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Ex Vivo Induction and Cytolytic Activity of *Theileria parva*-Specific CD8⁺ CTLs from ITM-Immunized Cattle Using Autologous Infected Cell Lines

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ABSTRACT

The apicomplexan blood parasite Theileria parva is the etiologic agent of East Coast fever (ECF), a highly lethal condition in cattle across Africa. The progress of vaccine design has been hampered by limited insight into the nature of protective immunity that arises after either natural infection or immunization using the infection and treatment method (ITM). This challenge mainly results from the absence of suitable techniques for assessing the memory T-cell populations after exposure. To resolve this issue, immune assays originally optimized for other intracellular organisms were modified to analyze T. parva, enabling detailed study of effector T-cell characteristics in immune animals and supporting vaccine improvement. In this investigation, peripheral blood mononuclear cells (PBMCs) from cattle immunized by ITM were stimulated with irradiated, autologous, T. parva-infected cells, provoking a proliferative recall reaction consisting of CD45R0+/CCR7− CD4+ and CD8+ subsets. When these cultures were later incubated with infected targets, cytotoxic T lymphocytes (CTLs) capable of destroying infected cells were detected. Removal of either CD4+ or CD8+ T cells showed that cytotoxic function was mainly due to the CD8+ fraction. Notably, PBMCs from all vaccinated steers exhibited strong proliferative responses in both T-cell types. This represents the first key outcome derived from the newly applied assay.

Keywords: Flow cytometry, East coast fever, Ex Vivo, Cytolytic activity

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Introduction

Theileria parva (T. parva), a tick-transmitted protozoan belonging to the phylum Apicomplexa, causes East Coast fever (ECF), which leads to the death of over one million head of cattle each year in sub-Saharan Africa [1]. The most severe losses occur among Bos taurus breeds and Bos indicus cattle living outside endemic areas, with smallholder herders most severely affected. Consequently, ECF-related illness and mortality remain a principal factor in rural poverty.

Following transmission by the tick Rhipicephalus appendiculatus, T. parva sporozoites rapidly invade bovine B and T lymphocytes, forming multinucleated schizonts [2]. These schizonts are free in the cytoplasm and induce malignant-like transformation of the host lymphocyte [3]. The parasite divides concurrently with the transformed host cell, and this stage is responsible for clinical disease. During acute infection, lymph nodes enlarge due to proliferation of infected and activated lymphocytes [4]. The infection leads to severe leukopenia, persistent fever, and anorexia. In advanced cases, distinct monocyte changes appear [5], and respiratory distress develops. Histological analysis of fatal infections shows that lung pathology involves widespread macrophage activation,

vascular infiltration, and vasculitis rather than simple lymphocyte proliferation [6]. These findings suggest that dysregulation of the innate immune response is linked to severe disease outcomes [7]. Although transformed lymphocytes appear to drive macrophage activation, the precise processes remain unclear.

Immunization by the infection and treatment method (ITM)—in which cattle are inoculated with crushed T. parva-infected ticks and simultaneously treated with oxytetracycline—provides strong protection against similar parasite strains but may not prevent infection with antigenically distinct ones [8]. Furthermore, ITM stabilates are costly, require antibiotic support, and must be stored in liquid nitrogen [9, 10]. Even so, ITM has shown that lasting protection is attainable and can be broadened through mixed isolate formulations [1]. The resulting immune cattle enabled exploration of host defense mechanisms. Transfer of $CD8^+$ T cells from an ITM-immunized calf to its genetically identical twin confirmed that protection is primarily mediated by $CD8^+$ cytotoxic T cells (CTLs) [11]. Attempts to achieve similar results using isolated schizont antigens have yielded partial success but also numerous challenges [12–15].

While these experiments clarified some aspects of T. parva immunity, the antigen-specific CTLs generated by immunization were consumed during standard assays, limiting detailed examination of protective mechanisms—a problem shared by earlier work [13]. Except for $CD8^+$ T-cell cytotoxicity against infected lymphocytes, no additional markers correlate reliably with immunity. Other measures, such as interferon-gamma secretion or cell proliferation, do not predict resistance to reinfection. This knowledge gap continues to obstruct vaccine development, as the essential features of protective immune responses remain undefined.

