Soluble BTLA (sBTLA) is Induced Following Targeting of BTLA *In-vivo* by ANB032, a Novel BTLA/HVEM Modulator Therapeutic Antibody for The Treatment of Autoimmune Disease

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Abstract

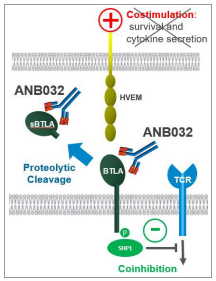
B and T lymphocyte attenuator (BTLA) is an immune checkpoint molecule that contributes to the regulation of T cell, B cell and dendritic cell function. The role of BTLA as a regulator of autoimmune disease and cancer has been demonstrated via genetics and animal models. More recently, studies have described the finding of soluble checkpoint molecules, including BTLA, in serum or in tumors of diseased patients that correlates negatively with disease outcome, suggesting immune suppression.

We previously disclosed ANB032, a novel anti-BTLA monoclonal antibody that modulates BTLA signaling. In cynomolgus monkeys, we observed elevated levels of soluble BTLA (sBTLA) in the serum that correlated in an anti-inflammatory manner to ANB032 exposure and reduced BTLA expression on T and B cells. The circulating sBTLA in serum represents the intact extracellular domain (ECD) of BTLA.

ANB032 was highly efficacious in a human PBMC-NOD-*scid IL2rγ*^{null} (NSG) graft versus host disease model. ANB032 reduced BTLA expression on human T cells and increased human sBTLA in the serum. Flow cytometric analysis of human T cells demonstrated that ANB032 dose-dependently reduced pathogenic T cell activation and expansion.

We found that BTLA is a substrate of PR3 and has a PR3 specific cleavage site in its ECD, suggesting that BTLA is naturally shed by proteolytic cleavage. We propose a mechanism whereby enzymatic cleavage of BTLA is enhanced by monoclonal antibody targeting, which may play a role in the anti-inflammatory therapeutic effect of ANB032.

Introduction: ANB032 modulates *trans* BTLA-HVEM interactions and induces the release of soluble BTLA *in-vivo*



BTLA was first identified as a negative immune regulator on T and B cells, most closely related to PD-1 and CTLA-4. BTLA expression levels vary substantially among different lymphoid and myeloid subtypes with highest expression on B cells but also significant expression on T cells as well as on mature dendritic cells, macrophages and natural killer cells (Han et al., 2004; Hurchla et al., 2005). The ligand of BTLA is Herpes-Virus entry mediator (HVEM). In the past decade, HVEM has emerged as a major and complex co-stimulatory signaling molecule.

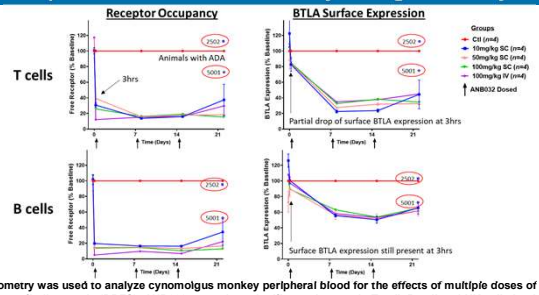
Engagement of BTLA and HVEM in trans results in bi-directional signaling, with coinhibition on the BTLA side and costimulation on the HVEM side. We've previously shown that ANB032 binds to BTLA on an epitope that does not disrupt trans HVEM interactions, stimulating agonism of BTLA that recruits SHP1 leading to co-inhibition on the BTLA side, while simultaneously disrupting HVEM signaling in trans leading to abrogation of costimulation.

Although the interactions of BTLA and HVEM have been characterized, recent evidence has identified the presence of soluble checkpoint molecules, including soluble BTLA (sBTLA) in numerous oncology settings where immune suppression is occurring.

Specifically, sBTLA is a predictor of negative outcome in CLL (Sordo-Bahamonde, et al., 2021), prostate cancer (Wang et al., 2020), hepatocellular carcinoma (Dong et al., 2020), pancreatic adenocarcinoma (Bian et al., 2019), renal cell carcinoma (Wang et al, 2019), and is a predictor of overall survival in patients undergoing immune checkpoint inhibitor therapy for solid malignancies (Gorgulho et al, 2021). Outside of oncology, in the setting of sepsis, sBTLA correlates with an increased risk of mortality (Lange et al, 2017). Although a mechanistic understanding of the induction of sBTLA has not been reported, proteolytic cleavage and shedding of a related checkpoint molecule, TIM-3 has been reported. Cleavage of TIM-3 at a juxtamembrane site by the serine protease proteinase 3 (PR3)(Vega-Carrascal et al. 2011) led us to investigate whether PR3 could potentially cleave BTLA in a similar manner.

