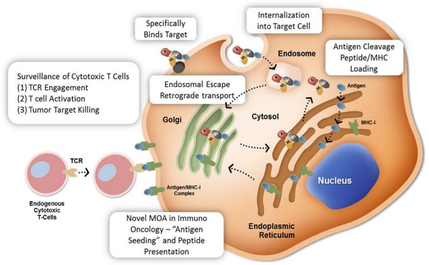
AACR 2018
Abstract 4912

Antigen Seeding Technology by Engineered Toxin Bodies Provides a Targeted Immuno-Oncology Approach for Treatment of Cancers

Brigitte Brieschke, Garrett L. Robinson, Aimee Iberg, Sangeetha Rajagopalan, Jack P. Higgins, Erin K. Willert, Hilario J. Ramos
Molecular Templates, Austin, Texas

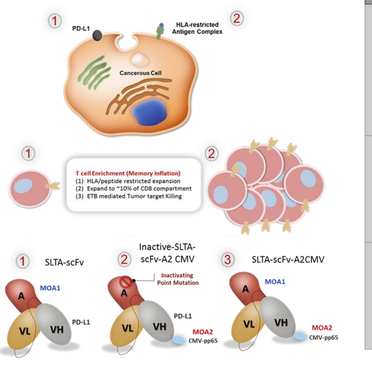
Background

Engineered Toxin Bodies (ETBs) are recombinant immunotoxins which utilize antibody-like targeting to deliver a modified Shiga-like Toxin A subunit (SLTA) to the cytoplasm of tumor cells for inhibition of ribosomal function and apoptotic cell death. Antigen Seeding Technology (AST) incorporates the fusion of an immunodominant antigenic peptide to our current ETB scaffold allowing for the delivery of intracellular peptide for subsequent MHC-I loading, surface presentation, and re-direction of an endogenous memory T cell response against the tumor. AST is a novel mechanism of action for re-directing immune responses to tumors. It is distinct from current Immuno-Oncology (IO) strategies, including ADCs, CAR-T's, and Bispecific antibodies, making ETBs an attractive treatment option for patients. MTEM's first candidate for AST development, MT-5594, targets PD-L1$^+$ tumors and delivers the HLA:A02 restricted peptide from the CMV pp65 tegument protein. Using an *in vitro* model for T cell and tumor co-culture we demonstrate a highly specific and potent T cell-redirection and tumor targeting by MT-5594. MTEM plans for clinical development of MT-5594 in 2019.



Antigen Seeding Technology Development

Antigen Seeding Model Characteristics And ETB Tool-Kit



Characteristic	Rationale
Target	(1) **PD-L1-scFv targeted** Enriched tumor cell expression Potential for immuno-modulation (2) **HLA:A02-peptide seeding** Highest prevalence in North American Populations Retrospective patient evaluation CMV, Influenza, HLA:A02 Immunodominant peptides
Antigen/CTL	**Cytomegalovirus-HLA:A02-pp65 restricted T cells** (1) >60% of adult population with T cell memory to CMV (2) Surveilling TEM populations (memory inflation) • Up to 10% of Memory CTLs CMV • 200-500 million available T cells for AST • Comparable to CAR-T therapeutic doses • AST drives full TCR-synapse engagement; not scFv-based
ETB Tool-Kit	**ETBs**: (1) MOA1: internalization and ribosomal inactivation (parental ETB without antigen) (2) MOA2: internalization and antigen seeding directed CTL lysis (loss of ribosome inhibition by point mutation) (3) MOA 1 + MOA 2: internalization, ribosome inactivation and antigen seeding technology (active ETB with antigen)

Methods

Flow Cytometry: Target cells were detached from culture vessels via trypsinization, washed and plated for antibody staining in standard FACs buffer (PBS + 1% BSA). Directly conjugated antibodies to human PD-L1 or HLA:A02 were co-incubated with target cells for 1hr at 4°C at concentrations recommended by the manufacturer. Flow cytometry was carried out on an Accuri C6 (BD Biosciences) or Athena (Cytek) and data was analyzed via FlowJo Software.

ETB On-cell Binding: For on-cell binding studies, ETBs were co-cultured with target cells for 1hr at 4°C followed by secondary antibody detection with a directly conjugated antibody against the SLTA portion of ETB. After 1hr at 4C, cells were harvested, washed and analyzed by flow cytometry as described above. EC50 and Bmax were calculated using non-linear regression analysis in GraphPad Prism software.

