RESEARCH ARTICLE

TIME-DEPENDENT SECRETION OF IFN-γ, TNF-α, PERFORIN, AND GRANZYME-B IN CTL-CONDITIONED MEDIUM FROM COLORECTAL CANCER PATIENT

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ABSTRACT

Background: Cytotoxic T lymphocytes (CTLs) are key effectors of adaptive immunity, exerting their functions through the release of pro-inflammatory cytokines and cytotoxic molecules. Conditioned medium (CM) derived from CTLs has emerged as a potential cell-free immunotherapeutic strategy; however, the temporal dynamics of its secreted mediators remain poorly defined.

Methods: Human CTLs were isolated, activated, and cultured in vitro. CM was harvested at defined intervals (days 5, 10, and 15). Concentrations of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), perforin, and granzyme-B were quantified by enzyme-linked immunosorbent assay (ELISA). Temporal secretion profiles were evaluated using ANOVA with post-hoc analysis.

Results: IFN-γ and TNF-α exhibited peak secretion at day 5, followed by a decline at later time points. In contrast, perforin and granzyme-B increased progressively, reaching maximal levels at day 15. All four mediators demonstrated significant time-dependent variation ($p < 0.05$).

Conclusion: CTL-derived CM displays distinct time-dependent secretion patterns, with cytokines predominating during early activation and cytotoxic molecules dominating later phases. These findings underscore the importance of optimizing CM collection timing to maximize its immunomodulatory and therapeutic potential, and provide a rationale for further translational development of CTL-CM in immunotherapy.

Keywords : Cytotoxic T lymphocyte, Conditioned medium, IFN-γ, TNF-α, Perforin, Granzyme-B, Immunotherapy.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are indispensable effectors of adaptive immunity, orchestrating the recognition and elimination of malignant or virus-infected cells.[1] Their activity is mediated through two synergistic mechanisms: secretion of immune-regulating cytokines and the direct induction of target cell apoptosis.[2] By combining immunomodulation with cytotoxicity, CTLs sustain immune surveillance and play a pivotal role in maintaining host defense.[3] The functional potency of CTLs relies on a tightly regulated network of effector molecules. Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) amplify immune responses by enhancing antigen presentation, recruiting immune effectors, and modulating the tumor microenvironment.[4] In parallel, perforin and granzyme-B form the cytolytic machinery that directly triggers programmed cell death in abnormal cells.[5] The temporal balance between these pro-inflammatory cytokines and cytotoxic molecules determines both the efficacy and safety of CTL responses. Dysregulated secretion may compromise tumor clearance or, conversely, promote immunopathology.[6]

In recent years, CTL-derived conditioned medium (CTL-CM) has emerged as a promising candidate for cell-free immunotherapy.[7] Containing a rich mixture of cytokines and cytotoxic proteins, CTL-CM may reproduce many of the beneficial effects of CTLs without the logistical, regulatory,

and safety barriers associated with adoptive cell transfer.[8] Its acellular nature offers advantages in terms of scalability, standardization, and reduced risk of immune rejection, positioning CTL-CM as an attractive biologic product for future clinical applications.[9] Despite this potential, a critical knowledge gap persists. The biological activity of CTL-CM is inherently dependent on the timing of its harvest, yet the temporal secretion dynamics of key mediators remain poorly defined.[10] Most existing studies focus on endpoint measurements, overlooking the fact that CTL effector functions evolve over time—from an early cytokine-dominated phase to a later cytotoxic phase.[11] Without systematic characterization of these kinetics, attempts to translate CTL-CM into therapeutic strategies risk inconsistency and suboptimal efficacy.[12]

To address this gap, the present study systematically investigates the time-dependent secretion of IFN-γ, TNF-α, perforin, and granzyme-B from activated human CTLs at defined culture intervals (days 5, 10, and 15). These time points were selected based on previous studies showing that CTLs undergo distinct functional phases during in vitro activation: an early effector phase (around day 5) characterized by rapid cytokine release, an intermediate phase (day 10) where cytokine secretion begins to decline while cytotoxic machinery is upregulated, and a late effector phase (around day 15) when perforin and granzyme-B reach peak levels of expression and activity.[13,14] Mapping mediator secretion across these intervals enables identification of the optimal window for conditioned medium (CM) harvest, which is essential for maximizing its biological activity.[15] By linking mechanistic insights into CTL kinetics with translational objectives, this study provides a scientific foundation for the rational development of CTL-CM as a standardized and clinically relevant form of cell-free immunotherapy.

