, differentiation and bone remodeling. As osteoclasts also have immune-suppressive capacity, Siglec-15 may play an important role in bone-related cancers (such as giant cell tumors) as well as cancer metastasis into the bone through mediating immune evasion in the bone microenvironment. Further studies will potentially expand the application of Siglec-15 based therapies to bone-related cancer indications.

What is the best biomarker for Siglec-15 based therapy?

As Siglec-15 has an opposing expression pattern compared to PD-L1, it is vital to determine which patients may benefit most from this therapy. Based on the current data from the NC318 phase I trial, which was conducted without any biomarkers, the therapeutic efficacy of Siglec-15 mAb is predominately observed in NSCLC(39). The mechanism for response remains to be elucidated but may be influenced by Siglec-15 expression level in the tumor site. In a cohort of more than 200 NSCLC samples, 25% of samples showed positive

Siglec-15 expression by IHC(11), which correlates with the objective response rate of NC318 in NSCLC (20%). It remains to be determined which biomarkers (Siglec-15 IHC or mRNA, PD-L1, M-CSF, and even soluble Siglec-15, etc.) could best guide patient selection, and more importantly, predict responses to anti-Siglec-15 therapy.

What is the best combinational therapeutic regimen for anti-Siglec-15 therapy?

Siglec-15 may represent a novel class of tumor-associated immune inhibitors with distinct expression and function to PD-L1. A deep understanding of its function in cancer immunity could provide potential solutions for future cancer immunotherapies. It is also important to explore the application of other therapeutic modalities, especially myeloid cell modulation reagents and/or T cell infiltration enhancing agents, which may provide potential synergy with anti-Siglec-15 therapy in the control of malignancy. Moreover, these concepts may help design bispecific antibodies or combination therapies to maximize tumor immunity and advance immunotherapy for tumors insensitive to anti-PD therapies.

9. Conclusion

Cancer immunotherapy in the post-anti-PD-1/PD-L1 era is facing challenges to overcome resistance to anti-PD therapy and extend therapeutic benefit to non-responders. Novel approaches targeting tumor-selective immune escape mechanisms may lead to a better understanding of the TME and the development of potential therapies with optimal efficacy but lower autoimmune toxicities. Siglec-15 may represent a novel pathway mediating cancer immune escape with the following critical features: 1) selective expression in the tumor-site, while almost absent in normal tissues, 2) a potent tumor-induced immune-suppressive mechanism and 3) a targetable pathway that normalizes cancer immunity in the TME upon blockade. Given these features, the phase I trial of Siglec-15 mAb showed good tolerance and an acceptable safety profile with encouraging efficacy as a single agent. Moreover, Siglec-15 represents an independent immune regulatory pathway from the PD-1/PD-L1 axis and shows a mutually exclusive expression profile with PD-L1 in human cancers, suggesting that it may provide a new therapeutic strategy for patients resistant to anti-PD therapy. Although a critical immune suppressive function of Siglec-15 has been characterized, the complete roles of Siglec-15 in immune regulation are yet to be elucidated. A more comprehensive understanding of this pathway in immune regulation, especially in cancer immunity, will inform the development of more effective mono- or combinational therapies.

