Sialoglycan – Siglec axis in the modulation of dendritic cells functions

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Jinyu Wang

Basel, 2022

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Heinz Läubli, Prof. Dr. Gerhard Christofori und Prof. Dr. Stephan von Gunten

Basel, den 14.12.2021

Prof. Dr. Marcel Mayor

Dekan

Sialoglycan – Siglec axis in the modulation of dendritic cells functions

Dissertation of Jinyu Wang, MSc. Cell Biology
Under the supervision of Prof. Heinz Läubli at the Laboratory of Cancer Immunotherapy, Department of Biomedicine, University of Basel and University Hospital of Basel, Switzerland
Submitted to the Faculty of Sciences of the University of Basel and the thesis committee,
Prof. Dr. Heinz Läubli, Prof. Dr. Gerhard Christofori, Prof. Dr. Stephan von Gunten, and Prof. Dr. Alfred Zippelius
on 19th October 2021, in Basel, Switzerland

Preamble

In accordance with paragraph 16 of the 'Promotionsordnung der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel' of 15th September 2015, this thesis contains unpublished work. I will first summerize the current knowledge and recent progress of anti-tumor immunity, cancer immunotherapy and sialoglycan - Siglec axis in the regulation of immune cells. Following is my unpublished project on inhibitory Siglec receptors regulate dendritic cells maturation and antigen presentation to CD4+ T cells.

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1. Introduction

In the following chapter, I will summarize the relationship among cancer immune surveillance, immune escape and cancer-associated glycosylation based on our current knowledge. Firstly, I will briefly discuss about the crosstalk between tumor cells and the host anti-tumor immunity. Secondly, I will discuss about the cancer-associated glycosylation and its influence on the balance between tumor and host immune system.

1.1. The immune system

1.1.1. Immune system and immune response

The fundamental function of the immune system is to fine-tune the host defensive barriers, and fight against exogenous pathogens and endogenous malignant transformations, which keeps the host in a healthy state. The mammalian immune system consists of multiple organs, different types of cells and various soluble factors that co-operate together to maintain the homeostasis of the host.

The major organs that involve in the mammalian immune system can be classified into primary lymphoid organs and secondary lymphoid organs. Primary lymphoid organs include thymus and bone marrow, where T lymphocytes and B lymphocytes differentiation take place respectively. Secondary lymphoid organs consist of spleen, lymph nodes and mucosal immune system, containing gut-associated lymphoid tissue and Peyer's patches, where T and B lymphocytes can encounter antigens and get activated for further immune responses (Murphy & Weaver, 2016).

In the cellular level, mammalian immune system also has multiple different types of cells. In general, these immune cells could be distinguished by their ability to recognize specific antigens. T and B lymphocytes are the major defense of the host immune system that could recognize specific antigens and carry out their function to eliminate those antigens. This specific recognition and killing is known as the adaptive immune system. On the other hand, some immune cells cannot distinguish specific antigen, which are known as the innate immune cells, mainly including Nature killer cells (NKs), Macrophages, Dendritic cells (DCs) and Granulocytes. These cells also carry out rather important functions, such as killing of target cells or pathogens, phagocytosis and antigen presentation to complete the process to trigger adaptive immune response. Other cells that involve in immune response includes gamma delta T cells, invariant NK T cells etc, whose functionality and characterization still need further invesitigation (Colombo et al., 2015; Murphy & Weaver, 2016). Except for direct cell-to-cell contact during immune response, various soluble factors also play crutial roles in the complicated immune regulating network in mammalian hosts. Different immunoglobulins (Igs) secreted by highly differentiated and functional plasma cells are potent weapons in the host humoral immunity. Specific chemokine production is capable of long-distance recruiting of distinct immune cells to migrate to the site of infection or malignant transformation for danger clearance. Numerous cytokines have the potential to participate in specific signaling pathways, guide distinct immune cell polarization, and stimulate immune cell maturation status. The complement system is the next defensive barrier which will be activated after pathogens breaking host epithelial barrier. It consists of over 30 proteins, mainly liver-derived, which can form complexes to fight pathogens either by directly killing or by facilitating phagocytosis and triggering inflammatory response (Murphy & Weaver, 2016).

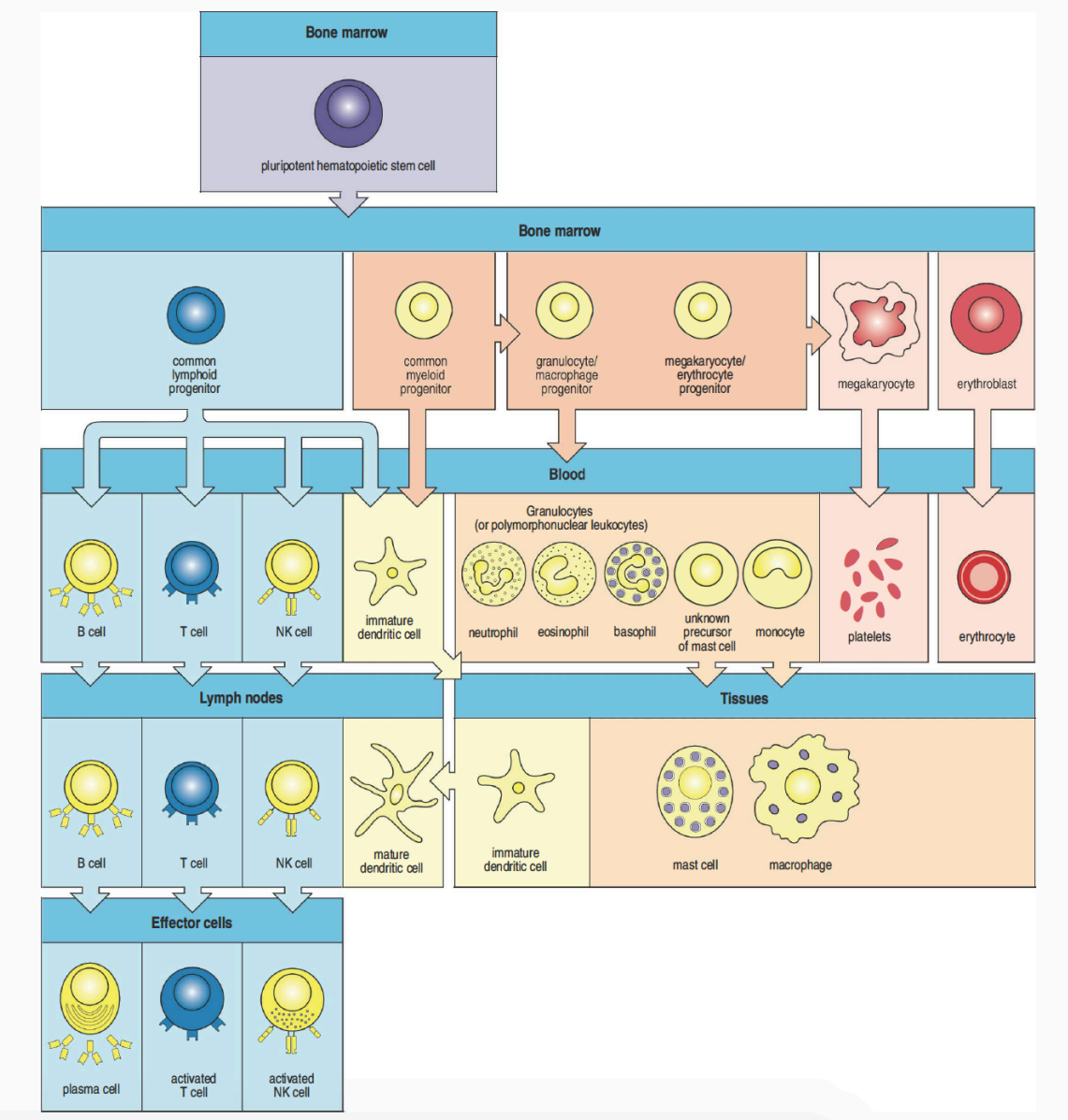


Figure 1.1 The distinct development pathways of different immune cells (Murphy & Weaver, 2016).

The cells of the immune system derived mainly from bone marrow pluripotent hematopoietic stem cells, and can be briefly classified into myeloid lineage and lymphoid lineage based on different development pathways (Figure 1.1). The myeloid lineage includes most cells from the innate immune system, while the

lymphoid lineage consists of adaptive immune cells and natural killer cells from innate immune system.

Successful immune response against pathogens and transformed cells requires co-operation of multiple immune cells and factors. Specific antigens derived from those pathogens or transformed cells should be recognized by the immune system, which then activates effector functions for the clearance of danger. Fine-tuned immune regulations are also essential to restrain immune responses at reasonable level, avoiding allergy and autoimmune diseases. The adaptive immune system also generates long-time immunological memories, and when encounter the same antigens again, the host will mount a quicker and even stronger immune response against danger signal (Murphy & Weaver, 2016).

1.1.2. Cellular crosstalk during T cell activation

Immune cells crosstalk during an adaptive T cell immune response requires sufficient interactions between T cells and antigen presenting cells (APCs). APCs provide three signals to prime antigen-specific T cells response (Figure 1.2). Major histocompatibility complex (MHC) molecules present antigen-derived peptides on the cell surface of APCs. The MHC:peptide (MHC:pep) complexes engage with T cell receptors (TCRs), providing the first signal for T cell activation. The second signal involves the interactions of adhesion and co-stimulatory molecules with their receptors, as shown in Figure 1.2, the binding of T cell CD28 molecules to APC CD80/CD86 molecules. The co-stimulatory secondary signal transduces activating signal to downstream cascade and stabilizes the immune synapse. The cytokines secreted by APCs comprise the third signals, guiding T cell differentiation or polarization to an effector phenotype (Gutcher & Becher, 2007).

However, there are also mechanisms in T cell physiology as negative feedback to regulate T cell activation. Cytotoxic T lymphocyte antigen 4 (CTLA-4) function as an inhibitory molecule, and is expressed constitutively on regulatory T cells (Tregs) and also activated conventional T cells (Tconv). Upon Tconv cells activation, CTLA-4 traffic from intracellular vesicles onto the cell surface, and compete for CD80/CD86 binding against CD28 (Leung et al, 1995). CTLA-4 protein has superior binding avidity to CD80/CD86, compared to CD28, and transduce inhibitory signals to suppress T cell function (Linsley et al, 1991,1994 and 1996; Walunas et al, 1994; Krummel et al, 1995).

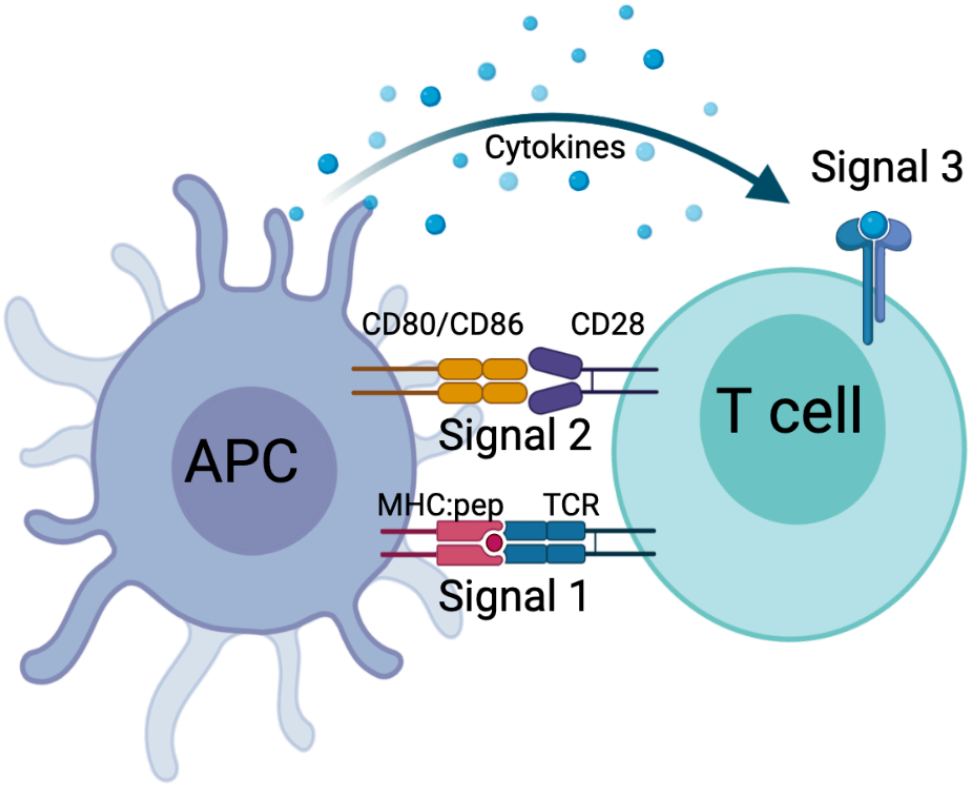


Figure 1.2 Three signals required for antigen-specific T cell activation. (Adapt from Gutcher & Becher, 2007).

1.1.3. Dendritic cell subsets and functions

Dendritic cells (DCs) were firstly discovered by Ralph Steinman and Zanvil Cohn in the 1970s (Steinman & Cohn, 1973 and 1974). They are a rare myeloid immune cell population, which play central roles in antigen presentation to initiate antigen-specific immunity and tolerance. Maturation of DCs links the innate immunity and adaptive immunity in response to various stimuli (Steinman, 2012). DCs are specialized APCs for naive T cell activation and initiating adaptive immune response (Steinman & Witmer, 1978; Nussenzweig et al, 1980).

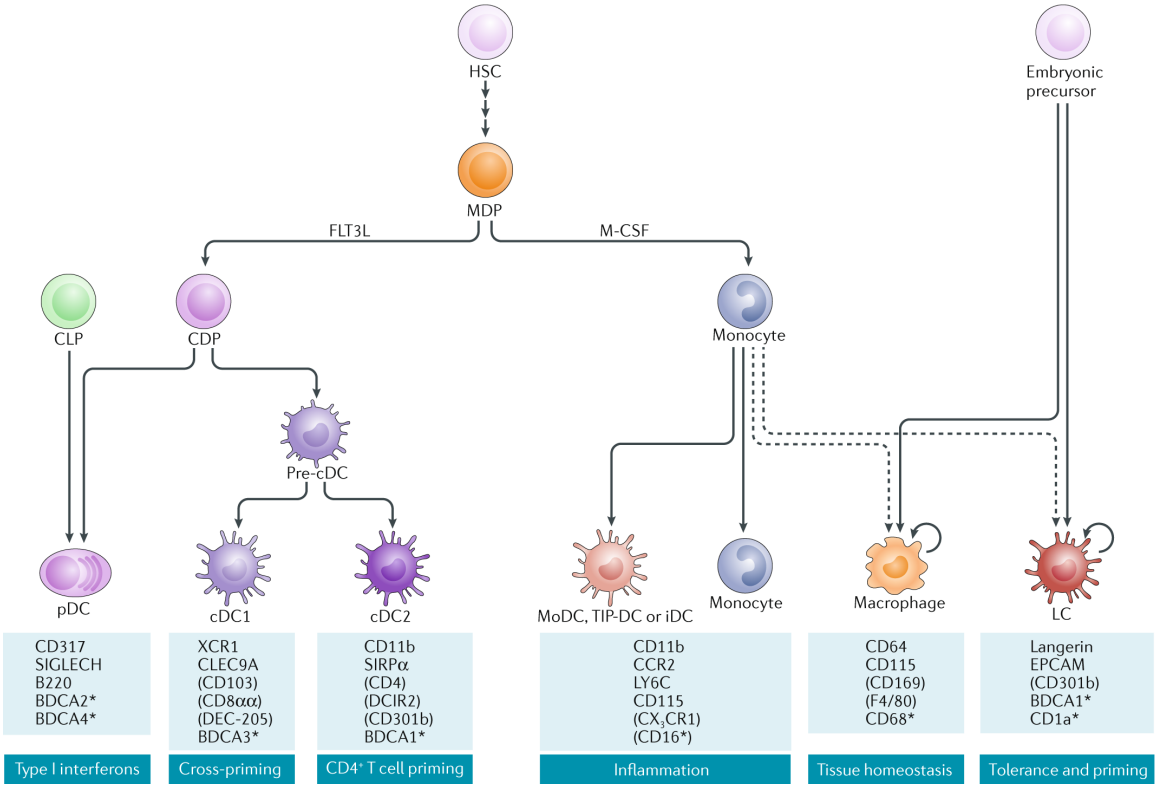


Figure 1.3 Functionally specialized conventional and non-conventional dendritic cell subsets and related lineages. (Eisenbarth, 2019) *HSC, haematopoietic stem cell; MDP, macrophage DC progenitor; CLP, common lymphoid progenitor; CDP, common DC precursor; pDC, plasmacytoid DC; cDC, conventional DC; MoDC, Monocyte-derived DC; TIP-DC, TNF/iNOS-*

*producing DC; iDC, inflammatory DC; LC, Langerhans cells. *Human-specific marker.*

DCs carry out important functions in antigen capture, processing and presentation, and they can be characterized as several different subsets based on their ontogeny. The majority of DCs are derived from myeloid cell lineage. (Figure 1.3). These DC subsets can be distinguished by their phenotypes and specific transcriptional network, which also correlate with distinct functions. Among these different DC subsets, conventional DCs (also known as classic DCs, cDCs) are the most important mediators of T cell priming. cDCs are further divided into two subgroups, Type 1 cDCs (cDC1s) and Type 2 cDCs (cDC2s). The development of cDC1s is dependent on transcriptional factors such as BATF3 and IRF8 (Hildner, 2008; Edelson et al, 2010), while cDC2s development requires the expression of transcriptional factor IRF4 (Schlitzer et al, 2013; Persson et al, 2013). Both cDC subsets can activate CD4+ and CD8+ T cells *in vitro*, although with different efficiency (Iyoda et al, 2002; Kamphorst et al, 2010). Under physiological conditions, these cDC subsets more preferentially activate specific T cell subsets, which is mainly determined by their distinct way of cytokine secretion and antigen presentation pathways. cDC1s are more potent in either priming naive CD8+ T cells through the engaging of TCR with the MHC class I (MHC-I) molecule and antigen peptide complex, or directing CD4+ T helper 1(Th1) cells differentiation through Interleukin-12 (IL-12) production. On the contrary, cDC2s are well-known for their capability to activate other CD4+ helper T cell subsets through the antigen peptides presented by MHC class II (MHC-II) molecules and other specific cytokines (Maldonado-López et al, 1999; Pulendran et al, 1999; Dudziak et al, 2007).

The plasmacytoid dendritic cells (pDCs) are partially derived from common DC precursors (CDPs), the same progenitors as cDCs, but with specific morphology

and carry out different functions. The pDCs are regarded as vital producer of Type I and III Interferons (IFNs), after recognizing intracellular DNA and RNA through pattern recognition receptors (PRRs). The pDCs also express MHC-II and costimulatory receptors CD40, CD80 and CD86. Therefore, pDCs are also capable of activate CD4+ T cells with low efficiency (Villadangos & Young,2008; Reizis et al, 2011; Swiecki & Colonna, 2015).

1.1.4. Dendritic cells and T cell activation

DCs stimulate T cells through the classical pathway depending on the three signals: recognizing specific MHC:pep complex by TCR, immunomodulatory molecules and receptors interaction, and secretion of cytokines (Figure 1.4).

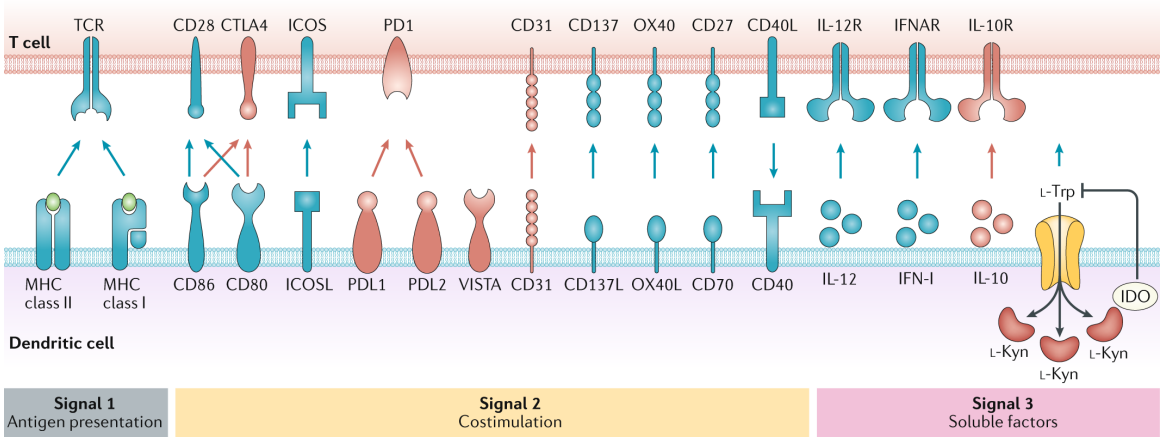


Figure 1.4 Induction of T cell-mediated immunity or tolerance by DCs. (Wculek et al, 2020)

As nicely reviewed in the figure above, depending on stimulatory or inhibitory functions provided by specific Signal 2 and Signal 3, DCs are capable to induce either T cell mediated immunity or tolerance (Wculek et al, 2020). However, the Signal 1 is of the most importance during an immune response. Lacking of Signal 1 would, to a large extent, impair the functionality of Signal 2 and 3. T

cells initiate distinct transcriptional programs responding to different concentrations of the MHC:peptide complex presented by DCs (Henrickson et al, 2008 and 2013).

