Correction of Underlying Lymphoid Glucose Utilization Defects with the BCG Microorganism: Implications for Type 1 Diabetes through Quantitative Method Development

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Introduction

- Human clinical trial data, including 8-year follow up of HbA1c after two BCG doses, shows long lasting blood sugar control in type 1 diabetes (T1D)
  - Vaccinated subjects had lymphoid restoration of glucose metabolism through a shift from oxidative phosphorylation to aerobic glycolysis, correcting defective glucose metabolism (Kuhtreiber et al. *Nature Vaccines* 2018 & *Cell Trends Endocrinol Metab* 2019)
  - This occurred via the BCG organism epigenetically reprogramming glucose utilization pathways (Kuhtreiber et al. *Cell iScience* 2019)
- We sought to better understand lymphoid defects in sugar utilization in T1D and present findings in T1D and non-diabetic controls (NDC)

Methods

- We investigated baseline defects in aerobic glycolysis in T1D versus NDC and evaluated the impact of BCG on glucose utilization *in vitro* and *in vivo* through a clinical study

Results

- T1D lymphocytes had defective baseline 2-NBDG uptake (indicating underactive aerobic glycolysis) and high oxygen consumption (indicating overactive oxidative phosphorylation) that was corrected after 24-hr exposure to BCG *in vitro* (**Fig. 1**)
  - Increased sugar uptake could also be observed in NDC subjects after BCG exposure, but the effect was more dramatic for T1D
- In T1D, the severity of the glucose utilization defect was most pronounced with age of onset (AOO) < 21 years compared to AOO > 21 years (**Fig. 1**)
- AOO influenced BCG's ability to accelerate glucose uptake in T1D both in culture and in an open-label study in the clinic (**Fig 2**)
- Prior exposure to BCG at birth resulted in life-long accelerated glucose uptake in NDC (**Fig. 3**), which was also observed:
  - In NDC with prior BCG treatment of bladder cancer
  - In T1D with latent tuberculosis infection

BCG corrects an underlying defect in glucose utilization in T1D monocytes; More severe defect in early age of onset (AOO)

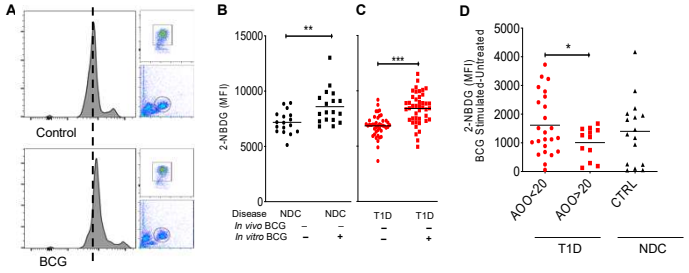


Fig 1. Glucose transport by T1D and NDC human primary monocytes as measured by 2-NBDG flow cytometry. All subjects were without childhood exposure to BCG. **(A)** The 2-NBDG assay for 4 hrs of labelled sugar quantifies the rate of glucose utilization by monocytes and flow cytometry. **(B,C)** Untreated baseline relative glucose uptake was significantly different for NDC (n=23) and T1D (n=43) patients (p<0.0043; 2-tailed, unpaired T-test). BCG increased relative glucose uptake for monocytes of NDC and T1D patients. 2-tailed, unpaired T-test revealed a significant difference between NDC and T1D patients (p=0.045). Untreated baseline and BCG treated baseline relative glucose uptake for T1D show a significant difference between untreated and BCG treated samples ($p=1.837 \times 10^{-5}$; 2-tailed, unpaired T-test). **(D)** Untreated baseline subtracted from BCG treated baseline relative glucose uptake for T1D patients separated by AOO < 20 and > 20 y of age compared to NDC. 1-tailed, unpaired T-test revealed a significant difference between untreated vs BCG-treated samples (p=0.02).

BCG decreases HbA1c in patients with T1D, but HbA1c effect is related to age of onset (AOO)

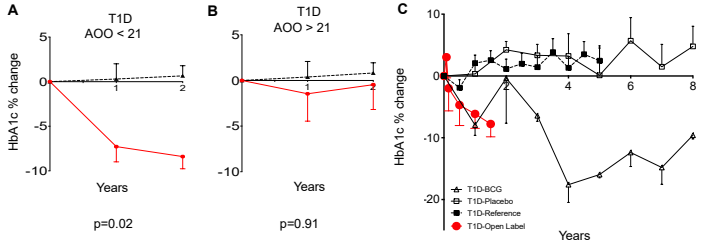
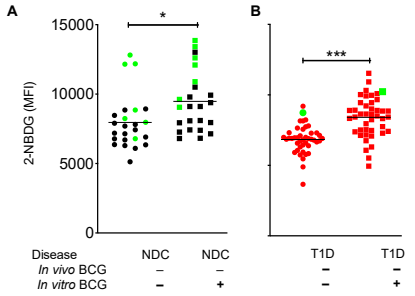


