Poster nr. 238

Exogenous GOT2 in CAR T cells improves metabolic function and preserves early memory T cell subsets

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Introduction

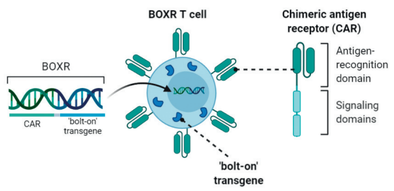
Despite unprecedented success in hematological malignancies, chimeric antigen receptor (CAR) T cell therapy has demonstrated limited efficacy against solid tumors. The solid tumor microenvironment (TME) is thought to suppress CAR T cell function through various mechanisms including competition for available nutrients and chronic stimulation resulting in limited T cell effector functions or dysfunctional states of T cell exhaustion or senescence[1]. Therefore, CAR T cells with enhanced metabolic fitness or more durable early memory phenotype could potentially improve the clinical outcome against solid tumors.

The enzyme glutamine oxaloacetate transaminase (GOT2) plays an important role in mitochondrial function and maintenance of redox homeostasis[2]. Co-expression of GOT2 in CAR T cells could boost metabolic fitness and improve function in the nutrient-poor TME. We have developed novel CAR T cells co-expressing a glypican-3 (GPC3)-targeting CAR and GOT2. *In vivo* preclinical studies have shown improved anti-tumor activity of GPC3 CAR + GOT2 (BOXR1030) T cells compared to Control (GPC3 CAR only) T cells[3]. A Phase I/II trial is ongoing to assess safety and preliminary efficacy of BOXR1030 in GPC3+ solid tumors (NCT05120271).

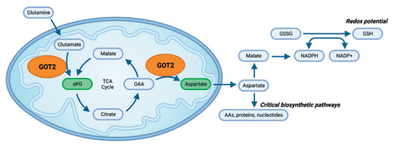
In the current study, we have characterized the T cell phenotype, effector functions and metabolic fitness of BOXR1030 T cells relative to Control GPC3 CAR T cells (which lack exogenous GOT2 co-expression) *in vitro*.

The BOXR Platform and BOXR1030

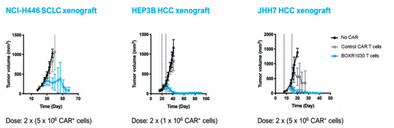
BOXR1030 T cells co-express a glypican-3 (GPC3)-targeting CAR and the enzyme GOT2.



GOT2 plays an important role in mitochondrial function and maintenance of redox homeostasis.



BOXR1030 T cells demonstrate superior *in vivo* efficacy relative to Control CAR T cells in multiple GPC3+ xenograft models.



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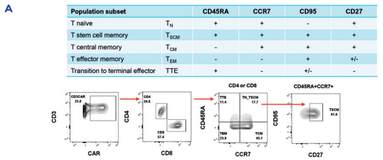
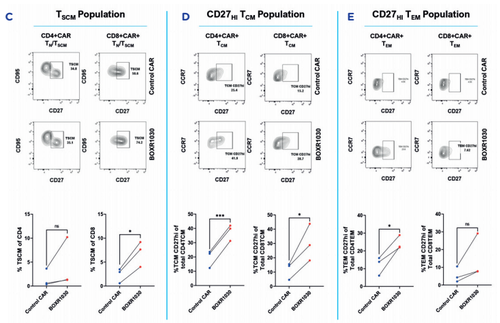
Results

Figure 1: BOXR1030 T cells exhibit a less differentiated phenotype than Control CAR T cells in drug product

Cell surface markers and gating strategy used to define T cell differentiation state



Within all memory T cell subsets, BOXR1030 CAR+ T cells exhibit less differentiated phenotype ($CD27_{HI}$) consistent with increased survival capability



T_N/T_{SCM} and T_{EM} populations are increased in BOXR1030 CAR+ T cells relative to Control CAR T cells

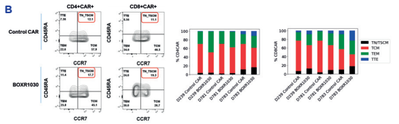
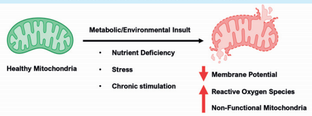