Comparable research on porcine reproductive and respiratory syndrome virus (PRRSV) in pigs and Mycobacterium species in cattle indicates that flow cytometry–based cytotoxicity systems may overcome such constraints [16, 17]. These methods enable examination of CTL-mediated killing of infected macrophages while preserving the effector cells for further study. The goal of the present research was to adapt and apply these cytotoxicity techniques to evaluate CTLs that arise in cattle after ITM immunization with T. parva.

Materials and Methods

Animals and cell lines

Four Holstein steers were experimentally challenged with *Theileria parva* using the infection-and-treatment protocol (ITM) following earlier studies [6, 7, 18, 19]. In short, a 1-mL subcutaneous inoculation containing homogenized material from ten infected tick salivary gland pairs of the Muguga sporozoite stabilate (Muguga genotype only, excluding the cocktail) was administered into the left parotid region. Simultaneously, long-acting oxytetracycline (20 mg/kg) was given intramuscularly. Clinical monitoring and supportive treatment were conducted according to established procedures [7].

Between 6 and 20 months after the primary infection, each steer received a booster injection with an identical dose of Muguga stabilate. No disease symptoms were observed at this stage, confirming established immunity to *T. parva*. Blood samples were taken from 3 to 12 weeks post-challenge for analysis of T-cell proliferation and to develop *T. parva*-specific cytotoxic T lymphocyte (CTL) lines, which were subsequently used in fluorescence-based cytotoxicity testing. Ethical clearance was granted by the Washington State University Institutional Animal Care and Use Committee (protocols ASAFs 4980 and 6622).

For each animal, lymphocyte cultures infected with *T. parva* were generated by infecting peripheral blood mononuclear cells (PBMCs) in vitro with Muguga sporozoites as previously reported [7, 15, 20, 21]. PBMCs were separated from whole blood via density-gradient centrifugation using Histopaque (Sigma-Aldrich, St. Louis, MO, USA). Infection success was verified between 14 and 21 days post-exposure through microscopic observation of *T. parva* schizonts in Giemsa-stained cytospin slides. Once established, cultures were maintained by sub-culturing at ratios of 1:2 or 1:3 every 2–4 days, depending on pH change (medium turning from red to yellow) and cellular density. Unless exposed to theilericidal drugs, *T. parva*-infected lymphocytes remain continuously proliferative and can be propagated long-term [22].

All cell lines were maintained in complete RPMI (cRPMI) medium consisting of RPMI 1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% calf serum, 20 mM HEPES (Gibco), 50 µM β-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), and 50 µg/mL gentamicin (Gibco). Cultures were incubated at 37 °C with 5% CO_2. All monoclonal antibodies (mAbs) applied are summarized in **Table 1**.

Table 1. Monoclonal and secondary antibodies employed in this study.

Antibody Designation	Isotype	Specificity	Source
ILA11A	IgG2a	Bovine CD4	WSUMAC, USA
7C2B	IgG2a	Bovine CD8	WSUMAC, USA
CACT80C	IgG1	Bovine CD8	WSUMAC, USA
ILA116A	IgG1	Bovine CD45R0	WSUMAC, USA
CACT116A	IgG1	Bovine CD25	WSUMAC, USA
GB21A	IgG2b	Bovine γδ TCR	WSUMAC, USA
3D12	IgG2a	Human CCR7	BD Pharmingen, USA
AKS8	IgG2a	Bovine CD335	Norwegian University of Life Sciences [23]
Goat anti-mouse	IgG2a PE-Cy5.5	—	ThermoFisher, USA
Goat anti-mouse	IgG1 Alexa Fluor 647	—	ThermoFisher, USA
Goat anti-mouse	IgG1 FITC	—	ThermoFisher, USA
Goat anti-mouse	IgG3 FITC	—	Southern Biotech, USA
Goat anti-rat	IgG FITC	—	Southern Biotech, USA
Goat anti-mouse	IgG2b PE	—	Southern Biotech, USA

Generation of T. parva*-specific effector T cells*

Effector T cells specific to *T. parva* were obtained following previously established methods [22] with slight modifications. Blood was drawn from *T. parva*-immune animals into tubes containing anticoagulant citrate dextrose (ACD). PBMCs were isolated by Histopaque density separation (1.077 g/mL; Sigma-Aldrich, St. Louis, MO, USA). Immune PBMCs were cultured in cRPMI and stimulated for 6 days with autologous, γ-irradiated (50 Gy) *T. parva*-infected lymphocytes at a 20:1 ratio under 37 °C/5% CO_2. After this first round of stimulation, cells were purified by density centrifugation to eliminate non-viable cells.