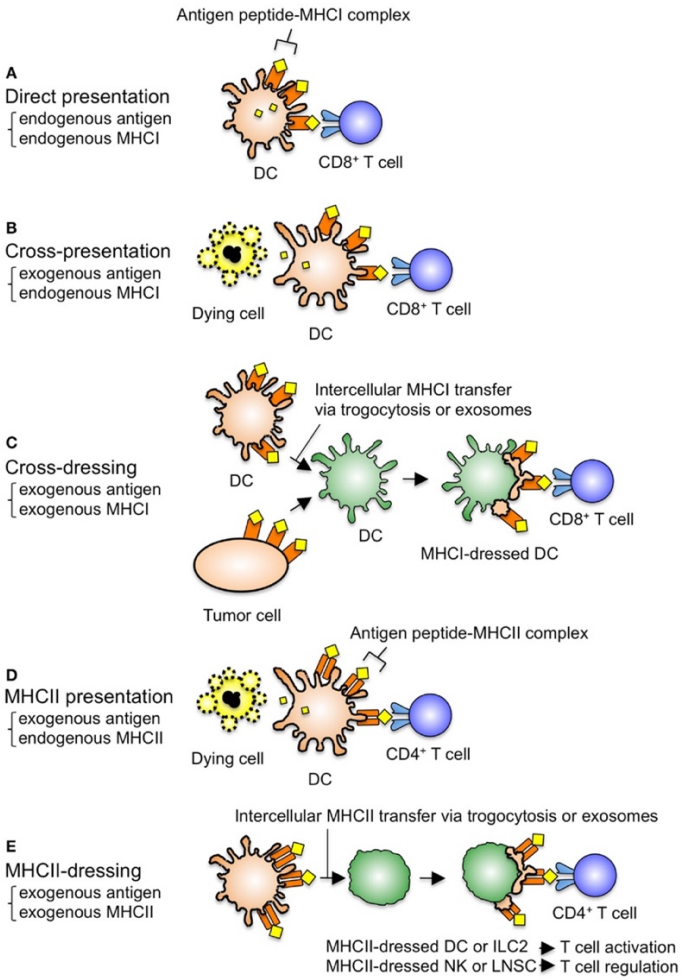


Figure 1.5 Canonical and non-canonical antigen presentation pathways. (Nakayama, 2015)

Several mechanism studies have been proposed to elucidate DC antigen presentation pathways during T cell activation (Figure 1.5). MHC-I molecules are involved in three different antigen presentation pathways, resulting in the priming of naive CD8+ T cells. Firstly, direct presentation defines the process of infected DCs presenting the antigen peptides derived from endogenous infectious source. This pathway is not very common, as direct infections to DCs

are required to initiate the direct presentation mechanism (Heath et al, 2004). Secondly, the most significant MHC-I molecule related antigen presentation pathway is termed as cross-presentation. Antigen cross-presentation occurs after engulfment and processing of exogenous cell-associated antigens, such as antigens derived from dying tumor cells and infected cells (den Haan et al, 2000; Iyoda et al, 2002; Schulz & Reis e Sousa, 2002). Thirdly, cross-dressing refers to the direct DC acquisition of MHC-I and peptide complex from neighboring DCs or tumor cells, through trogocytosis or exosomes (Harshyne et al, 2001 and 2003; Dolan et al, 2006; Smyth et al, 2008). On the other hand, MHC-II molecules function through MHC-II presentation and MHC-II-dressing, similar to MHC-I cross-presentation and cross-dressing, but to activate CD4+ T cells (Nakayama, 2015).

1.2. Cancer immunology and immunotherapy

1.2.1. Cancer progression and immune escape

In the mid-19th century, Rudolf Virchow discovered inflammatory infiltrates in solid tumors and suggested the possible correlation between chronic inflammation and tumorigenesis (Virchow, 1856, 1864 & 1881). Half a century later, Paul Ehrlich proposed the therapeutic possibility of harnessing immune system against cancer (Ehrlich, 1908). This proposal was first carried out by William Coley, who used Streptococcus pyogenes culture filtrates to treat patients with sarcoma (Coley, 1893). These first discoveries and trials suggest the potential of host immune system against cancer, and the cancer immunosurveillance theory was proposed (Figure 1.6).

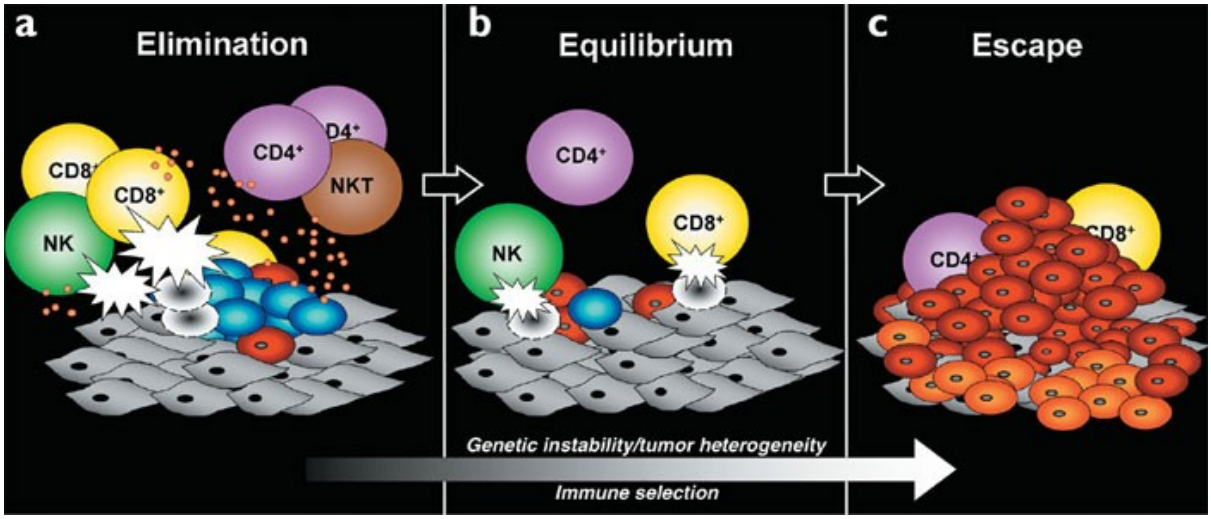


Figure 1.6. Cancer immunoediting encompasses three phases. (Dunn et al, 2002).

The cancer progression was further characterized into three phases: elimination, equilibrium and escape. In the elimination phase, tumor cells are recognized and attacked by immune cells. However, surviving tumor cells undergo cancer immunoediting to avoid being recognized by host immune system and thrive in the equilibrium phase. In the final escape phase, tumor cells accumulated enough mutations which overwhelm the host immune system, and showing uncontrolled growth (Shankaran et al, 2001; Dunn et al, 2002).

1.2.2. Tumor-associated antigen (TAA) presentation

The crosstalk between different immune cells, especially the APC and T cell interaction, is essential to initiate anti-tumor immune response (Figure 1.7, Garner & de Visser, 2020). In the tumor microenvironment (TME), both macrophages and DCs possess the capability to carry out TAA presentation function for the priming of TAA-specific T cell response. However, even infiltrated at higher abundance across various types of tumors, the tumor-associated macrophages (TAMs) are reported to be of low efficiency for APC

functions (Lewis & Pollard, 2006). On the contrary, cDCs, especially cDC1s, are reported to be the main mediator for cytotoxic T cell priming, followed by elimination of tumor cells through TAA-specific adaptive immune response (Broz et al, 2014; Roberts et al, 2016; Salmon et al, 2016; Spranger et al, 2017). In the TME with functional cDCs infiltration, tumor cell-associated antigen could be phagocytosed and processed by cDCs, before underwent MHC-I- or MHC-II-restricted antigen presentation. A subset of cDCs loading with different TAA-derived peptides and MHC molecules complex, migrate to draining lymph nodes (dLNs) and initiate naive T cells priming. The cDCs carrying self-antigen induce CD4+ T cell to polarize into regulatory T cell (Treg), while the cDCs carrying aberrant or mutated TAAs will prime antigen-specific cytotoxic T cells for anti-tumor immune response (Chen & Mellman, 2013; Garner & de Visser, 2020). Previous studies mainly focused on the cytotoxic CD8+ T cells and their priming by migratory subsets of cDC1s in anti-tumor immunity (Broz et al, 2014; Roberts et al, 2016; Salmon et al, 2016; Spranger et al, 2017). Despite that cDC2s are more heterogenous and less understood, researchers observed that TAA-derived peptide-bearing cDC2 subsets from TME are capable of migrating to dLNs and activating CD4+ conventional T cells. However, this process is suppressed by Tregs, and the suppression could be relieved by TME local Treg depletion (Binnewies et al, 2019). One recent study also demonstrated that cDC1s are also essential in early priming of CD4+ T cells through MHC-II and CD40 dependent manner (Ferris et al, 2020). Taken together, both cDC subsets carry out important TAA presentation functions, which is essential for specific tumor cell elimination by adaptive immune system.

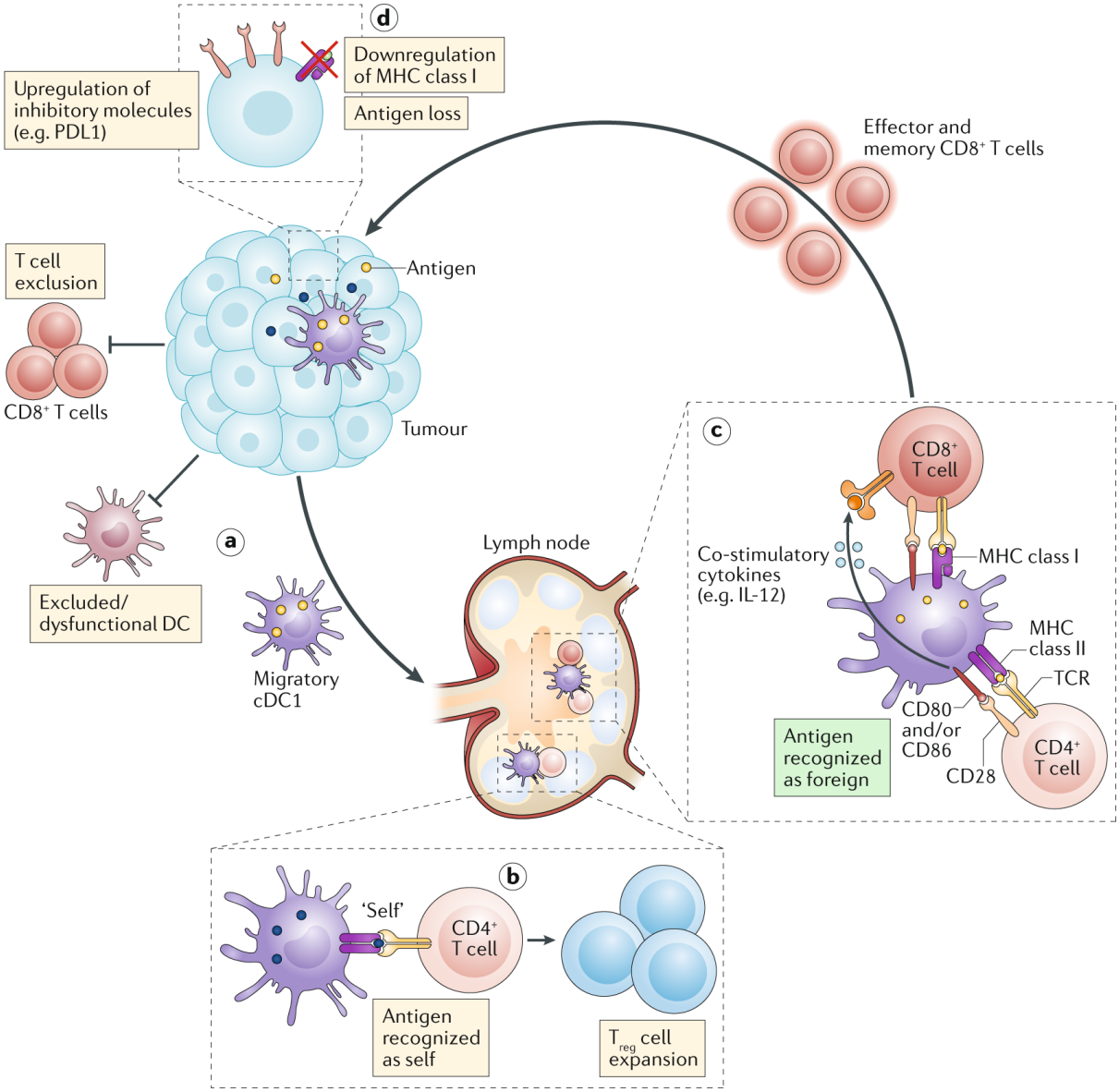


Figure 1.7 T cell priming to tumor antigens and subversion of this process.

(Garner & de Visser, 2020)

1.2.3. Innate immune sensing of tumor-derived danger-associated molecular patterns (td-DAMPs)

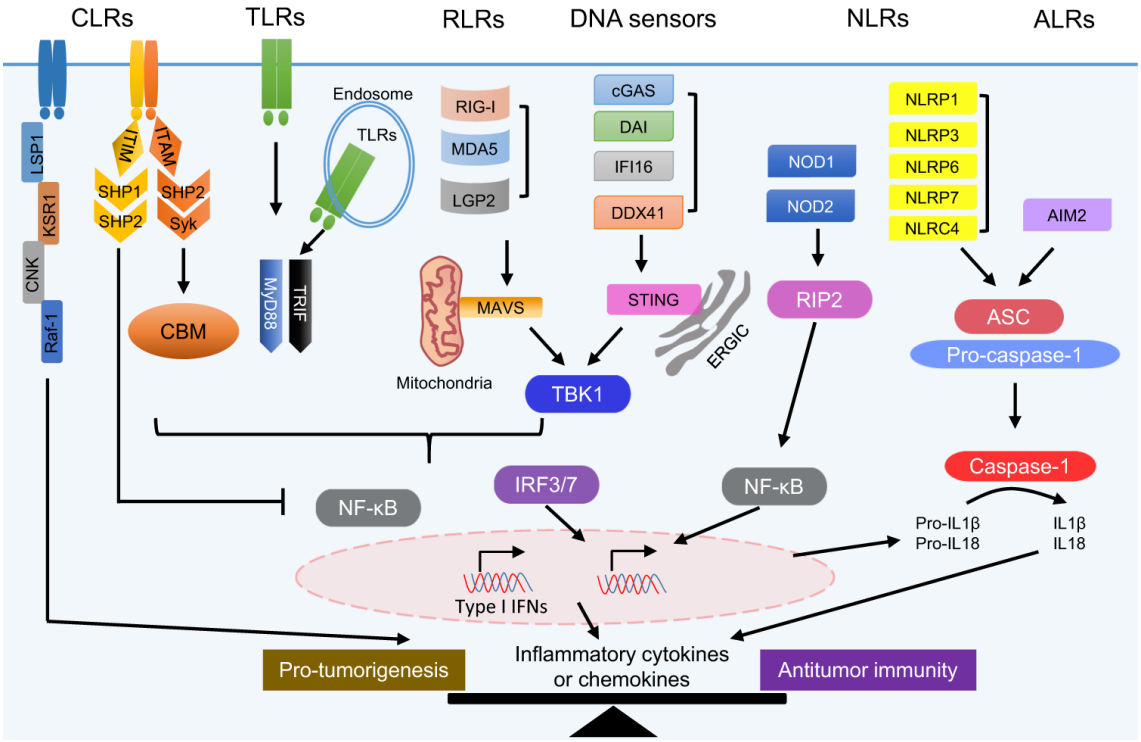


Figure 1.8 Innate immune sensing pathways in cancer. (Liu et al, 2020)

Prior to TAA presentation, innate immune sensing of td-DAMPs through pattern recognition receptors (PRRs) facilitates APC activation and maturation, resulting in optimized adaptive immune response (Figure 1.8). Innate immune sensing of DAMPs is activated through Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), AIM2-like receptors (ALRs), RNA sensor RIG-I-like receptors (RLRs) or DNA sensor cGAS-STING-axis related pathways (Cui et al, 2014; Liu et al, 2020). During tumorigenesis and conventional cancer therapies (including surgery, radiotherapy, chemotherapy and targeted therapy), stressed tumor cells undergo necrosis or apoptosis, and release different kinds of td-DAMPs (Rakoff-

Nahoum and Medzhitov, 2009). Innate immune sensing of td-DAMPs results in producution of inflammatory cytokines and chemokines. However, for some of these proteins, their functions are still controversial between tumorigenesis and anti-tumor immunity (Liu et al, 2020). Among them, Type I Interferon (IFN-I) secretion following TLR-3/4/7/8/9, NOD-1/2 or DNA/RNA sensors related pathways regulates innate and adaptive immune responses (Hervas-Stubbs et al, 2011; Rathinam and Fitzgerald, 2011). IFN-I stimulation promotes DC maturation and survival after encountering and engulfing antigens, which further enhances T cell priming (Lorenzi et al, 2011).

1.2.4. The suppressive tumor microenvironment (TME) impairs anti-tumor immunity

TME is a complicated system which contains different types of immune cells as well as stroma cells. Therefore, the immune regulation network in the TME is influenced by multiple factors. Suppressive compartments of the immune system, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), have been reported to negatively regulate anti-tumor immunity. Hypoxia status, aberrant cell metabolism and nutrients levels in the TME also correlate with the efficiency of immune response. Suppressive TME impairs anti-tumor immune response through excluding effector immune cells or promoting effector cells exhaustion (Binnewies et al, 2018).

Evidences suggest that T cell exclusion from tumor core is correlated with worse prognosis and survival in across different types of cancers (Naito et al, 1998; Zhang et al, 2003; Sato et al, 2005; Galon et al, 2006; Feig et al, 2013). Different theories have been proposed to elucidate effector T cells exclusion. Modifications and dislocations of key chemokines can misguide effector T cell

recruitment to retain in extracellular matrix (ECM) instead of TME (Molon et al, 2011; Feig et al, 2013). Induction and recruitment of suppressive immune cells (including TAMs, MDSCs and Tregs) or upregulation of T cell inhibitory receptors also efficiently decrease effector T cell infiltration in TME (Joyce & Fearon, 2015).

Apart from T cell exclusion, the immunosuppressive TME promotes effector T cell exhaustion. Exhausted T cells are characterized by impaired effector functions and diminished proliferative capacity, which are observed in chronic viral infections and cancers (Zippelius et al, 2004; Wherry et al, 2007; Baitsch et al, 2011). Exhausted T cells from TME upregulate co-inhibitory receptors including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene 3 protein (LAG-3), and T cell immunoglobulin domain and mucin domain protein 3 (TIM-3), which are also hallmarks of severely dysfunctional status (Pardoll, 2012; Crespo et al, 2013; Thommen et al, 2018). Metabolic defection and reprogramming of exhausted T cells have been observed, indicated by reduced mitochondrial respiration and glycolysis (Bengsch et al, 2016; Schurich et al, 2016; Sugiura & Rathmell, 2018).

1.2.5. Cancer Immunotherapy

Unleashing the anti-tumor potential of the immune system is the purpose of cancer immunotherapy, as many suppressive mechanisms and pathways are involved in the defective immune response during cancer progression. Most immune enhancement strategies amplify immune activation systemically, which leads to abnormal levels of the immune response. In immunocompetent patients, it remains higher likelihood to develop immune-related adverse events (irAEs) rather than durable objective responses. On the contrary, therapies, that are designed to restore defections and normalize immune response levels

suppressed by cancers, are achieving better outcomes in patient cohorts (Sanmamed & Chen, 2018). With numerous clinical trial ongoing in distinct directions, currently, Food and Drug Administration (FDA) approved cancer immune-based monotherapies include cytokine treatment, antibody-based checkpoint blockade and cell-based adoptive transfer. However, cytokine treatment with IL-2 in metastatic melanoma has proven to be highly toxic for decades (Rosenberg et al, 1988). Therefore, we will only discuss the other two treatment options, which are investigated intensively in recent years.

