iOTarg screening platform reveals novel mechanisms of colorectal cancer evasion from antigen-specific tumor cell killing by T cells

Authors and affiliations
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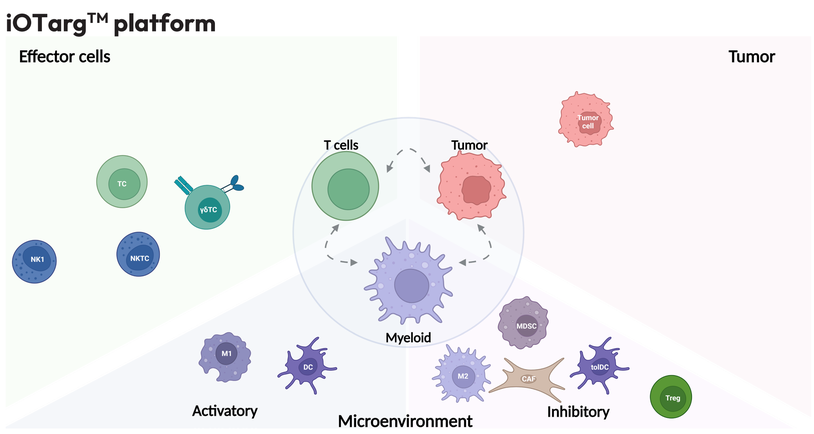
Introduction

We hereby introduce iOTarg™, a cutting-edge and versatile genetic screening platform designed to uncover immune resistance pathways within the tumor microenvironment. In this study, we focus on colorectal cancer (CRC), a disease where conventional immune-checkpoint blockade therapies have shown very limited succes so far. Therefore, iOTarg was engineered to reveal and analyze evasion mechanisms that might potentially be unique to the CRC setting in subverting antigen-specific T cell responses. This could unlock new avenues for future CRC treatments.

In a CRC cell line co-culture system (SW480, HCT116 or Colo-678) with different TC sources, we applied important aspects of tumor resistance to killing. Survivin-specific TCs cause strong tumor lysis, while tumor-infiltrating lymphocyte 412 (TIL412) show a weaker basal killing that reflects the situation in the tumor. Knockout of ß2-microglobulin blocked TC-mediated killing, while c-FLIP knockout increased tumor lysis significantly. Interestingly, PD-L1 knockout did not affect TC activation or killing. We screened over 6000 druggable genes revealing several critical candidates, including anti-apoptotic BIRC family members (BIRC2, 3), apoptosis regulators (Bcl-xL, XIAP), TNF signaling regulators (TRAF2, MAP3K7), and genes recently described as sensitization mediators (B3GNT3, CHMP4B). Caspase-8 knockout prevented TC-mediated killing of CRC cells, confirming its crucial role in immune-mediated tumor cell clearance. Further validation in secondary screenings reinforced a subset of genes as strong regulators of tumor cell killing, uncovering an array of CRC resistance mechanisms. Further validations are ongoing for selected novel hits in complex and disease-relevant models.

Our findings present a pioneering platform delineating resistance mechanisms against T cell-mediated colorectal tumor killing. We find known and new targets that could lead to new treatments and improve immune oncology options. This platform has the potential to discover new targets that may lead to new therapies targeting the tumor microenvironment, which could change how CRC is treated.

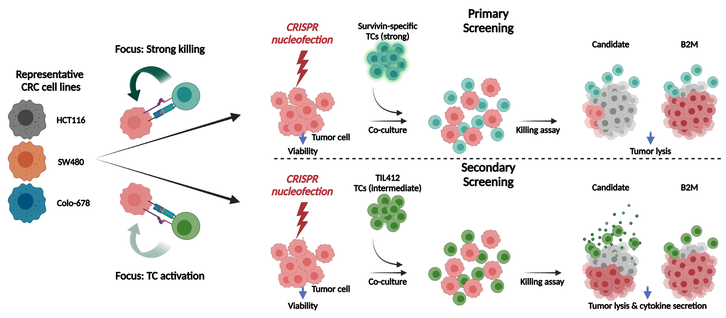


Results

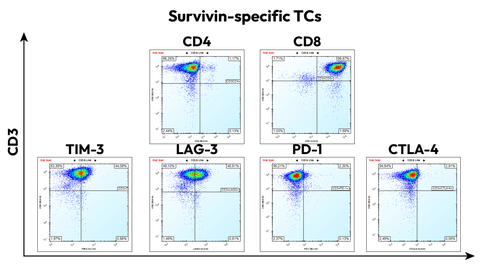
iOTarg™ platform provides different specificities, strengths and compositions of TC-TU interactions