Recovered lymphocytes were characterized and depleted of NK and γδ T cells using anti-NK and anti-γδ mAbs (1 µg/10^6 cells) **(Table 1)** together with rat anti-mouse IgG2a+b magnetic microbeads (Miltenyi Biotec, Waltham, MA, USA) per the manufacturer's recommendations. The remaining $CD4^+$ and $CD8^+$ cells were cultured again in cRPMI and restimulated for 5–6 days with the same autologous irradiated cell lines. Following the second activation, dead cells were removed, and viable cells were analyzed for phenotype and utilized either for CTL assays or to establish $CD4^+$ and $CD8^+$ subcultures. $CD8^+$ cells were positively isolated using a CD8-specific mAb (1 µg/10^6 cells) **(Table 1)** and rat anti-mouse IgG2a+b magnetic beads as previously detailed. Both $CD8^+$ and residual $CD4^+$ fractions were assessed for purity by flow cytometry. Expanded $CD4^+$ and $CD8^+$ cultures were grown in cRPMI containing bovine IL-2 (10 ng/mL; Kingfisher, USA) before being used in cytotoxicity evaluations.

Panels of monoclonal antibodies recognizing CD4, CD8, γδ T cells, CD45R0 (memory T-cell marker), CCR7, and CD25 were employed to determine cell composition after each stimulation and depletion step, after $CD8^+$ selection, and prior to co-culture cytotoxicity assays. Cells were labeled as previously described using fluorochrome-conjugated, goat anti-mouse, isotype-specific secondary antibodies [24]. Flow cytometric analysis was performed on a BD FACSCalibur instrument with CellQuest software (BD Immunocytometry Systems, San Jose, CA). Data analysis was conducted using De Novo FCS 6 software (DeNovo Software, Pasadena, CA, USA).

Flow cytometric cytotoxicity assay

A triple-color flow cytometric method was applied to evaluate the cytotoxic activity of both mixed parental lymphocyte cultures and purified *T. parva*-reactive $CD8^+$ and $CD4^+$ T-cell populations against autologous, infected target cells. Target cells were freshly prepared before each test. Infected cultures were harvested, cleaned of non-viable cells by density-gradient centrifugation, and adjusted to a concentration of 2×10^6 cells/mL in RPMI medium containing CellTracker Deep Red dye (Thermo Fisher, USA) at a dilution of 1:1000. Labeling was performed for 30 min at 37 °C, after which the cells were washed and transferred to complete RPMI (cRPMI).

Unfractionated and separated *T. parva*-specific T cells were also purified through centrifugation to remove dead cells and resuspended in cRPMI. The separated subsets, previously expanded in the presence of IL-2, were further purified using the same procedure before the assay.

Effector and labeled target cells were co-incubated for 6, 24, or 48 h. The effector-to-target (E:T) ratios were set at 40:1 for parental and $CD4^+$ lines and 20:1 for $CD8^+$ lines. A single well containing only stained target cells served as a background-death control. At each time point, one replicate was harvested, pelleted, and suspended in Annexin V binding buffer (BD Biosciences, USA), followed by staining with Annexin V–PE and 7-AAD (20 µL of each). Samples were incubated for 15 min at room temperature and analyzed by flow cytometry within 1 h. Data acquisition was restricted to CellTracker Deep Red-positive events, and at least 3×10^4 targets were recorded per sample.

