Immuno-STATs: Leveraging protein engineering to expand and track antigen-specific T cells *in vivo*

Zeigler S[1], Woodham A[2], Li M[1], Zeyang L[2], Kolifrath S[2], Cheloha R[2], Rashidian M[2], Chaparro R[3], Seidel R[3], Dearling J[2], Mesyngier M[2], O'Connor K[1], Celikgil A[1], Duddempudi P[1], Packard A[2], Garforth S[1], Goldstein H[1], Ploegh H[2], Almo S[1]

[1]Albert Einstein College of Medicine, Bronx, NY, USA; [2]Boston Children's Hospital/Harvard Medical School, Boston, Massachusetts, USA; [3]Cue Biopharma, Cambridge, MA, USA

Background

- Immunotherapies are highly promising and effective strategies for the treatment of cancer; however, continuing challenges persist, including **1)** untargeted global immune modulation, resulting in serious side effects; **2)** lack of therapeutics capable of in vivo expansion of tumor-specific T cells; **3)** inability to visualize in vivo tumor-specific T cell responses; and **4)** lack of flexible platforms to rapidly and efficiently explore new therapeutic strategies and immune-escape mechanisms.
- To address these challenges, we developed a novel class of precision biologics to treat cancer, autoimmune diseases and infectious diseases. We designed a modular platform constructed around an Fc-based covalent pMHC dimer, referred to as **synTac** (artificial synapse for T cell activation; also termed **Immuno-STATs™** for Selective Targeting and Alteration of T cells), which selectively delivers different cargoes, including costimulatory, coinhibitory or cytokine signals and other modalities, to primary T cells of defined specificity.
- Inclusion of costimulatory/immunomodulatory modules (e.g., targeting CD28 and 4-1BB pathways) allows for selective expansion of antigen-specific CD8⁺ T cells *in vitro* and *in vivo*.
- Coupling of positron emission-active nuclei (e.g., ^{64}Cu) enables Immuno-STAT scaffold-based immunoPET/CT to track disease-specific CD8⁺ T cells noninvasively *in vivo*, offering a promising theranostic platform.

Immuno-STAT design

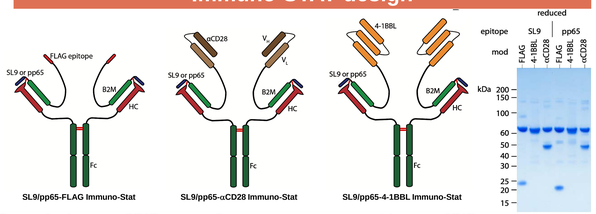


Figure 1 – Immuno-STAT design. Schematic representation of Immuno-STATs showing antigenic peptides (SL9 from HIV gag or pp65 from CMV) covalently attached to β2m, which is tethered to costimulatory domains (MODs: αCD28 scFv, single chain 4-1BBL trimer, or FLAG control). This chain is linked by engineered disulfides to the MHC heavy chain (HLA-A*0201) in the context of a dimeric bivalent Fc-fusion (IgG2a Fc).

SL9 Immuno-STATs selectively activate an SL9-specific CD8⁺ T cell clone

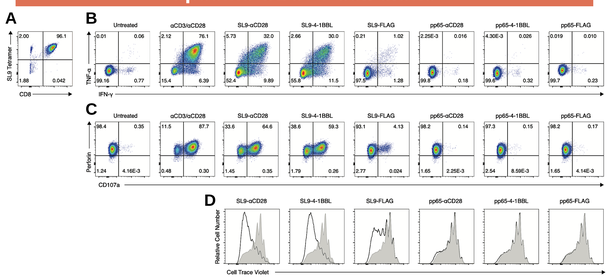


Figure 2 – SL9 Immuno-STATs selectively activate a CD8⁺ T cell clone expressing an SL9-specific TCR. A) SL9 (HIV gag protein) tetramer staining of the SL9-specific CTL. SL9 Immuno-STATs but not the non-cognate PP65 Immuno-STATs activate the SL9-CTL clone to promote **B)** IFN-γ and TNF-α production and **C)** perforin and CD107a degranulation markers. **D)** Only SL9 Immuno-STATs were able to stimulate proliferation of the SL9-CTL clone.