- Survivin-specific CD8⁺TCs recognize HLA-A2-positive CRC cell lines (SW480, HCT116, and Colo-678) and induce strong killing
- TIL412 is a mixture of CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes inducing intermediate killing of MART-1-pulsed CRC cell lines
- TIL412 show a highly exhausted memory phenotype whereas the Survivin-specific TCs are of a less exhausted effector phenotype

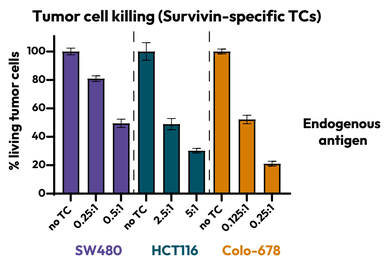
A. CRC iOTarg scheme

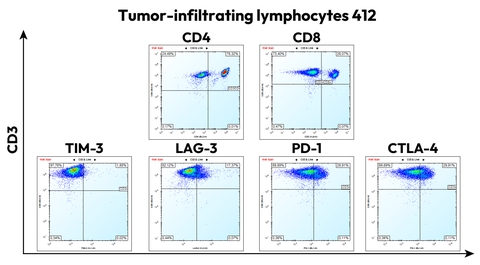


B. Effector TC phenotype



C. Co-culture system





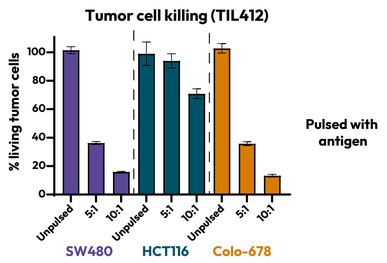


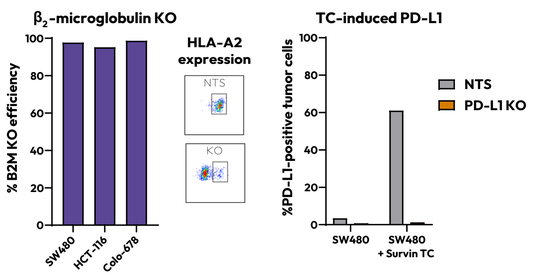
FIGURE I

A. Schematic representation of the iOTarg™ screening process. The primary screen involves co-culturing of three CRC cell lines (SW480, HCT116, or Colo-678) with enriched Survivin-specific CD8-positive TCs. The cell lines are nucleofected for a given gene and rested before co-culture. Tumor cell lysis is measured after 24 hours of killing. In the second part, the same cell lines are genetically modified and cultured with the TIL mixture 412 for 24 hours before measuring tumor cell killing and TC activation. **B**. FACS analysis of Survivin-specific TCs (top) and TIL412 (bottom) after expansion. Living immune cells were labeled for CD3, CD4, CD8, TIM-3, LAG-3, PD-1, and CTLA-4. Cells were also stained for naive, effector, and memory phenotype (data not shown). **C**. Representative killing assay of CRC cell lines SW480, HCT116, and Colo-678 with Survivin-specific TCs or TIL412 in different effector-to-target ratios. Tumor cells were seeded and rested before pulsing with MART-1 peptide (only for TIL412 co-culture). After co-culture with effector cells, tumor cell survival was measured by CTG. Bar graphs show mean +/- SEM.

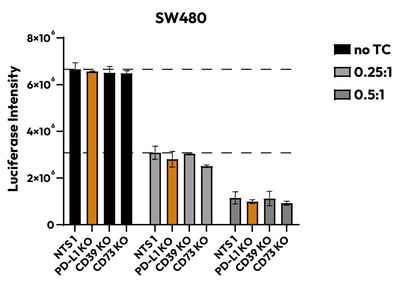
Assessment and establishment of relevant controls for evaluating the screen performance

- Arrayed nucleofection with RNP complexes induces around 80-90% knockout in control genes (RNA and protein)
- Knockout of classical immune checkpoints (PD-L1, CD39/73, and others) did not increase tumor cell susceptibility to killing
- Knockout of $ß_2$-microglobulin prevents MHC expression and completely abrogates tumor cell killing
- Knockout of intracellular resistance genes (to TC killing) significantly increased TC-mediated killing of CRC cell lines

