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Effector Cytokine Profiles of Ex Vivo Expanded CTLs in Colorectal Cancer HCT-116 Cells Co-Culture Models

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ABSTRACT

Background: Colorectal cancer (CRC) remains a major cause of cancer-related mortality worldwide, with limited benefit from conventional therapies in advanced disease. Cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells) are critical mediators of anti-tumor immunity, primarily through direct cytotoxicity and cytokine secretion. However, the dual roles of CTL-derived cytokines, particularly interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), in CRC progression remain incompletely understood. This study aims to analyze the cytokine profile (IFN-γ, TNF-α, and IL-6) in a CTL–CRC cell direct co-culture model. **Methods**: $CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) of CRC patients using magnetic negative selection and activated for 5 days with anti-CD3/CD28 beads and IL-2. Purity and viability were assessed by flow cytometry and morphology. Activated CTLs were co-cultured with the HCT116 CRC cell line at effector-to-target (E:T) ratios of 1:1, 5:1, and 10:1 under direct contact for 48 h. Supernatants were collected and cytokine levels (IFN-γ, TNF-α, and IL-6) were quantified using validated sandwich ELISA kits. **Results**: Direct co-culture with HCT116 cells significantly increased cytokine secretion in an E:T ratio-dependent manner. IFN-γ secretion rose from 3044.6±120 pg/mL at 1:1 to 4882.1±198 pg/mL at 5:1, plateauing thereafter. TNF-α levels remained relatively constant (628.6±67 pg/mL at 1:1 vs. 674.0±91 pg/mL at 10:1). IL-6, nearly undetectable at 1:1 (0.4±0.1 pg/mL), increased dose-dependently to 5.1±0.9 pg/mL at 10:1. **Conclusion**: Ex vivo expanded CTLs from CRC patients exhibit a distinct cytokine secretion profile characterized by robust IFN-γ release, stable TNF-α production, and a dose-dependent increase in IL-6 across different effector-to-target ratios.

Keywords: Co-Culture; Cytotoxic T Lymphocytes; HCT-116; Immunotherapy; Pro-inflammatory Cytokines

PENDAHULUAN

Colorectal cancer (CRC) is one of the most common malignancies worldwide, ranking as the third most frequently diagnosed cancer and the second leading cause of cancer-related deaths.(1) In 2020, there were an estimated 1.9 million new cases and 935,000 deaths globally. Despite advances in surgery, chemotherapy, and targeted therapy, the prognosis for advanced CRC remains poor, highlighting the need for better understanding of tumor–immune interactions to guide immunotherapy development. (2) The tumor microenvironment (TME) plays a pivotal role in CRC progression, consisting of tumor cells, stromal components, and infiltrating immune cells. (3) Among them, cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells) are central effectors of anti-tumor immunity. CTLs kill cancer cells directly through perforin–granzyme pathways and indirectly by releasing pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). These cytokines enhance antigen presentation, promote tumor cell apoptosis, and shape the immune contexture of the TME. (4) However, CRC cells and the surrounding stroma often counteract immune-mediated destruction by secreting immunoregulatory cytokines. Interleukin-6 (IL-6), a multifunctional cytokine frequently elevated in CRC patients, is linked to

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tumor progression through activation of the STAT3 signalling pathway, epithelial–mesenchymal transition, and resistance to apoptosis. (5) In a retrospective study of 162 CRC patients, serum IL-6 and TNF-α levels were significantly associated with lymph node metastasis, TNM stage, and tumor differentiation grade ($P < 0.05$), suggesting their value as markers of disease progression. (6) Another study with 30 CRC patients versus 30 healthy controls found significantly decreased serum TNF-α in CRC ($p = 0.016$), indicating complex, context-dependent roles of these cytokines in tumorigenesis. (7)

