Blockade of Siglec/Sialoglycan Axis Using *EAGLE* Technology to Potentiate Anticancer Immunity

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Introduction

The glyco-immune checkpoint (Siglec/Sialoglycan) axis has recently emerged as a new mechanism of cancer immune evasion. We have previously described an antibody-sialidase fusion platform technology named *EAGLE* (Enzyme-Antibody Glyco-Ligand Editing), using a bacterial sialidase for proof-of-concept studies, for glyco-immune checkpoint blockade. Since the bacterial sialidase poses immunogenicity concerns, we engineered and optimized a human sialidase to overcome its low expression and poor stability issues. We confirmed the antitumor activity of human sialidase-based *EAGLE* and identified predictive and correlative pharmacodynamic (PD) biomarkers to *EAGLE* treatment in preclinical tumor models. *EAGLE* with engineered human sialidase offers an effective immunomodulatory modality to overcome resistance to current immunotherapies by releasing immunosuppression of Siglec/Sialoglycan axis in the tumor microenvironment.

Glyco-Immune Checkpoints Suppress Innate and Adaptive Immunity

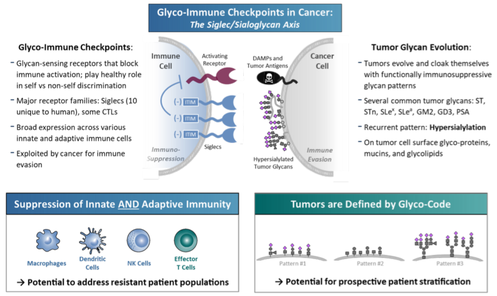
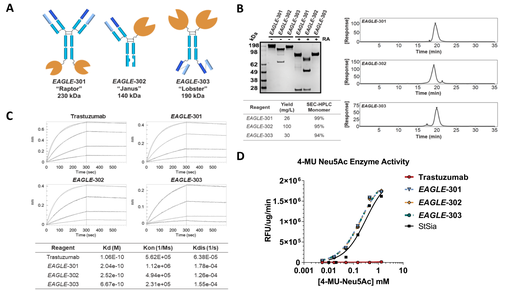


Figure 1. Schematic representation of glyco-immune checkpoints axis. At the immunological synapse, Siglecs (sialic acid-binding Ig-like lectins) on immune cells interact with tumor associated sialoglycans and recruit SHP phosphatases, dampening immune responses. Siglecs are expressed on macrophages, monocytes, DC, and NK cells, recognizing a variety of tumor associated glycans which contain a terminal sialic acid. The Siglec/Sialoglycan plays important roles in cancer cell killing by NK cells and macrophages, cancer antigen presentation, T cell priming and activation, and cancer cell killing by T effector cells.

Results

Construction of *EAGLEs* in Various Configurations (Enzyme-Antibody Glyco-Ligand Editing)



Reagent	Kd (M)	Kon (1/Ms)	Kdis (1/s)
Trastuzumab	1.06E-10	5.62E+05	6.38E-05
EAGLE-301	2.04e-10	1.12e+05	1.78e-04
EAGLE-302	2.52e-10	4.94e+05	1.26e-04
EAGLE-303	6.67e-10	2.31e+05	1.55e-04

Figure 2. Construction and characterization of *EAGLEs* in multiple configurations. "Janus" configuration has the highest expression yield. **A)** Configuration of *EAGLE* molecules. Estimated molecular weight of monomeric *EAGLE* molecule is indicated. **B)** SDS-PAGE and SEC-HPLC of purified *EAGLE* molecules. **C)** HER2 antigen binding of *EAGLE* molecules compared with Trastuzumab. **D)** Sialidase enzyme activity of *EAGLE* molecules.