A. Efficacy of nucleofection-based CRISPR knockouts



B. Classical immune checkpoints



C. Control performance for highthroughput screening

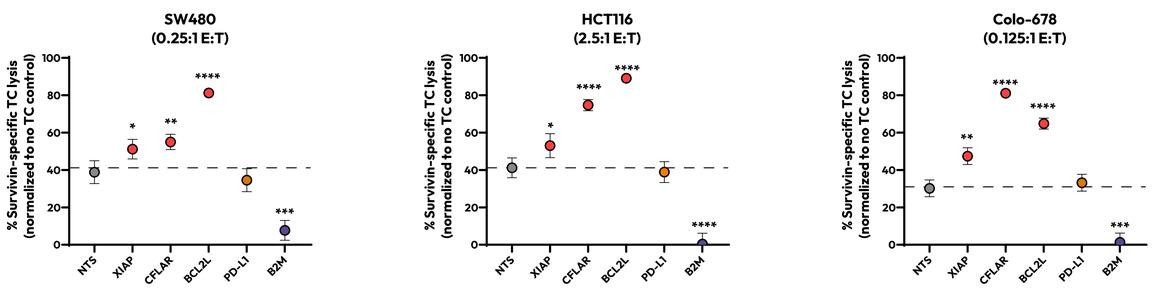


FIGURE II

A. Knockout efficacy of B2M and CD274 (PD-L1) in CRC cell lines was measured by FACS. Cells were rested for 72 hours after nucleofection, and B2M expression was normalized to expression on cells nucleofected with non-targeting sequences (NTS). For PD-L1 surface expression measurement, tumor cells were co-cultured with Survivin-specific TCs overnight. **B**. Killing assay with known immune checkpoints. SW480 cells were nucleofected with RNPs targeting PD-L1, CD39, and CD73 and co-cultured with Survivin-specific TCs for 24 hours. Surviving tumor cells were measured by CTG. **C**. Positive controls for high-throughput screening. Cells were CRISPRed as before and co-cultured with Survivin-specific TCs. Surviving tumor cells were measured by CTG, and tumor cell lysis was calculated by normalization to the no TC setting.

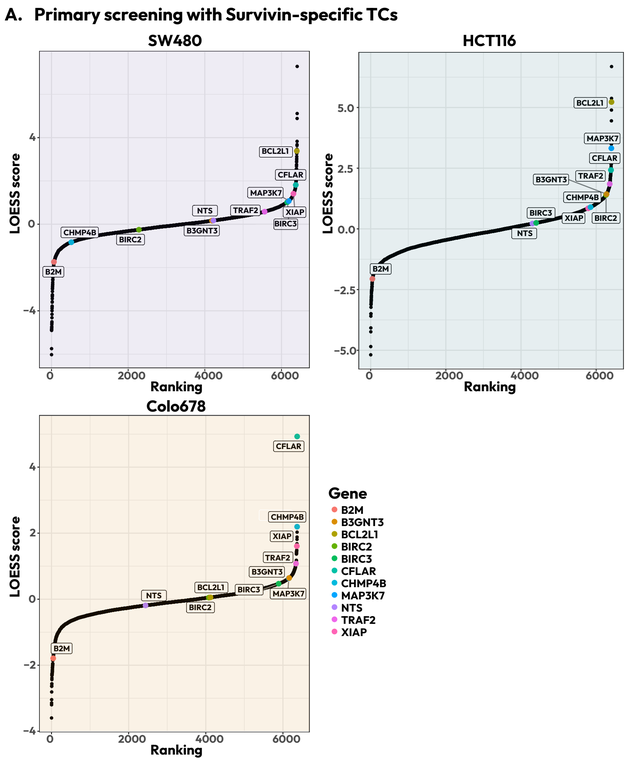
Statistical information

If not indicated otherwise, graphs are representative data from at least two independent experiments. Data points show mean ± SEM. Significance was calculated by two-tail Student's t-test.