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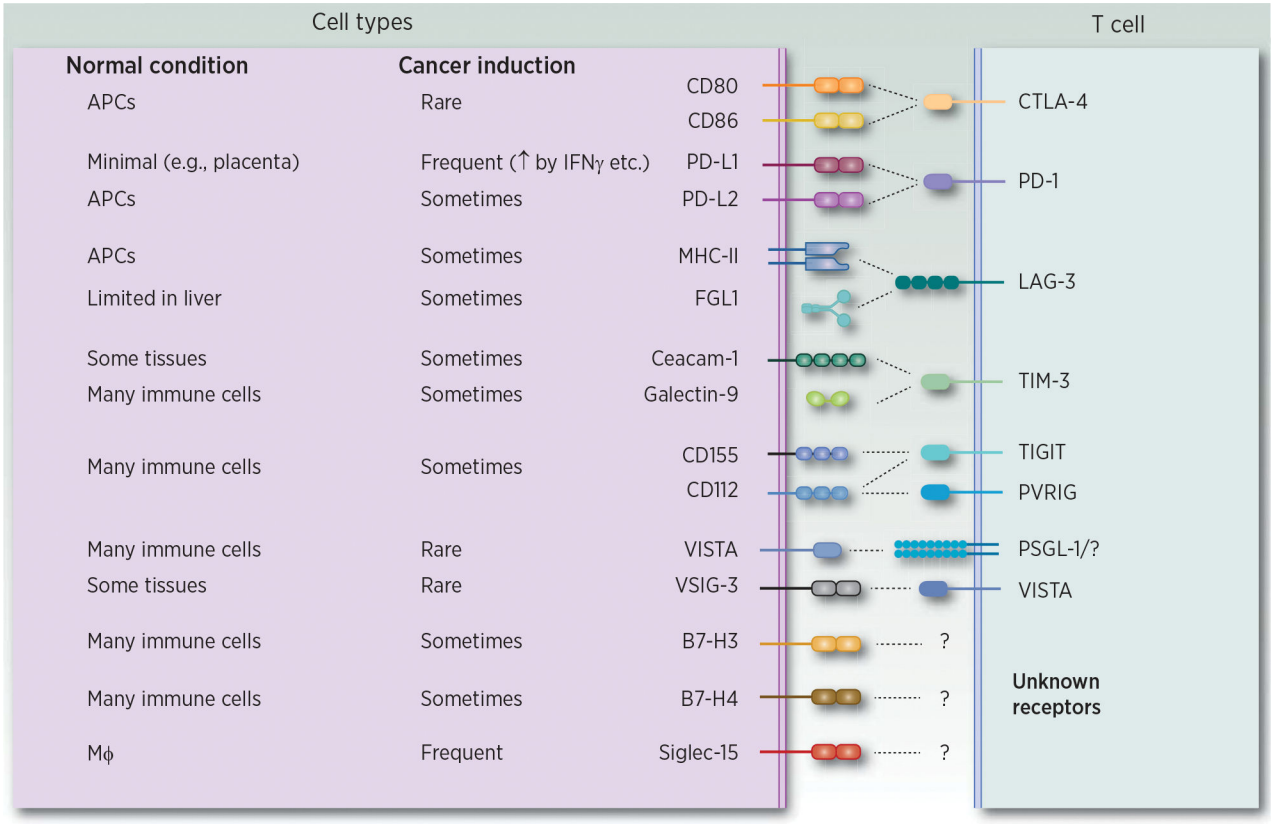


Figure 1. Expression profile of ligands in representative T cell coinhibitory pathways