Desialylation of Tumor Cells and Immunomodulation by *EAGLE*

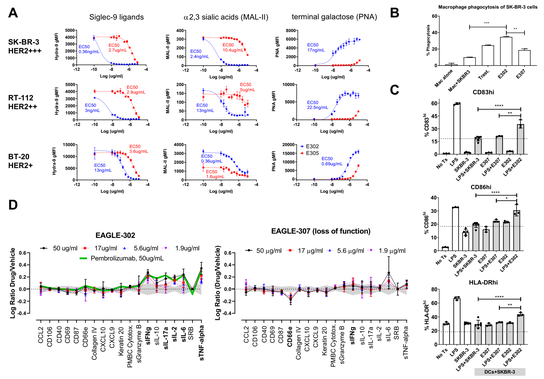


Figure 3. *EAGLE*-HER2 with bacterial sialidase demonstrates tumor desialylation and immunomodulation **A)** Cell surface desialylation experiments on cancer cells with varying levels of HER2 expression. E302 consists of a HER2-targeting trastuzumab arm, while E305 contains a dummy arm. **B)** BioMAP VascHT29 phenotypic profiling. Shaded grey envelope represents 95% significance envelope generated from historical vehicle controls. Bolded cytokines are significant at p<0.05 level. Bolded green line represents profile for Pembrolizumab at 50ug/mL. **C)** E302 treatment of SKBR-3 cells increases macrophage phagocytosis by tumor cell desialylation. Pre-treated SKBR-3 cells were tested in macrophage phagocytosis assay and analyzed for increased phagocytosis after tumor desialylation. **D)** E302 treatment of SKBR-3 cells leads to enhanced DC activation/maturation by tumor cell desialylation. DC activation/maturation was assessed by frequencies of CD83hi, CD86hi, and HLA-DRhi DCs. Data representative of 4 independent experiments.

Multiple *EAGLE* Formats Show Single Agent Complete Regressions in Preclinical Tumor Models

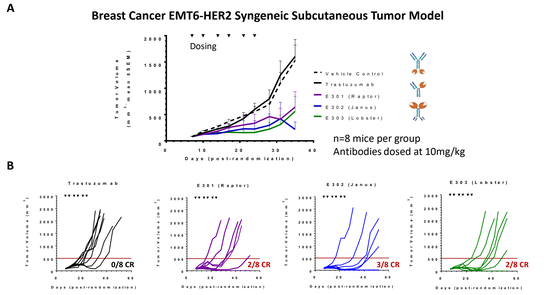
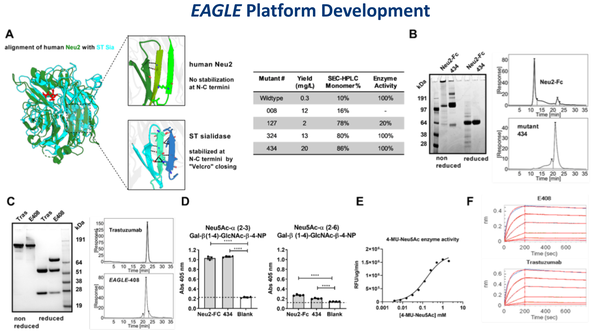


Figure 4. Efficacy studies of *EAGLEs* in syngeneic breast cancer EMT6-HER2 tumor model. Wild type BALB/c mice (n = 8 per group) were injected s.c. EMT6-HER2 cells. When tumor sizes reached about ~120 mm^3, mice were treated with *EAGLE*-301, 302, 303, Trastuzumab, or vehicle control twice a week for 3 weeks at 10mg/kg. The tumor growth was measured twice a week. **A)** Tumor growth curves of mean tumor sizes of each group. **B)** Tumor growth curves of individual mice.

Engineering of Human Sialidase for Improved Developability for *EAGLE* Platform Development



Mutant #	Yield (mg/L)	SEC-HPLC Monomer%	Enzyme Activity
Wildtype	0.3	10%	100%
008	12	16%	-
127	2	78%	20%
324	13	80%	100%
434	20	86%	100%

Figure 5. *EAGLE*-HER2 (E408) with engineered human sialidase as a therapeutic drug candidate. **A)** Structural comparison of bacterial Salmonella Typhimurium LT2 sialidase (ST Sia) versus human Neu2. Inset panels show the zoom-in view of the N- and C- termini. Table, Select Neu2 mutants demonstrated improved expression and reduced aggregation compared to wildtype Neu2. **B)** SDS-PAGE and SEC-HPLC comparison of wildtype Neu2-Fc versus optimized mutant 434. **C)** SDS-PAGE and SEC-HPLC comparison of *EAGLE*-408 versus Trastuzumab **D)** Substrate specificity of Neu2 and mutant 434 towards Sia-α(2-3/6)-Gal assayed by colorimetry using Neu5Ac-α(2-3)-Gal-β(1-4)-GlcNAc-β-pNP (top, p = <0.0001, N = 4) and Neu5Ac-α(2-6)-Gal-β(1-4)-GlcNAc-β-pNP (bottom, p = <0.0001, N = 4) **E)** Enzymatic activity of *EAGLE*-408 is comparable to enzyme-Fc alone, KM = 150uM **F)** HER2 antigen binding is similar between *EAGLE*-408 and Trastuzumab. KD=0.3nM for *EAGLE*-408 and 0.22nM for Trastuzumab

Human Sialidase-based *EAGLE*-HER2 Demonstrates Strong Efficacy in HER2-Low EMT6 Tumor Model

