Indoximod or ibrutinib/indoximod based clinical chemo-immunotherapy drives conversion of extra-tumoral circulating stem-like precursor CD8+ T cells into clonally expanded, rejuvenated effector cells

#LB200

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Background and Methods

Hypothesis

- Many of the effector CD8+ T cells that are "rejuvenated" by immunotherapy come from outside the tumor and derive from a circulating pool of "stem-like" memory or "precursor-exhausted" (T-PEX) cells.
  - These cells have been characterized in mice, but, despite their importance, circulating counterparts in humans have not yet been identified for study.
- We hypothesize that immunotherapy designed to enhance immunogenic antigen-presentation during chemotherapy might extensively reactivate these precursor T cells.
  - While the antigen-presentation step occurs in tissues, homing of the rejuvenated T cells to the tumor is via the circulation; thus, we hypothesize that they can be found in blood.

Immunotherapy targets expressed by dendritic cells

- The indoleamine 2,3-dioxygenase (IDO) pathway is an innate immunoregulatory mechanism in dendritic cells (DCs) that drives tolerance to apoptotic cells; IDO is exploited by tumors to evade immune responses.
- Bruton's Tyrosine Kinase (BTK) acts as a key upstream driver of the tolerogenic IDO pathway during chemotherapy (ref. *[1]*).
  - In tumor-associated dendritic cells (DCs), the BTK signaling pathway integrates local immunosuppressive signals such as TGFβ and CTLA-4 in the tumor microenvironment and allows these to over-drive and stabilize high IDO expression.
- BTK and IDO function together as a single, cell-intrinsic metabolic signaling pathway in DCs to block inflammatory DC maturation and suppress immunogenic cross-presentation of antigens from dying tumor cells – This is the BTK→IDO checkpoint (Fig 1).

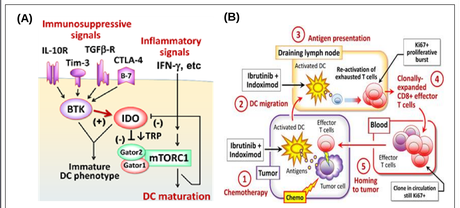

Figure 1. The BTK→IDO checkpoint in dendritic cells.
(A) Proposed BTK→IDO signaling model within dendritic cells (DCs), adapted from ref. *[1]*. (B) Temporospatial model for reactivation ('rejuvenation") of CD8+ T cells. Antigens are acquired locally in the tumor by activated DCs, which then migrate to tumor-draining lymph nodes (TDLNs) and cross-present tumor antigens to reactivate resting T-PEX cells. Normally this whole process would be suppressed by the BTK→IDO checkpoint, which prevents DC maturation. However, in the presence of ibrutinib/indoximod, the T cells are able to activate, proliferate, exit from the LNs, and home back to the tumor via the bloodstream. Circulating activated T cells readily re-enter the brain-tumor site in the presence of inflammation.

Chemo-immunotherapy treatments and patient selection

- Patients with pediatric brain tumors were selected from three clinical trials of chemo-immunotherapy:
  - Phase 1 trial (NCT02502708, NLG2105) of the IDO pathway-inhibitor indoximod plus oral temozolomide (TMZ) chemotherapy;
  - Phase 2 trial (NCT04049669, GCC1949) using indoximod + TMZ;
  - Phase 1 trial (NCT05106296, GCC2020) of dual immunotherapy using indoximod plus BTK-inhibitor ibrutinib, with oral cyclophosphamide and etoposide chemotherapy.
- Patients were selected retrospectively from the above trials for *post-hoc* analysis (not to analyze prespecified endpoints).
- Patients were chosen to give a broad representation of tumor histologies (recurrent medulloblastoma, ependymoma and glioblastoma; and newly-diagnosed DIPG), and to include a range of clinical responses.
  - Longitudinal blood samples (4-10 samples per patient) were obtained over a period of 6-24 months and analyzed by single-cell RNA-sequencing (scRNA-seq) with paired single-cell T cell receptor sequencing (scTCR-seq).