APC: antigen presenting cell; MΦ: macrophage

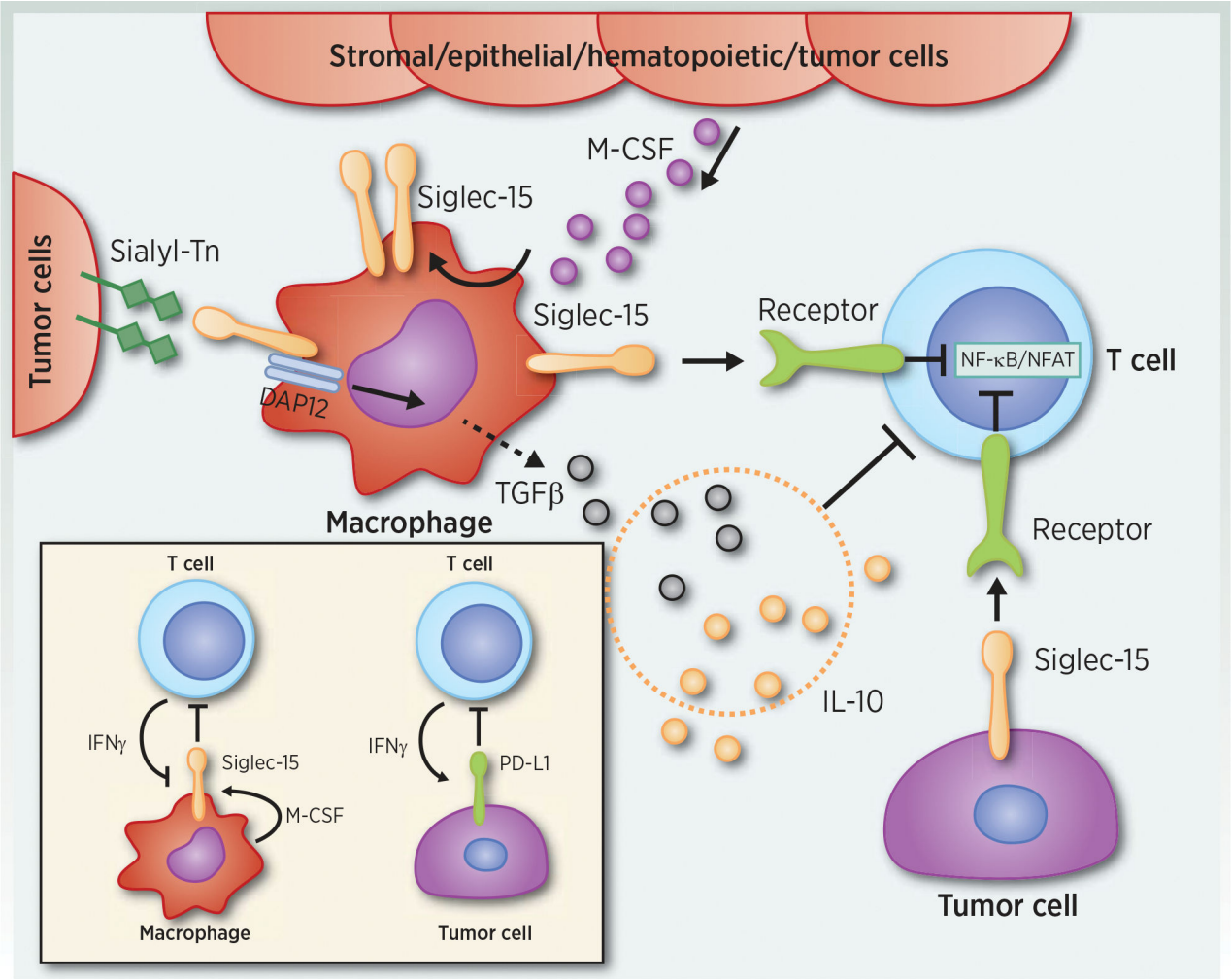
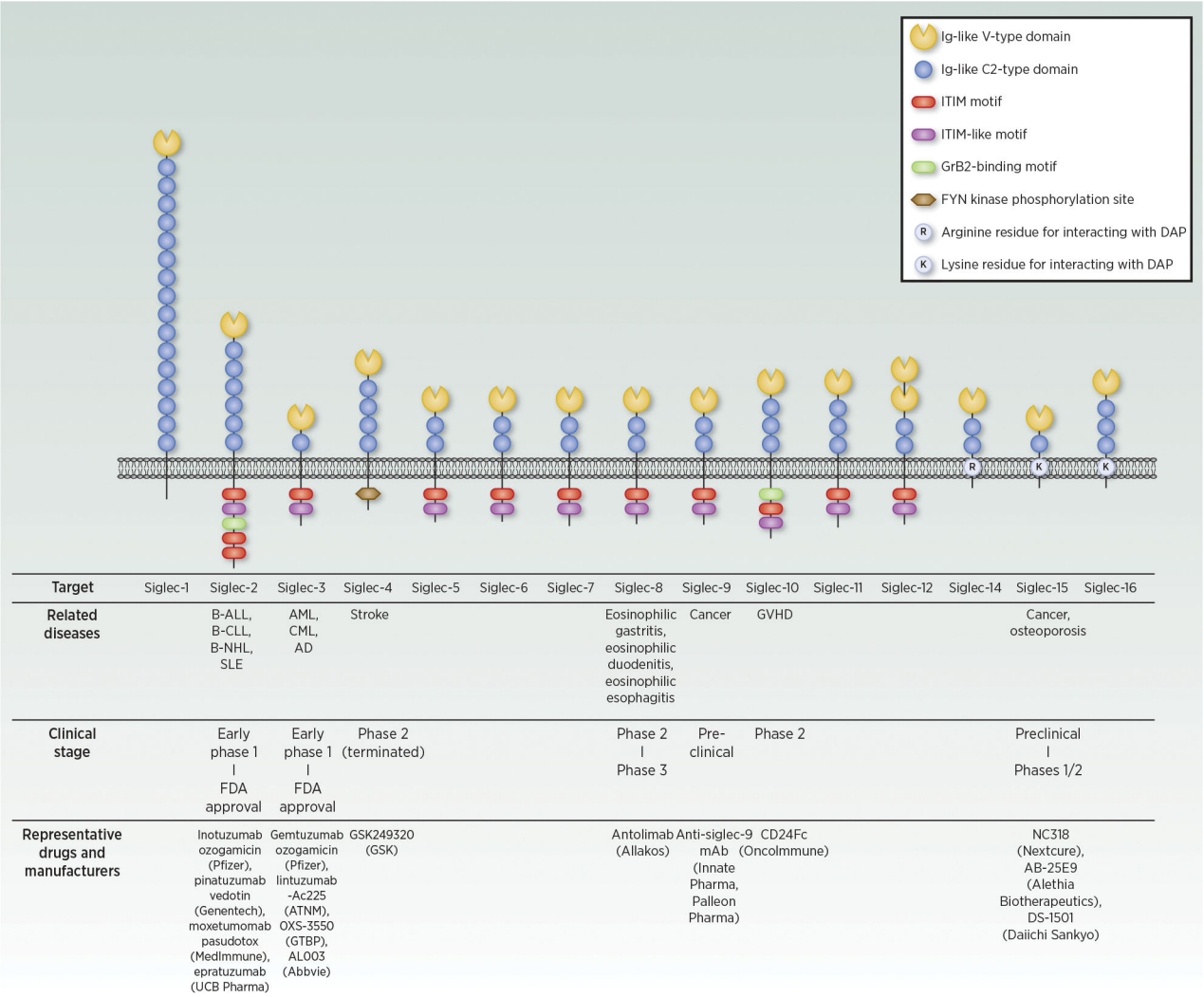