Immune checkpoint inhibitor (ICI) therapies

ICI therapies, especially the PD-1/PD-L1 axis and CTLA-4 targeting, have shown significant advantages in patients with different types of cancers in recent years (Sanmamed & Chen, 2018; Waldman et al, 2020). In non-regulatory naive T cells, both PD-1 and CTLA-4 are expressed only following the activation of T cells (Linsley et al, 1992; Freeman et al, 2000; Latchman et al, 2001). CTLA-4 binds to costimulatory molecules CD80/CD86 with higher avidity than CD28, transducing inhibitory signals to the activated T cells (Linsley et al, 1994). Similarly, the PD-1/PD-L1 axis inhibit effector functions of T cells after ligation based on the intracellular signaling of PD-1 (Freeman et al, 2000; Carter et al, 2002; Hui et al, 2017). Despite that only minimum progress has been reported of CTLA-4 blockade drug Ipilimumab on lung cancers, renal cell carcinoma and prostate cancer, it showed durable efficacy and survival benefit of patients with advanced melanoma (Yang et al, 2007; Lynch et al, 2012; Reck et al; 2013; Kwon et al, 2014; Maio et al, 2015; Schadendorf et al, 2015). New development of a humanized PD-1 blockade antibody Pembrolizumab showed even better response and less toxicity than Ipilimumab in advanced melanoma patients in phase III trial (Robert et al, 2015; Schachter et al, 2017). Furthermore, fully human PD-1 antibody Nivolumab showed nice response rates of patient cohorts with other cancers including

Hodgkin's lymphoma, renal cell carcinoma, head and neck squamous-cell carcinoma, colorectal cancer, hepatocellular carcinoma and urothelial carcinoma (Ansell et al, 2015; Motzer et al, 2015; Ferris et al, 2016; Overman et al, 2017; El-Khoueiry et al, 2017; Sharma et al, 2017).

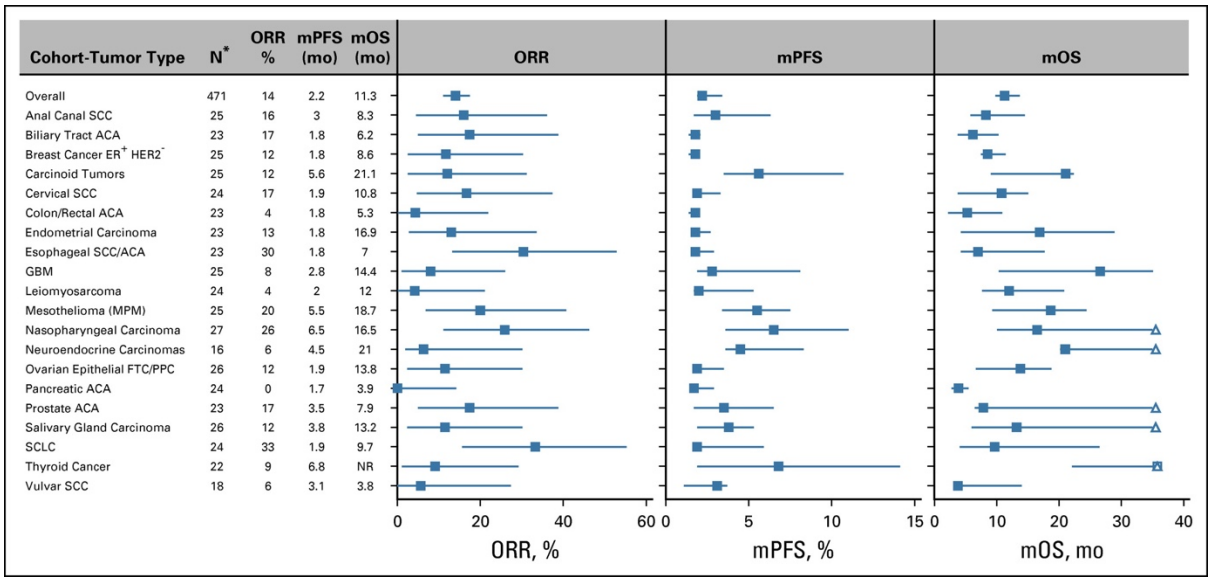


Figure 1.9 Objective response rate (ORR), progression-free survival (PFS), and overall survival (OS) for the total study cohorts of Pembrolizumab. (Ott et al, 2019)

Although ICI has emerged as the most promising therapeutic option for cancer, the cohorts that benefit from the treatment are still low. A large percentage of patients does not respond to the therapy, while some other patients failed to achieve sustained response, as observed in Pembrolizumab treatment for over 20 PD-L1+ solid tumor cohorts (Figure 1.9, Ott et al, 2019). These observations were further defined as primary or acquired resistance to ICI therapies. TME PD-L1 expression, tumor cell intrinsic key signaling pathways deficiency, mutational burden and microbiome all contribute to primary resistance to ICI. On the contrary, mechanisms for acquired resistance, which often leads to tumor relapse, are still not well understood yet (Bagchi et al, 2021). In the

meantime, ICI therapies often results in irAEs, due to the abnormal levels of immune response after disrupting negative regulation pathways (Bajwa et al, 2019; Martins et al, 2019). All of these drawbacks during ICI monotherapies suggesting the desire of further optimizations for achieving durable anti-tumor immunity with less side effects and toxicity. Countless ongoing therapeutic trials aim at overcoming the aforementioned bottlenecks, including combination therapies (most commonly, anti-PD1 and anti-CTLA4) and defining new checkpoints.

Adoptive cell transfer (ACT) therapies

ACT therapies aim at harnessing autologous or allogenic effector lymphocytes to treat cancer patients. Early study in leukemia patients demonstrated the efficacy of ACT of haematopoietic stem cells (Weiden et al, 1979). Allogeneic hematopoietic stem cell transplantation (HSCT) has evolved as the standard treatment for various leukemia patients (Rubnitz et al, 2010; Cornelissen et al, 2012; Hunger et al, 2012; Pession et al, 2013; Fielding et al, 2014; Rasche et al, 2018). Dr. Rosenberg was the pioneer to apply similar strategy in patients with advanced melanoma using *in vitro* expanded autologous tumor-infiltrating lymphocytes (TILs). In the first trials, durable response remained low. Optimized TIL transfer therapy following lymphodepletion demonstrated better outcome (Rosenberg et al, 1988, 1994 and 2011). Further applications of ACT T cell therapies were performed on patients with gynecologic malignancies, breast cancer, glioblastoma and ovarian cancer (Mayor et al, 2018; Fuentes-Antrás et al, 2020; Wang et al, 2020; Sarivalasis et al, 2021). Nevertheless, natural killer (NK) cell-based ACT therapies also showed its potential in the melanoma treatment (van Vliet et al, 2021).

However, successful TIL transfer therapies rely on presence of tumor-specific effector T cells. The limitations of TIL therapies further led to the development of genetically engineered chimeric antigen receptor (CAR) T cell therapies.

CARs usually contain extracellular TAA-targeting domain and intracellular signaling domain, allowing engineered cells to specifically recognize tumor cells in MHC-independent manners (Hong et al, 2020). CD19 targeting CAR-T cell therapies have been proved with great success in treating chronic lymphoid leukemia (CLL), acute lymphoblastic leukemia (ALL) and large B cell lymphoma (Porter et al, 2011; Brentjens et al, 2013; Maude et al, 2014; Neelapu et al, 2017). Application of CAR T cell therapy in solid tumors requires further investigation, as immunosuppressive TME, identification of specific target and tumor immunoediting negatively modulate CAR T cell effector function (Hong et al, 2020; Larson & Maus, 2021). Therefore, some researchers tried to engineer CAR T cells with helping effects, for example with interleukin-12 (IL-12) secretion, to overcome suppression (Waldman et al, 2020; Larson & Maus, 2021). Except for the difficulties of CAR T in solid tumors treatment, the CAR T therapy induced toxicity also deserves more attention. Different clinical trials reported CAR T therapies toxicity in multiple organs, which level differs from patient to patient. Cytokine release syndrome (CRS) and neurotoxicity are two hallmark side effects of CAR T therapies, due to potent and rapid activation and expansion of the CAR T cells. CRS usually happens in acute phase after ACT, which severity level ranging from fever to lethal symptoms. In cases of neurotoxicity, symptoms such as memory loss, confusion and hallucination were reported to be independent of CRS, suggesting distinct mechanisms of the two hallmarks (Brudno et al, 2016; Hay et al, 2017; Neelapu et al, 2018). Tumor resistance and relapse after CAR T therapies were also reported. Target antigen loss through different mechanisms is one of the reasons for tumor relapse (Orlando et al, 2018; Ruella et al, 2018; Hamieh et al, 2019). One other reported mechanism contributing to the resistance is CAR T exhaustion in cancer cells lacking death receptors, which caused prolonged CAR stimulation (Fraietta et al, 2018; Singh et al, 2020). To summarize, except for the high expenses and

limited accesses to CAR T cancer therapies, this therapeutic option still needs further investigation and, likely, decades of optimization.

1.3. Glycosylation and cancer

1.3.1. Universal glycosylation of living organisms

Every cell from unicellular and multicellular organisms are covered by a dense and complex layer of glycans after billions of years of evolution. Tissue mircoenvironment of multicellular organisms are characterized to be rich in secreted glycans and glycoconjugates. Similar observations were reported in the areas accumulated with unicellular organisms (Varki et al, 2015).

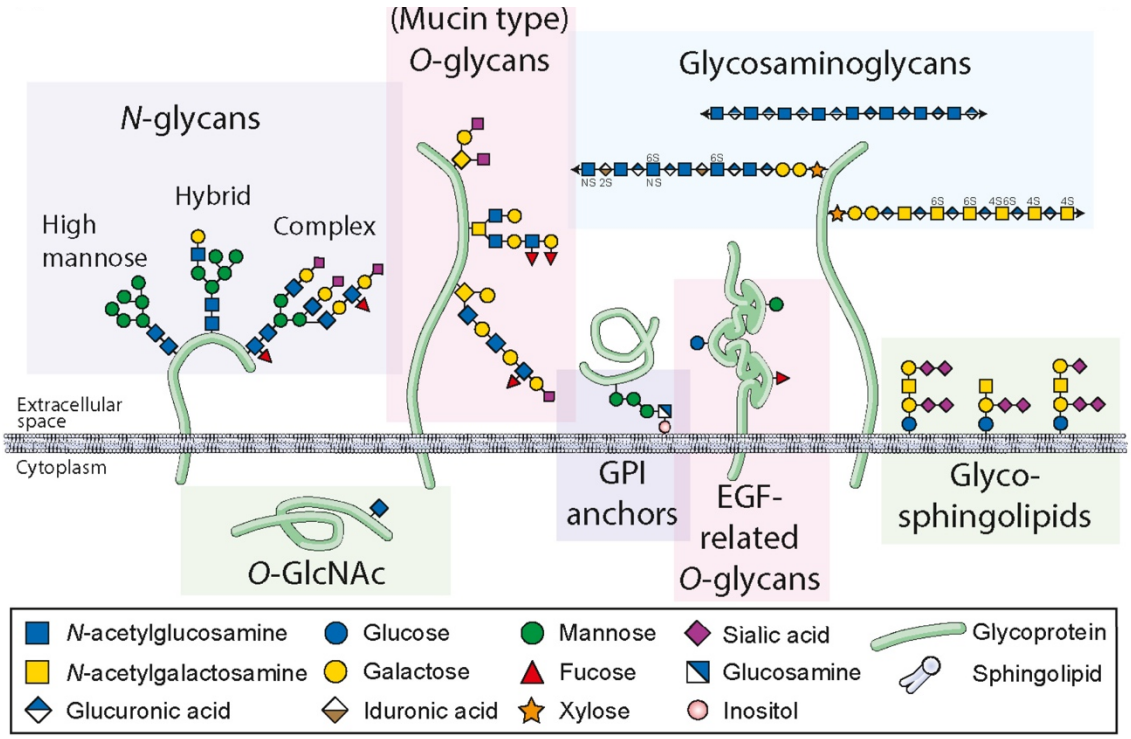


Figure 1.10 Common classes of glycoconjugates in human cells. (Mereiter et al, 2019)

Glycoconjugates, referring to the macromolecules which contain covalently linked glycans, include various classes, mainly glycosphingolipids, proteoglycans and glycoproteins. As shown in Figure 1.10, using human system as an example, N-linked and O-linked glycans are post-translational modifications commonly attached to specific amino acid residuals of extracellular proteins. N- glycans could be further characterized into categories with high mannose, hybrid or complex types. Mucin-type O-glycans are mainly secreted or cell surface glycoproteins. Other forms of glycosylation include GPI-anchored proteins, EGF-related O-glycans, glycosaminoglycans and glycosphingolipids. For intracellular proteins, O-linked N-acetylglucosamine (O-GlcNAcylation) is more commonly found (Mereiter et al, 2019). Glycosylation finetunes biological activities from molecular level to systemic level, empowering it to be an essential regulation pathway in physiological or pathological conditions (Pinho & Reis, 2015).

1.3.2. Alternations of glycosylation in cancer

Early discoveries of the correlation between malignant transformation and abnormal glycosylation were reported more than half a century ago (Ladenson et al, 1949; Hakomori & Murakami, 1968). Following-up research identified several frequently occurring aberrant glycosylation in various cancers, including terminal sialylation, truncated O-glycans, branched N-glycans, and diverse fucosylation (Figure 1.11, Pinho & Reis, 2015).
Changes in expression level of glycosyltransferases can lead to high α2,6- and α2,3-linked sialylation profile, which is positively related to cancer (Kim & Varki, 1997). Some sialylated antigens, such as SLe^a, SLe^x and Sia6LacNAc, are reported to be predictive for poor diagnose of cancers (Amado et al, 1998; Baldus et al, 1998; Lise et al, 2000). Expression and activities of certain

enzymes, including C2GnT and ST3Gal-I, are shown to correlate with truncated O-glycosylation (Dalziel et al, 2001; Gill et al, 2013). STn is one of the typical TAAs, which synthesis experienced O-glycan truncation and terminal sialylation. STn correlates with poor prognosis, and further emerged to be a target of cancer therapy (Sandmaier et al, 1999; Julien et al, 2009). Upregulation of branched N-glycans is owing to increased GnT-V, which overexpression is activated by cancer-associated RAS-RAF-MAPK signaling pathway (Dennis et al, 1987). Fucosyltransferases are responsible for glycan fucosylation, including terminal fucosylation and core fucosylation (Carvalho et al, 2010). Terminal fucosylation has proved to influence breast cancer, T cell leukemia and colorectal cancer, and core fucosylation is correlated with liver, breast and lung cancers (Sato et al, 1993; Matsuura et al, 1998; Hiraiwa et al, 2003; Potapenko et al, 2010; Liu et al, 2011; Trinchera et al, 2011).

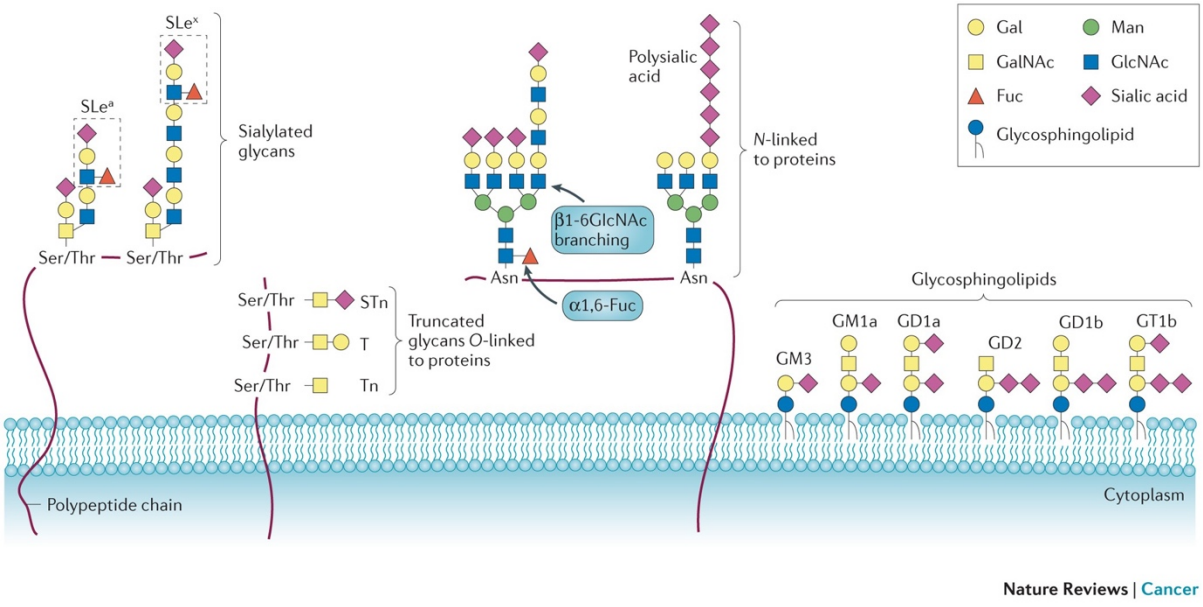


Figure 1.11 Important tumor-associated glycans. (Pinho & Reis, 2015)

Multiple malignant biological processes of cancer are influenced by glycosylation alternation, such as angiogenesis, invasion, metastasis and immunosuppression. Aberrant glycosylation is associated with changes of

interactions among cell to cell or cell to ECM. Basic cellular biological processes such as signaling and proliferative capacity are also under impacts of glycosylation alternation (Mereiter et al, 2019). In addition, altered glycosylation of glycoproteins (including CD44, CD133 and CD24) and signaling pathways (such as Notch, Hedgehog, Wnt/β-catenin and AKT) has also been reported in cancer stem cells. These changes may facilitate important biological functions of the cancer stem cells, including, but not limited to, self-renewal, metastasis, immune evasion and therapy resistance (Khan & Cabral, 2021).

1.3.3. Sialylation and immune regulation

Aberrant sialylation not only has influences on biology and functions of cancer cells / cancer stem cells, but also modulates immune response. Sialoglycan and sialic acid-binding immunoglobulin-like lectins (Siglecs) axis modulates immune cell responses from many aspects in healthy and pathological status. Terminal sialylation of glycoproteins is frequently reported in cancer, which can be recognized by the large family of immure regulatory Siglec receptors. As sialylation is observed in all cell types of mammals meanwhile not naturally found on majority of pathogens, this feature is mostly recognized by immune system as a 'self-associated molecular pattern (SAMP)', without triggering immune activation and facilitating immune evasion (Crocker et al, 2007; Varki, 2011; Macauley et al, 2014; Bärenwaldt & Läubli, 2019; Duan & Paulson, 2020).

Siglec receptors contain an amino-terminal V-set immunoglobulin domain that binds to sialic acid (SAs), and variable numbers of C2-set immunoglobulin domains (Figure 1.12). With deeper understanding of their structure similarity and conservation, Siglec-1 (CD169, sialoadhesin), Siglec-2 (CD22), Siglec-4

(myelin-associated glycoprotein) and Siglec-15 are reported to have clear orthologous in mammals, thus known as conserved Siglecs. Apart from those, the rest Siglecs are highly variable among species, termed as CD33-related Siglecs, Therefore, in different species, CD33-related Siglecs have different naming system. In humans, CD33-related Siglecs include Siglec-3/5/6/7/8/9/10/11/14/16, and in mice, consist of Siglec-3/E/F/G/H. Researches focused on their structures also suggested functional differences among these receptors. Based on the intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) or immunoreceptor tyrosine-based activating motif (ITAM), the Siglec receptors transduce different signals to inhibit or activate downstream pathways, except Siglec-1. Therefore, most of these receptors can also be grouped as inhibitory Siglecs and activating Siglecs (Crocker et al, 2007; Bärenwaldt & Läubli, 2019).

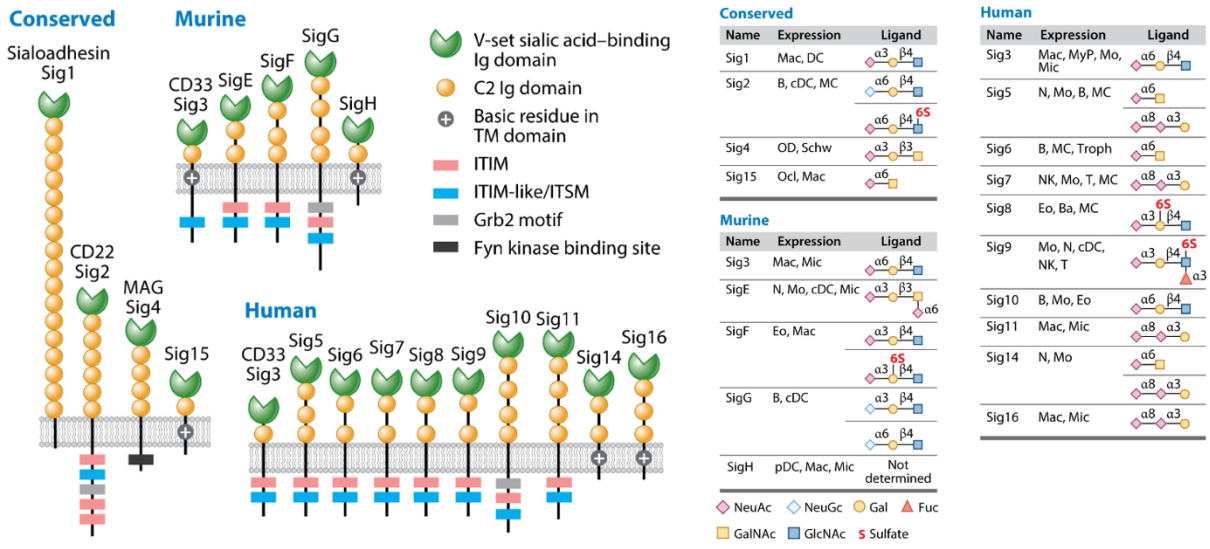


Figure 1.12 Human and murine Siglecs. (Adapted from Duan & Paulson, 2020)

Expression patterns of Siglecs are mainly observed in hematopoietic and immune system, except Siglec-4 and Siglec-6 (Crocker et al, 2007). Most

Siglec receptors are preferentially expressed on specific immune cell types, such as Siglec-1 on macrophages, Siglec-2 on B cells and Siglec-8 on eosinophils. Expression patterns of most Siglecs are complex and partially overlapping in distinct immune cell types. Changes of their expression patterns are reported to corelate with various diseases (Macauley et al, 2014; Duan & Paulson, 2020). During infections, some pathogens adapted sialylation as a surviving strategy to avoid immune attack, such as group B Streptococcus (Ali et al, 2014; Chang et al, 2014). However, for some other pathogens, elimination of sialylation through sialidases increases pathogenicity, such as streptococcus pneumoniae (Chen et al, 2011; Chang et al, 2012). Several other immune-related diseases are also reported under regulation of specific Siglecs, such as autoimmune disease, inflammatory lung diseases and neurodegeneration (nicely reviewed by Macauley, Crocker and Paulson, 2014).