Results

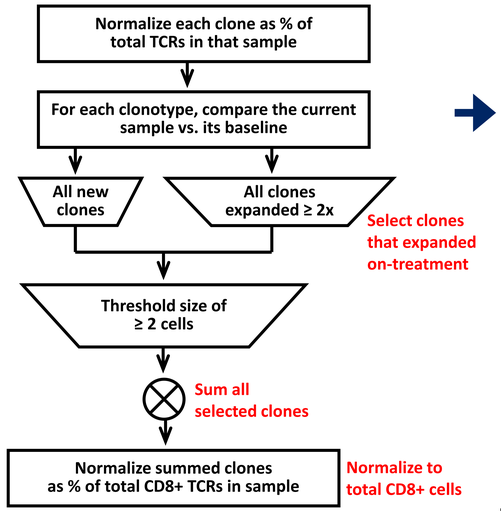

Figure 2. Quantitation of clonal expansion using the Clonal Expansion Index (CEI).
Indoximod or ibrutinib/indoximod based chemo-immunotherapy drives expansion of circulating CD8+ effector T cells. Single-cell RNA-sequencing (scRNA-seq) and scTCR-seq were performed on blood, and a Clonal Expansion Index (CEI) for each on-treatment sample was calculated as follows: the on-treatment sample was compared to the pre-treatment baseline, and TCR clonotypes of interest were defined as those with threshold clone size of at least 2 cells in the on-treatment sample, and that showed ≥ 2-fold expansion (or *de novo* appearance) compared to the baseline. The cells in all expanded CD8+ clonotypes were summed and expressed as the percentage of total CD8+ cells in that sample.

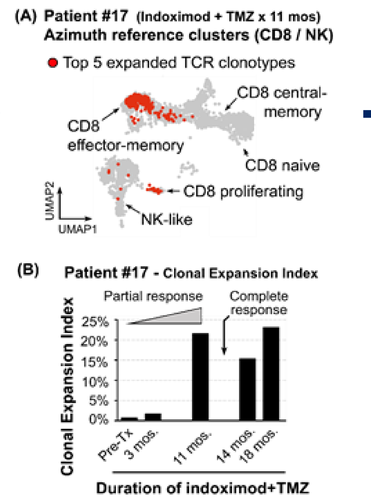

Figure 3. Expanded circulating T cell clones in a GCC1949 patient with a complete response (CR) to indoximod plus chemotherapy.
Patient 17 (recurrent medulloblastoma) had a complete response after 11 cycles of indoximod plus temozolomide (TMZ). (A) Cells with the top 5 TCR clonotypes that expanded, relative to the baseline sample, are shown as red dots projected onto the standard Azimuth reference populations for CD8+ T cells. The expanded clones were mostly either CD8+ effector cells or CD8+ proliferating cells. (B) Clonal Expansion Index (CEI) as described in Fig.2, during continued treatment. Up to 20% of the total CD8+ repertoire became treatment-induced expanded clones. Clinical iRANO tumor response is shown above the graph.

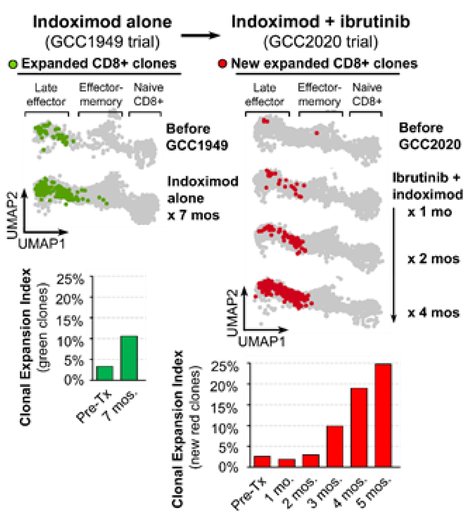

Figure 4. Expansion of new circulating T cell clones in response to ibrutinib/indoximod.
To address the key question of whether adding ibrutinib could reactivate T cell responses after tumor progression on indoximod, a patient with recurrent medulloblastoma crossed over to the GCC2020 trial due to progressive disease after 2 years (25 cycles) of indoximod plus chemotherapy on the GCC1949 trial. The green dots (projected onto the standard Azimuth reference populations for CD8+ T cells) and green bars show the initial clonal expansion (CEI) on indoximod alone; while the red dots and red bars show the robust expansion of additional new clones that were elicited by synergistic addition of ibrutinib. Of note, the old clones (green) from the prior therapy did not expand further, and hence did not contribute to the red dots.

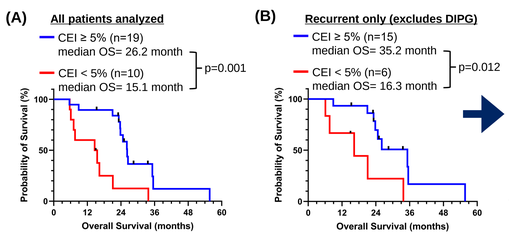

Figure 5. Elevated CEI correlates with improved survival.
(A) A Kaplan-Meier analysis of 29 patients treated with indoximod or ibrutinib/indoximod based therapy (recurrent medulloblastoma, ependymoma and glioblastoma; and newly-diagnosed DIPG; drawn from NLG2015, GCC1949, and GCC2020 trials), stratified by whether their CEI was above (blue line, n=19, median OS 26.2 months) or below (red line, n=10, median OS 15.1 months) 5% of total CD8+ T cells (p=0.001). (B) A Kaplan-Meier analysis of the 21 patients with recurrent disease from "A" above (excludes the DIPG patients), stratified by whether their CEI was above (blue line, n=15, median OS 35.2 months) or below (red line, n=6, median OS 16.3 months) 5% of total CD8+ T cells (p=0.012). Logrank test was used to calculate p values.