METHODS

Study Design

This study was a controlled in vitro experimental investigation aimed at characterizing the temporal secretion of IFN-γ, TNF-α, perforin, and granzyme-B in conditioned medium (CM) derived from human cytotoxic T lymphocytes (CTLs; $CD8^+$ T cells). Following isolation and activation, CTLs were cultured and CM was collected at three predetermined time points: day 5, day 10, and day 15. Each condition was performed in triplicate technical replicates. Randomization of plate layouts and sample processing order was applied using computer-generated maps, and analysts were blinded to group assignments during ELISA readings and statistical evaluation. All procedures were conducted under BSL-2 biosafety standards, and informed consent was obtained from all blood donors prior to sample collection.

T cell isolation

Peripheral blood samples (20–30 mL) were collected from 3 patients diagnosed with colorectal cancer (CRC) at SCCR, who met the inclusion criteria of not receiving immunomodulatory or cytotoxic therapy within 14 days prior to sampling. Patients with active infections, autoimmune diseases, or ongoing immunosuppressive treatment were excluded. Written informed consent was obtained from all participants before blood collection. 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%.

Cytokines and cytotoxic molecules assessment using ELISA

Cytokine and cytotoxic molecules levels were quantified using validated human sandwich ELISA kits specific for IFN-γ, TNF-α, perforin, and granzyme-B (Elabscience, Houston, TX, USA). Standards and samples were analyzed in duplicate, and standard curves were generated using a four-parameter logistic regression model ($R^2 \geq 0.95$). Assay reliability was confirmed by recovery testing, with intra-assay coefficients of variation (CV) ≤15% and inter-assay CV ≤20%. Results were expressed as cytokine concentrations in pg/mL, with optional normalization 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.

Ethical Clearance

The study received ethical approval from the Komisi Bioetika Penelitian Kedokteran/Kesehatan, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang (No. 427/IX/2025). All procedures were conducted in accordance with institutional policies and international ethical guidelines.

RESULTS

Morphological characteristics of cultured CTLs

Phase-contrast microscopy revealed dynamic morphological changes in CTLs during the 15-day culture period (Figure 1). On day 5, CTLs appeared predominantly as small, round cells forming compact aggregates, consistent

with early activation. By day 10, cells exhibited larger sizes and developed into more prominent spheroid-like clusters, reflecting active proliferation and expansion. On day 15, cultures showed dense, irregular aggregates with heterogeneous borders, indicative of advanced activation and effector differentiation. These progressive morphological transitions paralleled the functional changes observed in cytokine and cytotoxic molecule secretion.