Co-culture models provide an effective in vitro approach to investigate these immune–tumor interactions under controlled conditions. For example, co-culture of $CD8^+$ T cells with CRC cells such as HT29 has been shown to increase secretion of IFN-γ, TNF-α, and IL-6, while promoting cancer cell death in direct-contact systems. (8) Moreover, exposure of murine CRC cells (MC38) to activated T cells induced immunogenic cell death (ICD) markers such as HMGB1 and calreticulin, further supporting the importance of CTL-derived cytokines in modulating tumor immunity. (9) Importantly, synergistic signaling among IL-6, TNF-α, and IL-17A has been shown to activate STAT3 and NF-κB, creating a pro-tumorigenic inflammatory loop that fosters CRC growth. (10)

Taken together, these findings underscore the dual roles of CTL-secreted cytokines in CRC: while IFN-γ and TNF-α enhance anti-tumor immunity, IL-6 contributes to immune evasion and tumor progression. To further clarify these dynamics, this study aims to analyze the cytokine profile (IFN-γ, TNF-α, and IL-6) in a CTL–CRC cell direct co-culture model. Such insights may deepen our understanding of immune regulation in CRC and help identify therapeutic targets for immunomodulation.

MATERIALS AND METHODS

Study Design

This study was designed as a controlled in vitro experimental investigation aimed at quantifying the production of IFN-γ, TNF-α, and IL-6 in co-culture systems of human cytotoxic T lymphocytes (CTLs; $CD8^+$ T cells) and human CRC cell line (HCT116) under direct-contact conditions. The design incorporated three ratios (1:1, 5:1, 10:1) for 48h incubation time. Randomization was achieved through computer-generated plate maps and sample order processing, while analysts were blinded to group assignments during ELISA reading and statistical evaluation. Technical replication was set in triplicates per condition. All procedures were performed under informed consent and BSL-2 biosafety standards.

T cell Isolation

Peripheral blood samples (20–30 mL) were collected from CRC patient who had not received immunomodulatory treatment within 14 days. Blood was processed within 4 h, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation. $CD8^+$ T cells were enriched by magnetic negative selection kits, targeting ≥90% purity as confirmed by flow cytometry. Cells were activated for 5 days in RPMI-1640 supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, HEPES, and IL-2 (100 IU/mL), using anti-CD3/CD28 beads at a 1:1 bead-to-cell ratio. Beads were magnetically removed before co-culture, and activated CTLs were confirmed to maintain viability above 90%.

Cell Line and Co-culture

CRC cell line HCT-116 (ECACC) were maintained in McCoy's 5A supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. Cells were used at passages below 20. For co-culture, CRC cells were seeded one

day prior to experiments at 1×10^5 cells per well in 48-well plates to reach ~80% confluence. Activated CTLs were washed and added at designated E:T ratios under direct conditions. Controls included CRC-only, CTL-only, and medium blanks. Co-cultures were incubated for 48 h at 37 °C and 5% CO_2. Supernatants were collected at the time point, centrifuged, aliquoted, and stored at –20 °C.

Cytokines Assessment using ELISA

Cytokine levels were measured using validated sandwich ELISA kits for human IFN-γ, TNF-α, and IL-6 (Elabscience, Houston, TX, USA). Standards and samples were run in duplicate, and standard curves were fitted using a four-parameter logistic regression model ($R^2 \geq 0.99$). Assay quality was ensured through recovery testing, intra-assay CV ≤15%, and inter-assay CV ≤20%. Cytokine concentrations were reported in pg/mL and optionally normalized to viable cell counts per well.

Statistical Analysis

Statistical evaluation of the cell viability data from the combination treatment was performed using a one-way ANOVA, followed by Tukey's post hoc test, with SPSS version 24 software.

RESULTS

Purity and Viability of CTLs

$CD8^+$ T cells were successfully isolated from peripheral blood mononuclear cells (PBMCs) of CRC patients, as confirmed by morphological examination and flow cytometry. Following stimulation with anti-CD3/CD28 beads and IL-2 for 5 days, the CTLs displayed typical morphological changes, including small, round cell shape and the formation of compact spheroid-like clusters, indicative of cellular activation and proliferation (Figure 1A-1B). The gating strategy demonstrated that the majority of cells were $CD45^+$, with subsequent selection of $CD3^+$ lymphocytes, and further identification of $CD8^+$ (Tc) and $CD4^+$ (Th) subpopulations. The isolated $CD8^+$ T cells showed a high purity, consistently exceeding 80% (Figure 1C).