Here, we show that ANB032 increases the levels of circulating sBTLA *in-vivo* in cynomolgus monkeys and in a xenogeneic GvHD model in NGS mice where robust efficacy has been demonstrated. We observe that BTLA is shed from the surface of B and T cells indicating that sBTLA is a potential pharmacodynamic marker of ANB032 immune modulatory activity *in-vivo*.

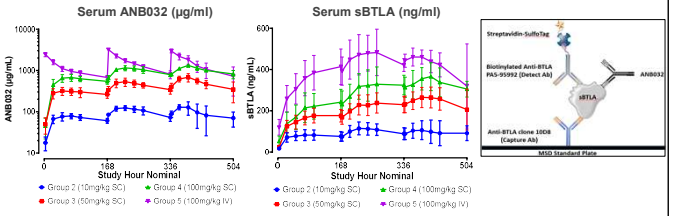
Figure 1: ANB032 achieves receptor occupancy and reduces BTLA expression on T and B cells in cynomolgus monkeys



Flow cytometry was used to analyze cynomolgus monkey peripheral blood for the effects of multiple doses of ANB032 in a dose range finding study (DRFS). ANB032 caused no significant changes in T cell, B cell and NK cell absolute counts, or percent distribution compared to vehicle control treated animals. Binding of fluorochrome labeled drug (ANB032-DyL488) was abrogated in all animals dosed with ANB032 (10mg/kg SC, 50mg/kg SC, 100mg/kg SC and 100mg/kg IV) compared to the vehicle control treated group, demonstrating receptor occupancy of BTLA with ANB032 to be >80% for T cells and >80% for B cells.

All animals dosed with ANB032 had reduced BTLA surface expression on T and B cells when detected with a fluorochrome labeled non-competing anti-BTLA antibody (clone 10D8, discovered by AnaptysBio), compared to vehicle control treated animals. Surface expression of BTLA on T and B cells was reduced ~75% and ~ 50%, respectively. Based on the detection of soluble BTLA in the serum in this and other ANB032 NHP studies, it is hypothesized that BTLA is shed from the surface of the cells following ANB032 binding. Notably, there is a difference in the kinetics of RO on T and B cells, which occurred very rapidly, compared to the loss of BTLA expression on T and B cells, which occurred relatively slowly suggesting an ANB032 dependent mechanism leads to BTLA shedding. Two animals that developed anti-drug antibodies (ADA) demonstrated some loss of RO and recovery of BTLA expression on T and B cells.

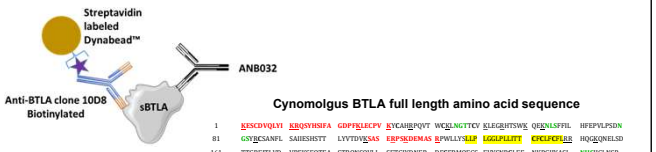
Figure 2: Serum concentrations of ANB032 and sBTLA in cynomolgus monkey were determined in a DRF study



Mean serum (SD) concentrations of ANB032 and cynomolgus monkey sBTLA per dose group were determined following weekly dosing with ANB032 either IV or SC. To measure sBTLA concentrations, an electrochemiluminescence (ECL) sandwich assay was developed. Clone 10D8 and ANB032 are capable of simultaneously binding separate sBTLA epitopes. The immunogen for human anti-BTLA polyclonal (PA5-95592) was located near the c-terminus of sBTLA and likely does not compete with 10D8 and ANB032 epitope binding. MSD plates are coated overnight with AnaptysBio human anti-BTLA antibody clone 10D8. Cynomolgus monkey serum samples containing sBTLA are added to the plate and detected with commercial human anti-BTLA polyclonal antibody (PA5-95592) labeled with biotin and streptavidin-sulfotag secondary detection reagent.