Figure 2. A working model of Siglec-15 in immune regulation

Siglec-15 is expressed on macrophages at a low level in physiological condition, but can be upregulated by M-CSF, which is released by various normal cell types in response to inflammatory cytokines or by tumor cells. It can also be expressed on different types of cancer cells. Siglec-15 directly inhibits NF-κB/NFAT signaling by engaging unknown receptor(s) and suppresses T cell proliferation and cytokine production. IL-10 is a mediator of Siglec-15's inhibitory function. Besides, Siglec-15 may also behave as a receptor on macrophages and produce TGF-β upon binding with its sialic acid ligand Sialyl-Tn on tumor cells. The increased level of IL-10 and TGF-β in the microenvironment will further magnify the immune suppressive effect of Siglec-15. Experimental evidence showed that Siglec-15 expression can be induced by M-CSF but suppressed by IFN-γ, in contrast to the induction mechanism of PD-L1 (panel on the bottom left).



Target	Siglec-1	Siglec-2	Siglec-3	Siglec-4	Siglec-5	Siglec-6	Siglec-7	Siglec-8	Siglec-9	Siglec-10	Siglec-11	Siglec-12	Siglec-14	Siglec-15	Siglec-16
Related diseases		B-ALL, B-CLL, B-NHL, SLE	AML, CML, AD	Stroke				Eosinophilic gastritis, eosinophilic duodenitis, eosinophilic esophagitis	Cancer	GVHD				Cancer, osteoporosis	
Clinical stage		Early phase 1 I FDA approval	Early phase 1 I FDA approval	Phase 2 (terminated)				Phase 2 I Phase 3	Pre-clinical	Phase 2				Preclinical I Phases 1/2	
Representative drugs and manufacturers		Inotuzumab ozogamicin (Pfizer), pinatuzumab vedotin (Genentech), moxetumomab pasudotox (MedImmune), epratuzumab (UCB Pharma)	Gemtuzumab ozogamicin (Pfizer), lintuzumab -Ac225 (ATNM), OXS-3550 (GTBP), AL003 (Abbvie)	GSK249320 (GSK)				Antolimab (Allakos)	Anti-siglec-9 mAb (Innate Pharma, Palleon Pharma)	CD24Fc (Oncolmmune)				NC318 (Nextcure), AB-25E9 (Alethia Biotherapeutics), DS-1501 (Daiichi Sankyo)	

Figure 3. Clinical application of Siglec family members

AD: Alzheimer's disease; ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; CML: chronic myeloid leukemia; GVHD: graft versus host disease; NHL: non-Hodgkin lymphoma; SLE: systemic lupus erythematosus.

Table 1:

Immune regulatory function of Siglec-15 in different experimental models

	Reagent/Modification	Model	Effect
In vitro	Siglec-15-Fc	Coated on plate or applied in soluble form in human PBMC or mouse splenic T cells	Suppress T cell proliferation and cytokine production
	Siglec-15 mAb	Applied in human PBMC with Siglec-15-Fc or BMDM co-culture with mouse T cells	Block T cell suppression mediated by Siglec-15-Fc or cell-associated Siglec-15
	Siglec-15 overexpressed on APCs	293T-K^bOVA cells co-cultured with OT-I T cells; THP-1 cells co-cultured with tumor cells expressing Sialyl-Tn	Suppress T cell proliferation, cytokine production and cytotoxicity; promote TGF-β production of THP-1 cells
	Siglec-15 KO on APCs	BMDM or tumor infiltrating CD11b+ cells co-cultured with mouse T cells	Promote T cell proliferation and cytokine production
In vivo	Siglec-15-Fc	EAE model	Aggravate EAE development
	Siglec-15 mAb	Mouse tumor models including B16-GM-CSF, MC38 or CT26 + BMDMs, and MC38 overexpressing Siglec-15	Supprress tumor growth and promote anti-tumor immunity
	Whole body Siglec-15 KO	a. OT-I in vivo activation model; b. EAE model; c. B16-GMCSF and GL261 tumor models	a. Promote OT-I proliferation and slow down contraction; b. aggravate EAE development; c. supprress tumor growth and promote anti-tumor immunity at tumor site
	LysM-Cre Siglec-15 KO	OT-I in vivo activation model; B16-GM-CSF and GL261 tumor models	Largely phenocopy the effects seen in whole body Siglec-15 KO
	Siglec-15 siRNA	Fungal infection by *Candida albicans*	Higher fungal burden associated with hyper-inflammation at infection site