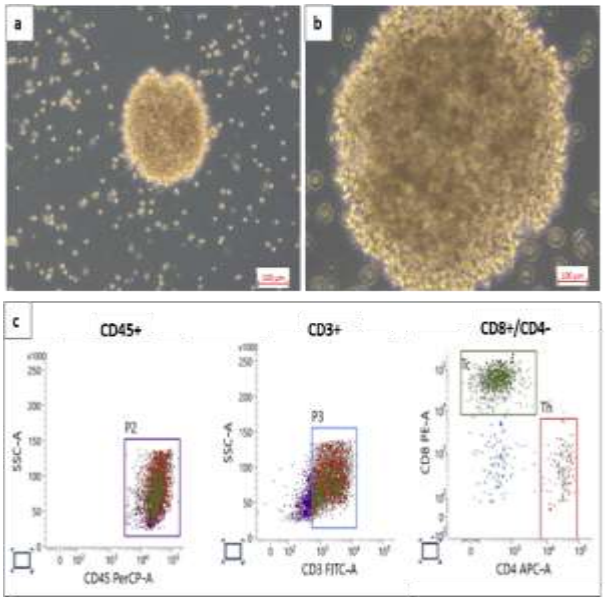

Figure 1. Morphology and flow cytometry analysis of isolated $CD8^+$ T cells. (a–b) Representative phase-contrast images showing activated CTLs after 5 days of stimulation with anti-CD3/CD28 beads and IL-2. CTLs exhibited a small, round morphology and formed compact clusters indicative of proliferation (scale bar = 100 µm). (c) Flow cytometry analysis demonstrating the gating strategy: $CD45^+$ leukocytes (left), $CD3^+$ T cells (middle), and subsequent identification of $CD8^+$ cytotoxic T cells (Tc, green) and $CD4^+$ helper T cells (Th, red) (right).

Cytokine Secretion in Co-Culture

Co-culture of CTLs with HCT116 cells resulted in a marked induction of pro-inflammatory cytokine secretion compared to single-culture controls. The co-culture of CTLs with HCT116 colorectal cancer cells induced measurable cytokine production that increased with higher effector-to-target (E:T) ratios (Figure 2). At E:T 1:1, IFN-γ levels reached 3044.6±120 pg/mL, TNF-α 628.6±67 pg/mL, and IL-6 was almost undetectable (0.4±0,1 pg/mL). At E:T 5:1, IFN-γ secretion rose markedly to 4882.1±198 pg/mL, while TNF-α remained stable at 628.6±82

pg/mL. IL-6 increased modestly to 3.3±0,2 pg/mL. At the highest ratio, E:T 10:1, IFN-γ plateaued at 4907.1±211 pg/mL, showing no further significant increase compared to 5:1. TNF-α increased slightly to 674.0±91 pg/mL, while IL-6 rose further to 5.1±0,9 pg/mL.

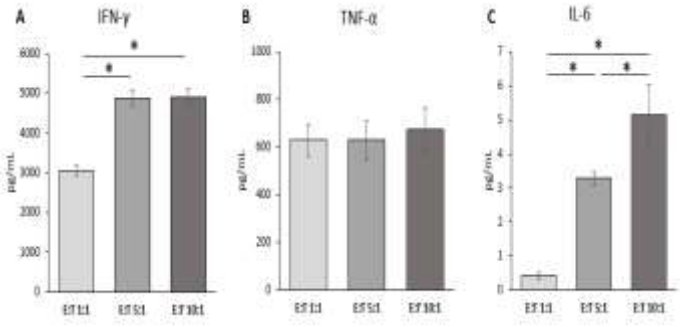

Figure 2. Cytokine secretion profile of CTLs at different effector-to-target (E:T) ratios. CTLs were co-cultured with target cells at E:T ratios of 1:1, 5:1, and 10:1 for 48 h, and cytokine levels in the supernatant were measured by ELISA. (A) IFN-γ secretion increased significantly at higher E:T ratios, reaching a plateau at 5:1. (B) TNF-α production remained relatively constant across E:T ratios. (C) IL-6 secretion showed a dose-dependent increase with higher E:T ratios. Data are presented as mean ± SD (n=3).