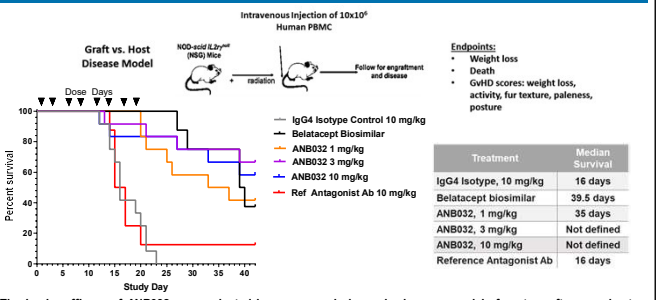
No measurable levels of sBTLA were detected in serum samples from cynomolgus monkeys Day 1, pre-dose or vehicle control treated samples. Shed sBTLA was detected in serum samples from all cynomolgus monkeys dosed with ANB032, supporting the hypothesis that BTLA is shed from the surface of B and T cells and that sBTLA is a pharmacodynamic marker of ANB032 activity *in-vivo*.

Figure 3: Immunoprecipitation and sequencing of sBTLA from cynos dosed with ANB032 identified peptides consistent with the extracellular domain of BTLA



To immunoprecipitate sBTLA, serum aliquots over numerous time points from one cynomolgus monkey dosed with 100 mg/kg in the DRF study were pooled. The presence of sBTLA was confirmed by ELISA. Biotinylated anti-BTLA clone 10D8 was added to the serum pool and incubated with rocking overnight at 4°C. Streptavidin coated Dynabeads™ were added and incubated with rocking overnight at 4°C. A magnet was applied to remove the beads from solution. Immunoprecipitated sBTLA sample was digested with trypsin and analyzed by nano-LC/LC-MS/MS at JadeBio. Raw spectra were searched against sequence databases, followed by data validation and normalization. The false discover rate was set to 0.1% for peptide and 1% for protein. Cynomolgus BTLA was identified in the sample, with matched peptides covering 17% of the sequence of full length cynomolgus BTLA (red text), or 35% of the predicted extracellular domain (ECD). All identified peptides come before the predicted single-pass transmembrane region (yellow highlight). The identified peptides are consistent with the presence of sBTLA ECD in the serum sample. Potential N-terminal glycosylation motifs are shown in green, and potential trypsin cleavage sites are underlined.

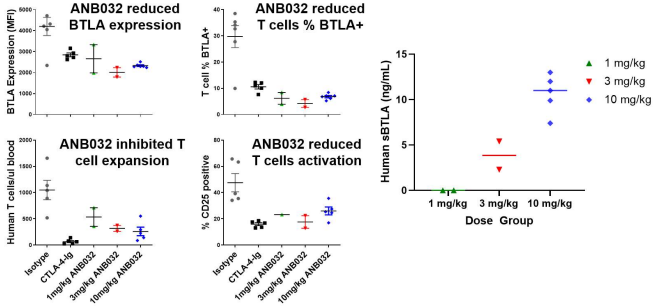
Figure 4: ANB032 is highly efficacious in a humanized murine GvHD model



Treatment	Median Survival
IgG4 Isotype, 10 mg/kg	16 days
Belatacept biosimilar	39.5 days
ANB032, 1 mg/kg	35 days
ANB032, 3 mg/kg	Not defined
ANB032, 10 mg/kg	Not defined
Reference Antagonist Ab	16 days

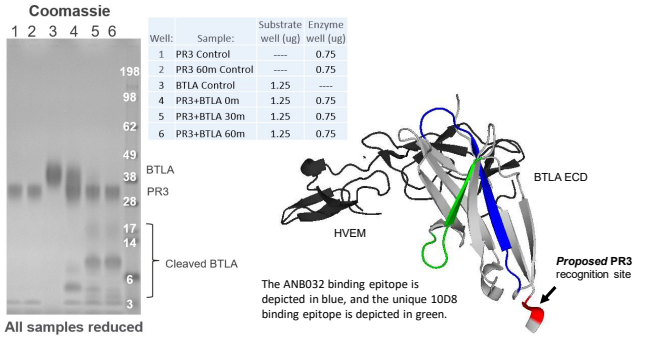
The *in-vivo* efficacy of ANB032 was evaluated in a xenogeneic humanized mouse model of acute graft versus host disease (GvHD). This model is a T cell-mediated disease induced by engraftment of human PBMCs in sub-lethally irradiated NSG mice. At the end of study on Day 42, animals treated with ANB032 at all doses showed statistically significant increased overall survival as compared with animals treated with isotype control antibody (10 mg/kg ANB032 dose group versus isotype, p=0.0019; 3 mg/kg ANB032 versus isotype, p=0.0002; 1 mg/kg ANB032 versus isotype, p=0.0001). There was not a significant difference in survival between the 10 mg/kg and 3 mg/kg ANB032 dose groups, suggesting that efficacy in the GvHD model may be maximal at doses equal to and greater than 3 mg/kg. No survival benefit was observed in animals treated with the reference antagonist antibody compared to isotype control treated animals.