1.3.4. Sialoglycan - Siglec axis modulates cancer immunosurveillance

Due to restricted expression patterns, Siglec-2 and Siglec-3 have been identified as therapeutic target for several leukemias and lymphomas for long time. Most recently, Siglec-2 CAR-T cells were applied in some clinical trials for treatment of pre-B cell acute lymphoblastic leukemia (Press et al, 1989; Feldman, 2005; Fry et al, 2018). Moreover, accumulating evidences showed that the sialoglycan - Siglec axis is involved in the regulation of host tumor immunosurveillance. In particular, commonly reported hypersialylation of cancers benefits their immune escape through ‘SAMP’ recognition by inhibitory Siglecs, resulting in poor prognosis (Fuster & Esko, 2005; Macauley et al, 2014; Büll et al, 2014). With deeper understandings of the regulation pathways, some previously identified cancer-associated markers were proved to be ligands for some inhibitory Siglecs, such MUC1, MUC16 and LGAL3BP, further highlighted the

critical roles of the sialoglycan - Siglec axis during cancer progression (Brockhausen et al, 1995; Belisle et al, 2010; Läubli et al, 2014a).
Several research works in the past decade have revealed regulation mechanisms of this axis in anti-tumor immunity from different aspects. In ovarian cancer patients, high levels of MUC16 expression increased MUC16-Siglec9 interactions on NK cells, B cells and monocytes. This ligation suppresses Siglec-9-expressing NK cell cytotoxic functions (Belisle et al, 2010). Further analysis of tumor-associated NK cells revealed broad expression patterns of ligands for both Siglec-7 and Siglec-9 by various human tumor cells, including several cell lines, acute myeloid leukemia, chronic lymphocytic leukemia and melanoma. Engagement of both inhibitory Siglecs on NK cells, through ligand interaction or agonistic antibodies, lead to reduced NK cell cytotoxicity functions. Desialylation of the target tumor cells restored the cytolytic capability of NK cells (Jandus et al, 2014). Interaction between cancer cell sialylation and NK cell Siglec-7 was investigated again through distinct approach, termed glycocalyx engineering. Hypersialylation status of tumor cells efficiently avoid immune attack from NK cells through a Siglec-7-dependent manner (Hudak et al, 2014). Unlike some myeloid cells, human T cells in naive state do not show significant Siglec expression, suggesting Siglecs are not crucial mediators for T cell biology (Duan and Paulson, 2020). In B16 mouse tumor model, hypersialylated tumor cells break the effector/regulatory T cell (Teff/Treg) balance to favor Treg polarization, consequently escaping the immunological tumor control. In comparison, engineered SA-low tumor cells enhanced ratio of effector T cells and their response, along with increased NK cell activation (Perdicchio et al, 2016a). Further research work demonstrated upregulation of several inhibitory Siglecs on tumor-infiltrating T cells from non-small cell lung cancer, ovarian cancer and colorectal cancer patients. Especially, a specific Siglec-9-expressing CD8+ T cell subset was identified, with co-expression of PD-1 and other inhibitory molecules, representing

exhaustion status. Tumor cells lacking key enzyme GNE and desialylated tumor cells, both with reduced sialylation, in turn enhanced CD8+ T cell activation. High levels of Siglec-E on mouse tumor-infiltrating T cells were also observed, with similar phenotype as human Siglec-9 positive CD8+ T cells, and mediate tumor immune escape. Knock-in Siglec-9 on mouse T cells specifically result in fastened tumor growth (Stanczak et al, 2018). Siglec-9 positive CD8+ T cell subset was also found in melanoma patient samples. Desialylation treatment of target cells rescued T cell mediated killing, which was restrained by the sialoglycan - Siglec axis (Haas et al, 2019).

In terms of the myeloid cell compartment, cancer cells with α2,3- or α2,6-linked sialylation ligation with Siglec-9 on human neutrophils *in vitro*, resulting in inhibited neutrophil functions. Siglec-9 blocking antibody, on the contrary, can rescue the suppression on neutrophils. *In vivo* tumor models confirmed that mouse neutrophils transgenic with human Siglec-9 expression showed reduced anti-tumor response. Similarly, neutrophils from mouse with deficiency of Siglec-E, the human Siglec-9 functional paralog, showed enhanced immune response against tumor (Läubli et al, 2014b). Glycosylation shapes both the phenotype and effector functions of TAMs through reprogramming metabolism in the TME (Mantuano et al, 2019). Cancer cells with sialylated MUC1 (MUC1-ST) expression can engage with Siglec-9-expressing macrophages, and guide their polarization to tumor-promoting TAM phenotype, with upregulation of checkpoint molecule PD-L1 (Beatson et al, 2016). Globally overexpression of CD24 on tumor cells is also reported to regulate the function of TAMs. By interact with Siglec-10 from TAMs, CD24 functions as a ‘don’t eat me’ signal to avoid the phagocytosis of macrophages (Barkal et al, 2019). Siglec-15, expressed by cancer cells, cancer stromal cells and cancer-infiltrated myeloid cells in human, is also reported to negatively regulate anti-tumor immunity. Siglec-15 deficient mice were highly resistant to implanted syngeneic tumor cells. Macrophage conditional Siglec-15 knockout results in better antigen-

specific T cell response (Wang et al, 2019). As for the other important myeloid cell type, the DCs, our understanding of Siglecs in the regulations of tumor-associated DCs is still limited. Previous research has pointed out that Siglec-G inhibits the formation of MHC and antigen peptide complex of DC cross-presentation function. Bone marrow-derived DC (BMDC) vaccination of DCs from Siglec-G knockout mice presented delayed tumor growth than wildtype mice BMDCs (Ding et al, 2016). Ovalbumin decorated by sialic acid-containing glycans favored Treg polarization during naive CD4+ T cells commitment in a DC-dependent manner, suggesting possible regulation pathways of DC function through sialoglycan - Siglec axis (Perdicchio et al, 2016b). Another group also reported that desialyltion of DCs themselves increase their maturation status, empowering them to better activate TAA-specific T cells. This discovery suggests a possibly cis-regulation pathway of sialylation and DC functions (Silva et al, 2016). However, systemic characterization and deeper understanding of the Siglecs on DCs in the tumor setting is still required.

2. Material and methods

2.1. Key resources table

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC-eFluor780 anti-human CD3 (SK7)	eBioscience	Cat# 47-0036-42
APC-eFluor780 anti-human CD19 (SJ25C1)	eBioscience	Cat# 47-0198-42
APC-eFluor780 anti-human CD56 (CMSSB)	eBioscience	Cat# 47-0567-42
BV711 anti-human CD45 (H130)	Biolegend	Cat# 304050
PE-cy7 anti-human CD11c (3.9)	Biolegend	Cat# 301608
FITC anti-human HLA-DR (L243)	Biolegend	Cat# 307604
PE-CF594 anti-human CD123 (7G3)	BD	Cat# 562391
BV421 anti-human CD1c (L161)	Biolegend	Cat# 331526
Percp-cy5.5 anti-human CD141 (M80)	Biolegend	Cat# 344112
PE anti-human Siglec-7 (6-434)	Biolegend	Cat# 339204
APC anti-human Siglec-8 (7C9)	Biolegend	Cat# 347106
APC anti-human Siglec-9 (191240)	R&D Systems	Cat# FAB1139A-100
PE anti-human Siglec-10 (5G6)	Biolegend	Cat# 347604
BV421 anti-mouse F4/80 (BM8)	Biolegend	Cat# 123132
BV421 anti-mouse GR-1 (RB6-8C5)	Biolegend	Cat# 108433
Percp-cy5.5 anti-mouse CD45 (30-F11)	eBioscience	Cat# 45-0451-82
BV711 anti-mouse CD11c (N418)	Biolegend	Cat# 117349
BV605 anti-mouse MHC-II (M5/114.15.2)	Biolegend	Cat# 107639
PE-cy7 anti-mouse MHC-II (M5/114.15.2)	eBioscience	Cat# 25-5321-80
APC anti-mouse MHC-II (M5/114.15.2)	Biolegend	Cat# 107614
BV510 anti-mouse CD11b (M1/70)	Biolegend	Cat# 101263
AF488 anti-mouse CD8a (53-6.7)	BD Biosciences	Cat# 557668
APC anti-mouse CD8 (53-6.7)	eBioscience	Cat# 17-0081-83
Continued		

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
AF488 anti-mouse CD103 (2E7)	Biolegend	Cat# 121420
PE-cy7 anti-mouse Siglec-E (M1304A01)	Biolegend	Cat# 677108
PE anti-mouse Siglec-F (E50-2440)	BD Biosciences	Cat# 552126
APC anti-mouse Siglec-G (SH2.1)	eBioscience	Cat# 17-5833-82
PE anti-mouse MHC-I (AF6-88.5)	Biolegend	Cat# 116508
APC anti-mouse CD40 (3/23)	BD Biosciences	Cat# 558695
BV605 anti-mouse CD80 (16-10A1)	Biolegend	Cat# 104729
PE anti-mouse CD80 (16-10A1)	Biolegend	Cat# 104708
PE anti-mouse CD83 (Michel-19)	Biolegend	Cat# 121507
APC anti-mouse CD86 (GL-1)	Biolegend	Cat# 105012
PE anti-mouse CD86 (GL-1)	Biolegend	Cat# 105008
PE-cy7 anti-mouse CD3 (145-2C11)	BD Biosciences	Cat# 552774
BV605 anti-mouse CD3 (145-2C11)	Biolegend	Cat# 100351
APC anti-mouse CD4 (GK1.5)	eBioscience	Cat# 17-0041-83
BV711 anti-mouse CD4 (GK1.5)	BD Horizon	Cat# 563050
FITC anti-mouse CD25 (PC61)	Biolegend	Cat# 102006
Percp-cy5.5 anti-mouse CD44 (IM7)	Biolegend	Cat# 103032
PE anti-mouse CD69 (H1.2F3)	Biolegend	Cat# 104522
Percp-cy5.5 anti-mouse CD69 (H1.2F3)	eBioscience	Cat# 12-0691-83
Chemicals Dyes		
CellTrace CFSE Cell Proliferation Kit	Invitrogen	Cat# C34554
CellTrace Violet Cell Proliferation Kit	Invitrogen	Cat# C34557
Zombie UV Fixable Viability Kit	Biolegend	Cat# 423108
Zombie NIR Fixable Viability Kit	Biolegend	Cat# 423106
Fixable Viability Dye eFluor 450	eBioscience	Cat# 65-0863-14
Proteins		
Recombinant murine M-CSF	Peprotech	Cat# 315-02
Recombinant murine GM-CSF	Peprotech	Cat# 315-03
EndoFit Ovalbumin	Invivogen	Cat# Vac-pova
Continued		

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
EasySep Mouse CD4+ T Cell Isolation Kit	Stemcell Technologies	Cat# 19852
EasySep Mouse CD8+ T Cell Isolation Kit	Stemcell Technologies	Cat# 19853
EasySep Mouse CD11c Positive Selection Kit II	Stemcell Technologies	Cat# 18780
Mouse Cytokine Array / Chemokine Array 44-plex (MD44)	Eve Technologies	

2.2. Experimental model and subject details

Patient samples

The local ethics committee in Basel, Switzerland, approved the sample collection and the use of the corresponding clinical data (Ethikkommission Nordwestschweiz, EKNZ, Basel Stadt, Switzerland). Informed consent was obtained from all patients prior to sample collection. Tumor samples were collected locally at the thoracic surgery of the University Hospital Basel, digested, processed and single cell suspensions were frozen in liquid nitrogen.

Animal strains

Siglec-E^{flox} mice were generated in collaboration with Biocytogen Company, and crossed with CD11c-cre mice kindly provided by Prof. Daniela Finke. Siglec-E systemic knockout (SigEKO) mice was obtained from Prof. Ajit Varki group. Siglec-9 transgenic mice were previously reported (Läubli et al. 2014). To generate higher frequency of Siglec-9-expressing mouse BMDCs, Siglec-9^{flox} mice were crossed with XCR1-cre mice as described previously (Ohta et al, 2016). All of these mouse strains were bred and backcrossed in-house to our

local C57BL/6 strain for over 9 generations. OT-I transgenic mice were kindly provided by Prof. Christoph Hess group, and OT-II transgenic mice were ordered from University of Zürich. All mouse experiments were approved by the local ethics committee (Basel Stadt, Switzerland) and performed in accordance with the Swiss federal regulations.

Cell lines

Mouse colorectal cancer cell line MC38 was kindly provided by collaborator from Hannover. tdTomato-expressing MC38 cell line was generated by our lab through lentiviral transduction, with the Luc2-tdTomato plasmid kindly provided by Prof. Gregor Hutter. OVA-expressing MC38 (MC38-OVA) cell line was kindly provided by Prof. Mark Smyth. B16D5 and EMT6 were kept by group of Prof. Alfred Zippelius. Chinese Hamster Ovary cell line with FMS-like tyrosine kinase 3 ligand secretion capability (CHO-Flt3L) was kindly provided by Dr. Panagiotis Tsapogas. Mouse immature dendritic cell line Sp37A3 was kindly provided by Merck KGaA.

2.3. Method details

Cell line culture.

Mouse cancer cell lines were maintained in Dulbecco's Modified Eagle Medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Germany), 1 mM sodium pyruvate (Gibco, USA), 1x MEM non-essential amino acid solution (Sigma, USA), and 100 µg/mL streptomycin & 100 U/mL penicillin (Gibco, USA).

CHO-Flt3L cells were maintained in Iscove′s Modified Dulbecco′s Medium (Sigma, USA) supplemented with 5% heat-inactivated fetal bovine serum (PAA Laboratories, Germany).

Sp37A3 mouse dendritic cell line and relative genetically modified lines were maintained in Iscove′s Modified Dulbecco′s Medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Germany), 1 mM sodium pyruvate (Gibco, USA), 1x MEM non-essential amino acid solution (Sigma, USA), 100 µg/mL streptomycin & 100 U/mL penicillin (Gibco, USA), 0.05 mM 2-mercaptoethanol (Gibco, USA), 20 ng/mL recombinant mouse GM-CSF (Peprotech, UK) and 20 ng/mL recombinant mouse M-CSF (Peprotech, UK).

Mice primary cell culture.

Mouse bone marrow derived DCs (BMDCs) were generated by plating 5 million bone marrow cells freshly isolated from tibia and femur into 10 cm dishes. Iscove′s Modified Dulbecco′s Medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories, Germany), 1 mM sodium pyruvate (Gibco, USA), 1x MEM non-essential amino acid solution (Sigma, USA), 100 µg/mL streptomycin & 100 U/mL penicillin (Gibco, USA), 0.05 mM 2-mercaptoethanol (Gibco, USA), and 10% filtered culture supernatant from CHO-Flt3L cells.

Animal tumor models

For tumor-bearing mice experiments, 7-12 weeks old mice were used. For wildtype MC38 and B16 cells, 5 x 10^5 tumor cells were injected subcutaneously into the right thoracic flank, while MC38-OVA tumor implanting experiments were performed using 1 x 10^6 cells. Tumor size and health score were measured and monitored three times per week. Perpendicular tumor diameters were measured by caliper and tumor volume calculated according to the following formula: tumor volume = (d^2xD)/2, where d and D represents the shortest and longest diameters of the tumors (in millimeter), respectively. For tumor growth experiments, mice were sacrificed once tumor size reached 1500 mm^3. For

tumor-infiltrating DC phenotype and functionality experiments, mice were sacrificed once tumor size reached 300-500 mm^3. Animals with ulcerated tumors were sacrificed and excluded from further analysis.

Tumor digests and PBMCs isolation

For the preparation of single cell suspensions from both human and mouse tumors, tumors were collected, surgical specimens were mechanically dissociated and subsequently digested using accutase (PAA Laboratories, Germany), collagenase IV (Worthington, USA), hyaluronidase (Sigma, USA) and DNase type IV (Sigma, USA) for 1 h at 37°C under constant agitation. Cell suspensions were filtered through 70-µm mesh twice and lysed for red blood cells using RBC lysis buffer (eBioscience, USA). PBMCs were isolated by density gradient centrifugation using Histopaque-1077 (Sigma, USA) from buffy coats. Mice splenocytes were isolated by mechanical disruption using the end of a 1 mL syringe, lysed for red blood cells using RBC lysis buffer. Then digested with Collagenase D (Roche, Switzerland) and DNase I (Roche, Switzerland). Samples were either used directly or frozen (in 90% FBS, 10% DMSO) and stored in liquid nitrogen until the time of analysis.

Generation of Siglec-E knockout Sp37A3 cells

Knockout of Siglec-E from Sp37A3 cells was performed using CRISPR/Cas9 mediated gene editing. Guide RNAs were designed online based on published data (http://greenlisted.cmm.ki.se/). Guide RNAs with the following sequences were synthesized by Microsynth (Switzerland): Forward: 5' - CAC CGG AGG GTC AGA ACC CCC AAG - 3', Reverse: 5' - AAA CCT TGG GGG TTC TGA CCC TCC - 3'. Then they were cloned into the lentiCRISPRv2 puro vector (Addgene plasmid #98290). Lentivirus with empty vectors or modified vectors were used to transduce the original Sp37A3 cell line. Single cell clones with

right phenotype were sorted into 96-well plates. After their recovery and expansion, individual clones were screened again for Siglec-E expression. Multiple clones were selected and pooled to avoid clonal selection.

Genetically modified Sp37A3 cell RNA sequencing analysis

Control empty vector transduced (CtrV) and Siglec-E knockout (EKO) Sp37A3 cells were taken from culture. 1 x 10^6 cells were seeded in 6-well plate and pulsed with 0.1 mg/ml EndoFit Ovalbumin (Invivogen, USA) for 2 hours. Then cells were washed and stimulated for maturation by 0.1 ug/ml Lipopolysaccharides (Sigma, USA) for 24 hours. Cells were washed, and RNA samples were enriched by RNeasy Plus Micro Kit (Qiagen, Germany). Sequencing and analysis were performed by Dr. Robert Ivanek from Bioinformatics Core Facility of University of Basel.

CtrV and EKO Sp37A3 cell cytokine/chemokine array analysis

CtrV and EKO Sp37A3 cells were seeded and pulsed same as in RNA sequencing analysis. After 36 hours of LPS stimulation, culture supernatant was collected, frozen, and sent in dry ice for a 44-plex Cytokine/Chemokine Array test (Eve Technologies, Canada). Cytokines and chemokines concentration were analyzed and presented by Eve Technologies.

Tumor-infiltrating DC (TiDC) sorting

Mouse subcutaneous tumors were freshly digested, and CD11c-positive cells were isolated by MACS (Stemcell, Canada), following with a FACS sorting to exclude dead cells and F4/80 or Gr-1 positive suppressive myeloid compartments.

DC and antigen-specific T cell co-culture.

BMDCs or Sp37A3 cells were seeded 4 x 10^4 cells per well in 96-well plate. Then cells were pulsed with 0.1 mg/ml OVA protein (Invivogen, USA) or left

unpulsed for 2 hours. DCs were washed and stimulated by 0.1 ug/ml LPS for overnight. OVA antigen-specific OT-I CD8+ T cells and OT-II CD4+ T cells were isolated from spleens of indicated mice respectively by MACS (Stemcell, Canada). T cells were labelled with CellTrace Violet (Invitrogen, USA) and added into wells, 2 x 10^5 cells per well. T cell activation and proliferation was checked after certain timepoints as described in each experiment. For TiDC experiments, freshly isolated TiDCs from MC38-OVA animal model were co-cultured with purified OT-I CD8+ T cells or OT-II CD4+ T cells at a ratio of 1:5. T cell proliferation was checked after 72 hours.

Multicolor flow cytometry

For multicolor flow cytometry, dead cells and doublets were excluded in all analyses. Corresponding isotype antibodies or fluorescence-minus-one (FMO) samples were used as a control, in particular for the Siglec stainings. All tumor samples were analyzed with a Fortessa LSR II flow cytometer (BD Biosciences). For infiltration analysis, mice were euthanized, and tumors were mechanically dissociated and digested as described for the human sample preparation.

Statistical analysis

Statistical analysis was performed using Prism 9 (GraphPad, USA). Different comparison strategies were indicated in each specific figure respectively.