Immuno-STATs selectively stimulate *in vitro* expansion of functional SL9-specific CD8⁺ T cells from HIV⁺ PBMCs

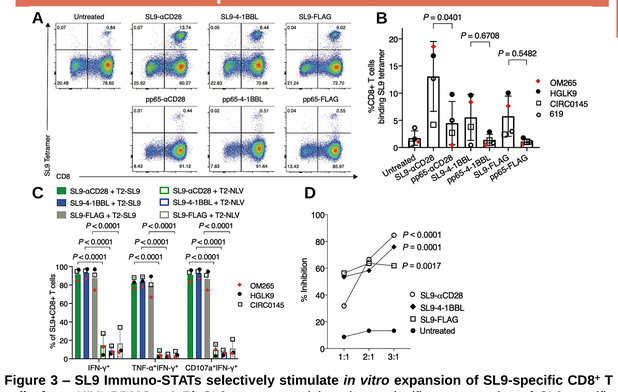


Figure 3 – SL9 Immuno-STATs selectively stimulate *in vitro* expansion of SL9-specific CD8⁺ T cells from HIV⁺ PBMCs. A-B) SL9 tetramer staining shows significant expansion of SL9-specific CD8⁺ T cells across multiple donors only in response to SL9 Immuno-STATs. **C)** Enhanced IFN-γ, CD107a and TNF-α expression was detected from SL9-specific CD8⁺ T cells expanded with Immuno-STATs then restimulated with T2 cells pulsed with SL9 but not PP65 peptides. **D)** SL9-specific CD8⁺ T cells were expanded by SL9 Immuno-STATs then co-cultured with autologous PBMCs infected with an infectious molecular clone of HIV expressing luciferase as a surrogate marker for productive infection. Inhibition of Infection was measured by reduction of luciferase signal.

Selective *in vitro* expansion of PP65-specific CD8⁺ T cells from CMV⁺/HIV⁺ PBMCs

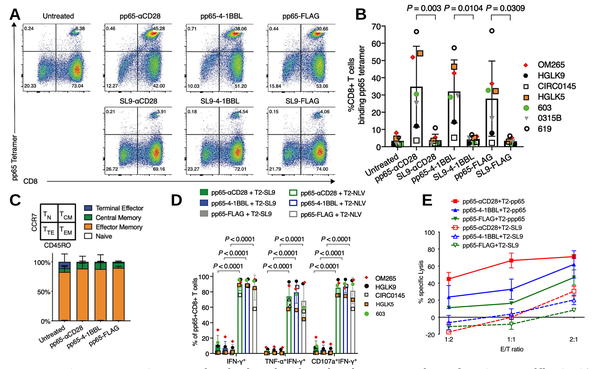


Figure 4 – PP65 Immuno-STATs selectively stimulate *in vitro* expansion of PP65-specific CD8⁺ T cells from CMV⁺/HIV⁺ PBMCs. A-B) PP65 tetramer staining shows significant expansion of PP65-specific CD8⁺ T cells across multiple PBMC donors only when treating with PP65 Immuno-STATs. **C)** Impact of Immuno-STATs on CD8⁺ T cell differentiation. **D)** Enhanced IFN-γ, CD107a and TNF-α expression was detected from PP65-specific CD8⁺ T cells expanded with Immuno-STATs and then restimulated with T2 cells pulsed with PP65 but not SL9 peptides. **E)** Immuno-STAT treatment enhanced cytolytic activity of CD8⁺ T cells against T2 cells pulsed with PP65 peptide.

