Balanced Diakine™ Targeted Cytokines Localize within the Immunological Synapse and Augment Immune Response in Combination with T Cell Engagers

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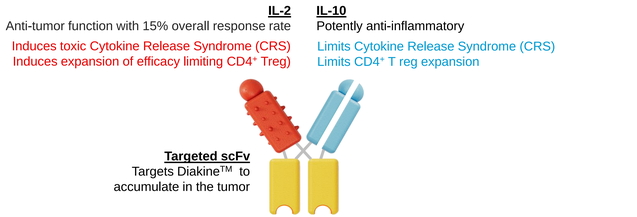
Abstract #951

BACKGROUND

- Diakines™ are a best in class of fusion proteins coupling two complementary cytokines *via* a targeting scFv scaffold that enhances potency and concentration of the molecule to the intended target cells.
- Diakines™ adopt a highly stable antibody-like conformation where the cytokines and the targeting complementarity determining regions oriented to opposite sides of the structure, which enables functional bridging between immune and target cells (**Fig. 1**).
- In pre-clinical studies, targeting DK2^{10} (EGFR) to cell surface receptors permits the concentration and sustained retention of the molecule at the cell surface and ultimately in the tumor microenvironment, resulting in a more effective molecule (**Fig. 3**).
- Initial clinical biomarker proof of concept for DK2^{10} (EGFR), which couples wild-type (“wt”) IL-2 with IL-10 and targets EGFR bearing cells establishes the balanced uncoupling of T cell activation, proliferation, increased peripheral T cell repertoire diversity from IL-2 associated toxicity and Treg expansion.
- This immune response profile has prompted further evaluation of the potential for Diakines™ to combine with T cell engagers (“TCE”).

***FIG. 1* – Structure of DK2^{10} (EGFR): Cytokines and targeting system orient to opposite sides of the three-dimensional structure.**



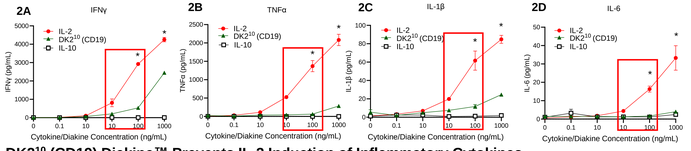
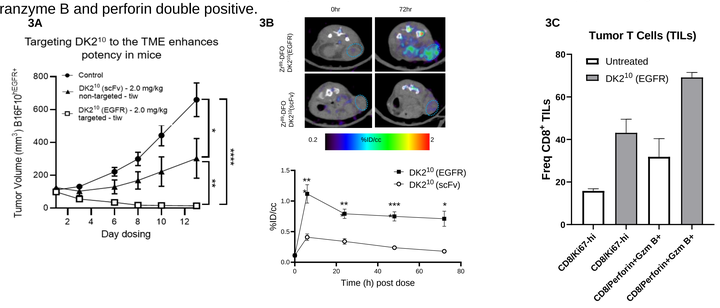


***FIG. 2* - DK2^{10} (CD19) Diakine™ Prevents IL-2 Induction of Inflammatory Cytokines**
Coupling high affinity IL-10 (haIL-10) to wild-type full strength IL-2 blocks IL-2 mediated induction of, IFNγ (**2A**), TNFα (**2B**), IL-1β (**2C**) and IL-6 (**2D**) from human PBMC as measured using multiplex ELISA. Red boxes indicate therapeutic range.

***FIG. 3* – Targeting Improves Function and Tumor Retention.**
(3A) DK2^{10} (EGFR) 2 mg/kg dosing exhibits 100% cures vs. ~50% tumor growth control with non-targeted DK2^{10}. (**3B)** DK2^{10} (EGFR) is retained in the tumor for at least 3 days post administration 1 log higher than non targeted control. 2 mg/kg dose in mice leads to ~400 ng/mL targeted DK2^{10} retention in the TME for **at least 3 days,** which is 2-4-fold higher than levels required for maximal T cell stimulation (*in vitro* is achieved at 100 – 200 ng/mL).
(3C) Tumor Infiltrating Lymphocytes (TILs) from DK2^{10} (EGFR) treated mice exhibited ~2-fold more proliferating CD8$^+$ T cells that were granzyme B and perforin double positive.