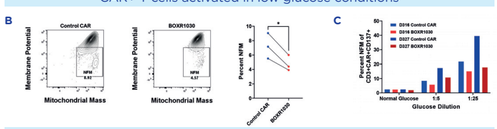
Figure 1. BOXR1030 and Control (GPC3 CAR only, no GOT2) T cells were manufactured by retroviral transduction of PBMCs from healthy donors. Following thaw, cells were analyzed by flow cytometry with a cocktail of antibodies recognizing CD3, CD4, CD8, GPC3 CAR, CD45RA, CCR7, CD27, CD95 and Live/Dead dye. **(A)** Different T cell subsets were defined using the cell surface phenotype[4,5] illustrated in the table and representative gating example. **(B)** Representative flow plots of the different T cell subsets gated on CD4 CAR+ and CD8 CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from three donors are summarized in the stacked bar plots. **(C-E)** Assessment of the degree of terminal differentiation or senescence was performed by evaluating CD27 expression in BOXR1030 and Control CAR T cells within the Naïve/T stem cell memory **(C)**, T central memory **(D)** and T effector memory **(E)** cell subsets. Loss of CD27 is associated with terminal differentiation and progression to replicative senescence[6]. Samples were analyzed using an Attune Nxt flow cytometer (Thermo Fisher Scientific). Data were analyzed using FlowJo v.10 and OMIQ. Statistical analysis was performed using GraphPad Prism 9 with paired t-test (n=3 donors), ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Figure 2: BOXR1030 T cells demonstrate improved metabolic health and survival in low glucose conditions relative to Control CAR T cells

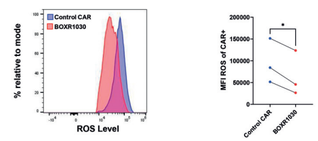
Environmental and metabolic stressors impair mitochondrial function in T cells



Lower frequency of non-functional mitochondria is observed in BOXR1030 CAR+ T cells activated in low glucose conditions



Decreased ROS levels are seen in BOXR1030 CAR+ T cells activated in low glucose conditions



Higher frequency of live, proliferating cells is observed in BOXR1030 CAR+ T cells activated in low glucose conditions

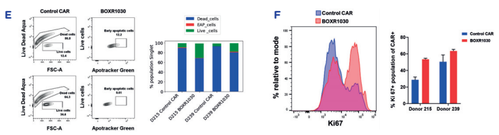
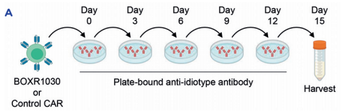


Figure 2. BOXR1030 and Control (GPC3 CAR only, no GOT2) T cells were activated in normal (37mM glucose) or low glucose conditions. **(A)** Model highlighting various stressors resulting in Non-functional Mitochondria (NFM)[7]. **(B-D)** NFM were quantitated in CAR+ cells in BOXR1030 and Control CAR T Cells using MitoTracker Green and MitoTracker Red dyes for mitochondrial mass and mitochondrial membrane potential, respectively. **(B)** Cells were stimulated with plate-bound GPC3 CAR anti-idiotype antibody for 48 hours in low glucose (0.37mM) media and assessed for NFM. Representative flow plots for NFM gated on CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 3 donors are summarized in the graph. **(C)** Cells were activated with GPC3+ Hep3B tumor cell spheroids in normal glucose (37mM) or low glucose (1:5 diluted = 7.4mM glucose, 1:25 diluted = 1.48mM glucose) conditions for 7 days. Activated (CD137+) CAR+ cells were assessed for NFM. Results from 2 donors are summarized in the graph. **(D)** ROS level were assessed in CAR+ cells in BOXR1030 and Control CAR T cells using CellROX® Green dye following stimulation with plate-bound GPC3 CAR anti-idiotype antibody for 48 hours in low glucose (0.37mM) media. Representative flow plots of ROS levels gated on CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 3 donors are summarized in the graph. **(E)** Frequency of Live, Early apoptotic and Dead cells were quantified in CAR+ cells in BOXR1030 and Control CAR T cells using Apotracker Green dye following stimulation with plate-bound GPC3 CAR anti-idiotype antibody for 48 hours in low glucose (0.37mM) media. Representative flow plots showing the 3 populations gated on CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 2 donors are summarized in the graph. **(F)** Ki67 levels were quantitated in CAR+ cells in BOXR1030 and Control CAR T cells following stimulation with plate-bound GPC3 CAR anti-idiotype antibody for 48 hours in low glucose (0.37mM) media. Representative flow plots of Ki67 levels gated on CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 2 donors are summarized in the graph. Samples were analyzed using an Attune Nxt flow cytometer (Thermo Fisher Scientific). Data were analyzed using FlowJo v.10 and OMIQ. Statistical analysis was performed on graphs with n=3 donors with paired t-test, *p<0.05.