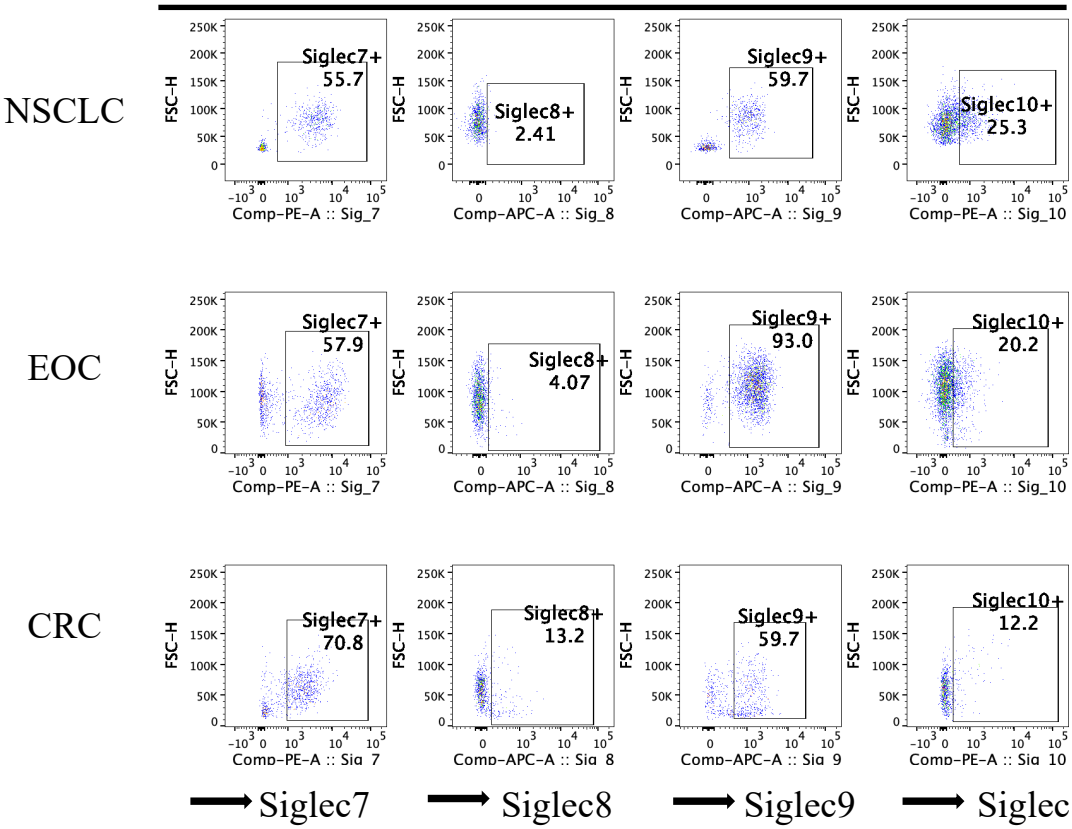
3. Results

3.1. Tumor-infiltrating conventional dendritic cells express inhibitory Siglec receptors in humans

Previous reports have shown that Siglec receptors are expressed on myeloid cells, including cDCs (Lübbers et al, 2018; Bärenwaldt & Läubli, 2019). However, the expression and functions of these receptors on intratumoral cDCs from patients with different types of cancer are poorly understood. Therefore, we first tested the expression of several inhibitory Siglec receptors on tumor-inflitrating conventional DCs (Ti-cDCs) from different types of cancers by flow cytometry (Gating strategy shown in Figure1A-1B and Supplementary Figure S1). We found that a significant proportion of both type 1 and type 2 cDC expressed inhibitory Siglec receptors (Figure 1C-1E). Across different cancer types that we tested, Siglec-7 and Siglec-9 constantly expressed at more pronounced levels by Ti-cDCs. In comparison, Siglec-10 showed low to intermediate expression levels on Ti-cDCs, while Siglec-8 expression was even less frequent. Similar expression patterns of these Siglec receptors were also obeserved on plasmacytoid dendritic cells (pDCs), although to a lower level (Supplementary Figure S2). Taken together, this data demonstrates that inhibitory Siglecs are expressed on human cancer-associated cDCs and could be involved in the regulation of these cells.

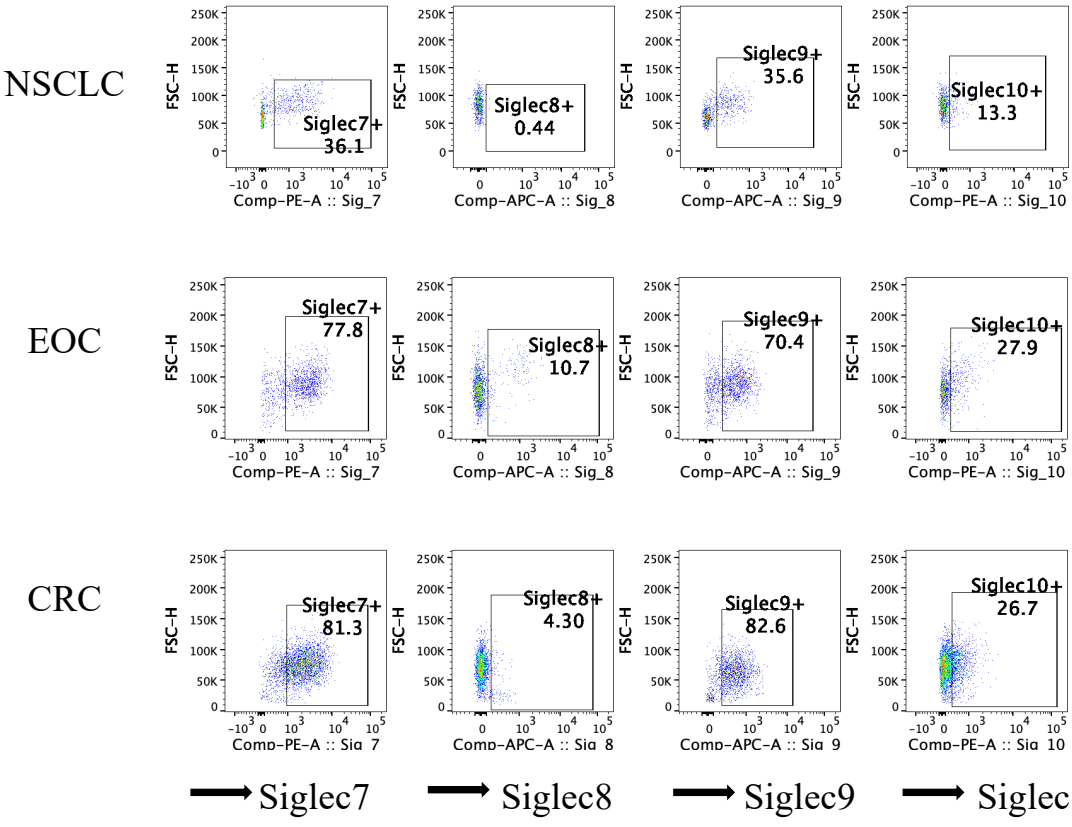
A

Tumor-infiltrating CD141+ cDC1s

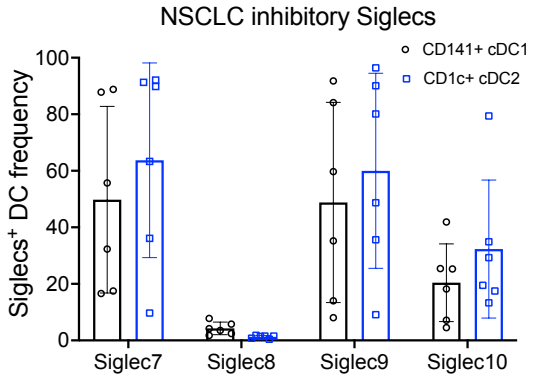


B

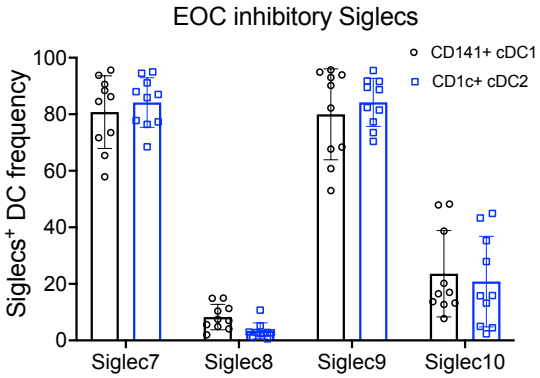
Tumor-infiltrating CD1c+ cDC2s



C



D



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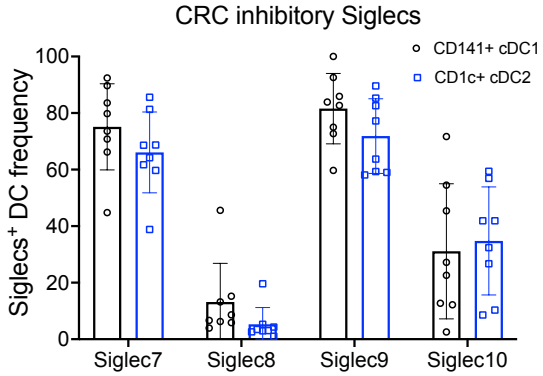


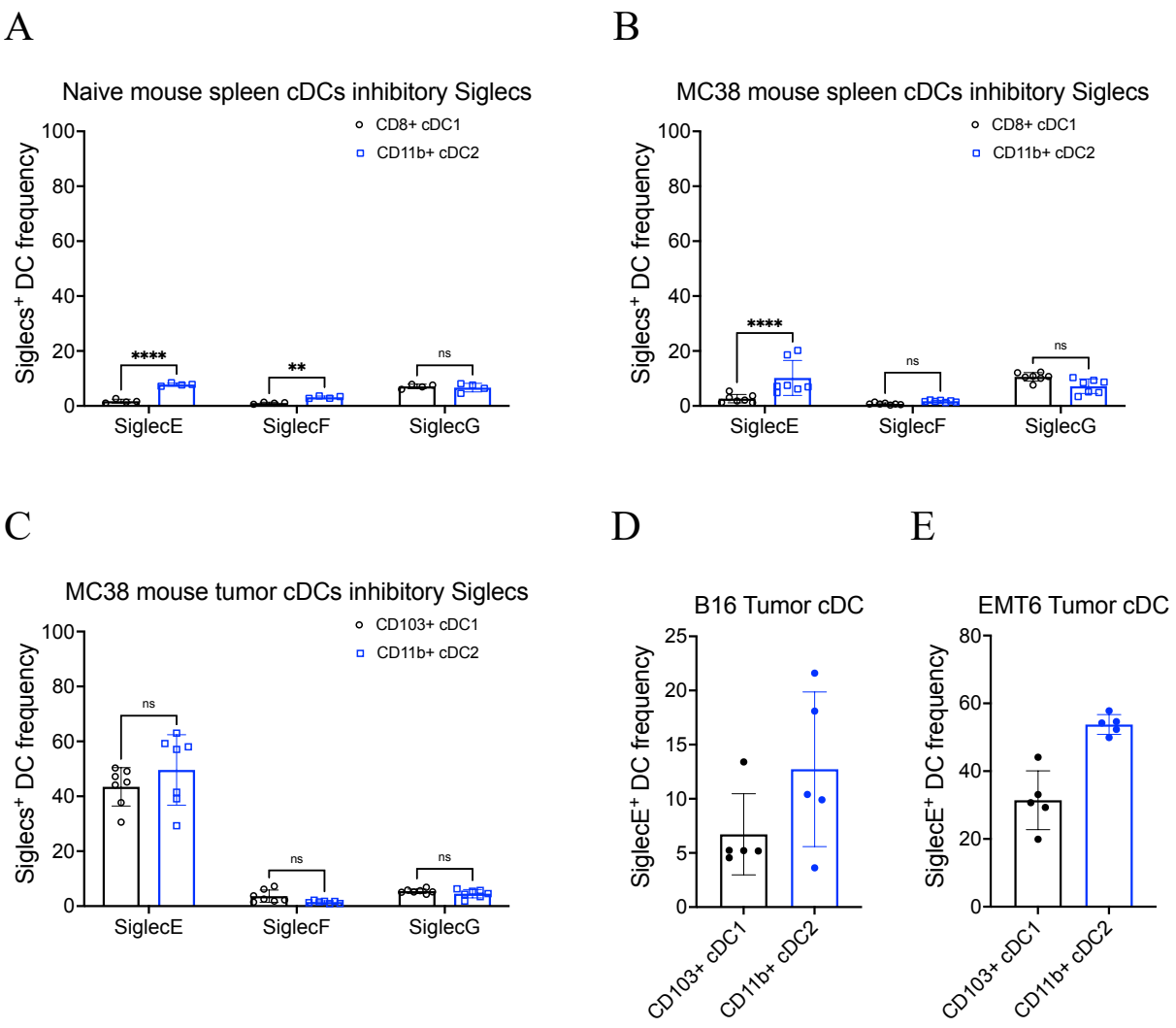
Figure 1. Expression of inhibitory Siglecs on human Ti-cDCs.

(A-B) Flow cytometry gating of different Siglecs from human Ti-cDC subsets. (C-E) The expression patterns of inhibitory Siglecs from patient tumor samples of non-small cell lung cancer (NSCLC), epithelial ovarian cancer (EOC) and colorectal cancer (CRC). Data are presented as mean (± SD).

3.2. Siglec-E expression is upregulated on Ti-cDC subsets during mouse cancer progression

To further investigate the functions of Siglec receptors on cDCs during cancer progression, we next analyzed the expression of several murine inhibitory Siglec receptors on mice. Healthy C56BL/6 wildtype mice spleen cDCs, or spleen and intratumoral cDCs from subcutaneous MC38 tumor-bearing mice were isolated and the expression of several inhibitory Siglecs were identified by flow cytometry (Figure 2A-2C, Gating strategy shown in Supplementary Figure S3). We found out that in healthy state, naive mouse spleen cDCs only express these inhibitory molecules at very low levels (Figure 2A). Similarly, in MC38 tumor-bearing mice, spleen cDCs still kept the low expression rates of these Siglecs (Figure 2B). Intriguingly, Siglec-E, the functional paralog of human Siglec-9, was more frequently observed on both Ti-cDC subsets (Figure 2C). The expression of Siglec-E on Ti-cDCs was also confirmed by other mouse subcutaneous tumor models including C57BL/6 mouse B16 melanoma and Balb/c mouse EMT6 breast cancer (Figure 2D-2E). In order to understand whether Siglec-E expression influences cDC functions, we first tried to use mouse bone marrow derived DC (BMDC) models. We obtained mouse bone marrow cells from wildtype (WT) mice or systemic Siglec-E knockout (SigEKO) mice, and culture the cells seven days *in vitro* supplemented with either Granulocyte-macrophage colony-stimulating factor (GM-CSF) or FMS-like tyrosine kinase 3 ligand (FLT3L). However, we did not observe significant Siglec-E expression on naive BMDCs from WT mice in either cytokine group (Figure 2F). Stimulation of BMDC maturation by lipopolysaccharide (LPS) also showed no improvement of Siglec-E expression (data not shown). Taken together, it suggests that healthy state DCs might only reprensent minimum inhbitory Siglec-E expression, while cDCs isolated from tumor sites showed unusually high Siglec-E expression. To explore

the possible functions of Siglec-E expression on DCs, we next screened several mouse DC cell lines for the Siglec-E expression levels. Among them, an immature DC cell line Sp37A3, generated from C57BL/6 mouse spleen (Bros et al, 2007), showed significant expression of Siglec-E (Figure 2G). This cell line provides us a very nice tool to study the functions of the inhibitory Siglec-E on DCs. Thus, we tried to use a CRISPR-Cas9-based lentivirus transduction system to knockout the Siglec-E expression from Sp37A3 cells. After pooling several single clones that were confirmed to have ultra-low Siglec-E expression, we successfully generated Siglec-E knockout (EKO) Sp37A3 line, along with an empty control vector transduced (CtrV) Sp37A3 line (Figure 2H).



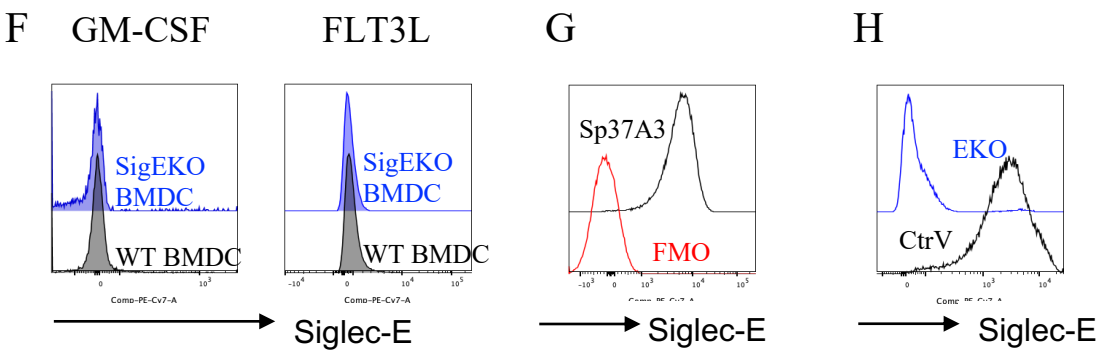


Figure 2. Inhibitory Siglec-E expression is significant on mouse tumor-associate DCs.

(A-C) The expression patterns of several murine inhibitory Siglecs on cDCs isolated from (A) naive C57BL/6 mice spleens, (B) MC38 tumor-bearing mouse spleens and (C) tumors were analyzed by flow cytometry. (D-E) Siglec-E expression on tumor cDC subsets from (D) B16 melanoma and (E) EMT6 breast cancer mouse models. (F) Siglec-E expression of BMDCs from wildtype (WT, black line) and systemic Siglec-E knockout (SigEKO, blue line) mice after 7-day *in vitro* culture supplemented with GM-CSF or FLT3L. (G) Expression of Siglec-E on Sp37A3 cell line (black line) versus FMO control (red line). (H) Siglec-E expression of Siglec-E knockout (EKO) Sp37A3 cells and empty control vector (CtrV) transduced Sp37A3 cells. Data are presented as mean (± SD), and two-way ANOVA was used for two-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

3.3. Siglec-E-deficient DCs showed elevated activation and maturation status

Next, we want to explore whether ablation of Siglec-E from DCs could influence DC activation and maturation. Thus, we first tried to analyze markers of DC maturation on the newly generated CtrV Sp37A3 and EKO Sp37A3 cell lines (Figure 3A-3C). DC maturation makers including MHC-I, MHC-II and CD40 showed significant upregulation on EKO Sp37A3 cells compared to the Siglec-E-expressing Sp37A3 cells. This data suggests that DCs lacking inhibitory Siglec-E expression might be more activated. To test this hypothesis *in vivo*, we generated a subcutaneous MC38 mouse tumor model in conditional DC Siglec-E knockout mice ($CD11c^{cre/wt}SigE^{flox/flox}$ mice, abbr. $CD11c^{cre}SigE^{fl}$), in order to avoid some possible influences from other Siglec-E-deficient bystander cells. Interestingly, we also observed significant increases of these maturation markers of tumor-infiltrating cDCs lacking Siglec-E expression compare to their littlemates, in particular on tumor-infiltrating cDC1s (Figure 3D-3F). As our group previously reported that $CD11c^{cre}SigE^{fl}$ mice showed delayed MC38 subcutaneous tumor growth compared to their Siglec-E-sufficient littermates (Stanczak et al., 2021), this data supports a correlation between better maturation status of DCs with Siglec-E deficiency and a stronger anti-tumor immune response of the host.

Previous research demonstrated that DC maturation includes upregulation of surface markers (‘phenotypic maturation’) and activation of cytokines production (‘functional maturation’). DCs lacking either phenotypic or functional maturation (‘semi-maturation’) are closely relevant to impeded functionality (Dudek et al, 2013). Therefore, we were wondering, to what extent, DC maturation status is influenced by Siglec-E-deficiency when encountering activating stimuli. We pulsed CtrV and EKO Sp37A3 DCs with ovalbumin (OVA) antigen, followed by DC maturation stimulation with TLR4 ligand lipopolysaccharide (LPS). Then we

performed bulk RNA sequencing of both DC cell types (EKO and CtrV). Interestingly, the EKO Sp37A3 cells upregulated mRNA levels of multiple cytokines (IFNb1, IL1a, IL1b, IL6, IL12b, IL23a), chemokines (CCL3, CCL4, CCL5, CCL22, CXCL2), co-stimulatory molecules (CD40, CD80) and DC specific markers (CCR7, Flt3) (Figure 4A). GeneSet Enrichment Assay (GSEA) suggests that several hallmark pathways changed significantly between the two Sp37A3 lines. The most striking changes were related to several pathways involving antigen presenting cell functions. EKO Sp37A3 cells showed stronger upregulation of type I and II Interferon (IFN-α and IFN-γ)-related responses, tumor necrosis factor alpha (TNF-α) response and general inflammatory response (Figure 4B). Taken together, the results reveal that Siglec-E-deficient DCs might represent better response to stimulation. To verify these findings from protein level, we collected cell culture supernatant of OVA-pulsed LPS-stimulated Sp37A3 cells, and performed a Mouse Cytokine / Chemokine Array assay. Among the 44 cytokines and chemokines, the levels of Interleukin (IL)-1β, IL-12 or IL-23 p40 subunit (IL12/IL23 p40), CXCL2 and CCL22, showed different secretion patterns between the two Sp37A3 lines (Figure 4C and Supplementary Figure S4A-S4B). Elevated cytokine and chemokine production demonstrates that Siglec-E-deficient Sp37A3 cells also represent enhanced functional maturation status in respond to stimulation. Phenotypic analysis of surface maturation markers and co-stimulatory molecules also showed that EKO Sp37A3 cells are easier activated by stimuli compared to CtrV Sp37A3 cells (Figure 4D and Supplementary Figure S4C). Taken together, these results further supported our findings on transcriptome level and implied that the EKO Sp37A3 cells represent better maturation status in response to stimuli, both phenotypically and functionally, compared to the Sp37A3 cells with high Siglec-E expression. Particularly, elevated co-stimulatory molecules might correlate with stronger T cell priming. Therefore, we hypothesized that DCs lacking the inhibitory Siglec-

E expression might be more efficiently carrying out antigen-presentation cell functions.

