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Botensilimab modulates innate and adaptive gene expression programs resulting in superior immune stimulation relative to a first-generation anti-CTLA-4 antibody

Poster # 941

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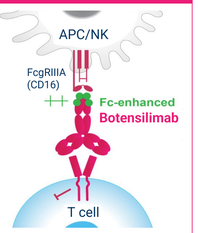
Background

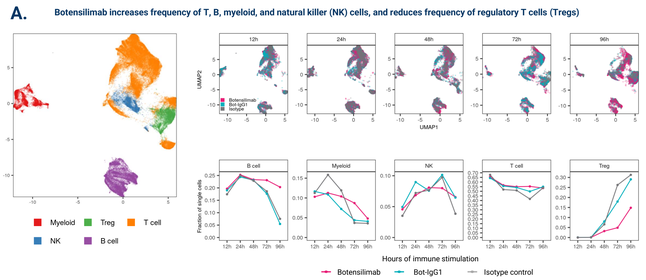
Botensilimab is a next-generation fragment crystallizable (Fc)-enhanced anti–cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody that promotes optimized T cell priming, activation, memory formation, and intratumoral regulatory T cell depletion. In patients with advanced solid tumors, botensilimab alone and in combination with balstilimab (anti-programmed cell death protein 1 [PD-1] antibody) demonstrated durable clinical responses in nine different immunotherapy-resistant or poorly immunogenic tumor types. We hypothesize that botensilimab engages multiple immune cell types through its enhanced Fc region, to drive deep and broad clinical responses.



Here, we applied high-resolution single-cell profiling to demonstrate the differentiated molecular mechanisms of botensilimab relative to a first-generation IgG1 (non-Fc-enhanced) anti-CTLA-4 antibody (bot-IgG1).

High-res map of human immune stimulation captures broad cellular & molecular activity of botensilimab

Fig 1. Botensilimab drives a differentiated immune response



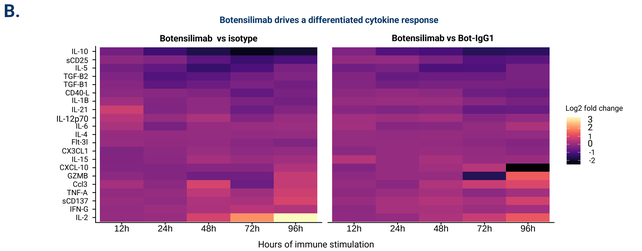


Fig 1. Human peripheral blood mononuclear cells (PBMCs) were isolated from eight donors and stimulated with 10ng/ml staphylococcal enterotoxin A (SEA) peptide together with 5μg/ml botensilimab, first generation aCTLA4 (bot-IgG1) or hIgG1 Fc-enhanced isotype control. Samples were collected and analyzed at multiple time points throughout the four-day reaction. (A) Samples were analyzed using scRNA-seq by 10x Genomics 3'GEX with dead cell removal on an Annexin V column. Demultiplexing, barcode processing, and gene counts were performed using CellRanger. Data from all eight donors was pooled, visualized by UMAP, and clustered based on UMAP distance. Clusters were labeled based on the following markers: T cell = CD3D/E/G, NK = KLRD1, KLRB1, NKG7, Myeloid = CD68, CD80, HLA-DRA, B Cell = CD19, MS4A1, Treg = CD4, FOXP3, IL2RA. Average relative frequencies of each major immune subset were calculated. Changes in cell populations detected by scRNA-seq, including reduced Tregs (Fig 2B), and increased B and NK cells (data not shown), were corroborated by flow cytometry. (B) Supernatants were collected and analyzed by Luminex bead array to quantify cytokine secretion from the gross PBMC population.

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A human in vitro study comparing botensilimab to a first generation anti-CTLA-4 reveals mechanisms of superior immune stimulation by botensilimab