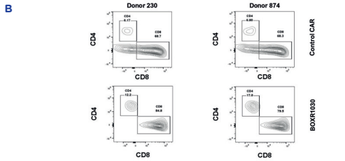
Results

Figure 3: T stem-like/memory populations with superior capacity for persistence and proliferation are preserved in BOXR1030 T cells following chronic stimulation

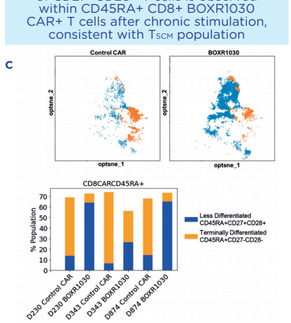
Schematic of chronic stimulation experiment



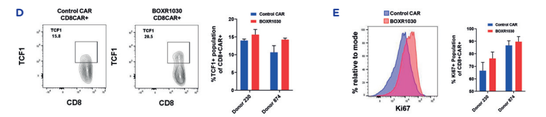
Higher frequency of CD4 and $CD8_{HI}$ subsets observed in BOXR1030 cells after chronic stimulation



Dramatically increased population of CD27+CD28+ T cells is observed within CD45RA+ CD8+ BOXR1030 CAR+ T cells after chronic stimulation, consistent with T_{SCM} population



BOXR1030 T cells exhibit higher TCF1 expression (associated with T_{SCM}) and increased proliferative capacity compared to Control CAR T cells after chronic stimulation



Higher number of mitochondria per cell and more tubular appearance (associated with memory T cells) are observed in BOXR1030 T cells after chronic stimulation

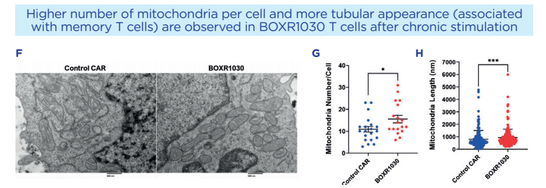
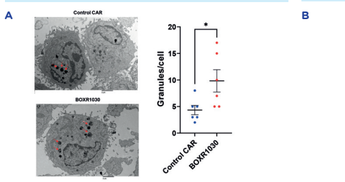


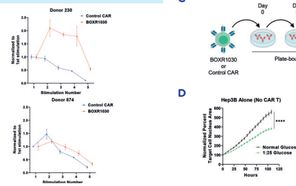
Figure 3. BOXR1030 and Control (GPC3 CAR only, no GOT2) T cells were repeatedly stimulated with plate-bound GPC3 CAR anti-idiotype antibody. **(A)** Schematic representation of stimulation with anti-idiotype antibody coated plate. **(B)** Frequency of CD4+ and CD8+ populations were assessed in CAR+ cells in BOXR1030 and Control CAR T cells following 5 repeated stimulations over 15 days. Representative flow plots of BOXR1030 and Control CAR T cells are shown. **(C)** Expression of CD27 and CD28 within the CD8+ CAR+ CD45RA+ population was evaluated in BOXR1030 and Control CAR T cells following 5 repeated stimulations over 15 days. The t-SNE plot shows less differentiated[5] $CD27^+CD28^+$ (blue) and terminally differentiated[8] $CD27^-CD28^-$ populations (orange) in BOXR1030 and Control CAR T cells and results from 3 donors are summarized in the stacked bar plots. **(D)** Levels of transcription factor TCF1 (which are associated with T_{SCM}[9]) were quantitated in CD8+ CAR+ cells in BOXR1030 and Control CAR T cells following 3 repeated stimulations over 9 days. Representative flow plots of TCF1 gated on CD8+ CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 2 donors are summarized in the graph. **(E)** Ki67 levels were quantitated in CD8+ CAR+ cells in BOXR1030 and Control CAR T cells following 3 repeated stimulations over 9 days. Representative flow plots of Ki67 gated on CD8+ CAR+ populations in BOXR1030 and Control CAR T cells are shown and results from 2 donors are summarized in the graph. **(F)** Mitochondrial morphology in BOXR1030 and Control CAR T cells was examined by electron microscopy following 4 repeated stimulations over 12 days. Higher number of mitochondria with tubular appearance are associated with memory T cells[10]. Representative images are shown and mitochondrial numbers/cell and mitochondrial length data from 20 independent images (n=1 donor) are summarized in the graph. Samples were analyzed using an Attune Nxt flow cytometer (Thermo Fisher Scientific) or transmission electron microscope. Images/data were analyzed using ImageJ, FlowJo v.10 and OMIQ. Statistical analysis was performed on mitochondrial data from 20 independent images with unpaired non-parametric student t test; *p<0.05; ***p<0.001.