Data processing

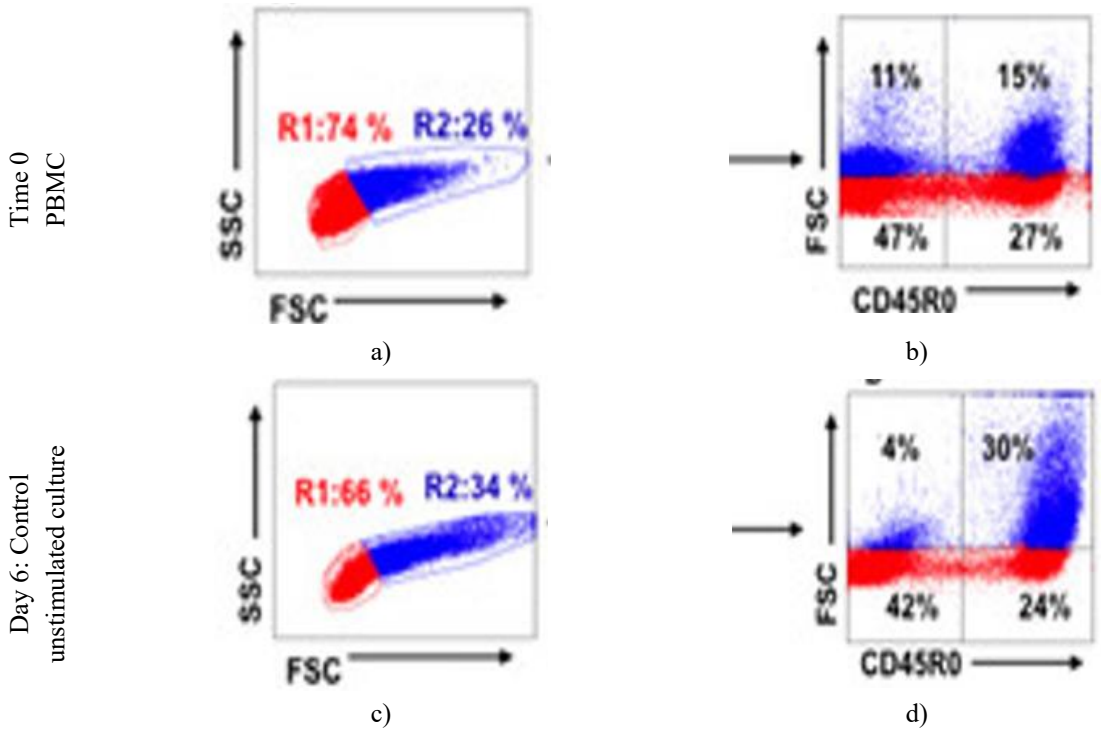
Flow cytometry data were interpreted using FCS Express (De Novo Software, Los Angeles, CA, USA). Statistical evaluations were carried out in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Group comparisons were made using a one-tailed Student's *t*-test, and differences were considered significant when $p < 0.05$.

Results and Discussion

Recall response of PBMC from T. parva*-immune steers to stimulation with autologous infected cells*

To establish a flow cytometric approach for assessing cytotoxic recall activity, peripheral blood mononuclear cells (PBMC) from ITM-immunized steers were exposed to autologous, γ-irradiated *T. parva*-infected lymphocytes. Final cell preparations used in assays were cleared of non-viable cells and characterized to confirm the presence of both $CD4^+$ and $CD8^+$ T cells or pure subsets of each.

As illustrated in **Figure 1**, PBMC were displayed on side-scatter (SSC) versus forward-scatter (FSC) plots, where color-coded electronic gates identified proliferative activity. Region R1 represented small, quiescent lymphocytes, while R2 included larger cells undergoing blastogenesis [20, 21]. Additional gating in FSC vs. CD45R0 plots was used to quantify antigen-responsive memory T cells. Comparison between initial PBMC populations **(Figures 1a and 1b)**, unstimulated cultures maintained for six days **(Figures 1c and 1d)**, and cultures stimulated with infected cells for the same period **(Figures 1e and 1f)** demonstrated a clear expansion of antigen-specific memory T cells following stimulation. This pattern was consistent across PBMC from all four immune steers **(Figure 1g)**.



