Anti CLEC2D-TLR9 agonist conjugate binds to and internalizes CLEC2D on myeloid cells, plasmacytoid Dendritic cells and B cells leading to robust TLR pathway activation and inflammatory cytokine production

Poster #1131

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Background

Current immunotherapies fail to provide benefit to tumors poorly infiltrated with T and NK cells. However, activation of myeloid cells and B cells can lead to recruitment of functional T and NK cells turning the "cold" tumor microenvironment (TME) into "hot". CLEC2D is broadly expressed on germinal center B cells, activated primary plasmacytoid DCs (pDC) and tumor associated macrophages (TAMs)[1] (**Fig.1**). Upon internalization, CLEC2D acts as a vehicle to deliver histone/CpG complexes to endosomal Toll Like Receptor 9/ TLR9, stimulating an inflammatory response[2]. Furthermore, CLEC2D is the ligand for CD161 which is an immune checkpoint expressed on both T and NK cells, making it a target for additional immunotherapeutic intervention.

Immunitas has developed a novel ISAC (Immune-stimulatory antibody conjugate) that targets CLEC2D protein and delivers CpG/TLR9 agonist (**Fig.2**). This CLEC2D-TLR9 ISAC is a dual-mechanism therapeutic:

1. Activates myeloid and B cells to turn "cold" tumor into hot, recruiting T cells/NK cells
2. Blockade of CLEC2D binding to CD161 unleashes the activation of infiltrating CD161+ T/NK cells

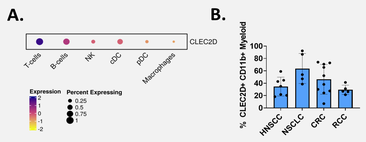

Figure 1. CLEC2D is highly expressed in tumor infiltrating myeloid and B cells. (**A**) scRNAseq of ovarian tumor samples show CLEC2D expression on B-Cells, cDCs, pDCs relevant for TLR9 Activation. (**B**) CLEC2D is consistently expressed in the Tumor-Infiltrating Myeloid cells across multiple solid tumors by flow cytometry.

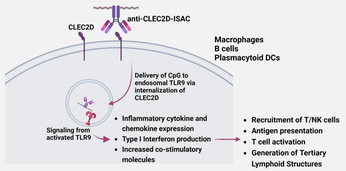

Figure 2[A]. CLEC2D Internalization as the Delivery Mechanism for TLR9 Agonist (CpG) resulting in activation and recruitment of T and NK cells in TME

Generation of CLEC2D-CpG-ISAC (Immune-Stimulatory Antibody Conjugate)

Immunitas has developed highly specific antibodies to human CLEC2D. The antibody selected can bind to CLEC2D expressing engineered cell line with high affinity at an EC50 of 0.68 nM (**Fig.3A**). It blocks interaction of CLEC2D to its soluble CD161, its ligand at an IC50 of 1.04nM (**Fig.3B**). To assess internalization by the anti CLEC2D antibody, the antibody is labeled with pH sensitive dye that fluoresce in the low pH of lysosomes indicating intracellular internalization (**Fig.3C**).

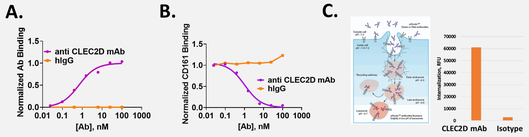

Figure 3. Immunitas antibody binds to CLEC2D with high affinity (**A**), blocks CD161/CLEC2D interaction (**B**), and internalizes on CLEC2D+ cell lines (**C**) making this an ideal candidate for generation of TLR9 agonist conjugate

To generate ISAC i.e. Antibody conjugated to CpG DNA, a transglutaminase-based conjugation method was adopted. The antibody was first deglycosylated with PNGase F to expose the Q295 site. Then, an amine-PEG-azide linker was conjugated to Q295 site-specifically with microbial transglutaminase (mTGase). This was followed by a click chemistry reaction between the azide group and DBCO-CpG oligo, leading to the final product with 2 oligos per antibody molecule. The molecule used in this study has an average of ~2.5 CpG per molecule or 2.5 OAR (Oligo Antibody Ratio).