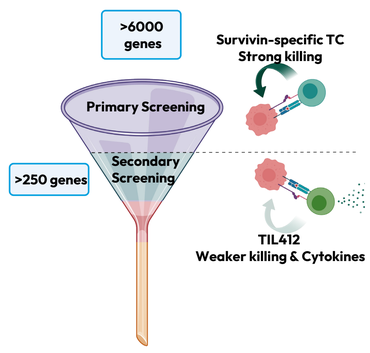
iOTarg™ platform discovers known and novel regulators of TC-mediated killing in CRC

- More than 6000 druggable genes were screened in co-culture of CRC cell lines and Survivin-specific TCs
- iOTarg™ rediscovered several known regulators of TC-mediated killing (e.g., BIRC family, c-FLIP and XIAP)
- Top hits from the primary screenings were rescreened in a confirmatory screen focusing on weaker killing and TC activation

A. Primary screening with Survivin-specific TCs



B. Secondary Screening Workflow



C. Secondary Screening Output

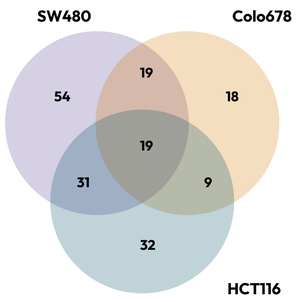


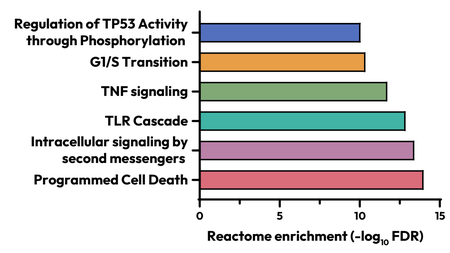
FIGURE III

A. High-throughput iOTarg™ Screening in three cell lines (with Survivin-specific TCs) to identify novel immune checkpoints in colorectal cancer. Tumor cells were nucleofected with a CRISPR library targeting approximately 6000 genes and co-cultured with Survivin-specific TCs or medium alone. Tumor viability was measured using CTG readout, and gene ranking was based on impact on TC-mediated tumor lysis. Z-scores were calculated using the CTG values from cytotoxicity (with TCs) and viability (without TILs) setup. Local regression (LOESS) was used to fit cytotoxicity to viability, and LOESS score-ranked results are shown for SW480, HCT116, and Colo678. **B**. Scheme of the secondary screening workflow. More than 250 genes selected in the primary screening were tested using new sgRNA sequences. The CRC cell lines were nucleofected, pulsed with MART-1 antigen, and co-cultured with TIL412. Tumor cell lysis was measured as before, and in addition, IFNg and IL-2 secretion of TCs was determined by ELISA. **C**. Venn diagram showing the overlap of targets in all secondary screenings.

CRC resistance mechanisms against TC-mediated killing include downstream TNF signaling

- Hits of the CRC iOTarg™ screening are enriched in pathways of apoptosis regulation and TNF signaling
- Pathways enriched in the screening hits have several interaction nodes
- Several novel genes with previously unreported function in regulating tumor's resistance to immune attack were identified

A. Pathways and neworks enriched in screening hits



B. Network map of pathways

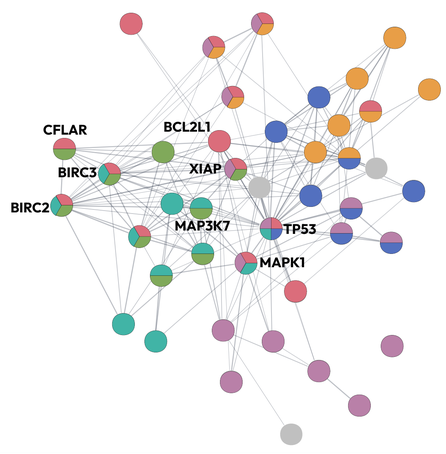


FIGURE IV

A. Pathways and regulatory nodes were significantly enriched in the iOTarg™ CRC screening. Hits from the secondary screening were analyzed with g:Profiler for enrichment in the Reactome pathway dataset. Pathways with more than 500 genes were excluded. **B**. Network visualization of A. Data was visualized using the EnrichmentMap App for Cytoscape. Enrichment significance was calculated as -log10 of False Discovery Rate.

Conclusion

- iOTarg™ is a novel genetic screening platform that uncovers immune resistance pathways in the TME
- The platform was applied to CRC due to the suboptimal response rate of most patients to immune-checkpoint blockade
- The screening reconfirmed several known tumor-intrinsic regulators of TC-mediated killing (e.g., BIRC family, c-FLIP, and XIAP).
- The role of intratumoral TNF signaling in desensitizing tumors to immune cell attack, as indicated by emergence of hits associated with the TNF downstream pathway was reinforced
- Identification of several new genes with previously unreported roles in desensitizing tumors against TC-mediated killing gives us confidence in the ability of our discovery platform

Contact