PP65-specific CD8⁺ T cells prevent CMV infection in fibroblasts after Immuno-STAT treatment and restimulation

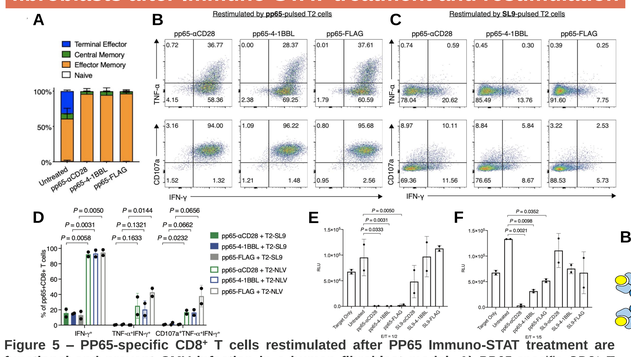


Figure 5 – PP65-specific CD8⁺ T cells restimulated after PP65 Immuno-STAT treatment are functional and prevent CMV infection in a human fibroblast model. A) PP65-specific CD8⁺ T cells were enhanced for effector memory cells following PP65 Immuno-STAT treatment. PP65-specific CD8⁺ T cells were treated with PP65 Immuno-STATs and restimulated by T2 cells loaded with **B)** PP65 or **C)** SL9 peptide showed **D)** significant IFN-γ, CD107a and TNF-α expression only when restimulating with PP65-pulsed T2 cells. **E-F)** PBMCs were treated with Immuno-STATs for 7 days then co-cultured with an HLA-A*0201-restricted human fibroblast line infected with a recombinant Towne strain of HCMV that expresses luciferase, allowing for detection of HCMV infection by luciferase activity. Only PBMCs treated with PP65 Immuno-STATs inhibited HCMV infection.

Immuno-STATs stimulate *in vivo* expansion of PP65-specific CD8⁺ T cells from human PBMCs transferred to mice

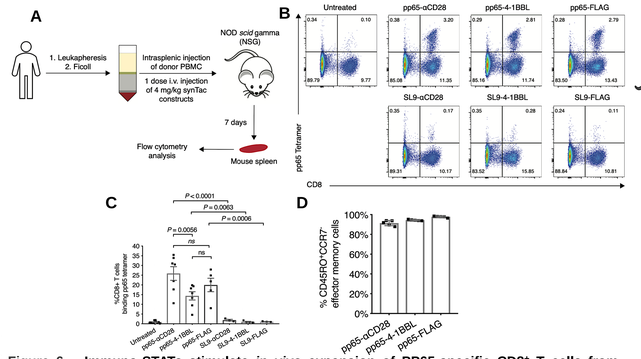


Figure 6 – Immuno-STATs stimulate *in vivo* expansion of PP65-specific CD8⁺ T cells from human PBMCs transferred to mice. A) Experimental setup for *in vivo* expansion studies. NOD *scid IL2Rγ*$^{-}$ (NSG) mice were intrasplenically injected with PBMCs from a CMV⁺/HIV⁻ donor and given one dose (4 mg/kg) of Immuno-STAT before harvesting spleens for flow cytometry. **B)** PP65 tetramer staining shows **C)** significant expansion of PP65-specific CD8⁺ T cells only when treating with PP65 Immuno-STATs. **D)** The majority of expanded PP65-specific CD8⁺ T cells were effector memory cells (CD45O⁺/CCR7⁻).

Immuno-STATs selectively stimulate *in vivo* expansion of CD8⁺ T cells from a CMV⁺/HIV⁺ human donor

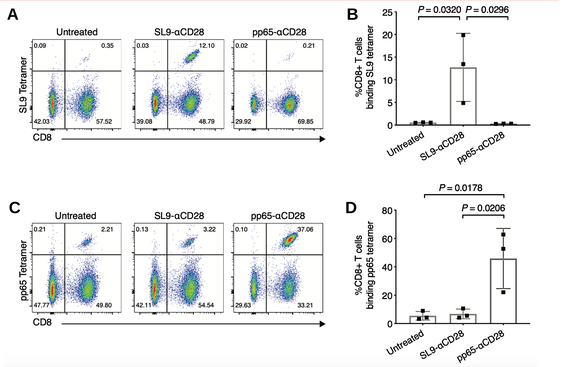


Figure 7 – Immuno-STATs stimulate *in vivo* expansion of SL9 or PP65-specific CD8⁺ T cells from human CMV⁺/HIV⁺ PBMCs transferred to mice. PMBCs from a human CMV⁺/HIV⁺ donor were intrasplenically injected into immunodeficient NSG mice, which then received one dose (4 mgs/kg) of either SL9 or CMV Immuno-STATs with an anti-CD28 scFv MOD. **A-B)** SL9 tetramer staining demonstrated significant expansion of SL9-specific CD8⁺ T cells only when using the SL9-αCD28 Immuno-STAT. **C-D)** Similarly, significant PP65-specific CD8⁺ T cell expansion was only detected when mice were treated with the PP65-αCD28 Immuno-STAT.

