Toll-like receptor 9 (TLR9) belongs to the family of pattern recognition receptors in the innate immune system and is predominately expressed in human B cells and plasmacytoid dendritic cells (pDCs). CpG oligonucleotides present in specific nucleic acid sequence contexts induce immune responses via stimulation of TLR9.

Novel spherical nucleic acid (SNA) configuration of TLR9 agonist oligonucleotides are designed to trigger innate and adaptive immune responses against tumor cells in cancer patients. SNAs are densely-packed, radially-oriented 3-dimensional arrangements of oligonucleotides surrounding a liposomal nanoparticle. This 3D-architecture increases cellular uptake compared to conventional “linear” oligonucleotides that are not in SNA configuration. SNAs enter cells and localize to endosomes, which is where TLR9 proteins are localized, making SNAs ideal TLR9 agonists.

Immune checkpoints are inhibitory pathways crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses. However, tumors use immune-checkpoint pathways, particularly the PD-1 / PD-L1 pathway, as a major mechanism of immune resistance.

Here, we investigated the ability of TLR9-agonist SNAs to synergize with an anti-PD-1 checkpoint inhibitor to produce long-term, specific anti-tumor immunity.

### RESULTS

#### SNA Is Taken Up More Than Linear Oligonucleotides

- **Figure 1.** Cellular uptake of SNA and linear oligonucleotides. (A) Primary human foreskin keratinocyte (HFK) cells were treated with 100 nM Cy5-labeled oligonucleotide in linear or SNA format. At 24 hours the uptake was assessed by fluorescence microscopy. (B) Healthy human volunteer peripheral blood mononuclear cells (PBMCs) were isolated from whole blood. PBMCs were treated for 24 hours with fluorescein-labeled oligonucleotide in linear or SNA format. Flow cytometry was used to assess the uptake of oligonucleotides, and was performed in the presence of 2 mg/mL trypan blue to quench extracellular fluorescein. Mean ± SEM of N=4 PBMC donors is shown. 2-way RM ANOVA with Holm-Sidak’s multiple comparisons correction -values: ** < 0.01, *** < 0.001, **** < 0.0001.

#### TLR9-Agonist SNA Induces TLR9-Dependent Immunostimulation in Mice

**Figure 2.** Cytokine induction by SNAs in WT and TLR9-KO mice

Male C57BL/6 mice aged 17-18 weeks, either wild-type (WT) or lacking TLR9 (TLR-KO), were injected subcutaneously with 3 mg/kg SNA or vehicle (PBS). At 10 hours post-administration, blood was drawn and processed to serum. Serum cytokine levels were quantified using a multiplex ELISA (Quansys). Mean ± SEM of N=4 mice is shown.

#### TLR9-Agonist SNA Induces Immunostimulation in Non-Human Primates

**Figure 3.** Immunostimulation by SNAs in non-human primates

Cynomolgus macaques were injected subcutaneously with SNA and blood was subsequently drawn for assessment of serum cytokine levels and activation of immune cell subsets. Mean ± SEM of N=4 (2 male and 2 female) macaques per group. (A) Macaques were treated with TLR9-agonist SNA or a non-CpG negative control SNA at 3 mg/kg. Serum cytokine levels were quantified using a Lumixin panel. (B) Macaques were treated with TLR9-agonist SNA or a non-CpG negative control SNA at the indicated dose levels. Flow cytometry was used to quantify changes in immune cell activation at 24 hours post-administration. Activated plasmacytoid dendritic cells (pDCs) were defined as CD3/6/14/20-HLADR+ CD11c+ CD123+ CD86+. Activated B cells were defined as CD3- CD20+ CD86+.

#### TLR9-Agonist SNA Treatment Upregulates PD-1 and PD-L1 Expression in EMT-6 Tumors

**Figure 4.** PD-1 axis expression in EMT-6 tumors

On study day 0, BALB/C mice were subcutaneously inoculated with EMT-6 cells to establish flank tumors. On study day 10 (when tumor volume reached ~ 100 mm³) and study day 18, mice were treated with 1.2 mg/kg SNA or PBS vehicle administered intratumorally, with 10 mg/kg anti-PD-1 antibody administered intraperitoneally, or both. At 9 days after initiating treatment (study day 19), the tumors were removed and the expression of PD-1 and PD-L1 mRNA was assessed using qRT-PCR. mRNA levels were normalized to GAPDH using the 2^-ΔΔCt method.

### CONCLUSIONS

- TLR9-agonist SNAs induced potent, TLR9-dependent TH1-type immune responses in mice and monkeys and increased checkpoint protein expression in the tumor.
- SNA plus anti-PD-1 combination therapy induced tumor-specific immunity and memory.
- These data support the clinical investigation of SNAs in immuno-oncology. One such SNA, AST-008, is undergoing a Phase 1a clinical trial and is planned for testing in cancer patients combined with an anti-PD-1 antibody.