DISCUSSION

In the present study, we successfully isolated and expanded $CD8^+$ cytotoxic T lymphocytes (CTLs) from peripheral blood samples of colorectal cancer (CRC) patients. Flow cytometry analysis confirmed a high purity of $CD45^+CD3^+CD8^+$ cells, consistently exceeding 90%, which is in line with previous reports demonstrating the feasibility of isolating tumor-reactive T cells from peripheral blood (11). Following stimulation with anti-CD3/CD28 beads and IL-2, the CTLs underwent robust activation, as reflected by their morphological transformation into small, round cells and the formation of dense spheroid-like clusters. Such morphological features have been well described as hallmarks of activated T cells undergoing proliferation and clonal expansion (12). These results highlight the reliability of our approach to generate sufficient numbers of functional CTLs for potential immunotherapeutic application in CRC.

The functional activity of the expanded CTLs was further validated through cytokine secretion profiling at different effector-to-target (E:T) ratios. IFN-γ production increased markedly with higher E:T ratios and reached a plateau at 5:1, suggesting that CTL activation was saturated beyond this threshold. IFN-γ is a central effector molecule in anti-tumor immunity, enhancing antigen processing, promoting Th1 polarization, and directly exerting cytotoxic effects on tumor cells (13). The robust IFN-γ secretion observed in this study indicates that expanded CTLs retained strong effector functionality and could potentially overcome the immunosuppressive tumor microenvironment in CRC.

TNF-α secretion, on the other hand, remained relatively stable across the tested E:T ratios. TNF-α is known to mediate direct tumor cell apoptosis as well as augment antigen presentation and recruitment of additional immune cells (14). The lack of a dose-dependent increase in TNF-α may suggest that its production is more tightly regulated than IFN-γ, possibly reflecting a controlled response to prevent excessive systemic inflammation. This observation is consistent with previous studies showing that TNF-α release often occurs early during T-cell activation and may not scale proportionally with effector density (15-16).

Interestingly, IL-6 secretion showed a clear dose-dependent increase with higher E:T ratios. While IL-6 is commonly implicated in tumor-promoting processes such as angiogenesis, metastasis, and resistance to apoptosis (17), accumulating evidence suggests that IL-6 also plays context-dependent roles in T-cell biology, including supporting T-cell survival,

proliferation, and differentiation (18). The elevated IL-6 observed in our study may therefore reflect an autocrine or paracrine mechanism that sustains CTL expansion during prolonged activation. However, given the dual role of IL-6 in cancer immunity, further studies are warranted to delineate whether its induction in this setting contributes to therapeutic efficacy or to potential pro-tumorigenic pathways.

Taken together, these findings demonstrate that CTLs isolated from CRC patients can be efficiently expanded ex vivo with high purity and retain robust effector functions, particularly in terms of IFN-γ secretion. The relatively stable TNF-α response and the dose-dependent increase in IL-6 highlight the complexity of CTL cytokine regulation and suggest that different effector molecules may be governed by distinct activation thresholds. These results not only validate the feasibility of generating patient-derived CTLs for adoptive immunotherapy but also provide important insights into the functional heterogeneity of CTL responses, which should be considered in the optimization of therapeutic strategies. Future studies integrating phenotypic, transcriptomic, and functional profiling will be essential to further characterize the quality of expanded CTLs and their potential to improve clinical outcomes in CRC patients.