Fig 2. Botensilimab relieves immunosuppression

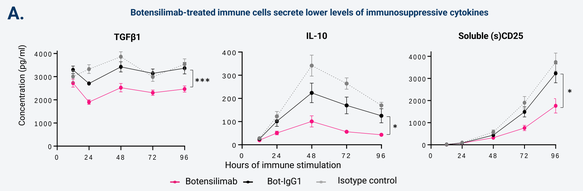
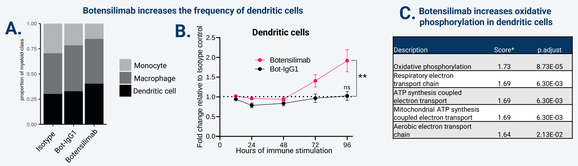
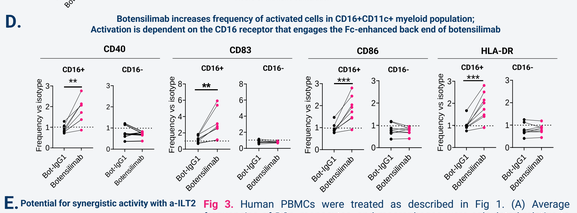


Fig 2. Human PBMCs were treated as described in Fig 1. (A) Longitudinal assessment of immunosuppressive cytokines in the supernatant by Luminex. Each point represents average of eight donors. (Error bars = standard error of mean (SEM)). One-Way ANOVA followed by Tukey's test for multiple comparisons *p<0.05, ***p<0.001. (B) Longitudinal assessment of CD3+CD4+CD127loCD25+ Tregs and CD3+CD4+CD25- non-Tregs by flow cytometry. Each point represents average of eight donors. (Error bars = SEM). One-Way ANOVA followed by Tukey's test for multiple comparisons. **p<0.01, ***p<0.001. (C) Correlation of Treg frequency and sCD25 concentration.

Fig 3. Botensilimab increases the frequency and metabolic activity of dendritic cells (DC) in the myeloid compartment



Description	Score*	p.adjust
Oxidative phosphorylation	1.73	8.73E-05
Respiratory electron transport chain	1.69	6.30E-03
ATP synthesis coupled electron transport	1.69	6.30E-03
Mitochondrial ATP synthesis coupled electron transport	1.69	6.30E-03
Aerobic electron transport chain	1.64	2.13E-02



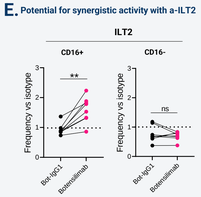


Fig 3. Human PBMCs were treated as described in Fig 1. (A) Average frequencies of DCs, monocytes, and macrophages were calculated relative to the total myeloid population. (B) The increased frequency of DCs was confirmed by flow cytometry. DCs were defined as CD3-CD19-CD14-CD11c+HLA-DR+ of CD45. One-Way ANOVA followed by Tukey's test for multiple comparisons, **p<0.01. (C) Differential gene expression was calculated for dendritic cells at the 72h timepoint between botensilimab and bot-IgG1. Ranked list gene set enrichment was calculated. The top five most significant gene sets are shown. (D) Quantification of activation markers CD40, CD83, CD86, and HLA-DR by flow cytometry in the CD19-CD3-CD16+CD11c+ myeloid population after 72h of immune stimulation. Each point represents the fold change over isotype for an individual donor. Dotted line = isotype. Unpaired, two-tailed T test. **p<0.01, ***p<0.001. (E) Quantification of the inhibitory receptor ILT2 on the myeloid population in (D) after 72h of immune stimulation. Each point represents the fold change over isotype for an individual donor. Dotted line = isotype. Unpaired, two-tailed T test. **p<0.01.

Fig 4. Enhanced T cell priming by botensilimab leads to upregulation of TCR, IL12, and type I interferon signaling