Generation of Positron Emission-active variants of the Immuno-STAT scaffold with sortase A

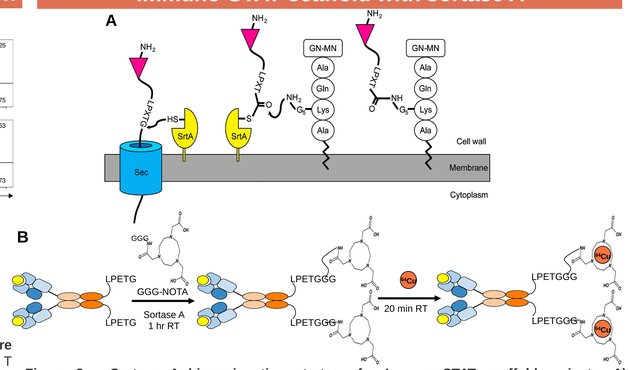


Figure 8 – Sortase A bioconjugation strategy for Immuno-STAT scaffold variants. A) Mechanism of sortase A-mediated cell sorting of surface proteins in *Staphylococcus aureus*. **B)** Sortase A-mediated instillation of tri-glycine-NOTA, a Cu-specific chelator. This intermediate is purified and loaded with PET-active ^{64}Cu to yield Immuno-STAT scaffold conjugates for PET/CT imaging. These variants are based on the murine H-2D^{b} class I MHC and lack modulatory domains.

Detection of influenza A-specific CD8⁺ T infiltration into the respiratory tract via immunoPET using a murine surrogate of the Immuno-STAT scaffold

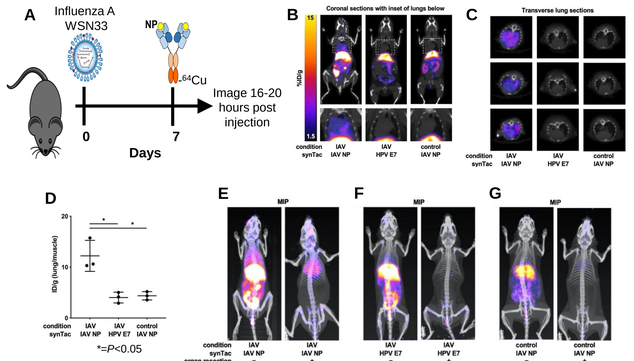
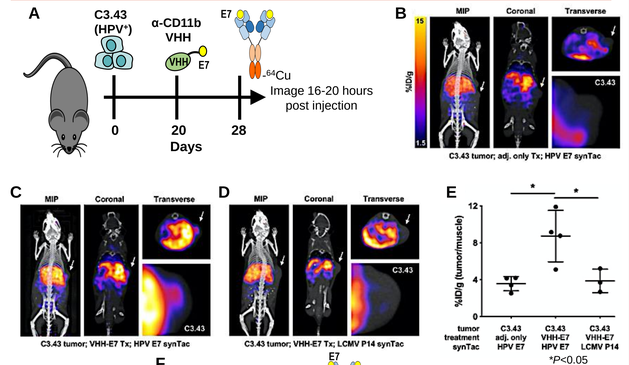


Figure 9 – Detection of influenza A (IAV)-specific CD8⁺ T infiltration into the respiratory tract via immunoPET with variants of the Immuno-STAT scaffold. A) Dosing strategy for influenza A challenge. **B)** Coronal and **C)** transverse lung images show **D)** significant lung recruitment only in IAV-challenged mice imaged with the IAV Immuno-STAT variants bearing a nucleoprotein (NP)-derived peptide. **E-G)** Abdominal organ resection removes nonspecific liver signal and emphasizes lung and respiratory tract recruitment following IAV challenge.