Figure 4: BOXR1030 T cells exhibit superior effector function under stress conditions as compared to Control CAR T cells

More cytotoxic granules/cell are observed in BOXR1030 T cells relative to Control CAR T cells after chronic stimulation



BOXR1030 T cells retain greater capacity to produce Granzyme B after chronic stimulation



BOXR1030 T cells exhibit superior tumor cell killing compared to Control CAR T cells after chronic stimulation and in low glucose culture

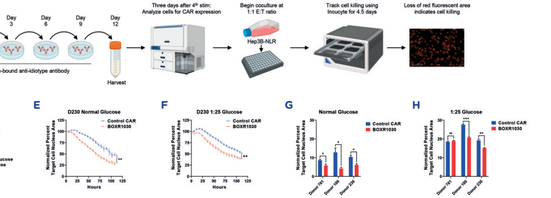


Figure 4. Effector function of BOXR1030 and Control (GPC3 CAR only, no GOT2) T cells following culture in stress conditions such as repeated stimulation or low glucose was examined. **(A)** Cytotoxic granules (red arrows) present in individual T cells was quantified in BOXR1030 and Control CAR T cells by electron microscopy following 4 repeated stimulations with plate-bound GPC3 CAR anti-idiotype antibody over 12 days. Representative images are shown and granule numbers/cell from 7 independent images (n=1 donor) were quantified by ImageJ and summarized in the graph. **(B)** Granzyme B (GNB) levels in supernatants of BOXR1030 and Control CAR T cells following 5 repeated stimulations with plate-bound GPC3 CAR anti-idiotype antibody over 15 days was determined by MSD (Meso Scale Diagnostics) assay. Graphs summarize data from 2 donors. **(C)** Schematic of GPC3+ Hep3B-NLR cell killing assay in low glucose conditions. BOXR1030 and Control CAR T cells were repeatedly stimulated with plate-bound GPC3 CAR anti-idiotype antibody over 12 days and then cocultured at 1:1 effector:target ratio with Hep3B-NLR cells in normal glucose (37mM) or low glucose (1:25 diluted = 1.48mM glucose) media. Cell killing was monitored every six hours for 4.5 days using a Sartorius IncuCyte. Fluorescent area of NLR protein restricted to the nucleus of target cells was quantified using IncuCyte analysis software. **(D)** Growth of Hep3B-NLR cells cultured without CAR T cells in normal or low glucose media. Red-fluorescent area of Hep3B-NLR nuclei was normalized to start of coculture. Percent increase of red fluorescent area normalized to the beginning of coculture (t=0h) indicates cell proliferation. **(E-H)** Analysis of Hep3B killing by BOXR1030 and Control CAR T cells in normal or low glucose media following multiple stimulations according to the schematic (C). Red-fluorescent area of Hep3B-NLR nuclei was normalized to start of coculture. Percent loss of red fluorescent area relative to the beginning of coculture (t=0h) indicates cell killing. Representative plots from one donor showing Hep3B-NLR killing over time by BOXR1030 and Control CAR T cells in normal and low glucose conditions are shown **(E, F)** and graphs summarize Hep3B tumor killing endpoint data for BOXR1030 and Control CAR T cells in low and normal glucose conditions (mean+/-SEM for 3 donors). Statistical analysis was performed on killing curves using 2-way Repeated Measures ANOVA **(E,F)**, or endpoint summary data using student's t-test **(G, H)**, ns, not significant, *p<0.05, **p<0.01, ***p<0.001.

Conclusions

- **Following manufacture, BOXR1030 T cells have a higher frequency of less differentiated T_{SCM} population and more $CD27^+$ cells in all memory T cells subsets (indicating fewer terminally differentiated cells) compared to Control CAR T cells lacking the GOT2 transgene.**
- **When exposed to stressful conditions such as low glucose and chronic stimulation, BOXR1030 T cells exhibit (i) improved metabolic fitness, (ii) increased cell survival and proliferative capacity, (iii) multiple characteristics of less differentiated/early memory T cell populations and (iv) retention of effector functions relative to Control CAR T cells.**
- **The enrichment of early memory T cell subsets and resistance to terminal differentiation in BOXR1030 are likely the result of reinforced metabolic fitness from exogenous GOT2 and suggests that BOXR1030 T cells will exhibit superior persistence and durable function in the treatment of solid tumors.**
- **These results support the ongoing Ph1/2 BOXR1030 clinical study and warrant future investigation of GOT2 and other transgenes to enhance metabolic fitness of cellular therapies.**

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Disclosures

All authors are current employees of SOTIO Biotech Inc.