T cell enrichment: PBMCs from HLA-typed donors were isolated from Leukopaks (All Cells) by density centrifugation. For CMV-CTL enrichment, cells were treated with 1µM of appropriate peptide (e.g. CMV-pp65 for HLA:A02) in the presence of cytokine cocktail. Cells were expanded for 1-2 weeks and were split according to viability and density of culture. CMV-CTL expansion was measured by flow cytometry with fluorophore conjugated TCR-binding, MHC-I pentamers (Pro-Immune).

Co-culture Assays: Target cell lines were plated at 20-30K cells/well of 96 well plates and incubated with ETBs as described. After 4hrs, free-ETB was washed away and cells were either co-cultured with T cells or media alone. MDA-MB-231 cells expressing a fluorescent tag (Essen Bio) or HCC1954 and A375, pre-stained with Cytolight Red dye (Essen Bio) were used as targets for IncuCyte assays. Cell viability over time was measured on the IncuCyte-S3-Live cell imager (Essen Bio) and phase and fluorescent images were captured every 4-6hrs. Percent viability was measured by fluorescent cell counts via IncuCyte-S3 software package. Data was plotted as total fluorescent cell counts and normalized to T=0. IC50 was calculated by non-linear regression and plotted using GraphPad Prism Software.

ELISA: For detection of Human IFN-γ, supernatant was harvested from co-culture experiments at 48hrs post treatment and cytokine was detected using ELISA Max kits (Biolegend) via manufacturer's protocol. Changes in absorbance at 450nM were compared against a standard curve and analyzed via GraphPad Prism Software.

Website: www.mtem.com Contact: hilario.ramos@mtem.com

PD-L1 Targeted ETBs Have Potent and Predictable On-Target Activity

Tumor cell lines were screened for expression of PD-L1 by flow cytometry with commercially available antibodies or PD-L1-targeted ETBs derived from a POC validated binding domain (SLTA-scFv1) or internally identified binding domain (SLTA-scFv3; Table 2). Surface expression and ETB binding to high (HCC1954), mid-range (MDA-MB-231), and low PD-L1 (A375 or MDA-MB-468) expressing tumor cell lines is shown (Figure 1A, Table 2). Expression of surface PD-L1 directly correlated with ETB binding (Bmax; Table 2). Addition of CMV antigen to ETB scaffold (SLTA-scFv3-A2CMV; MT-5594) did not change on-cell binding (Figure 1B) or MOA1 cell-kill of targets (Figure 1C, Table 2) which was dependent on PD-L1 expression for activity.

Figure 1. ETB Bindng (FACs) and Cell Viability (Cell-Titer-Glo)

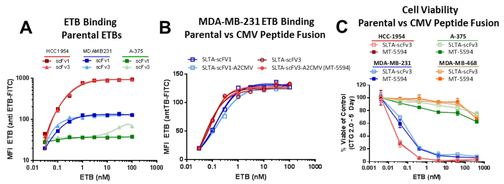


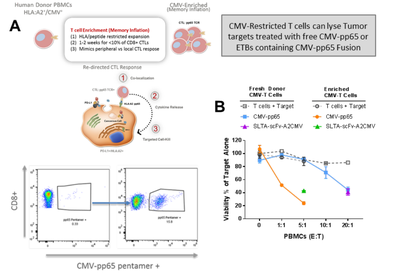
Table 2: PD-L1 Target Expression and ETB interaction with Target Cell Lines

Target Cell line	Target Expression (MFI)	ETB Binding Bmax (MFI)		IC_{50} (pM) Day 5 (CTG 2.0)	
		scFv3	MT-5594	scFv3	MT-5594
HCC1954	48,378	999	938	8.4	9.6
MDA-MB-231	28,345	192	179	77	37
A-375	1137	37	41	>1000	>1000
MDA-MB-468	< 500	DNT	DNT	>1000	>1000