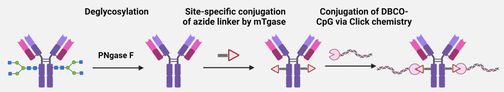

Figure 4A. Transglutaminase based conjugation of CpG to anti-CLEC2D antibody.

CLEC2D-ISAC can deliver CpG to Myeloid cells and activate TLR9 pathway: Activation of IRF and NFκB signaling

TLR9 activation triggers the production of pro-inflammatory cytokines and type I interferons through the NFκb and IRF pathways, respectively.

THP-1 dual reporter cell line when treated with TLR9 agonists leads to activation of both NFκB and IRF signaling pathways that can be detected by respective reporters. Treatment of this cell line with 100nM of CLEC2D–ISAC molecule activated TLR9 dependent NFKB (**Fig.5A**) and IRF (**Fig.5B**) pathways. Conversely, both CLEC2D antibody alone and CpG alone did not lead to activation of TLR9.

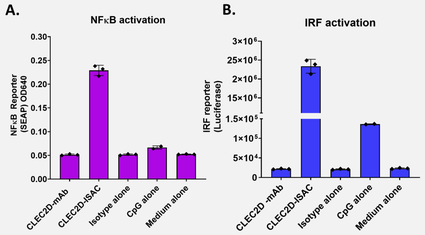

Figure 5. NFkb (**A**) and IRF (**B**) pathways are activated upon CLEC2D-ISAC treatment of THP1-TLR9 Dual reporter cell line.

CLEC2D-ISAC treatment leads to B cell proliferation an enhanced expression of co-stimulatory molecules

B cells play a critical role in anti tumor immunity. Activation of B cells leads to potent cellular and humoral immune responses to cancer. They are Antigen presenting cells that capture and present tumor antigens. B cells also promote tertiary lymphoid structures (TLS) that drive B and T cell interactions and primes effective anti-tumor response.

We have observed that stimulated B cells express high levels of CLEC2D (**Fig.6A**). When these cells are treated with CLEC2D-ISAC, it leads to B cell proliferation as shown by increased %KI67+ B cells (**Fig. 6B**). Furthermore, CLEC2D-ISAC treatment leads to enhanced surface expression of co-stimulatory molecules including CD86, HLA-DR and CD40 (**Fig. 6C**). Overall, CLEC2D-ISAC stimulation leads to sustained activation of B cells which can be highly beneficial for anti tumor immune response.

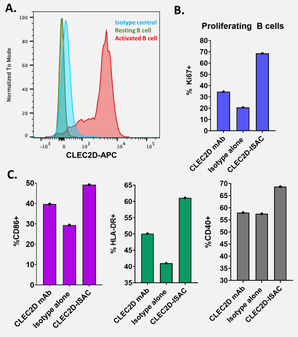

Figure 6. Stimulated B cells express high levels of CLEC2D (**A**) and CLEC2D-ISAC Treatment Leads to Enhanced B cell proliferation (**B**), and expression of co-stimulatory molecules (**C**).

CLEC2D-ISAC treatment leads to enhanced Type I IFN production by plasmacytoid DCs

Plasmacytoid DCs (pDCs) are one of the highest expressers of TLR9. Activation of the TLR9 pathway in pDCs leads to production of Type I IFNs which are critical for initiating anti tumor immunity.

CLEC2D is highly expressed on fully matured human plasmacytoid Dendritic cells (**Fig. 7A**) There is a dramatic increase in production of IFN-α by pDCs through activation by CLEC2D-ISAC molecule, in comparison to free CpG and antibody alone (**Fig. 7B**).