Description	Score*	p.value	p.adjust	Top enriched genes (maximum of 20 shown)
TCR signaling	1.52	5.80E-06	0.001745	PSMB8, PSMB9, PSMB1, CD3E, CD3D, PSMB10, UBE2D2, PSME2, PLCG2, PTPRC, PSMA5, PSME1, MALT1, HLA-DPB1, TRAT1, LCK, PAK2, CD3G, LAT, PSMA1...
Downstream TCR signaling	1.51	1.50E-05	0.003772	PSMB8, PSMB9, PSMB1, CD3E, CD3D, PSMB10, UBE2D2, PSME2, PSMA5, PSME1, MALT1, HLA-DPB1, TRAT1, LCK, CD3G, PSMA1, UBC, BCL10, NFKBIA, UBB
IL-12 family signaling	1.54	9.86E-05	0.011299	STAT1, CDC42, MIF, JAK1, RAP1B, PSME2, HNRNPDL, P4HB, SOD1, PAK2, ARF1, SNRPA1, PDCD4, PPIA, IL27RA, HSPA9, LCP1, ANXA2, CANX, RALA...
IL-12 signaling	1.53	0.000157	0.015786	ANXA2, CANX, RALA, AIP, TALDO1, HNRNPF, HNRNPA2B1, IL6ST, CAPZA1, GSTO1, CFL1, IL12RB1, SOD2, PITPNA
JAK-STAT signaling after IL-12 stimulation	1.52	0.000676	0.03213	CDC42, MIF, RAP1B, PSME2, HNRNPDL, SOD1, PAK2, ARF1, SNRPA1, PDCD4, PPIA, HSPA9, LCP1, ANXA2, RALA, AIP, TALDO1, HNRNPF, HNRNPA2B1, CAPZA1 ...
Interferon alpha/beta signaling	1.62	1.16E-06	0.000582	IFITM1, STAT1, PSMB8, RSAD2, HLA-C, MX1, IFI6, ISG15, JAK1, IFIT3, BST2, ISG20, SAMHD1, HLA-F, IRF1, HLA-E, IFIT2, IRF7, IRF2, OAS1...
Scavenging by Class A Receptors	1.73	1.18E-08	1.78E-05	HSP90B1, CALR, FTL, FTH1
Binding & Uptake of Ligands by Scavenger Receptors	1.63	4.88E-06	0.001745	HSP90B1, CALR, FTL, FTH1, APOL1
Antigen presentation with class I MHC	1.54	0.001026	0.03213	HLA-C, CALR, PDIA3, HLA-F, HLA-E, HSPA5, HLA-B, TAP2, BCAP31, TAP1, B2M, CANX, ERAP2, HLA-A, SEC31A
ER-Phagosome pathway	1.52	2.22E-05	0.004777	PSMB8, HLA-C, PSMB9, PSMB1, CALR, PSMB10, PSME2, PDIA3, HLA-F, HLA-E, PSMA5, PSME1, PSMA1, HLA-B, UBC, TAP2, TAP1, SEC22B, UBB, PSMD12

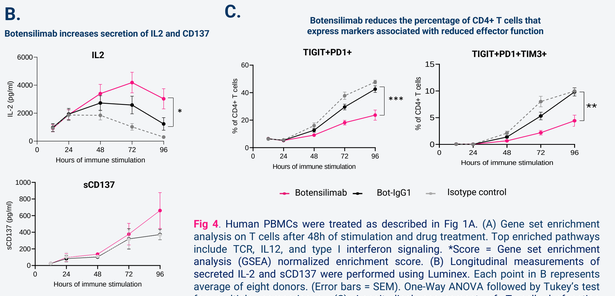


Fig 4. Human PBMCs were treated as described in Fig 1A. (A) Gene set enrichment analysis on T cells after 48h of stimulation and drug treatment. Top enriched pathways include TCR, IL12, and type I interferon signaling. *Score = Gene set enrichment analysis (GSEA) normalized enrichment score. (B) Longitudinal measurements of secreted IL-2 and sCD137 were performed using Luminex. Each point in B represents average of eight donors. (Error bars = SEM). One-Way ANOVA followed by Tukey's test for multiple comparisons. (C) Longitudinal assessment of T cell dysfunction phenotypes PD1+TIGIT+ and PD1+TIGIT+TIM3+ on CD4 T cells by flow cytometry.

Fig 5. Botensilimab upregulates metabolic pathways that correlate with enhanced immune activity

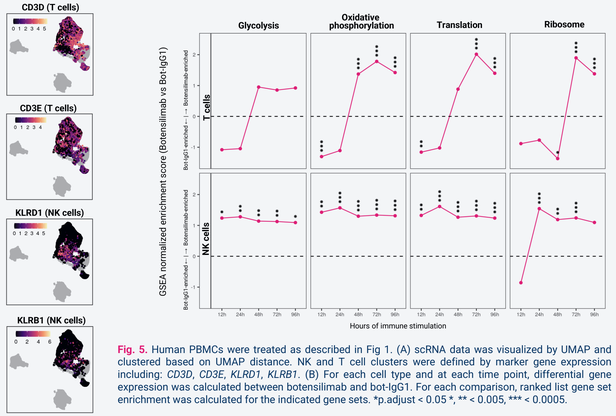


Fig. 5. Human PBMCs were treated as described in Fig 1. (A) scRNA data was visualized by UMAP and clustered based on UMAP distance. NK and T cell clusters were defined by marker gene expression including: *CD3D*, *CD3E*, *KLRD1*, *KLRB1*. (B) For each cell type and at each time point, differential gene expression was calculated between botensilimab and bot-IgG1. For each comparison, ranked list gene set enrichment was calculated for the indicated gene sets. *p.adjust < 0.05 *, ** < 0.005, *** < 0.0005.