Several limitations should be acknowledged in this study. First, the experiments were performed using ex vivo expanded CTLs from a limited number of CRC patients, which may restrict the generalizability of the findings (19). The small sample size also precludes definitive statistical conclusions. Second, while cytokine secretion (IFN-γ, TNF-α, and IL-6) was evaluated as a functional readout, other important aspects of CTL biology such as cytotoxicity assays (e.g., granzyme B, perforin release, or direct tumor cell killing) (20) were not included and should be assessed in future work to provide a more comprehensive functional profile. Third, although IL-6 secretion was observed to increase in a dose-dependent manner, its precise role in supporting or hindering CTL-mediated antitumor activity remains unclear and requires further mechanistic studies. Finally, the current study did not investigate the influence of the tumor microenvironment, which is known to impose strong immunosuppressive signals and may significantly affect the functionality of expanded CTLs in vivo.

CONCLUSION

This study demonstrated that ex vivo expanded CTLs from CRC patients exhibit a distinct cytokine secretion profile characterized by robust IFN-γ release, stable TNF-α production, and a dose-dependent increase in IL-6 across different effector-to-target ratios.

Funding
None

Conflict of Interest
The authors declare that they have no conflict of interest

Author Contribution
SI: Methodology, Investigation, Data Analysis, Writing – Original Draft. **AP:** Conceptualization, Project Administration, Supervision, Funding Acquisition, Writing – Review & Editing. **NH:** Laboratory Work, Data Analysis, Visualization. **DC:** Formal Analysis, Literature Review, Writing – Review & Editing.

REFERENCES

1. Hou, W., Yi, C., & Zhu, H. (2022). Predictive biomarkers of colon cancer immunotherapy: Present and future. *Frontiers in Immunology*, *13*, 1032314. https://doi.org/10.3389/fimmu.2022.1032314

2. Zhao, W., Jin, L., Chen, P., Li, D., Gao, W., & Dong, G. (2022). Colorectal cancer immunotherapy-Recent progress and future directions. *Cancer Letters*, *545*, 215816. https://doi.org/10.1016/j.canlet.2022.215816
3. Ghazi, B., El Ghanmi, A., Kandoussi, S., Ghouzlani, A., & Badou, A. (2022). CAR T-cells for colorectal cancer immunotherapy: Ready to go? *Frontiers in Immunology*, *13*, 978195. https://doi.org/10.3389/fimmu.2022.978195
4. Weng, J., Li, S., Zhu, Z. *et al.* (2022). Exploring immunotherapy in colorectal cancer. *J Hematol Oncol* **15**, 95. https://doi.org/10.1186/s13045-022-01294-4
5. Braumüller, H., Mauerer, B., Andris, J., Berlin, C., Wieder, T., & Kesselring, R. (2022). The Cytokine Network in Colorectal Cancer: Implications for New Treatment Strategies. *Cells*, *12*(1), 138. https://doi.org/10.3390/cells12010138
6. Geng, S., Zhu, L., Wang, Y., Liu, Q., Yu, C., Shi, S., & Yu, S. (2024). Co-Colorectal cancer stem cells employ the FADS1/DDA axis to evade NK cell-mediated immunosuppression after co-cultured with NK cells under hypoxia. *International Immunopharmacology*, *143*, 113535. https://doi.org/10.1016/j.intimp.2024.113535
7. Bhat, A. A., Nisar, S., Singh, M., Ashraf, B., Masoodi, T., Prasad, C. P., Sharma, A., Maacha, S., Karedath, T., Hashem, S., Yasin, S. B., Bagga, P., Reddy, R., Frennaux, M. P., Uddin, S., Dhawan, P., Haris, M., & Macha, M. A. (2022). Cytokine- and chemokine-induced inflammatory colorectal tumor microenvironment: Emerging avenue for targeted therapy. *Cancer Communications*, *42*(8), 689-715. https://doi.org/10.1002/cac2.12295
8. Xie, Q., Liu, X., Liu, R. *et al.* (2024). Cellular mechanisms of combining innate immunity activation with PD-1/PD-L1 blockade in treatment of colorectal cancer. *Mol Cancer* **23**, 252. https://doi.org/10.1186/s12943-024-02166-w
9. Pramil, E., Dillard, C., & Escargueil, A. E. (2020). Colorectal Cancer and Immunity: From the Wet Lab to Individuals. *Cancers*, *13*(7), 1713. https://doi.org/10.3390/cancers13071713
10. Wang, Z., Ahmed, S., Labib, M., Wang, H., Wu, L., Shokri, N., Blanco, S., Karim, S., Sargent, E. H., McGray, A. J., De Perrot, M., & Kelley, S. O. (2023). Isolation of tumour-reactive lymphocytes from peripheral blood via microfluidic immunomagnetic cell sorting. *Nature Biomedical Engineering*, *7*(9), 1188-1203. https://doi.org/10.1038/s41551-023-01023-3
11. Knörck, A., Schäfer, G., Alansary, D., Richter, J., Thurner, L., Hoth, M., & Schwarz, E. C. (2022). Cytotoxic Efficiency of Human CD8+ T Cell Memory Subtypes. *Frontiers in Immunology*, *13*, 838484. https://doi.org/10.3389/fimmu.2022.838484
12. Butkute, A., Baltramonaitis, M., Malmige, S., Darinskas, A., Pasukoniene, V., & Mlynska, A. (2025). Targeting stemness pathways modulates macrophage polarization and reprograms the tumor microenvironment. *Frontiers in Immunology*, *16*, 1513404. https://doi.org/10.3389/fimmu.2025.1513404
13. Jeffrey Eka Wijaya, Albertus Ari Adrianto, & Awal Prasetyo. (2025). Dismantling Immunosuppression in Colorectal Cancer: A Systematic Review and Meta-Analysis on Phyllanthus niruri as a Potent Antagonist of the IL-10 Axis in the Tumor Microenvironment. *Bioscientia Medicina : Journal of Biomedicine and Translational Research*, *9*(10), 9031-9047. https://doi.org/10.37275/bsm.v9i10.1404