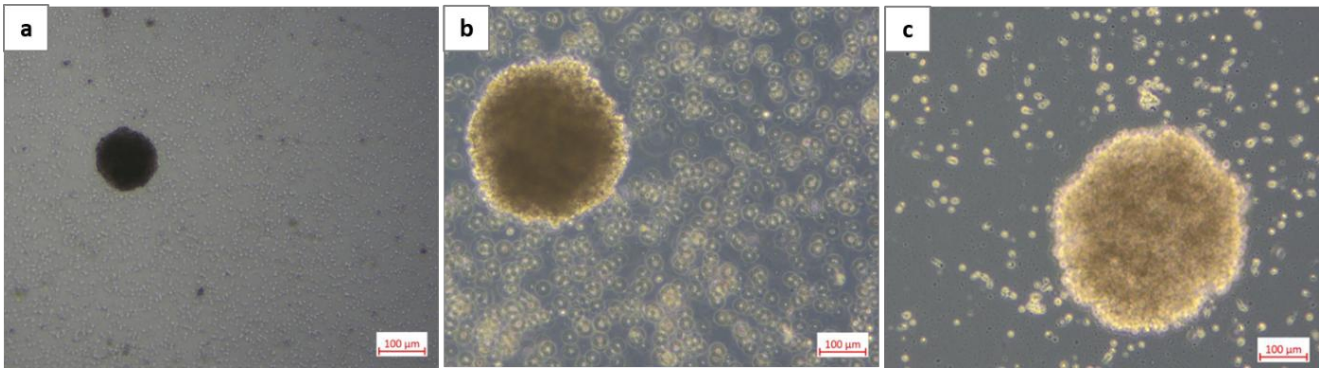

Figure 1. Morphological progression of cultured human CTLs over 15 days. Representative phase-contrast microscopy images of CTLs at different culture intervals. (a) Day 5: small, compact aggregates with round morphology. (b) Day 10: increased cell size and formation of larger spheroid-like clusters. (c) Day 15: dense, irregular aggregates with heterogeneous borders, indicative of advanced activation. Scale bar = 100 µm.

Conditioned medium (CM) collected from activated CTLs exhibited distinct time-dependent secretion patterns of cytokines and cytotoxic effector molecules (Fig. 2). IFN-γ concentrations peaked at day 5, followed by lower levels at day 10 and day 15, although these differences were not statistically significant ($p > 0.05$). TNF-α showed a different temporal profile, with minimal secretion at day 5, a marked peak at day 10 ($p < 0.05$ vs. day 5), and a significant decline by day 15 ($p < 0.05$ vs. day 10). In contrast, perforin levels were low at day 5, increased substantially at day 10, and decreased again at day 15, indicating a transient cytotoxic effector phase. Granzyme-B displayed a similar trend, rising significantly at day 10 and reaching its highest levels at day 15 ($p < 0.05$). Overall, statistical analysis confirmed significant time-dependent differences for three mediators ($p < 0.05$ vs. baseline). Taken together, these findings suggest that cytokine secretion predominates during the early activation phase of CTLs, while cytotoxic effector molecules accumulate later, highlighting distinct functional windows that may shape the immunotherapeutic potential of CTL-derived CM.

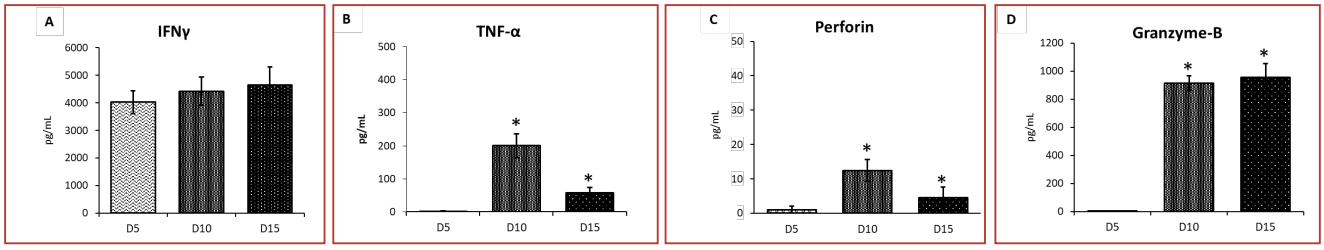

Figure 2. Time-dependent secretion of cytokines and cytotoxic molecules by activated human CTLs. Conditioned medium (CM) was collected at days 5, 10, and 15 of culture and analyzed by ELISA. (A) IFN-γ secretion peaked on day 5 and declined thereafter. (B) TNF-α followed a similar pattern, with highest levels detected at day 5. (C) Perforin showed a progressive increase, reaching maximum levels on day 15. (D) Granzyme-B secretion also increased over time, with peak concentrations at day 15. Bars represent mean ± SD of triplicate experiments (n=3). *$p < 0.05$ versus day 5.

DISCUSSION

In this study, we demonstrated that activated human cytotoxic T lymphocytes (CTLs) exhibit distinct temporal patterns of cytokine and cytotoxic molecule secretion during in vitro culture. Specifically, IFN-γ and TNF-α secretion peaked early at day 5, while perforin and granzyme-B accumulated progressively, reaching maximum levels at day 15. These findings highlight the dynamic nature of CTL effector function and suggest that the timing of conditioned medium (CM) collection critically influences its molecular composition and potential immunotherapeutic value.