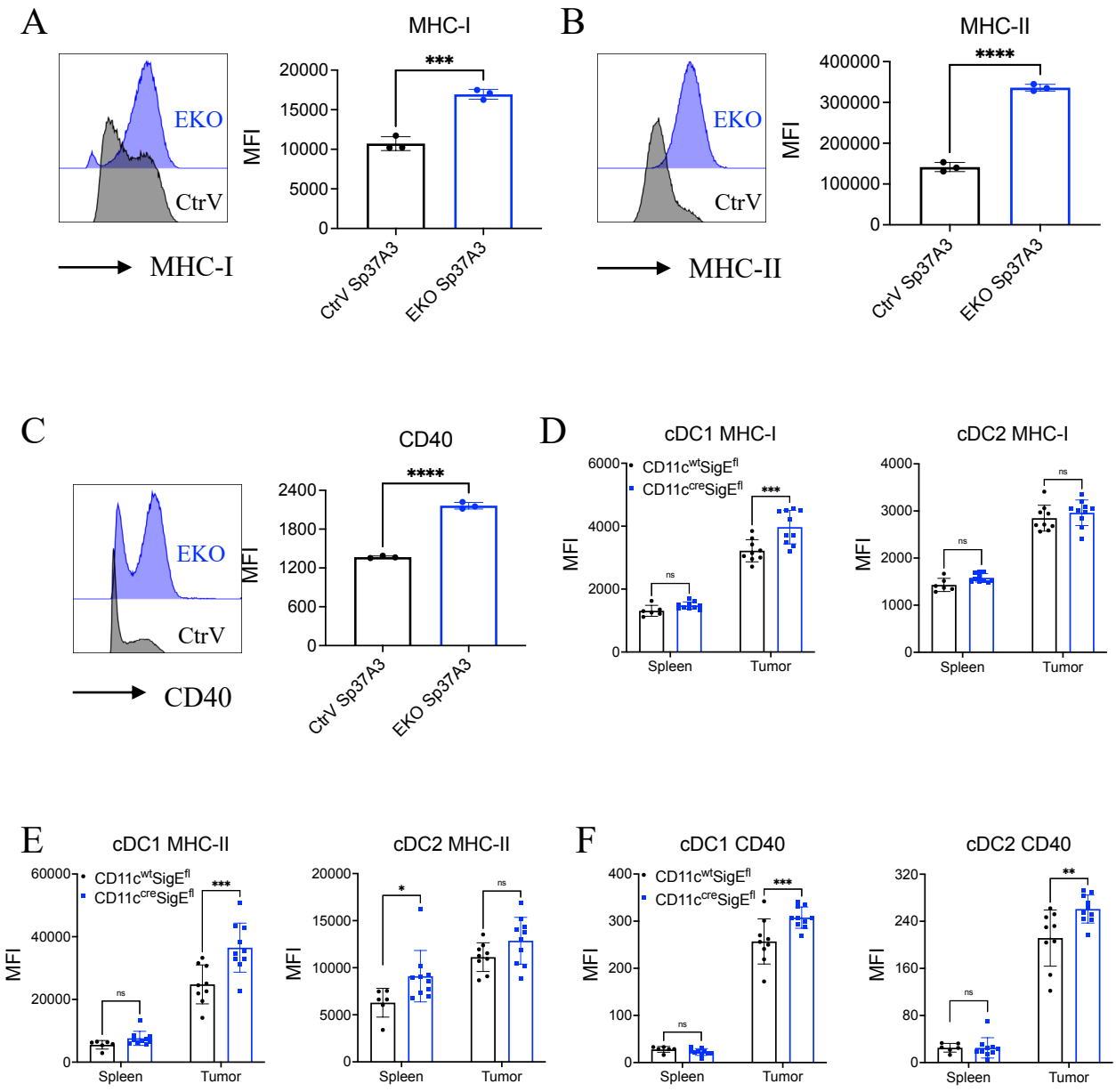


Figure 3. Siglec-E-deficient DCs showed enhanced phenotypic maturation.
(A-C) Flow cytometry analysis of the expression levels of several DC maturation markers on CtrV Sp37A3 cells (black) and EKO Sp37A3 cells (blue), including (A) MHC-I, (B) MHC-II and (C) CD40. (D-F) Maturation markers on spleen and tumor-infiltrating cDCs isolated from MC38 subcutaneous tumor models of

$CD11c^{cre}SigE^{fl}$ mice (blue) and littermates ($CD11c^{wt}SigE^{fl}$, black) by flow cytometry. Data are presented as mean (± SD). Two-way ANOVA was used for two-way comparisons, and unpaired t test was used for one-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

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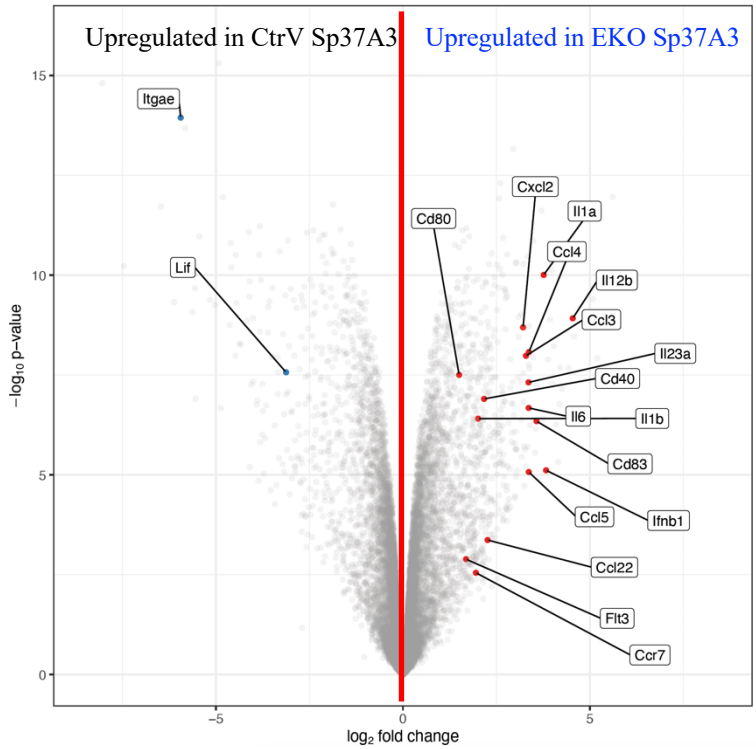

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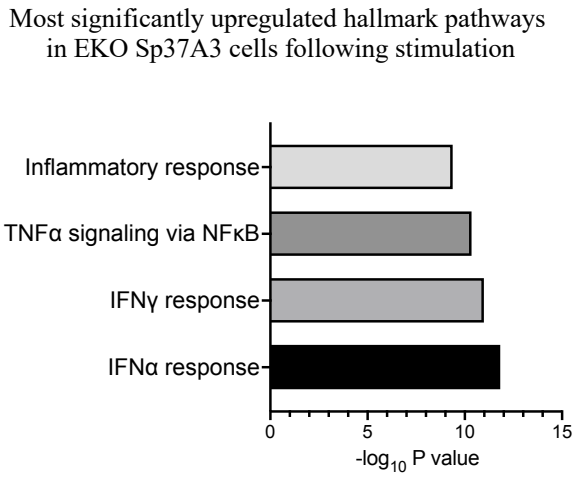


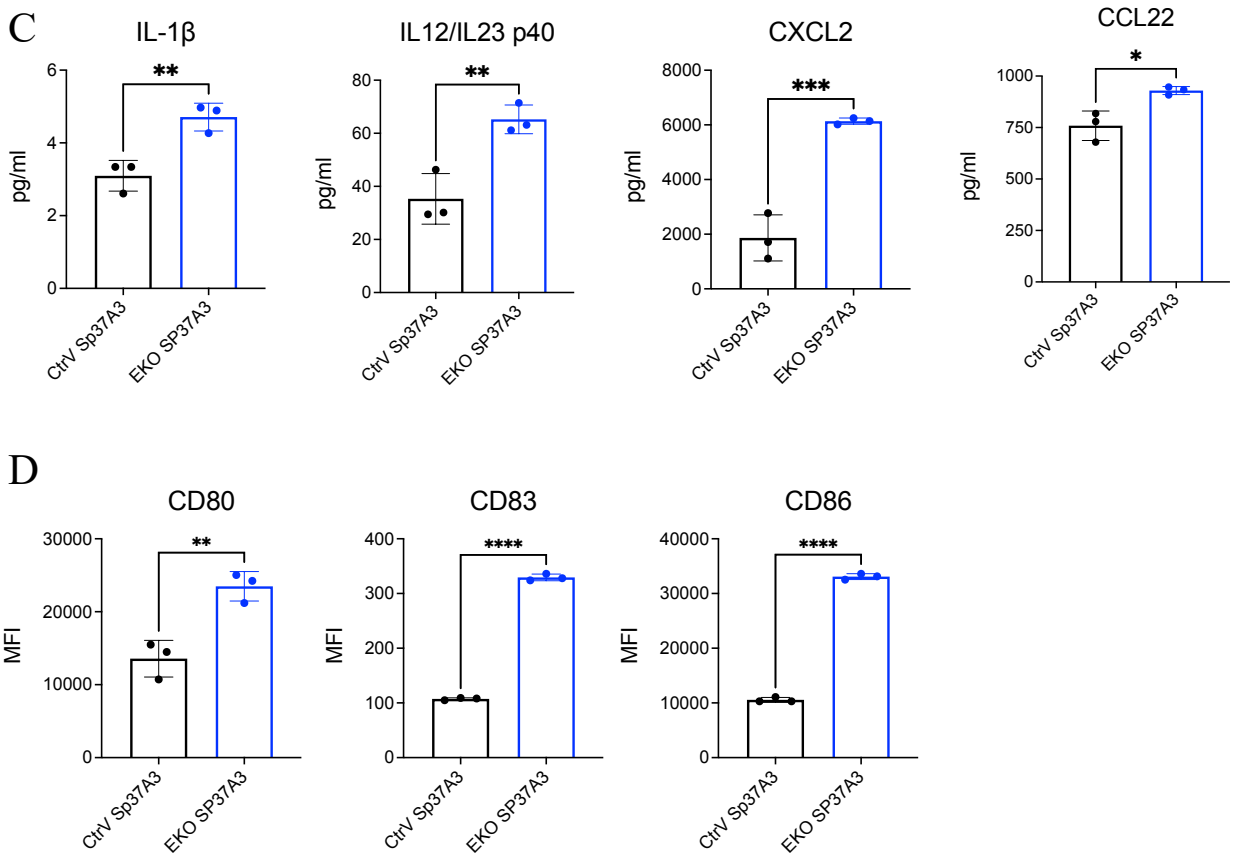


Figure 4. Siglec-E-deficient DCs showed elevated maturation status upon stimulation.

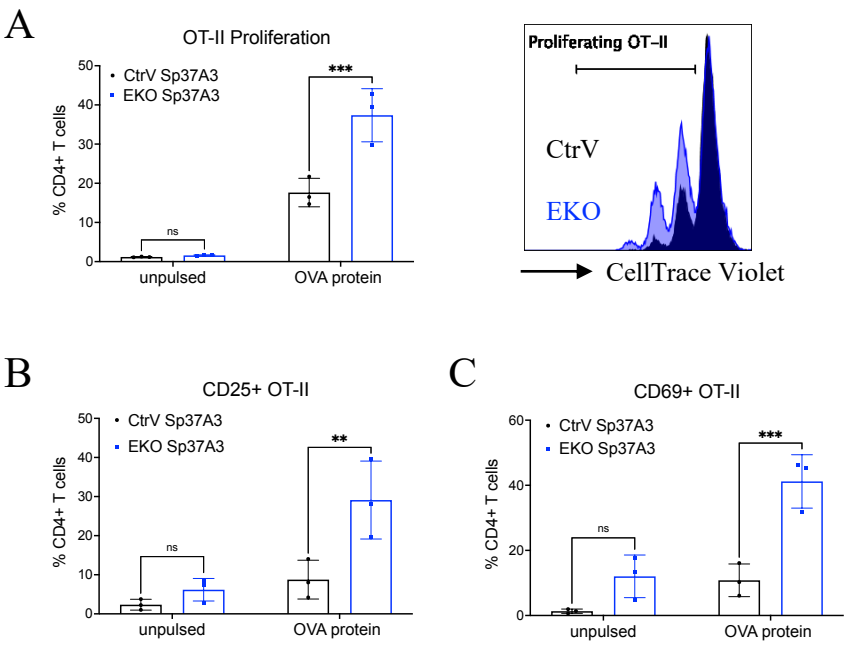
(A) Volcano plot of genes upregulation on CtrV and EKO Sp37A3 cells after stimulation. (B) GSEA analysis of the most significantly activated pathways in EKO Sp37A3 cells. (C) Cytokines, chemokines production and (D) activatory, co-stimulatory markers of CtrV (black) and EKO (blue) Sp37A3 cells.

Data are presented as mean (± SD), and unpaired t test was used for one-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

3.4. Inhibitory Siglecs impair DC antigen presentation to CD4+ T cells

In order to understand whether the antigen presentation functionality of DCs is affected by the expression of Siglec-E, we performed antigen uptake, antigen processing and presentation assays to monitor the antigen handling process. Firstly, we analyzed antigen endocytosis of the Sp37A3 cells with fluorescent-labelled soluble OVA antigen or tumor cell-associated antigens. During the indicated timepoints, we did not observe any difference of antigen uptake between these two DC lines (Supplementary Figure S5A-S5B). Furthermore, we co-cultured the Sp37A3 cells with live fluorescent-labelled or auto-fluorescent MC38 tumor cells. In line with the previous results, neither the frequency of fluorescent-positive DCs nor the mean fluorescence intensity (MFI) showed any significant change (Supplementary Figure S5C-S5D). Since antigen uptake was not affected by the Siglec-E expression, we then investigated antigen processing. We harnessed the reagent DQ-OVA, which shows fluorescent signals after being processed. We observed the MFI fold changes from several timepoints, and found out that after certain time of processing, the EKO Sp37A3 DCs showed stronger fluorescent signals (Figure S5E). This result suggests that the DCs with Siglec-E-deficiency were more efficient in antigen processing. To understand whether this leads to better antigen presentation, we used OVA antigen pulsed Sp37A3 cells to co-culture with either antigen-specific CD4+ T cells from OT-II transgenic mice or CD8+ T cells from OT-I transgenic mice. T cell proliferation was analyzed by CellTrace Violet proliferation dye, and their activation was shown by surface CD25 and CD69 expression. Even though both MHC-I and MHC-II molecules are expressed at higher levels in EKO Sp37A3 cells, only OT-II CD4+ T cells showed enhanced activation and proliferation during co-culture (Figure 5A-5F). Since DCs are also known to prime CD8+ T cells through cross-presentation of cell-associated antigens, we also used heat-shocked wildtype or OVA-expressing MC38 tumor cells (MC38-wt or MC38-OVA) to replace

soluble OVA antigen. Heat-shocked MC38-OVA cells induced strong OT-I CD8+ T cells activation and proliferation, but we still failed to observe any difference between the two Sp37A3 lines (Supplementary Figure S5F). Taken together, the inhibitory Siglec-E expression on Sp37A3 DCs dampens their functions to activate antigen-specific CD4+ T cells, without affecting CD8+ T cell priming. To investigate whether the Siglec-E functional paralog human Siglec-9 also influences DC antigen presentation, we generated mice expressing the human Siglec-9 transgene. Naive BMDCs generated from these transgenic mice showed high expression of human Siglec-9. Similar to our observations on Siglec-E-expressing DCs, CD4+ OT-II cells co-cultured with OVA antigen pulsed Siglec-9 positive BMDCs represented less activation and proliferation (Figure 5G-5I). No difference of CD8+ OT-I cells proliferation and activation was observed between Siglec-9 positive and Siglec-9 negative BMDC co-culture (data not shown). Taken together, these results suggested that the expression of inhibitory Siglecs could diminish DC and CD4+ T cell crosstalk through impaired antigen processing and presentation.



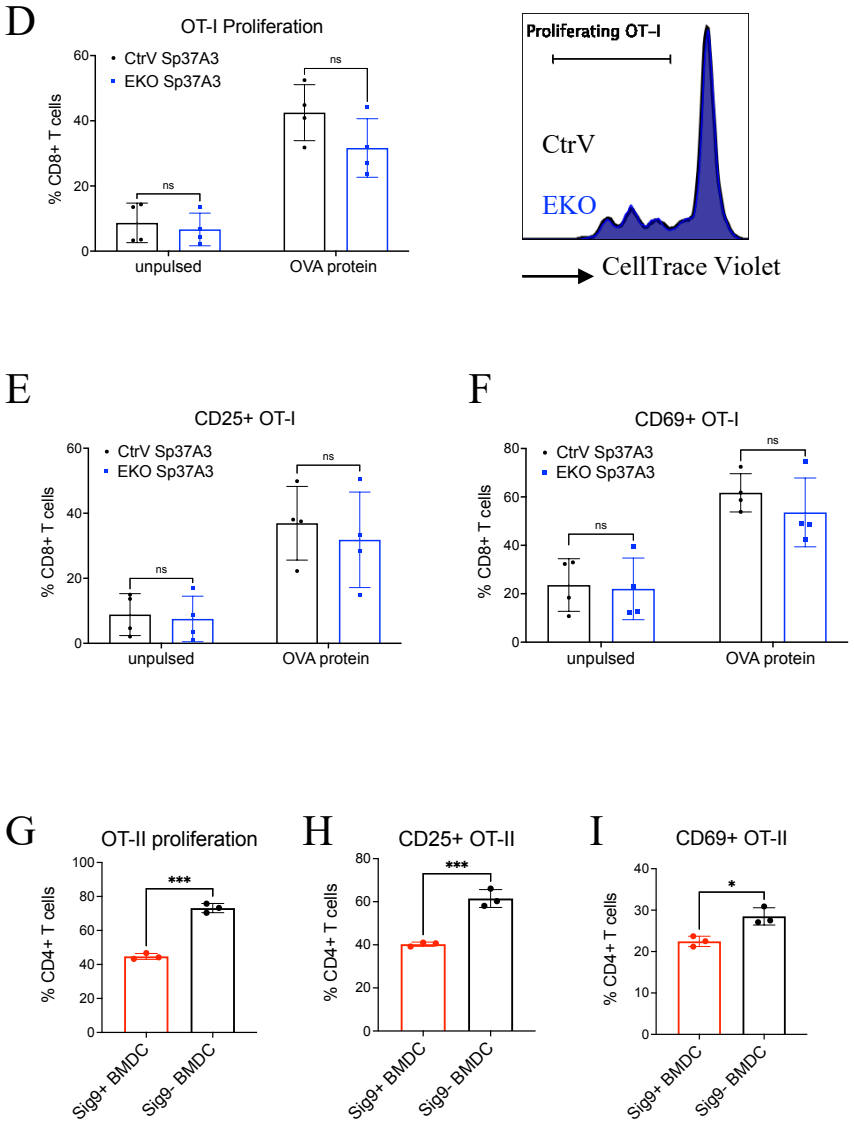


Figure 5. Inhibitory Siglecs impair DC antigen presentation to CD4+ T cells. (A-F) 48-hour *in vitro* co-culture of OVA-pulsed CtrV or EKO Sp37A3 cells with (A-C) CD4+ OT-II T cells or (D-F) CD8+ OT-I T cells. (G-I) 72-hour *in vitro* co-culture of OVA-pulsed Siglec-9 positive (red) or Siglec-9 negative (black) BMDCs with CD4+ OT-II T cells. Data are presented as mean (± SD). Two-way ANOVA was used for two-way comparisons, and unpaired t test was used for one-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

4. Supplemental information

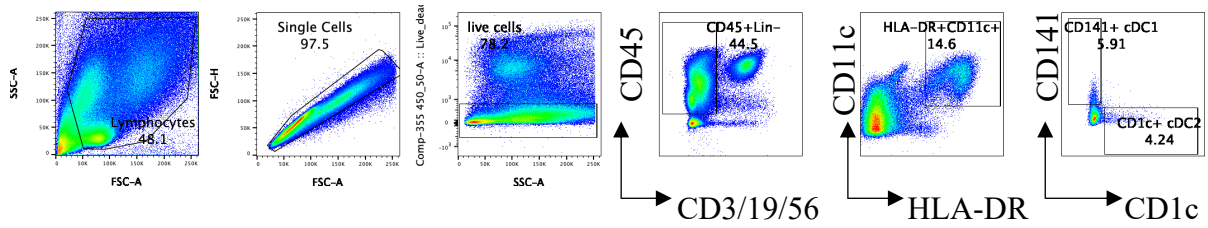


Figure S1. Gating strategy of human intratumoral dendritic cell subsets.