Translating mechanistic signatures to clinical biomarkers

Objective

As a class, immunotherapies deliver durable and even curative responses, but many patients experience limited or no benefit. Biomarkers that predict patient response to immunotherapy are needed to enable personalized treatment regimens. Their discovery is complicated by the complexity of the immune system and its interactions with tumor and tumor microenvironmental factors. We hypothesized that deep, mechanistic profiling of immunotherapy drugs would drive discovery of novel biomarkers. Agenus' VISION (Virtual Systems in Immuno-Oncology) platform is designed to derive markers and signatures of clinical drug activity from translational assay models through the application of computational and statistical methods across disparate measures such as single cell RNA sequencing, cytokine levels, flow markers, among others. Here, we defined the cellular and molecular characteristics of an immune response driven by botensilimab based on the stimulation of healthy donor PBMCs, derived significant underlying mechanisms of response, and projected those features onto patient samples from the Phase I clinical trial of botensilimab.

Fig 6. A mechanistic gene expression signature predicts clinical response to botensilimab in a retrospective analysis of Phase I data

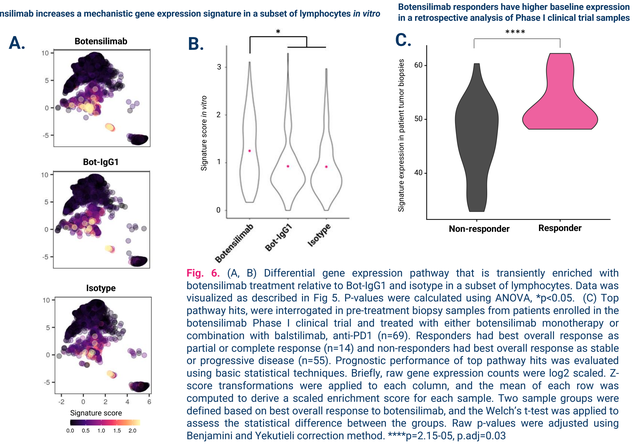


Fig. 6. (A, B) Differential gene expression pathway that is transiently enriched with botensilimab treatment relative to Bot-IgG1 and isotype in a subset of lymphocytes. Data was visualized as described in Fig 5. P-values were calculated using ANOVA. *p<0.05. (C) Top pathway hits, were interrogated in pre-treatment biopsy samples from patients enrolled in the botensilimab Phase I clinical trial and treated with either botensilimab monotherapy or combination with balstilimab, anti-PD1 (n=69). Responders had best overall response as partial or complete response (n=14) and non-responders had best overall response as stable or progressive disease (n=55). Prognostic performance of top pathway hits was evaluated using basic statistical techniques. Briefly, raw gene expression counts were log2 scaled. Z-score transformations were applied to each column, and the mean of each row was computed to derive a scaled enrichment score for each sample. Two sample groups were defined based on best overall response to botensilimab, and the Welch's t-test was applied to assess the statistical difference between the groups. Raw p-values were adjusted using Benjamini and Yekutieli correction method. ****p=2.15-05, p.adj=0.03

Conclusions

- By performing deep single cell profiling on stimulated human PBMCs, we demonstrated mechanisms by which botensilimab is superior to first-generation anti-CTLA-4 in driving deep and broad immune responses, including:
  - relief of immunosuppression
  - expansion of dendritic cells
  - reprogramming of immunometabolism
  - enhanced TCR signaling
- Botensilimab is advancing in phase I/2 clinical studies alone and in combination with balstilimab, anti-PD-1(NCT03860272), AGEN2373, anti-CD137(NCT04121676) and AGEN1571, anti-ILT2(NCT05377528)
- Increased expression of ILT-2 in botensilimab-treated immune subsets supports the combination strategy with AGEN1571 (anti-ILT2) currently under clinical investigation
- Signatures of the botensilimab immune response identified in the laboratory may predict clinical outcomes for patients on treatment.

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