METHODS

- The DK2^{10} (CD19), Diakine™ combines wtIL-2 and haIL-10 with a CD19 targeting moiety to deliver immune response directed against B cell targets.
- Healthy donor PBMCs and/or CD8$^+$ T cells were treated with DK2^{10} (CD19), anti-CD20 TCE, or the combination of the Diakine™ and the TCE and co-cultured with CD19$^+$ CD20$^+$ GFP+ Raji tumor cells as targets at an effector to target ratio of 10:1. Cytolysis was assessed by measuring fluorescence signal from target tumor cells using Incucyte live cell imager over 5 days in the presence of Diakine™ treated CD8$^+$ T cells. CD8$^+$ T cells were harvested from culture and reintroduced to fresh tumor cultures at 5-day intervals to measure persisting cytolysis.
- Pro-inflammatory cytokines and checkpoint inhibitory molecules in culture supernatants were quantified by multiplex ELISA. Intracellular cytokines were analyzed using flow cytometry.
- Immune-target cell synapse was visualized using spinning disk confocal microscopy. Effector-target avidity was measured using acoustic force microscopy.

RESULTS

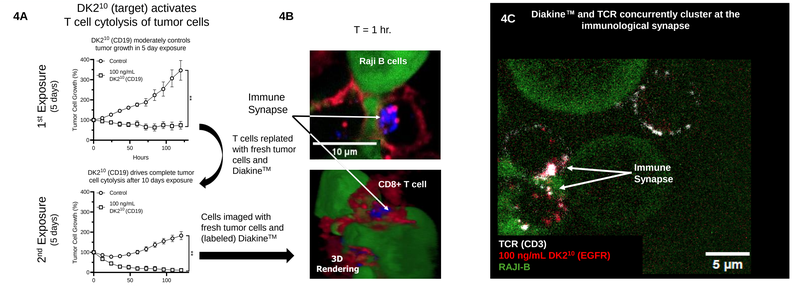


***FIG. 4* – Tumor cell surface targeting of DK2^{10} (CD19) permits sustained cytolysis and sequestration of the Diakine™ into the immune synapse.** A cytolytic assay was performed and assessed by live cell imaging. Immune-target cell synapse was visualized using spinning disk confocal microscopy. **(4A)** CD8$^+$ T cells co-cultured with Raji tumor cells in the presence of DK2^{10} (CD19) lysed/controlled target tumor cells growth over a 5-day period during a first exposure. Following the first exposure, fresh tumor target cells were re-exposed to CD8$^+$ T cells from the 1st exposure, resulting in complete cytolysis of Raji cells *in vitro*. This suggests that continued exposure of CD8$^+$ T cells to DK2^{10} (CD19) maintains CD8$^+$ T cell cytotoxicity as well as preventing activation induced cell death (AICD). The ability of the CD8$^+$ T cells to cytolyze the tumor target cells is accomplished in an MHC I dependent manner (data not shown). **(4B)** Confocal microscopy of a cytolytic event between a CD8$^+$ T-cell and a tumor target Raji cell localizes and clusters DK2^{10} (CD19) to the immunological synapse. **(4C)** Confocal microscopy of a cytolytic event between a CD8$^+$ T-cell and a tumor target Raji cell illustrating the colocalization of TCR and Diakine™ to the synapse.

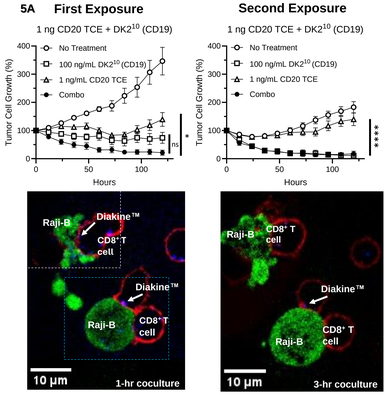
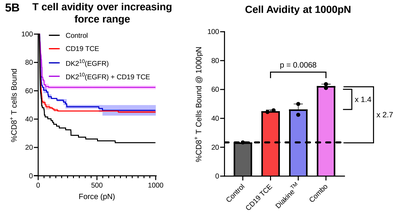


***FIG. 5* – Diakine™ enhances TCE mediated cytotoxicity.**
(5A) Combination of DK2^{10} (CD19) and CD20 TCE enables 10-fold reduction in TCE concentration, while still driving anti-tumor function. The Diakine™ sequesters into the immunological synapse in the presence of TCE. Diakine™ also treatment permits ~2-hr immune synapse. **(5B)** Tumor cell surface targeting of the Diakine™ permits both independent and TCE combinatorial avidity enhancement of CD8$^+$ T cells for tumor cells and interactions exceeding 2 hrs as measured using acoustic force microscopy.