Figure 5: ANB032 demonstrated inhibition of T cell expansion, reduced BTLA expression and induction of sBTLA *in-vivo* in a humanized murine GvHD model .



On day fourteen of the xenogeneic humanized mouse model of acute GvHD, blood from a limited number of animals from each group was obtained via cardiac puncture. Circulating human T cells were analyzed by flow cytometry to characterize BTLA expression, enumerate human T cell numbers, and characterize the activation marker CD25. ANB032 reduced BTLA expression on human T cells, inhibited T cell expansion in a dose-dependent manner and reduced expression of the activation marker CD25 at all doses. Plasma concentrations of human sBTLA were measured by ELISA and shown to increase in a dose-dependent manner. Based on robust efficacy observed in this model, the ANB032 effects on pathogenic human T cells, and the observation of sBTLA in the serum, our hypothesis is that BTLA is shed from the surface of T cells and that sBTLA is a pharmacodynamic marker of ANB032 activity *in-vivo*.

Figure 6: BTLA ECD is cleaved by the serine protease PR3 at a proposed recognition site near the transmembrane domain



Well:	Sample:	Substrate well (ug)	Enzyme well (ug)
1	PR3 Control	----	0.75
2	PR3 60m Control	----	0.75
3	BTLA Control	1.25	----
4	PR3+BTLA 0m	1.25	0.75
5	PR3+BTLA 30m	1.25	0.75
6	PR3+BTLA 60m	1.25	0.75

Proteinase 3 (PR3) is a neutrophil serine protease that is released into the extracellular space upon neutrophil activation. PR3 has previously been shown to cleave the checkpoint receptor T-cell immunoglobulin and mucin domain 3 (TIM-3), reducing the levels of TIM-3 on the surface of cells. The ability of PR3 to cleave BTLA *in vitro* was tested by co-incubating purified recombinant PR3 and BTLA-ECD for up to 60 minutes. Following co-incubation, soluble BLTA-ECD is cleaved into multiple smaller fragments (lanes 5 and 6, Bis-Tris gel) demonstrating that PR3 has the ability to cleave BTLA. An analysis of the sequence proposes at least one PR3 recognition motif towards the C-terminus of the BTLA-ECD (structure figure, red). This data suggests that like TIM-3, BTLA may be subject to cleavage via serine proteases on the surface of cells.

Conclusions

- **Studies have described soluble checkpoint molecules, including sBTLA, in serum or in tumors of diseased patients that correlates negatively with disease outcome, suggesting that soluble checkpoint molecules such as sBTLA are biomarkers of immune suppression, effectors of immune suppression, or both.**
- **In cynomolgus monkeys treated with ANB032, we observed elevated levels of sBTLA in serum that correlated to ANB032 exposure and reduced BTLA expression on T and B cells. The discovered sBTLA represents the intact extracellular portion of BTLA.**
- **ANB032 was found to be highly efficacious in a xenogeneic GvHD model in NSG mice. ANB032 led to anti-inflammatory regulation of pathogenic human T cells in this model, with reduced T cell activation, reduced T cell expansion, reduced T cell BTLA expression and induction of human sBTLA in the serum, in a dose-dependent manner. This finding supports the hypothesis that sBTLA is a biomarker of ANB032 activity *in-vivo*.**
- **We found that BTLA is cleaved by PR3, similar to what has been published for TIM3, suggesting that BTLA is naturally shed by proteolytic cleavage, likely upon ligation in trans *in-vivo*. We propose a mechanism whereby enzymatic cleavage of BTLA is enhanced by monoclonal antibody targeting, which may play a role in the anti-inflammatory therapeutic effect of ANB032.**