Tracking HPV E7-specific CD8⁺ T infiltration to HPV E7-expressing C3.43 tumors via Immuno-STAT immunoPET



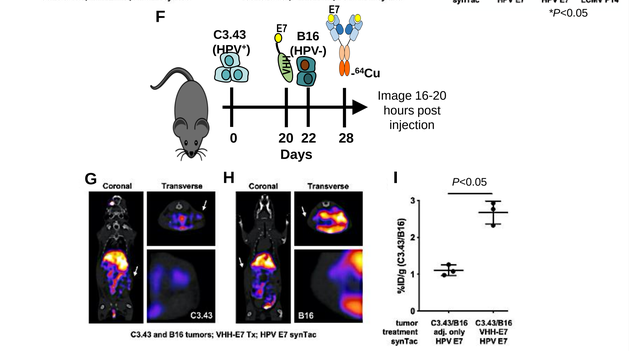


Figure 10 – Tracking HPV E7-specific CD8⁺ T infiltration to HPV E7-expressing C3.43 tumors via Immuno-STAT immunoPET. A) Dosing strategy for C3.43 tumor imaging. **B)** C3.43 tumors are immunosuppressive and require anti-CD11b VHH-E7 immunization to expand E7-specific CD8⁺ T cells for intratumoral detection. ImmunoPET of C3.43 tumors probed by **C)** cognate E7 Immuno-STAT or **D)** non-cognate LCMV Immuno-STAT resulted in **E)** significant intratumoral PET signal only for the cognate Immuno-STAT. **F)** Dosing strategy for cognate (C3.43) versus non-cognate (B16) tumor responses in mice probed with the E7 Immuno-STAT. Coronal and transverse images of the **G)** C3.43 and **H)** B16 tumors demonstrate **I)** significant tumor signal only in the cognate C3.43 tumor.

Detection of splenic expansion of LCMV-specific CD8⁺ T cells and in vivo exposure of Immuno-STAT scaffold

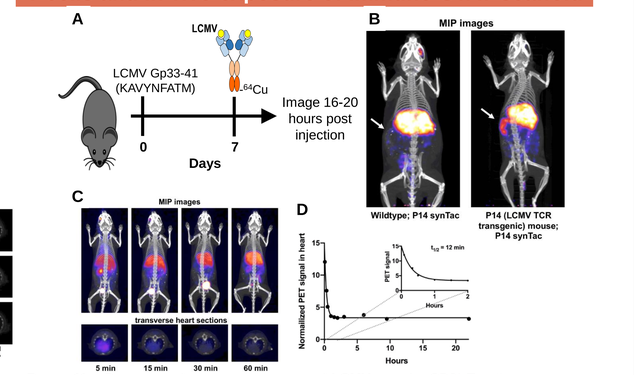


Figure 11 – Detection of splenic expansion of LCMV-specific CD8⁺ T cells and assessment of murine Immuno-STAT scaffold half-life. A) Dosing strategy of transgenic P14 mice (TCRα$^{-/-}$ /LCMV GP_{33-41} TCR knock-in) to simulate acute LCMV infection. **B)** Strong splenic signal in P14 mice imaged with the LCMV Immuno-STAT variant. **C)** Measurement of Immuno-STAT scaffold $T_{1/2}$ by tracking ^{64}Cu signal in the heart. **D)** Quantification of heart signal shows the murine Immuno-STAT scaffold $T_{1/2}$ is approximately 12 minutes.

Conclusions

- Immuno-STATs direct selective expansion of targeted CD8⁺ T cell populations in vitro resulting in functional CTLs with antiviral activity against CMV and HIV.
- Immuno-STATs direct selective in vivo expansion of CMV and HIV-targeted CD8⁺ T cells, consistent with therapeutic applications.
- Immuno-STAT variants bearing PET-active nuclei provide the first instance of antigen-specific CD8⁺ localization in vivo, suggesting diagnostic and prognostic capabilities.
- Localization of the Immuno-STAT scaffold in the disease tissues is highly significant, as it affords opportunities for antigen-selective restimulation in the target tissue, which may be critical for the efficacy of antigen-specific immunotherapeutic strategies for cancer and infectious diseases.

Presented at The Society for Immunotherapy of Cancer's 35th Anniversary Annual Meeting (SITC 2020)