14. Yi, M., Li, T., Niu, M., Zhang, H., Wu, Y., Wu, K., & Dai, Z. (2024). Targeting cytokine and chemokine signaling pathways for cancer therapy. *Signal Transduction and Targeted Therapy*, *9*(1), 1-48. https://doi.org/10.1038/s41392-024-01868-3
15. Raskov, H., Orhan, A., Christensen, J. P., & Gögenur, I. (2020). Cytotoxic CD8+ T cells in cancer and cancer immunotherapy. *British Journal of Cancer*, *124*(2), 359-367. https://doi.org/10.1038/s41416-020-01048-4
16. Vecchio, F. D., Martinez-Rodriguez, V., Schukking, M., Cocks, A., Broseghini, E., & Fabbri, M. (2021). Professional killers: The role of extracellular vesicles in the reciprocal interactions between natural killer, CD8+ cytotoxic T-cells and tumour cells. *Journal of Extracellular Vesicles*, *10*(6), e12075. https://doi.org/10.1002/jev2.12075
17. Zeng, J., Chen, H., Liu, X., Xia, H., Chen, L., Lin, D., Wang, N., Weng, C., Guan, G., & Zheng, Y. (2024). Cuproptosis in microsatellite stable colon cancer cells affects the cytotoxicity of CD8+T through the WNT signaling pathway. *Chemico-Biological Interactions*, *403*, 111239. https://doi.org/10.1016/j.cbi.2024.111239
18. Philip, M., & Schietinger, A. (2022). CD8+ T cell differentiation and dysfunction in cancer. *Nature Reviews Immunology*, *22*(4), 209-223. https://doi.org/10.1038/s41577-021-00574-3
19. Hoekstra, M. E., Vijver, S. V., & Schumacher, T. N. (2021). Modulation of the tumor micro-environment by CD8+ T cell-derived cytokines. *Current Opinion in Immunology*, *69*, 65-71. https://doi.org/10.1016/j.coi.2021.03.016
20. Zhang, H., Lv, X., Kong, Q., & Tan, Y. (2022). IL-6/IFN-γ double knockdown CAR-T cells reduce the release of multiple cytokines from PBMCs in vitro. *Human Vaccines & Immunotherapeutics*, *18*(1), 1–14. https://doi.org/10.1080/21645515.2021.2016005