AST: CMV-T Cells Can Be Re-directed to Kill PD-L1/HLA:A2$^+$ Target Cells

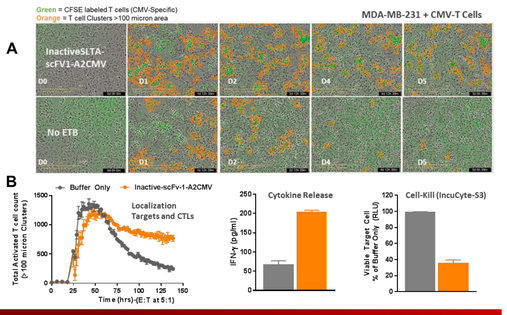
Donor PBMCs were isolated from whole blood for use directly in assays, or enriched for 1-2 weeks with CMV-pp65(HLA:A02) to mimic memory inflation. Frequency of antigen specific cells was measured by TCR-pentamer staining and showed an ~50 fold expansion of antigen specific CTLs after enrichment (Figure 2A). Treatment of MDA-MB-231 targets with free CMV-pp65 peptide or ETBs containing a fused CMV-pp65 peptide (A2CMV) led to a comparable antigen seeding response and dose-dependent target lysis (Figure 2B). To further evaluate an AST response, we generated an ETB carrying a fused CMV-pp65 peptide with a point mutation in SLTA, rendering it inactive for MOA1 (Inactive-SLTA-scFv-A2CMV; Table 1) for use in co-culture assays with MDA-MB-231 (PD-L1 intermediate, HLA:A02$^+$) targets. For these studies, targets were co-cultured with enriched CTLs in the presence or absence of Inactive-SLTA-scFv-A2CMV (Fig. 3A). T cell activation and target cell-kill was measured by live cell imaging on a fluorescent based imager (IncuCyte-S3). Co-culture in the presence CMV-containing ETB led to (1) prolonged association of CTLs with targets, (2) secretion of IFN-γ and (3) re-directed lysis of targets (Figure 3) thus demonstrating AST in the absence of MOA1.

Figure 2: Modeling CMV-pp65 CTL Response in CTL/MDA-MB-231 Co-culture Model



CMV-Restricted T cells can lyse Tumor targets treated with free CMV-pp65 or ETBs containing CMV-pp65 Fusion

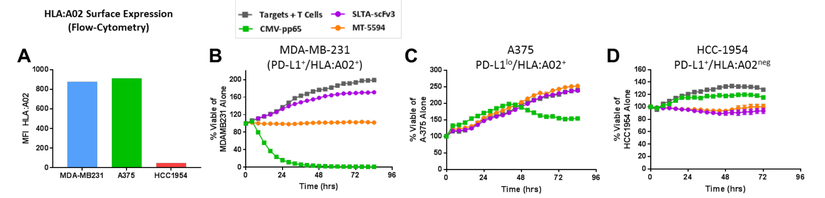
Figure 3: Inactive ETB Delivering CMV-Peptide Re-Directs CTL Response to Tumor cells



MT-5594 Displays Potent and Target-Specific Activity

The AST response is highly selective and requires both the targeting receptor (PD-L1) and matched HLA-restriction between target and effector CTLs. We examined surface expression of PD-L1 (Table 2) and MHC-I HLA:A02 (Figure 6A) by flow cytometry followed by initiation of a stringent confluence model with our lead ETB, MT-5594. MDA-MB-231 represent a target cell line expressing both required surface markers (PD-L1$^+$/HLA:A2$^+$). A375 express matched HLA but lack appreciable PD-L1 (PD-L1 ^{lo}/HLA:A2$^+$). Finally HCC1954 represent a line expressing the targeting receptor PD-L1 but mismatched HLA (PD-L1$^+$/HLA:A2^{neg}). Target lines were cultured with HLA:A2 restricted (A2CMV) CTL at 2:1 in the presence or absence of ETBs (37 nM) or pp65-CMV peptide. Free CMV-pp65 peptide which can be loaded both exogenously and through endogenous processing elicited potent T cell mediated lysis of both HLA positive lines (MDA-MB-231, A375) but not HLA negative line, HCC1954 (Figure 6B,C,D). In contrast, the ETB-driven AST response was not observed on targets lacking either PD-L1 (A375; 6C) or HLA:A2 (HCC1954; 6D) consistent with requirement for both receptors for AST responses.

Figure 6: AST Response Requires Matched Target and HLA Expression



MT-5594 Promotes a Potent and Dose-Dependent Response Under Stringent Conditions

In order to assess the potency of MT-5594 under stringent conditions, MDA-MB-231 target cells were plated at near confluency and co-cultured with CMV-enriched CTLs at a 2:1 (E:T) ratio in the presence or absence of active ETBs with or without the HLA:A02-CMV (A2-CMV) fusion (Figure 7). ETBs were administered in a full dose curve, either as a single administration (Figure 7A) or two subsequent doses (at D=0 and D=4) and potency was assessed by IC_{50} calculation. Target cell kill (IncuCyte-S3, 120hrs post dose) and cytokine release (IFN-γ secretion, 48hrs post dose) were dependent on delivery of the CMV-A2 peptide and showed dose-dependent activity (Figure 7A, B, C). Cell-kill via MOA1 was consistent between ETBs with or without the A2 CMV however, addition of T cells led to a shift in IC_{50} potency and absolute kill in the assay. After 4 days in culture, tumor targets were re-administered ETB to mimic *in vivo* dosing strategies. After dose 2 in the presence of ETB containing A2-CMV and cognate CTLs, we observed a marked reduction in the population of live targets which correlated with an increased magnitude of IFN-γ secretion (Figures 7B, C; Table 3).