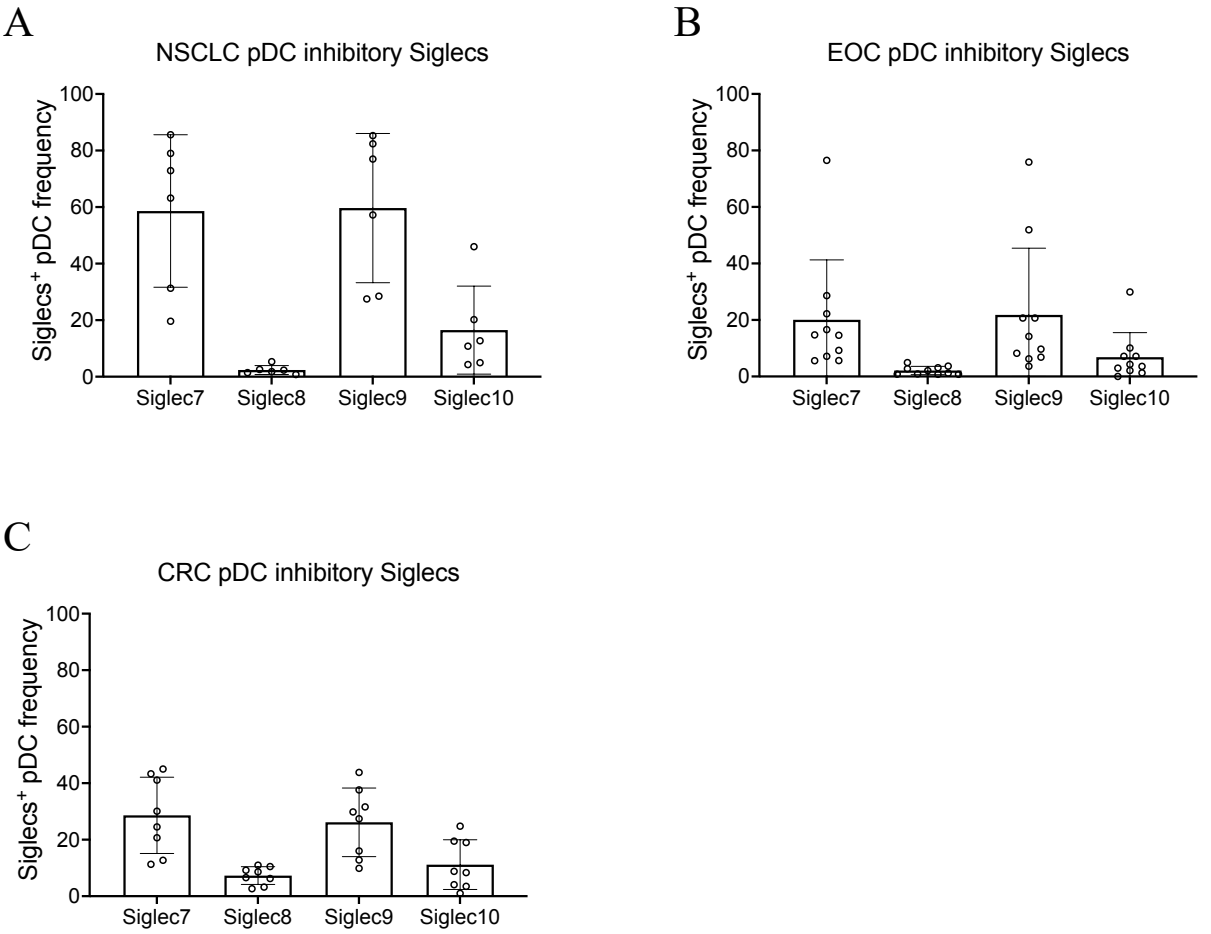
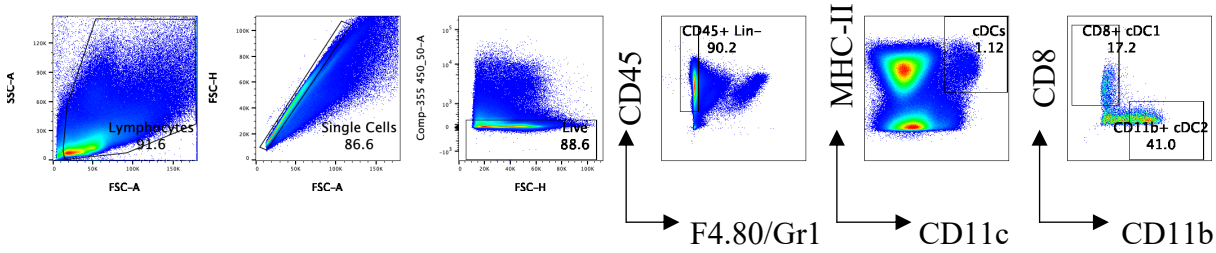


Fig S2. Expression of inhibitory Siglecs on human tumor-infiltrating pDCs.

A



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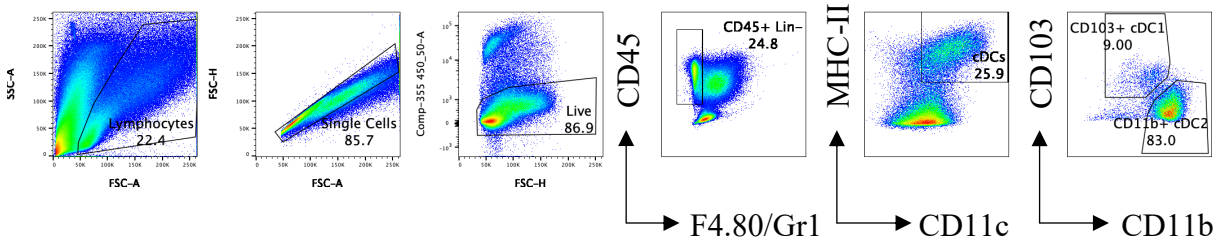
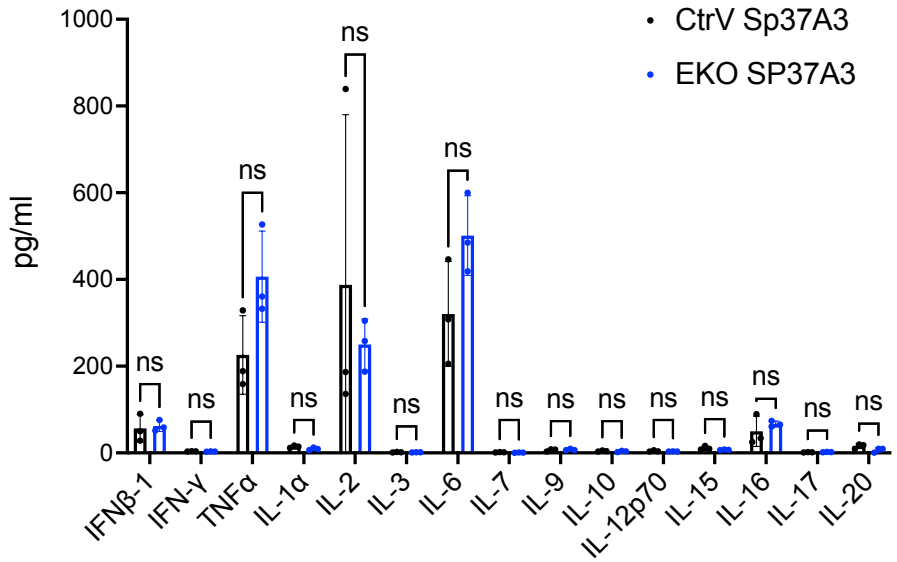


Figure S3. Gating strategy of mouse cDCs.

Mouse cDC subsets gating strategies of samples from (A) spleens and (B) tumors.

A



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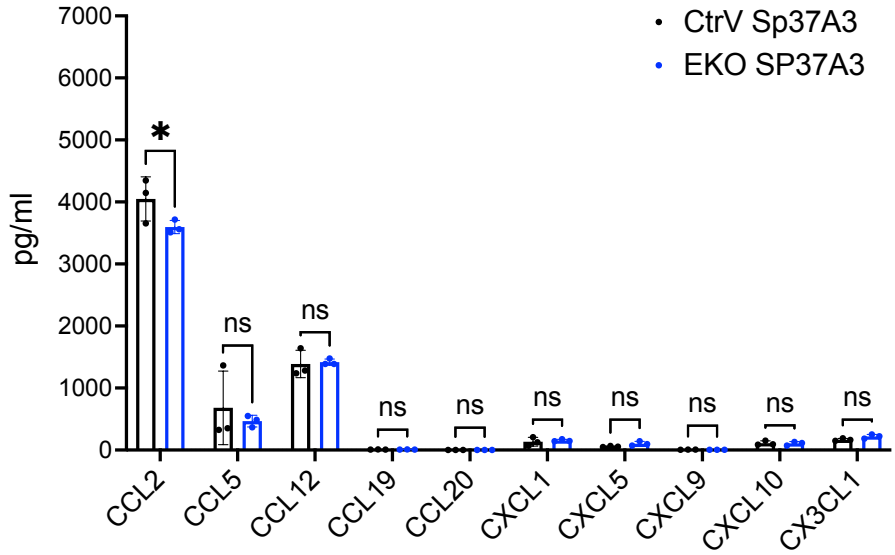

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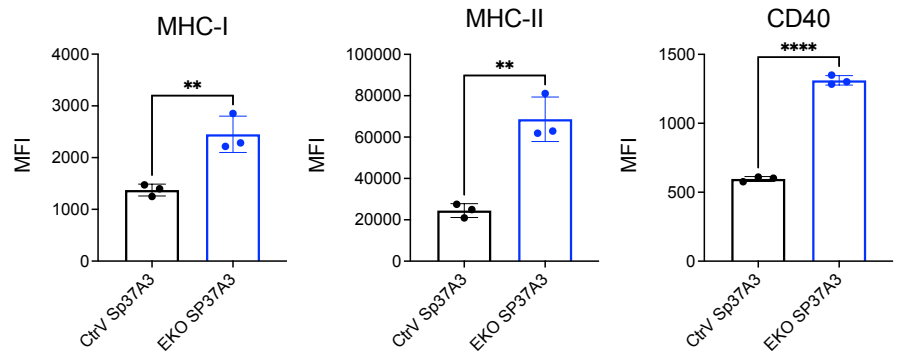
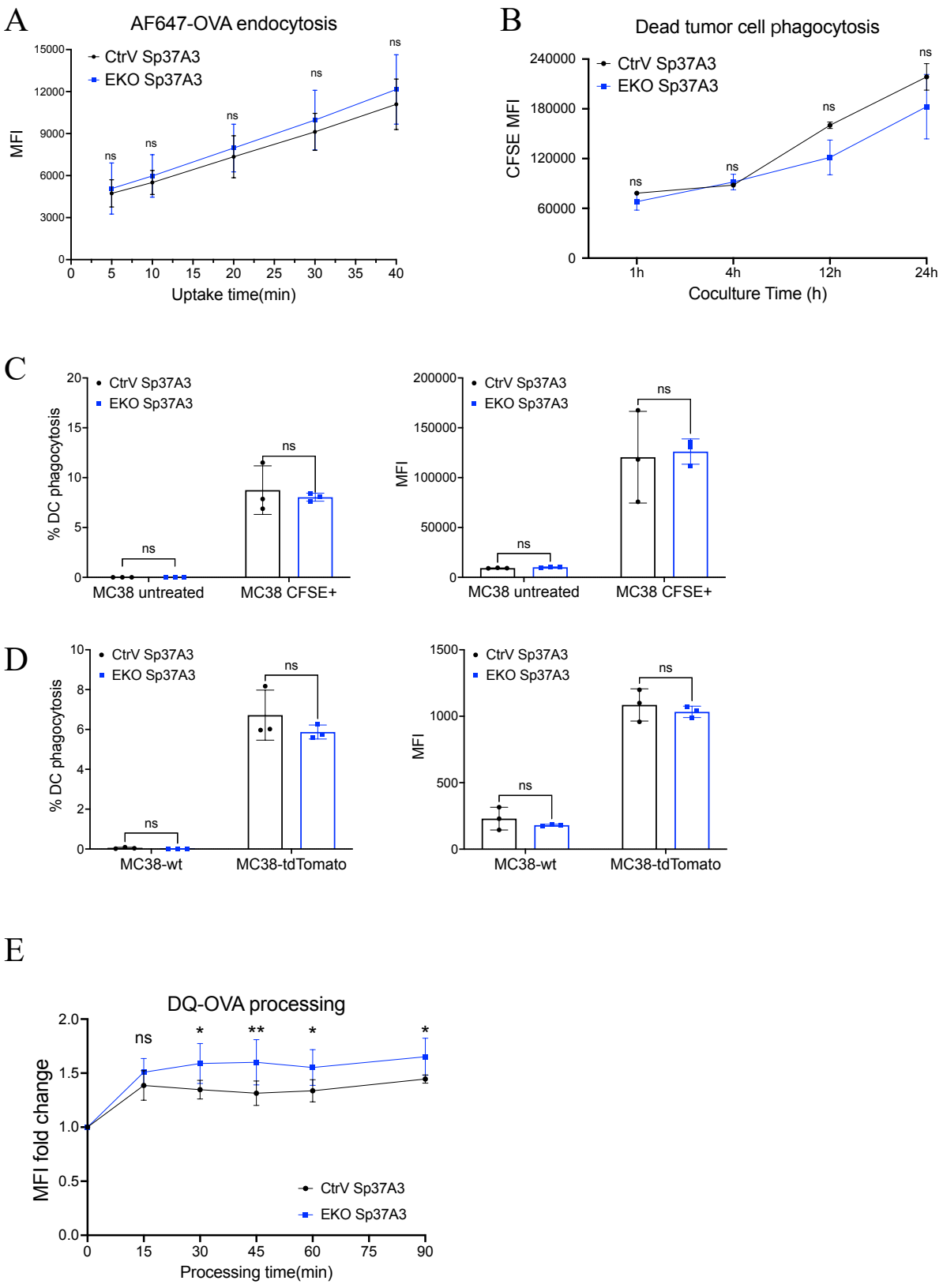


Figure S4. Siglec-E-deficient DCs showed elevated maturation status upon stimulation.

(A-B) Secretion levels of other cytokines and chemokines. (C) Phenotypic maturation markers of CtrV and EKO Sp37A3 cells. Data are presented as mean (± SD), and unpaired t test was used for one-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).


A
AF647-OVA endocytosis
CtrV Sp37A3
EKO Sp37A3
MFI
Uptake time(min)
B
Dead tumor cell phagocytosis
CFSE MFI
Coculture Time (h)
C
% DC phagocytosis
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MC38 CFSE+
D
MC38-wt
MC38-tdTomato
E
DQ-OVA processing
MFI fold change
Processing time(min)
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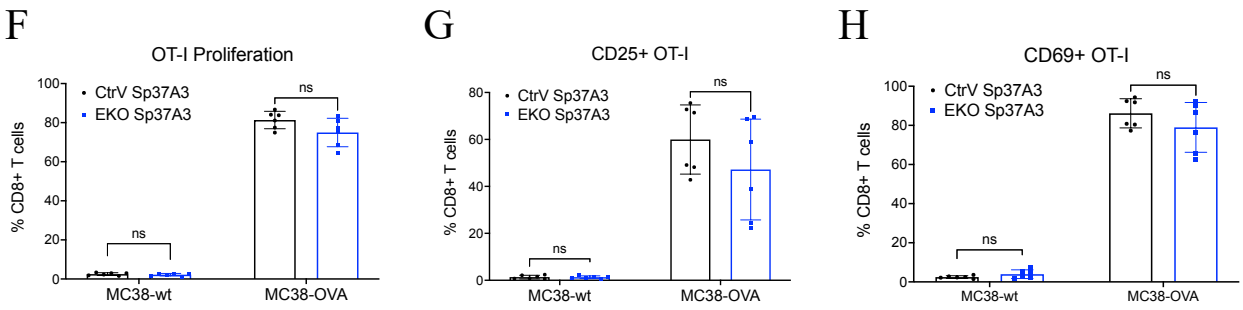

Figure S5. Siglec-E expression affects only antigen processing.

(A-B) Phagocytosis of (A)soluble AF647-conjugated OVA antigen and (B) CFSE-labelled heat-shocked MC38 cells by Sp37A3 DCs. (C-D) *Sp37A3 endocytosis of live fluorescent tumor cells.* Sp37A3 DCs were co-cultured 4 hours at 1:2 ratio with (C) untreated MC38 cells or CFSE-labelled MC38 cells, (D) wildtype MC38 (MC38-wt) cells or tdTomato+ MC38 cells. (E) DQ-OVA antigen processing assay. (F-H) Sp37A3 DCs were pulsed with heat-shocked wildtype MC38 cells or MC38-OVA cells. Then co-culture with OT-I cells for 48h to test antigen cross-presentation efficiency. Data are presented as mean (± SD) and two-way ANOVA was used for two-way comparisons (*P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

5. Discussion and outlook

In the past decade, many desperate cancer patients have benefited from the newly thrived cancer immunotherapies, especially immune checkpoint inhibitors and CAR T cell therapies. However, considering the cancer patient population, only small cohorts of patients have shown durable clinical responses. Limitations of these therapies still exist, such as resistant to therapies and toxicity to the body (Waldman et al, 2020; Bagchi et al, 2021; Larson & Maus, 2021). The immune enhancement therapies, aiming at amplify the basic immune response, frequently end up with immune-related adverse events (irAEs), possibly due to the extremely high response rate compared to common physiological state. Therefore, the 'immune normalization' theory has been proposed to unleash the suppressed potential of the immune system, rather than recklessly increasing it (Sanmamed & Chen, 2018).

Aberrant glycosylation, including terminal sialylation, truncated O-glycans, branched N-glycans, and diverse fucosylation, represents one of the commonly observed features of cancer cells, indicating the 'abnormal' status of the malignantly transformed cells (Pinho & Reis, 2015). Hypersialylation profile of cancer cells, along with the broad expression patterns of sialic acid-binding immunoglobulin-like lectins (Siglecs) by the immune system, hints possible functions of the sialoglycan - Siglec axis in the regulation of immune response against cancer. Previous researches proved this axis negatively modulates functions of NK cells, T cells, neutrophils, and macrophages (Belisle et al, 2010; Läubli et al, 2014b; Jandus et al, 2014; Beatson et al, 2016; Perdicchio et al, 2016a; Stanczak et al, 2018; Haas et al, 2019; Barkal et al, 2019; Wang et al, 2019). As the Siglec receptors are involved in the regulation network of host anti-tumor immune response, increasing attention has been focused on their therapeutic manipulation. Current success and drawbacks of harnessing

PD1/PDL1 axis as immune checkpoints in cancer treatment give rise to viewing Siglecs as novel checkpoints (Macauley et al, 2014; Duan & Paulson, 2020). However, to design optimized therapeutic reagents, better characterization and elucidation of the regulation network by the sialoglycan - Siglec axis is still required.

The aim of this work was to characterize expression pattern of inhibitory Siglecs from dendritic cells (DCs), especially tumor-infiltrating DCs, and further identify possible regulation pathways of DC functions by inhibitory Siglecs. Since hypersialylation is now regarded as one of the cancer hallmarks, deeper understandings of the effects of the sialoglycan - Siglec axis on DCs, the bridge between the innate and adaptive immune system, are essential.

Previous researches on antigen or DC-self sialylation status and the inhibitory Siglec signaling indicated the essential regulatory roles of DC functions by the sialoglycan - Siglec axis (Ding et al, 2016; Perdicchio et al, 2016b; Silva et al, 2016). However, comprehensive characterization of the inhibitory Siglecs on tumor-associated DCs and their functions is still lacking. Therefore, we first tried to identify the expression of several inhibitory Siglecs on the cell surface of tumor-infiltrating conventional DCs (Ti-cDCs) from non-small cell lung cancer (NSCLC), epithelial ovarian cancer (EOC) and colorectal cancer (CRC) patient samples. Interestingly, it turns out that very similar expression patterns of these Siglecs were observed in the three cancer types we tested. Siglec-7 and Siglec-9 were expressed by high frequencies of both Ti-cDC subsets, while Siglec-10 expression was intermediate level and Siglec-8 only showed minimum expression. We hypothetically assumed that highly similar expression pattern might correlate with some very conserved regulation pathways for DC functional modulations during the cancer progression. Since several murine inhibitory Siglecs have been reported to be the functional equivalent of human Siglecs, to test our hypothesis, we then tried to identify whether we could

observe expression of those murine inhibitory Siglecs on tumor-associated cDC subsets.