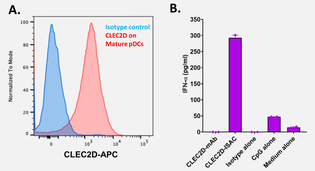

Figure 7. High expression of CLEC2D on pDCs (**A**) leads to stronger uptake of CLEC2D-ISAC by pDCs resulting in Type I IFN production (**B**)

Treatment of inhibitory macrophages with CLEC2D-CpG-ISAC Restores T-Cell Proliferation

Macrophages are the most predominant and immunosuppressive immune cell population present in most solid tumors. Ability to reprogram them could be highly beneficial for immunotherapy non-responders.

CLEC2D is highly expressed on anti-inflammatory macrophages as compared to immune-activating M1 like inflammatory macrophages (**Fig.8A**).Treatment of *in vitro* generated and highly anti-inflammatory TAMs (Tumor Associated Macrophages) with CLEC2D-ISAC result in reversal of inhibition of T cell proliferation (**Fig.8B**) and both IFN-γ (**Fig.8C**) and Granzyme B (GzmB) (**Fig.8D**) production. This suggests that CLEC2D ISAC treatment of TAMs can reprogram them to become T cell activating.

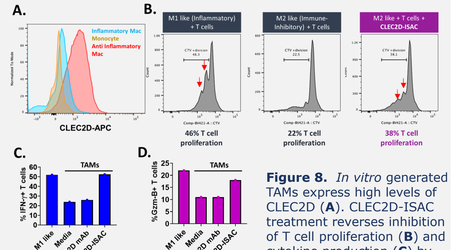

Figure 8. *In vitro* generated TAMs express high levels of CLEC2D (**A**). CLEC2D-ISAC treatment reverses inhibition of T cell proliferation (**B**) and cytokine production (**C**) by in vitro generated TAMs

Favorable Preliminary Safety Profile of CLEC2D-ISAC in Cytokine release assay using normal donor PBMCs

Early safety assessment of the ISAC molecules in human PBMCs shows that these molecules do not lead to release of inflammatory cytokines that cause Cytokine Release Syndrome such as IL-6, IFN-γ, IL-2, TNF-α, IL-1β and GM-CSF.

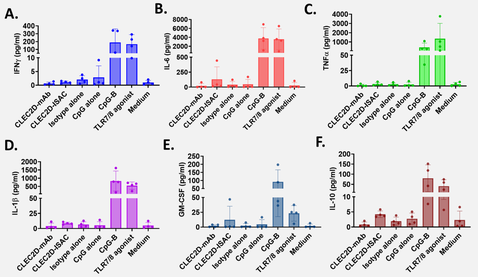

Figure 9. Unstimulated PBMC from four independent healthy donors were cultured for 48h in presence of 100nM CLEC2D-ISAC and control stimulants. Supernatants were quantified for cytokine production by MSD

Discussion

- Immunitas has developed fully human antibodies to CLEC2D that bind with high affinity and can internalize on CLEC2D+ cell lines and primary immune cells.
- CLEC2D-ISAC molecules can activate TLR9 pathway, and we have Proof of Concept (POC) for activation of anti-inflammatory myeloid cells and B cell by CLEC2D-ISAC enabling induction of sustained T cell immunity.
- Early safety assessment of the CLEC2D-ISAC molecules in human PBMCs shows that these molecules do not lead to release of inflammatory cytokines
- Ongoing plans to assess anti tumor efficacy of the CLEC2D-ISAC molecules in humanized mouse models with enhanced myeloid infiltration
- We believe this highly differentiated approach can lead to TME modulation resulting in activation of T and NK cells resulting in anti tumor benefit in patients that otherwise do not respond to immunotherapy.

1. Braud V et al: LLT1-CD161 interaction in Cancer: Promises and Challenges: Front Immunol (2022): Volume 13; https://doi.org/10.3389/fimmu.2022.847576
2. Jiann-Jyh Lai, et.al: Immune Sensing of Cell Death through Recognition of Histone Sequences by C-Type Lectin-Receptor-2d Causes Inflammation and Tissue Injury. Immunity (2020): 52, 123–135.
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