Figure 7. Single and Dual Cycle Dosing Response in Full Confluence Robust MDA-MB-231 Model
-Fully Established Monolayer Conditions
-Intermediate PD-L1 Expressing Target Line

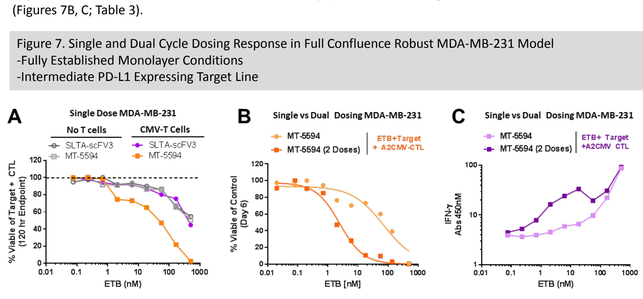


Table 3. Potency Characteristics AST ETBs

ETB	CMV-CTL In Co-culture	IC50 Dose 1 (nM)	IC50 Dose 2 (nM)
SLTA-scFv3	-	654	581
SLTA-scFV3 A2CMV	-	670	568
SLTA-scFv3	+	559	351
SLTA-scFV3 A2CMV	+	72	2.5

MT-5594 Is a PD-L1 Targeted ETB Selected for Lead AST Development

MDA-MB-231 target cells were co-cultured with CMV-enriched CTLs at a 2:1 ratio (E:T) in the presence or absence of PD-L1 targeting ETBs. ETB sets consisted of HLA:A2-CMV peptide fusions and constructs +/- inactivating mutations in SLTA rendering ribosomal inhibition non-functional (Figures 4 and 5). Co-culture of target cells and HLA:A2 restricted CTLs led to cell kill (Figure 4A) and IFN-γ secretion (Figure 4B) dependent on delivery of CMV peptide by ETB. Inactive ETBs failed to elicit these responses in the absence of CTLs but demonstrated substantial potency in the presence of CTLs. Similar results were obtained for a second HLA:A2 donor (data not shown). In contrast CTLs derived from an HLA:A01 restricted donor did not elicit AST in the presence of HLA:A02 delivering ETBs. MT-5594, a derivative of our scFv3 targeted ETB family, with on target selectivity and high potency was selected as a lead for development of the AST platform.

Figure 4: Evaluating AST Response with Active and Inactive Lead ETBs

MDA-MB231 +/- CMV-T Cells (2:1) +/- ETBs (500 nM)

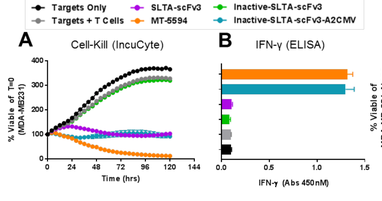
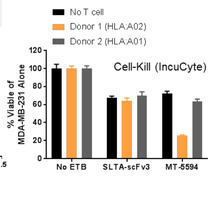


Figure 5: Evaluating HLA Restriction and AST Response



Conclusions

PD-L1 is a Validated Target on Tumor Cells

- Targeting PD-L1 improves outcomes to a variety of tumors
- Direct cell-kill approach obviates need for infiltrating T-Cells
- MT-5594 has potent direct cell-kill activity on PD-L1+ tumor lines independent of T cell recognitions

Antigen Seeding Technology is Built for Next Generation Immuno-Oncology

MT5594: PD-L1 targeting and HLA:A02-CMV-pp65 restriction

- Capitalizes on Robust memory CMV-CTL response in individuals
- Endogenous CMV-Response parallels benchmarks in CAR-T dosing strategies
- Leverages IO success in PD-L1 responsive indications
- Novel and complimentary to current IO therapeutics (Table 4)

In vitro model systems developed to mimic conditions we expect to observe in the clinic

- Robust and stringent mid PD-L1 expressing MDA-MB-231 model system
- MT-5594 exhibits low nM potency and selective-on-target specificity
- MT-5594 is under *in vivo* evaluation; clinical development by is expected in 2019

AST Unique MOA for T cell Re-direction

- Dual MOA: ribosomal inhibition and CTL Lysis (IO)
- No need for direct activity on T cells; Focuses on tumor modulation
- Recruits naturally occurring memory T cell pool
- Paints Tumor as foreign (peptide) and may expand memory

Table 4. AST is a Novel and Clinically Relevant Strategy for IO

Therapeutic Strategy	Targeting MOA	T Cell Requirements
Bispecific	T cell and Tumor	Polyclonal T Cell engagement May be restricted by local exhausted TIL
CAR-T	Tumor	Exogenous engineering required Difficult in vivo expansion
AST (ETB)	Tumor	Co-opts Endogenous memory Surveilling and expansion competent