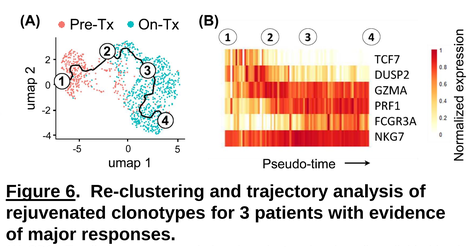

Figure 6. Re-clustering and trajectory analysis of rejuvenated clonotypes for 3 patients with evidence of major responses.
(A) CD8+ T cell clones were pooled and re-clustered, using all available blood samples (n=22) from 3 patients with either (i) massive clonal expansion of activated CD8+ T cells on therapy (expanded clones reaching 16-25% of total CD8+ T cells); (ii) complete radiographic tumor response (CR) on treatment; or (iii) both. These 3 patients included the two shown in Figs. 3 and 4, plus one additional patient who had a near-complete response on GCC1949, followed by CR on GCC2020. Colors show whether the individual cells derive from a pretreatment or on-treatment sample for that clone. Superimposed line shows Monocle trajectory analysis. (B) A heatmap shows expression of selected genes (normalized for each gene) over the pseudo-time differentiation trajectory shown in (A). Results from a representative patient are shown in (A) and (B).

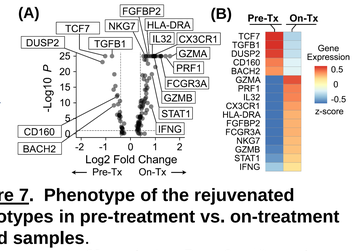

Figure 7. Phenotype of the rejuvenated clonotypes in pre-treatment vs. on-treatment blood samples.
(A) Using the set of n=22 samples from Figure 6, a differential gene expression analysis was performed using pooled clonotypes from pre-treatment and on-treatment samples (volcano plot; vertical axis shows Log10 corrected P value). (B) Normalized relative expression (z-score) of selected genes is shown; p<0.001 for each gene shown. At earliest appearance, each clonotype showed a "hybrid" combination of genes associated with immaturity/arrest (BACH2, DUSP2, LTB, IL7R, CD160) and effector/memory (NKG7, GZMK, GZMA). Within each responding clone, this "precursor" phenotype progressively transitioned into a mature effector phenotype (PRF1, GZMB, GZMH, FGFBP2, KLRB1, IFNG). Results from a representative patient are shown in (A) and (B).

Conclusions

We hypothesize that:

- For patients treated with chemo-immunotherapy, the relevant site to look for re-activated T cells is the peripheral blood.
- Expansion of effector CD8+ clones during treatment is associated with better outcome.
- By tracing TCR clonotypes sequentially across multiple cycles of treatment, we can observe the entire sequence of reactivation, from resting stem-like precursors (T-PEX) to fully activated late effector T cells.

Future Directions

- This is a rich source of mechanistic data on the key transition from exhausted to rejuvenated effector cells and may reveal new actionable targets.
- Ongoing trials are actively enrolling patients with pediatric brain cancer :
  - GCC1949 phase 2 trial (NCT04049669), indoximod plus oral temozolomide.
  - GCC2020 phase 1 trial (NCT05106296), indoximod plus ibrutinib, with oral cyclophosphamide and etoposide.
  - For referrals, contact: thjohnson@augusta.edu; tayking@augusta.edu

Acknowledgements

Funding was provided by:

- Janssen Scientific Affairs, LLC (provided ibrutinib)
- Lumos Pharma, Inc. (provided indoximod)
- NIH (R01 CA229646)
- Alex's Lemonade Stand Foundation
- Beloco Foundation
- Cannonball Kids' cancer Foundation
- Hyundai Hope on Wheels Foundation
- Miriam Lloyd Halsey Foundation
- Northern Nevada Children's Cancer Foundation
- Press On Foundation
- Trial Blazers for Kids Foundation

References

[1] Sharma MD, Pacholczyk R, Shi H, Berrong ZJ, *et. al.* Inhibition of a BTK-IDO-mTOR axis promotes differentiation of monocyte-lineage inflammatory dendritic cells and enhances anti-tumor T cell immunity. *Immunity*. 2021 Oct 12;54:2354-2371.

Presented at the 2023 Annual Meeting of the American Association for Cancer Research. April 18, 2023. Orlando, Florida.