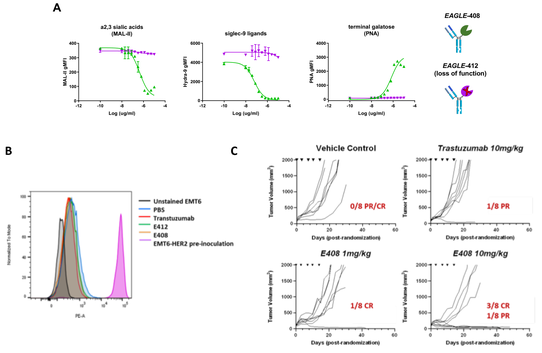


Figure 6. Efficacy studies of human sialidase-based *EAGLE*-HER2 (E408). **A)** Desialylation experiments on HER2-positive breast cancer SKBR-3 cells. **B)** FACS characterization of HER2 expression of EMT6 tumor cells grown *in vivo* and *in vitro*/pre-inoculation) and **C)** *In vivo* efficacy experiments in EMT6-HER2 model. *EAGLE*s and trastuzumab were dosed twice a week for 2 weeks at 10mg/kg.

The Mechanism of Action of *EAGLE* Involves Innate and Adaptive Immunity

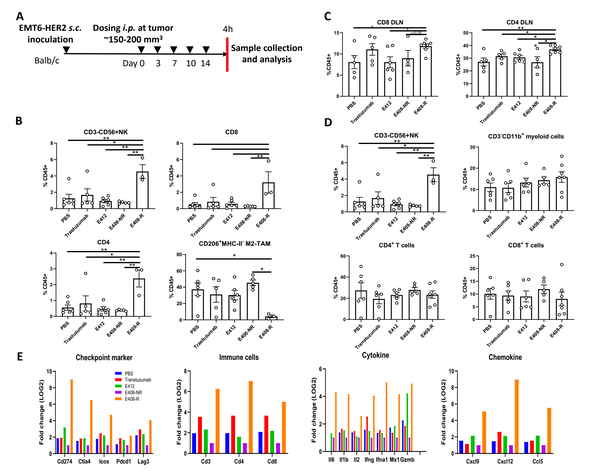


Figure 7. *EAGLE*-HER2 (E408) treatment potentiates innate and adaptive immune response. **A)** Sketch of MoA and pharmacodynamic study design. **B)** Immune cell infiltration in the tumor. **C)** Frequencies of CD4 and CD8 cells in the DLN. **D)** Frequencies of immune cells in the circulation. **E)** Nanostring characterization of gene expression profile in the tumor. (E408-NR: E408 non-responders; E408-R: E408 responders)

EAGLE Demonstrates a Wide Margin of Safety

- The safety of the *EAGLE* platform was evaluated by testing an *EAGLE*-Sialidase molecule, a human sialidase-Fc fusion without a tumor-associated antigen-targeting arm, in rats and cynomolgus monkeys
- *EAGLE-Sialidase* is tolerated up to 100 mg/kg in rats and cynomolgus monkeys
  - 14-day non-GLP toxicity studies, two doses at 10, 50, and 100 mg/kg, q1W, *i.v.* injection
  - No significant toxicity findings in rat and monkey toxicity studies from in-life assessment, clinical chemistry (except for a mild transient elevation of ALP only observed in rats), and histology analysis
  - Observation of pharmacodynamic effects of desialylation of immune cells
- *EAGLE* doesn't cause cytokine release in human PBMC assays

Conclusions

- **Siglec/Sialoglycan axis (glyco-immune checkpoint) plays critical roles in cancer immune evasion**
  - Inhibit innate antitumor immune response
  - Suppress adaptive antitumor immunity
- ***EAGLE* demonstrates immunomodulation and compelling monotherapy efficacy in preclinical tumor models, a wide margin of safety in rats and monkeys, and enhancement of anti-cancer immune responses of NK cells, macrophages, DCs, and T cells in human cell systems mimicking tumor-mediated immunosuppression**
  - Single-agent complete regressions at 1 and 10 mg/kg with immunological memory
  - No-observed-adverse-effect-level (NOAEL) >= 100 mg/kg (two doses) in monkeys with PD effects
  - Potentiation of innate and adaptive anti-tumor responses
- ***EAGLE* offers new opportunities to treat cancer targeting Siglec/Sialoglycan axis**
  - Overcome the heterogeneity challenges of tumor sialoglycans
  - Disable immunosuppressive glycan functions within tumor microenvironment
  - Transform existing tumor targeting mAbs into immune-modulating agents