We harnessed animal subcutaneous tumor models to analyze the expression of murine inhibitory Siglecs. By comparing with healthy mouse spleen cDCs and MC38 tumor-bearing mouse spleen cDCs, we found out that only mouse Ti-cDC subsets showed significant expression of Siglec-E, but not Siglec-F and Siglec-G. Expression of human Siglec-8 and its murine paralog Siglec-F is mainly observed on eosinophils, and our results confirmed that they also do not activate expression on tumor-associated cDCs (Duan & Paulson, 2020). Human Siglec-10 has been reported to affect macrophage phagocytosis, while its paralog murine Siglec-G is related to DC cross-presentation (Ding et al, 2016; Barkal et al, 2019). However, neither of them showed expression on Ti-cDCs as well, suggesting they are not key regulators of cDC functions during cancer progression. Intriguingly, the functional equivalent of human Siglec-9, murine Siglec-E shared highly identical expression pattern to Siglec-9, which hints similar and essential modulatory roles on Ti-cDCs functions. In human cancers, Siglec-9 already showed superior suppressive influences through reducing NK cell cytotoxity, inducing CD8+ T cell dysfunction / exhaustion, dampening neutrophil reactive oxygen species (ROS) production and favoring tumor-promoting macrophage polarization (Belisle et al, 2010; Läubli et al, 2014b; Jandus et al, 2014; Beatson et al, 2016; Stanczak et al, 2018; Haas et al, 2019). These facts encouraged us to further try to discover the possibility of specific inhibitory pathways of Siglec-9 and Siglec-E on DCs.

To further investigate whether these inhibitory Siglecs expression has influence on DCs, we tried to analyze the functions of Ti-cDCs. We restimulated the Ti-cDCs *in vitro* with lipopolysaccharide (LPS), and performed FACS analysis of the production of several cytokines, including IL-6, IL-10, IL-12 and CXCL9, but failed to observe activated production (Data not shown. DC restimulation protocol described here: Barilla et al, 2019). We then tried to isolate ovalbumin

antigen (OVA)-experienced live CD11c+F4/80-Gr.1- Ti-cDCs from OVA-expressing MC38 (MC38-OVA) subcutaneous tumor. Co-culture of these Ti-cDCs with OVA-specific CD8+ T cells (OT-I) or CD4+ T cells (OT-II) represented neither activation nor proliferation (Data not shown). Collectively, we believe that Ti-cDCs from our mouse models are dysfunctional and not suitable for functional studies. Therefore, we ran across this bottleneck to find appropriate models for mechanism study.

We then generated mouse bone marrow-derived DCs (BMDCs) with either GM-CSF or Flt3L as supplement. However, similar to the naive spleen cDCs, BMDCs does not show any Siglec-E expression, even after LPS stimulation. Therefore, we turned to screen mouse DC cell lines and found the Sp37A3 cell line, derived from wildtype C57BL/6 mouse spleen, express Siglec-E at significantly high level (Bros et al, 2007). Through CRISPR-Cas9 genomic editing, we successfully generated Sp37A3 cells with Siglec-E-deficiency (EKO Sp37A3) and its control (CtrV Sp37A3). By analyzing several phenotypic maturation markers of DCs, including MHC-I, MHC-II and CD40, we found out that EKO Sp37A3 line showed significant upregulation of these maturation markers compared to the CtrV line. These observations resemble the findings on Ti-cDCs from Siglec-E DC conditional knockout ($CD11c^{cre}SigE^{flox/flox}$) mice, in particular, the Ti-cDC1 subset, which showed elevated maturation status compared to Siglec-E-sufficient littermates. As demonstrated previously, steady state immature DCs continuously endocytosed ‘self-antigens’ and responsible for immunotolerance role of adaptive immune system (Sallusto et al, 1995). Meanwhile, DC maturation is characterized by phenotypic maturation, including activation of surface co-stimulatory and MHC molecules, and functional maturation, with secretion of inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23 and TNF. Impairment of either leads to anergy, tolerance, and, specifically in tumor conditions, pro-tumorigenicity (Dudek et al, 2013). Therefore, we determined to investigate the changes of DC maturation status

when encountering stimulation. Strikingly, the EKO Sp37A3 cell line still presented even significant enhanced maturation status, both phenotypically and functionally. Upregulation of co-stimulatory molecules, MHC molecules, and increased secretion of several cytokines and chemokines related to T cell migration and differentiation were observed in the EKO Sp37A3 DC line in mRNA level and protein level. Since most of these upregulated proteins are reported to involve in DC migration, antigen presentation and education of T cell polarization, we find the necessity to analyze the relative functions of DCs, and DC / T cell crosstalk.

As DC antigen presentation procedure contains antigen capture, antigen process and antigen present, we first tested the antigen uptake capacity of the Siglec-E sufficient and deficient Sp37A3 DCs. We compared the capacity of endocytosis with soluble antigen, cell-associated antigens and live tumor cells by EKO and CtrV Sp37A3 DCs, without finding any significant difference. Then, we tried to harness DQ-OVA as the indicator of antigen processing, and it appeared that Siglec-E deficiency on DCs increased their antigen processing. Previously inhibitory role of mouse Siglec-G has been reported to impede MHC:pep complex formation and further negatively influenced antigen cross presentation to CD8+ T cells. Moreover, Siglec-G knockout BMDC vaccines bearing tumor-associated antigen showed better tumor control *in vivo* (Ding et al, 2016). Now that we did not observe increased levels of Siglec-G from Ti-cDCs, we assumed the pronounced expression of Siglec-E might influence DC antigen presentation in a similar manner.

EKO Sp37A3 DCs showed elevated levels of hallmarks of T cell priming Signal 1 (MHC-I/MHC-II), Signal 2 (CD80/CD86) and multiple cytokines and chemokines reported to correlate with T cell trafficking, activation and helper T cells polarization, suggesting possible regulation pathways during the crosstalk of DCs and T cells. Thus, we next focused on identifying whether the difference of antigen processing further leads to difference of antigen presentation to T

cells. We took advantages of the OVA and OT-I / OT-II system to test naive T cell activation. Strikingly, for OT-II cells, OVA-pulsed EKO Sp37A3 DCs induce much stronger activation and proliferation compared to co-culture with the CtrV Sp37A3 DCs. However, we did not observe any difference on OT-I activation, either pulse DCs with soluble OVA or with cell-associated OVA antigen. These discoveries indicated that loss of inhibitory Siglec-E on DCs facilitates antigen-specific CD4+ T cells priming, without affecting CD8+ T cells activation. These findings are interesting as previous works on CD4+ T cell functions in antitumor immunity are mostly focused on the suppressive Treg population, while the conventional CD4+ T cells functions during carcinogenesis were highly underappreciated. Only until recently, Prof. Krummel's group demonstrated that migratory cDC2 population is vital in conventional CD4+ T cell activation and acquisition of Type 1 helper T cell (Th1)-like phenotype. However, migratory cDC2 functions are suppressed by TME Tregs, while TME local Treg depletion unleashed the cDC2 functional potential, which resulted in CD4+ T cell-dependent tumor rejection (Binnewies et al, 2019). Same year, another important discovery by Prof. Schreiber's group pointed out the key role of CD4+ T cell help in mice respond to ICI therapy. MHC-II-restricted neoantigen presentation to was emphasized, regarding to the CD4+ T cells help both in CD8+ priming and cytotoxicity maturation (Alspach et al, 2019). Another recent research from Prof. Murphy's lab showed surprising new findings that early CD4+ T cell priming requires cDC1s in cancer. This cDC1 and CD4+ T cell crosstalk in tumor is dependent on MHC-II and CD40 signaling pathway on cDC1s (Ferris et al, 2020). With the newly reported tamoxifen-inducible ThPOK$^{CreERT2.hCD2}$ line to specifically target conventional CD4+ T cells without affecting CD8+ T cells and Tregs, CD4+ T cell research will foreseeably be more feasible (Andrews et al, 2021).

Finally, in order to confirm whether our finding of Siglec-E functions on DCs is also reflecting a similar role of Siglec-9, we generated BMDCs from human

Siglec-9 transgenic mice and co-cultured with antigen-specific T cells. Impeded CD4+ T cell activation in Siglec-9-expressing BMDC group during co-culture is confirmed. Taken together, expression of the inhibitory Siglec-E, and its human functional paralog Siglec-9, on DCs suppresses DCs antigen presentation to CD4+ T cells. These results revealed the highly identical role of these homologous inhibitory Siglecs in regulating DCs functions. Future research could further focus on regulation of DC and CD4+ T cell crosstalk, especially the immunological synapse formation and stability, through the sialoglycan – Siglec axis. One recent work revealed desialylation of DCs prolonged the half-life of DC surface MHC-I molecules, result in increased immunological synapse stability with autologous T cells (Silva et al, 2020).

To summarize, in our project, we identified the expression patterns of several human and mouse inhibitory Siglecs on Ti-cDC subsets. Among them, human Siglec-9 and its functional paralog murine Siglec-E both expressed by high frequencies of cDCs. These observations caught our interest to investigate possible regulation pathways of cDC functions through the sialoglycan - Siglec axis. Through mouse DC cell lines and BMDCs, we discovered the underappreciated DC and CD4+ T cell crosstalk was hampered by inhibitory Siglecs expression. However, we still have not figured out deeper mechanisms of which signaling pathway or network is involved in regulating the antigen presentation through inhibitory Siglecs. Despite that, our work still shed light on finetuning DC and T cell crosstalk and designing of new therapeutic approaches aiming to unleash this suppressed potential of DC functions.

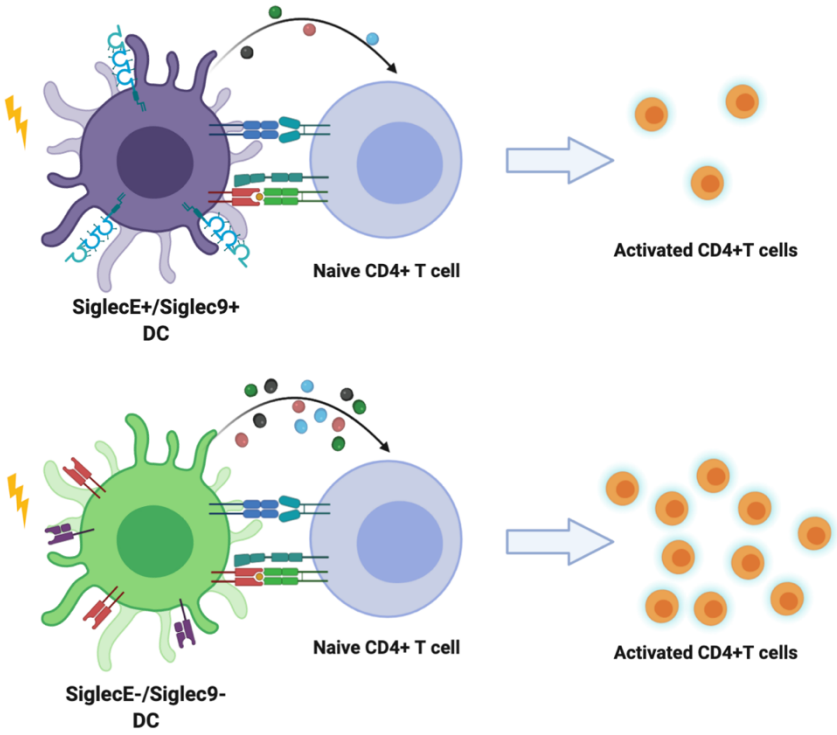

Figure 5.1 Graphical summary. *Hypersialylation is one of the hallmarks of cancer cells. Sialoglycan - Siglec axis has previously been reported to regulate functions of various immune cell types, including NK cells, neutrophils, macrophages and T cells, during tumor progression. Inhibitory Siglecs are found on tumor-infiltrating DCs. Expression of the inhibitory Siglec-E or Siglec-9 on DCs dampens DC maturation and further negatively modulate CD4+ T cells activation.*

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7. Acknowledgements

First and foremost, I would like to thank my supervisors Prof. Heinz Läubli and Prof. Alfred Zippelius, for their kindness and patience over the last years. Thank you for creating such a nice atmosphere in the lab that everyone enjoys working here. I felt very comfortable during the whole PhD period, for all the support, encouragement and trust you gave me and the freedom of performing my ideas. I am also really grateful for all the scientific guidance, suggestions and inspirations you shared with me, without which I would never make it till today. I am impressed by your passion for career and enthusiasm towards research, which reflect how to become a better person and scientist. I would always keep in mind of all the good qualities I learned from you. Especially, I owe a delayed apology to Heinz, that I was lost and did not achieve much progress in the first two years of PhD. Thank you so much for putting up with me and pushing me back to the right track on time.

I would particularly thank all my PhD committee members, Prof. Gerhard Christofori and Prof. Stephan von Gunten, for always willing to spend time for me to discuss about my project and provide precious inputs. All of your kind advice provides us the opportunity to think out of the box, which leads to the current accomplishments of this project. Special thanks to Prof. Daniel Ricklin for kindly admit to chair the final defense of my project in short notice, and thanks to all of my committee professors for accompanying me until the last mile.

I want to express my gratefulness to all of the previous and present members of Cancer Immunotherapy Lab and Cancer Immunology Lab, for all the help and support in my PhD career and life ever since the day when I come to visit the

lab for the first time. Particularly thanks to Dr. Michal Stanczak, Dr. Marcel Trefny, Dr. Abhishek Kashyap, Dr. Anne Bärenwaldt, Dr. Nicole Kirchhammer, Dr. Natalia Rodrigues Mantuano, Dr. Michela Manni, Laura Fernandez Rodriguez, Philip Schmassmann, Mélanie Buchi, Béatrice Dolder, Petra Herzig, Claudia Gärtner-Pelham, Dr. Yang Liu, Reto Ritzchard and Dr. Marta Trüb, for all the scientific suggestions and technical support you kindly provided in this project. Last but not least, thanks to all our lab members for maintaining such a comfortable working atmosphere. It was a great pleasure to know and work with every one of you over the last four years.

Thanks to the stuff of DBM flow cytometry team, DBM animal facility for the platform and service you kindly provide us. Thanks to Dr. Panagiotis Tsapogas, Prof. Tsuneyasu Kaisho, Prof. Christoph Hess, Dr. Jonas Lötscher, Philippe Dehio, Dr. Marianne Dölz, Edit Horvath, Dr. Ruize Gao and Dr. Qinmei Yang for providing us the reagents or animals in this study. Thanks to Dr. Robert Ivanek's group and Philippe Demougin for helping us perform and analyse the bulk RNA sequencing results.

Last but not least, I would like to show my love and gratitude for my family members, especially my grandparents, my parents, Siqi Wang and our pet kitten Sunny for their understanding, love, encouragement and hope for me, even though I cannot come back to visit you for long time. Without any of you, I would not be the same person as I am today. I would again thank my previous supervisor Prof. Rui Sun and Prof. Zhigang Tian, for guiding me to the field of immunology and teaching me the basic rules of being a scientist with curiosity and integrity.

List of abbreviations

ACT	Adoptive cell transfer
ALL	Acute lymphoblastic leukemia
ALR	AIM2-like receptor
APC	Antigen presenting cell
BMDC	Bone marrow-derived DC
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
$CD11c^{cre}SigE^{fl}$	Dendritic cell conditional Siglec-E knockout mouse
$CD11c^{wt}SigE^{fl}$	Siglec-E-expressing littlemates for $CD11c^{cre}SigE^{fl}$ mice
cDC	Conventional/Classic dendritic cell
cDC1	Type 1 cDC
cDC2	Type 2 cDC
CDP	Common DC precursor
CLL	Chronic lymphoid leukemia
CLR	C-type lectin receptor
CRC	Colorectal cancer
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T lymphocyte antigen 4
CtrV & EKO	Control vector-tranduced & Siglec-E knockout Sp37A3
DC	Dendritic cell
dLN	Draining lymph node
ECM	Extracellular matrix
EOC	Epithelial ovarian cancer
FDA	Food and Drug Administration
FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	Fluorescence-minus-one

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	GeneSet Enrichment Assay
HSCT	Hematopoietic stem cell transplantation
ICI	Immune checkpoint inhibitor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
irAE	Immune-related adverse event
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LPS	Lipopolysaccharide
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MHC:pep	MHC:peptide
NK	Nature killer cell
NLR	NOD-like receptor
NSCLC	Non-small cell lung cancer
OVA	Ovalbumin
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
RLR	RIG-I-like receptor
ROS	Reactive oxygen species
SA	Sialic acid
SAMP	Self-associated molecular pattern

Siglec	Sialic acid-binding immunoglobulin-like lectin
SigEKO	Siglec-E systemic knockout mouse
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
Tconv	Conventional T cell
TCR	T cell receptor
Td-DAMP	Tumor-derived danger-associated molecular pattern
Th	CD4+ T helper cell
Ti-cDC	Tumor-inflitrating conventional DC
TiDC	Tumor-infiltrating dendritic cells
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
WT	Wildtype

Curriculum Vitae

Name: Jinyu Wang
E-mail: jinyu.wang@unibas.ch
Tel.: +41 78 884 38 92
Nationality: China
Date of birth: 1992/05/10
Address: Hebelstrasse 20, Basel, Switzerland

EDUCATION BACKGROUND

- **B.Sc. Degree** (Aug.2010 - Jun.2014)
  University of Science and Technology of China
  School of Life Sciences
  Major: Biological Science
- **M.Sc. Degree** (Jul.2014 - Nov.2016)
  University of Science and Technology of China
  School of Life Sciences
  Major: Cell Biology
- **Doctoral Degree** (May.2017 – Dec.2021)
  University of Basel
  Department of Biomedicine
  Major: Cancer Immunology

LANGUAGE SKILLS

- Chinese (simplified, Mandarin): native
- English: fluent

EXPERIMENTAL SKILLS

Animal tumor models / Cell culture / Flow cytometry / Western blot / Immunohistochemistry / qRT-PCR / Genomic Editing / …

RESEARCH INTERESTS

- Immune regulation network in the tumor microenvironment.
- Antigen presenting cell dysfunction and differential polarization during tumor progression.
- Inducing myeloid cell polarization plasticity potential to improve host antitumor immunity.

RESEARCH EXPERIENCE

- **Liver Immunology and Cancer Dec. 2012-Jan. 2017**
  Institute of Immunology, USTC
  Research Supervisor: Prof. Rui Sun & Prof. Zhigang Tian
  1. Constructed patient-derived xenograft model of human hepatocellular carcinoma.
  2. Sorafenib treatment in the patient-derived xenograft model.
  3. NK cell-based adoptive cell therapy in the patient-derived xenograft model.

- **Cancer Immunology May. 2017- present**
  Department of Biomedicine, University of Basel
  Research Supervisor: Prof. Heinz Läubli, Prof. Alfred Zippelius & Prof. Gerhard Christofori
  1. Generating Siglec conditional knock-out mouse model and Siglec knock-out dendritic cell(DC) cell line for in vivo and in vitro studies.
  2. Using different mouse tumor models to study the influences of tumor microenvironment hypersialylation on DC antigen presentation function.
  3. Using in vitro DC cell lines and bone marrow-derived DCs to study the Siglec expression changes on DCs, and how they affect DC and T cell crosstalk.

CONFERENCE PRESENTATIONS AND AWARDS

1. 2019 Society for Glycobiology (SFG) Annual Meeting. 'Dissecting dendritic cell sialic acid-mediated interactions in antitumor immunity.' [Poster & Travel Award]
2. 34th Conference of the European Macrophage and Dendritic cell Society (EMDS). 'Expression of Siglec-E on dendritic cells induces cell dysfunction and facilitates cancer progression.' [Poster]

PUBLICATIONS

1. Sun, C., Xu, J., Song, J., Liu, C., **Wang, J.,** Weng, C., ... & Tian, Z. (2015). The predictive value of centre tumour CD8+ T cells in patients with hepatocellular carcinoma: comparison with Immunoscore. *Oncotarget*, *6*(34), 35602.
2. Sun, C., Xu, J., Huang, Q., Huang, M., Wen, H., Zhang, C., **Wang, J.,** ... & Tian, Z. (2017). High NKG2A expression contributes to NK cell

exhaustion and predicts a poor prognosis of patients with liver cancer. *Oncoimmunology*, *6*(1), e1264562.

3. Sun, C., Lan, P., Han, Q., Huang, M., Zhang, Z., Xu, G., Song, J., **Wang, J.,** ... & Tian, Z. (2018). Oncofetal gene SALL4 reactivation by hepatitis B virus counteracts miR-200c in PD-L1-induced T cell exhaustion. *Nature communications*, *9*(1), 1-17.
4. Wu, Y., **Wang, J.,** Zheng, X., Chen, Y., Huang, M., Huang, Q., ... & Sun, C. (2020). Establishment and preclinical therapy of patient-derived hepatocellular carcinoma xenograft model. *Immunology letters*, *223*, 33-43.
5. Stanczak, M. A., Mantuano, N. R., Kirchhammer, N., Sanin, D. E., **Wang, J.,** Trefny, M. P., ... & Läubli, H. (2021). Targeting cancer glycosylation repolarizes tumor-associated macrophages allowing effective immune checkpoint blockade. *bioRxiv*.
6. **Wang J.,** Manni M., Bärenwaldt A., Wieboldt R., … & Läubli H. (2021) Siglec receptors modulate dendritic cell activation and antigen presentation to T cells in cancer. **[*in revision*]**