RESULTS

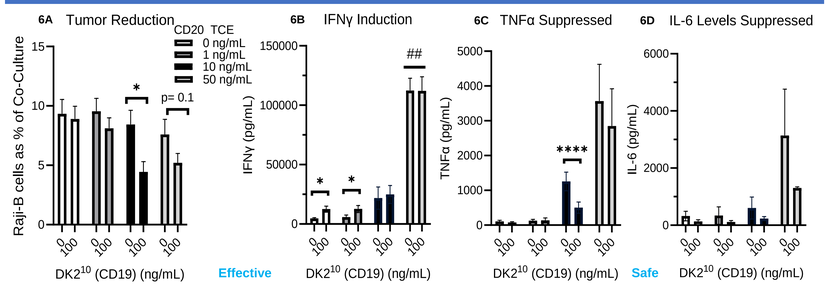
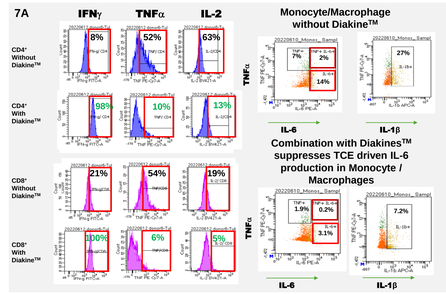


***FIG. 6* – PBMCs treated with Diakine™ and TCE improved anti-tumor function while suppressing hallmark pro-inflammatory cytokines.** The DK2^{10} (CD19), Diakine™ combines wtIL-2 and haIL-10 with a CD19 targeting moiety to deliver immune response directed against B cell targets. PBMC’s were bulk co-cultured with Raji tumor cells at an E:T of 10:1 in the presence of DK2^{10} (CD19) in combination with a CD20 TCE at 0 (white), 1 (gray), 10 (black), and 50 (light gray) ng/mL for 48hrs. Pro-inflammatory cytokines were quantified by multiplex ELISA. Combining Diakine™ treatment with TCE leads an approximate 50% cytolysis of the tumor population **(6A)**, with associated induction of IFNγ levels correlative with anti-tumor efficacy (##: data above upper limit of quantitation) **(6B).** Combining Diakine™ with TCE also leads to the suppression and control of pro-inflammatory cytokines typically associated TCEs while enhancing anti-tumor efficacy **(6C & 6D)**.



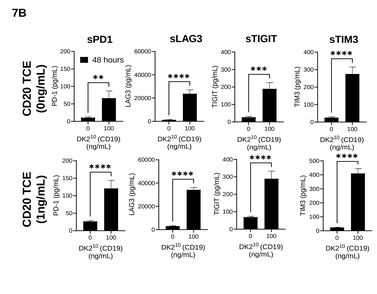


***FIG. 7* – Diakines™ are capable of suppressing TCE mediated pro-inflammatory cytokines. (7A)** Bulk cultures of PBMCs were co-cultured with TCE, tumor cells, and with Diakine™. Diakine™ exposure suppresses intracellular TNFα and IL-2, while enhancing intracellular IFNγ from CD4$^+$ and CD8$^+$ T cells compared to T cells treated only with TCE. Intracellular TNFα, IL-6, and IL-1β is suppressed from antigen presenting cells when exposed to Diakine™, which limits the magnitude of CRS and TCE mediated inflammation. (**7B**) Soluble immune checkpoint markers were measured using multiplex ELISA following second exposure of CD8$^+$ T cells to tumor cells showing significant shedding of sPD-1, sLAG-3, sTIGIT, and sTIM-3 in the presence of Diakine™.

CONCLUSIONS

The Diakine™ platform combines complementary cytokines with a targeting scFv to enrich on the tumor cell surface. Combining IL-2 with IL-10 in the Diakine™ platform creates a molecule that has potent anti-tumor efficacy with a limited CRS response. Indeed, DK2^{10} (CD19) and DK2^{10}(EGFR) enhance the cytolytic function of CD8$^+$ T cells and augment both the duration and avidity of effector-to-target cell interactions while residing in the immunological synapse. *This structure-function relationship of the cytokines and targeting moiety in the Diakine™ platform represents a paradigm shift for how to use cytokines to both safely stimulate the immune system and significantly enhance T cell interactions with tumor cells.* These results provide mechanistic rationale for the Diakine™ platform to be combined with TCEs to potentially improve therapeutic index. Combining the Diakine™ platform with TCEs enhance anti-tumor effect of the TCE, while eliminating the toxicity typically observed with TCE treatments.