The early dominance of cytokine secretion reflects the classical role of CTLs in rapidly shaping the immune microenvironment during initial antigen encounter.[16] IFN-γ and TNF-α are well-established mediators of antitumor and antiviral immunity, promoting macrophage activation, enhancing antigen presentation, and contributing to

apoptosis of target cells.[16,17] Our observation that these cytokines peak around day 5 is consistent with previous reports showing maximal transcriptional activity and effector cytokine release in the early activation phase of $Cd8^+$ T cells.[18,19]

In contrast, perforin and granzyme-B secretion followed a delayed but sustained pattern, with maximal release at day 15. This aligns with previous studies indicating that the acquisition of cytotoxic granules requires extended activation and proliferation.[20,21] Granzyme-B, in particular, has been reported to accumulate progressively in mature effector CTLs and memory subsets, enabling potent and targeted killing of tumour or infected cells.[22] Our findings reinforce this temporal separation between cytokine-driven immunoregulation and granule-mediated cytotoxicity, underscoring the functional plasticity of CTLs during their activation trajectory.

From a translational perspective, these dynamics have important implications for developing CTL-derived CM as a cell-free immunotherapy.[23] CM collected during the early activation window (day 5) is enriched in pro-inflammatory cytokines, which may be more effective in immune stimulation and priming. In contrast, CM collected at later intervals (day 15) is enriched in perforin and granzyme-B, potentially favouring direct cytotoxic activity. Such compositional differences suggest that therapeutic applications could be tailored based on the intended outcome-either immune activation or targeted cytotoxicity.[24,25] This concept is consistent with recent efforts to exploit T cell-derived secretomes as biologically active products in cancer immunotherapy.[26,27]

Nevertheless, several limitations should be acknowledged. First, our study was limited to in vitro activation of CTLs using a standardized protocol, which may not fully recapitulate the complexity of in vivo antigen-driven activation.[28] Second, only four mediators were examined, while CTLs are known to secrete a broader range of cytokines, chemokines, and exosomes that could contribute to the overall therapeutic effect. Future work should expand the profiling to include additional effector and regulatory molecules, as well as functional assays testing the bioactivity of CM on target tumor or immune cells.[29] Furthermore, in vivo validation will be essential to confirm the translational potential of CTL-derived CM as a safe and effective cell-free immunotherapy.

This study demonstrates that cytotoxic T lymphocyte (CTL)-derived conditioned medium exhibits distinct time-dependent secretion dynamics. Pro-inflammatory cytokines, particularly IFN-γ and TNF-α, peaked at day 5, reflecting an early immune-activating phase, whereas cytotoxic effector molecules, perforin and granzyme-B, progressively increased and reached maximal levels at day 15, representing a late cytotoxic phase. These findings emphasize that the timing of conditioned medium collection is critical in determining its molecular composition and functional potential. Harvesting CTL-CM at earlier intervals may favor immunomodulatory and stimulatory applications, while later collection may be more suitable for cytotoxic purposes. Collectively, these insights provide a scientific foundation for optimizing CTL-CM as a standardized, cell-free immunotherapeutic strategy and support further preclinical and translational investigations to validate its efficacy and safety in vivo.

CONCLUSION

In conclusion, this study provides novel insights into the time-dependent effector dynamics of human CTLs. The distinct temporal profiles of cytokine versus cytotoxic molecule secretion underscore the importance of optimizing the timing of CM collection. These findings lay the groundwork for rationally designing CTL-derived secretome-based therapies, bridging fundamental T cell biology with translational immunotherapy development.

ACKNOWLEDGEMENT
We thank the Stem Cell and Cancer Research (SCCR) Laboratory for providing access to their research facilities.

FUNDING
None

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest

AUTHOR CONTRIBUTION
SI and AP conceptualized the study and drafted the initial manuscript. AFK and DKN provided critical revisions, significantly enhancing the intellectual content of the manuscript. NH and DKN contributed to formatting adjustments, content revisions, and translation, ensuring clarity and consistency throughout the manuscript. All authors were actively involved in the study design, critically reviewed the data, and provided final approval of the manuscript.

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