Fig. 2. (A) HbA1c % change from baseline in T1D patients receiving open-label BCG treatment with AOO < 21 y (n=5) compared to a T1D reference population not receiving BCG treatment (n=62) for 2 years. **(B)** HbA1c % change from baseline in T1D patients receiving open-label BCG treatment with AOO > 21 y (n=8) compared to a T1D reference population not receiving BCG treatment (n=62). **(C)** HbA1c % change from baseline in T1D patients receiving open-label BCG treatment (n=5) (red line) compared to previously published data of T1D from an earlier double-blinded, placebo-controlled study, verifying in the open-label trial the trends and effects based on juvenile versus older AOO in T1D subjects.

BCG increases relative glucose uptake in T1D and NDC human primary monocytes; Past BCG exposure results in life-long augmentation



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Subject	Indication	Country at Birth	BCG Strain	Current Age	Dosing Age
1	Newborn Vaccination	India	India	25	Birth
2	Bladder Cancer	USA	TICE	71	Adult
3	Newborn Vaccination	Monaco	Sanofi Pasteur	42	Birth
4	Newborn Vaccination	Hungary	Danish	39	Birth
5	Newborn Vaccination	India	India	25	Birth
6	Newborn Vaccination	Ukraine	Bulgaria	49	Birth
7	Bladder Cancer	USA	TICE	71	Adult
8	Newborn Vaccination	Hungary	Danish	67	Birth

Fig. 3. (A) Glucose uptake in monocytes of NDC patients (n=23) following 24-h incubation with or without *in vitro* BCG treatment. Samples marked in **green** are from individuals with previous BCG vaccinations in childhood or who received BCG as treatment for bladder cancer. Samples marked in **black** show the effect of BCG treatment *in vitro* on glucose uptake with 4 hrs of labelled 2-NBDG compound. In NDC, sugar update is accelerated. The same experimental conditions were applied to NDC who had BCG at birth or had received high dose BCG for bladder therapy. *In vivo* therapy with BCG augmented sugar utilization. **(B)** Glucose uptake in monocytes of T1D patients (n=43) following 24-h incubation with or without *in vitro* BCG treatment. The sample marked in **green** represents an individual with T1D with remarkable diabetes management (63-y history of excellent HbA1c control; AOO 12 y; current age 75 y) and a lung mass suspicious for latent *M. tuberculosis* infection (signs of tuberculosis treated 20 years earlier on chest X-ray). Samples shown in **red** show the *in vitro* response of BCG exposure on monocytes followed by 4 hrs of measured 2-NBDG uptake. **(C)** Summary of past BCG exposures, strain of BCG and current ages of subjects in which BCG appears to keep the cells under excellent aerobic conditions. The long-lasting and near life-time effect of BCG treatment (vaccine or bladder infusion) may permanently modify monocyte glycolysis for accelerated aerobic glycolysis.

Conclusions

- We conclude that NDC monocytes have different levels of basal lymphoid sugar utilization compared to T1D
  - T1D lymphocytes have lower levels of basal lymphoid sugar utilization compared to NDC, but in culture this is corrected with BCG
- AOO of T1D influences the severity of glucose utilization defects *in vitro* and *in vivo* and influences *in vivo* correction of blood sugars with BCG vaccination
- Prior BCG vaccinations at birth appear to "permanently" reset monocyte glucose utilization, thus influencing sugar utilization patterns

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Optimizing TNFR2 Antagonism for Cancer Immunotherapy: Antibody Structure & TNFR2 Density on Target Cells Drive Tumor Microenvironment Specificity

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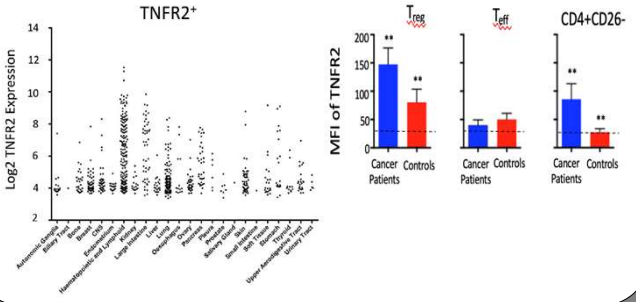
Introduction

- Most current immunotherapies lack regulatory T cell (Treg) specificity for tumor-residing cells or tumor-specific killing for broadly expressed oncogenes
- Tumor necrosis factor receptor 2 (TNFR2) antibody antagonism may be an attractive cancer immunotherapy because it has shown tumor microenvironment specificity
  - Human TNFR2 is massively overexpressed on tumor-residing Tregs, under-expressed on tumor residing T effectors (Teffs) and found directly on some cancer cells (Yang et al. *J Leukoc Biol*. 2020)

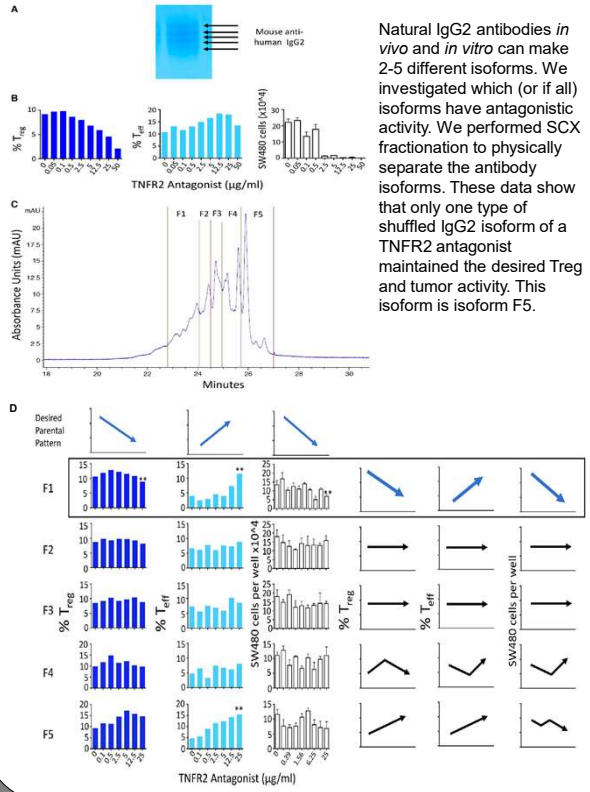
Methods

- We screened 791 human tumor cell lines from diverse cancer tissues, finding frequent and aberrant TNFR2 expression (*below, left*) and examined the density of TNFR2 on Tregs, Teff and cancer cells in cutaneous T cell lymphoma vs normal controls (*below, right*)
- Using 3 cell-based tumor microenvironment assays, we tested a novel human-directed TNFR2 antagonist placed on different human isoform frameworks
- Using mutagenesis techniques, we tested different mutations to our novel TNFR2 antagonist on human chimeric structures to optimize function



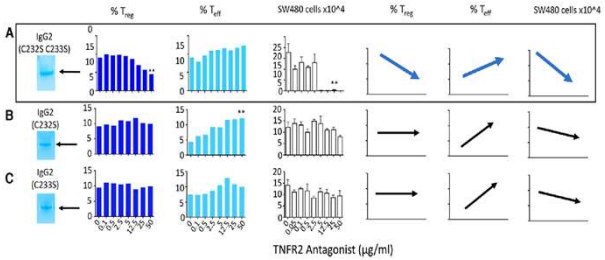
Results

Use of an IgG2 antibody reduced the dependency on Fc receptor function



Natural IgG2 antibodies *in vivo* and *in vitro* can make 2-5 different isoforms. We investigated which (or if all) isoforms have antagonistic activity. We performed SCX fractionation to physically separate the antibody isoforms. These data show that only one type of shuffled IgG2 isoform of a TNFR2 antagonist maintained the desired Treg and tumor activity. This isoform is isoform F5.

Mutated hinge variants of IgG2 produce single and stable isoforms with different functional activity in cell-based tumor microenvironment assays



Chimeric human IgG2 antibodies were constructed with hinge mutations at **(A)** C232S and C233S, **(B)** C232C only, and **(C)** C233S only. **(A)** Human IgG2 isoform with hinge stabilization mutants C232S, C233S showed a single band on a nonreducing gel and restored Treg killing, Teff proliferation and SW480 tumor cell killing. **(B-C)** Human IgG2 isoform with hinge stabilization mutant C232S or C233S showed a single band on a nonreducing gel, but no Treg killing, good Teff activity, and sluggish SW480 tumor killing. A representative experiment is shown for the Treg and Teff experiments, performed >5 times on different normal blood donors. For the SW480 cells, the error bars represent 8 separate experiments. Data are mean ± SEM.

Conclusions

- These findings suggest that the ideal TNFR2 antagonists are the human IgG2 isoform, have hinge stabilization and have wider separation of antibody arms to bind to newly synthesized TNFR2 on rapidly growing tumor cells or Tregs specific to the tumor microenvironment
- When bound to TNFR2, antagonistic antibodies with these characteristics form a non-signaling cell surface dimer that functions as an effective immunotherapy with high tumor microenvironment specificity and desired modulations

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