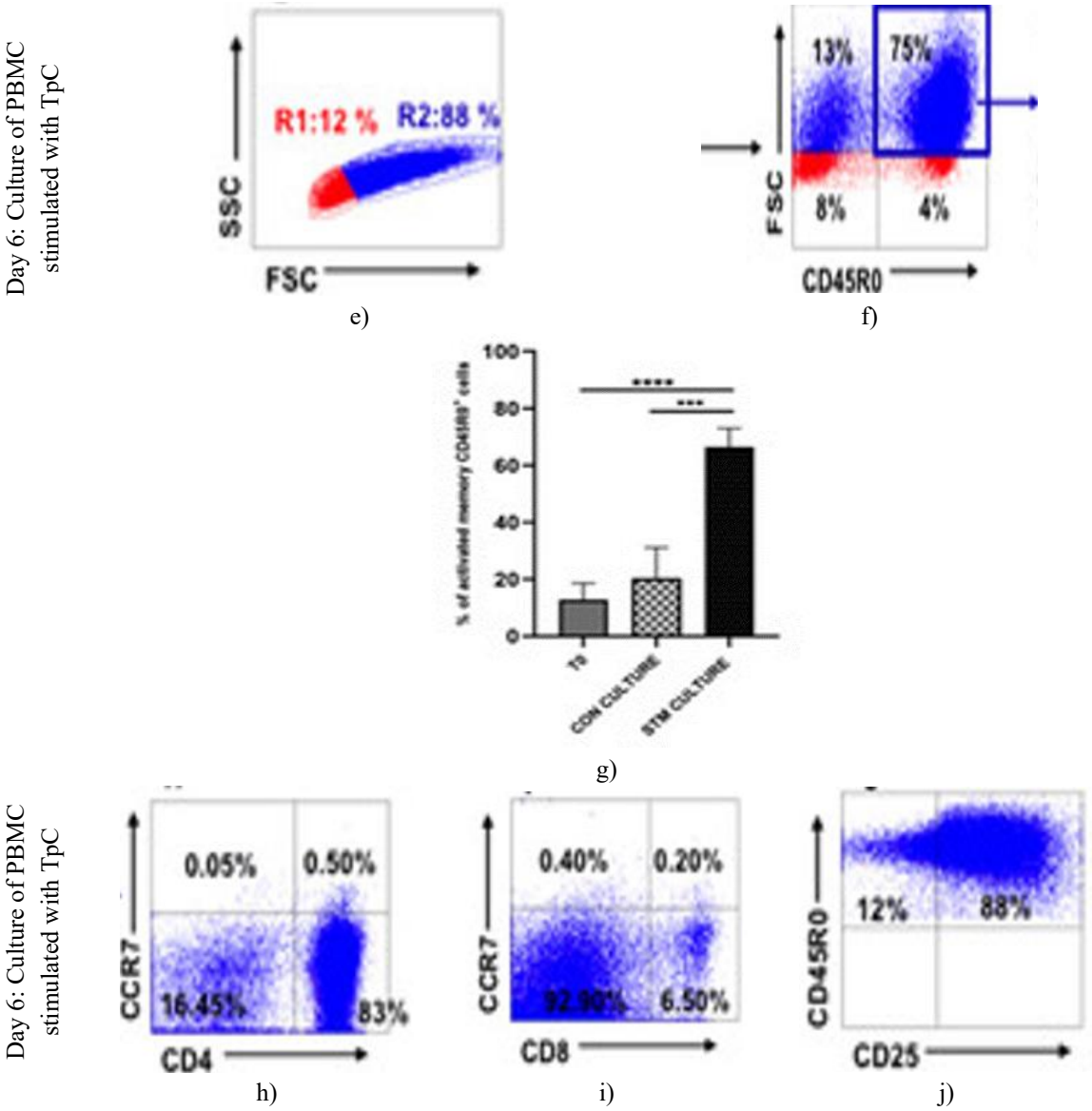


Figure 1. Flow-cytometric characterization of the proliferative T-cell response following exposure to autologous, γ-irradiated T. parva-infected lymphocytes. Gates R1 and R2 differentiate resting from enlarging, dividing cells. Additional gating (FSC vs. CD45R0) identifies the memory subset proliferating after antigenic stimulation. Representative plots compare PBMC at baseline (a, b), after six days without stimulation (c, d), and after six days with infected-cell stimulation (e, f), indicating enhanced memory T-cell expansion. (g) summarizes recall responses from all four steers. (h, i) show representative phenotypes of proliferating $CD4^+$ (h) and $CD8^+$ (I) memory cells. (j) demonstrates that most proliferating antigen-specific memory cells expressed CD25 by day 6. Control cultures = unstimulated (CON CULTURE); stimulated cultures = infected-cell-stimulated (STM CULTURE). Statistical analysis by one-tailed t-test compared memory T-cell frequencies at baseline (T_0) with unstimulated ($p < 0.0001$, ***) and stimulated cultures ($p = 0.0003$, ****).

Further examination of the proliferating memory population revealed that $CD4^+$ T cells predominated. Both $CD4^+$ and $CD8^+$ cells lacked CCR7 (CD197), a characteristic consistent with effector-memory phenotypes **(Figures 1h and 1i)**. Most antigen-reactive memory cells also expressed the activation marker CD25 **(Figure 1j)**.

Flow cytometric evaluation of cytotoxic activity of T cells against autologous T. parva–infected targets

A flow cytometry–based assay was designed to quantitatively assess the ability of cytotoxic T lymphocytes (CTLs), derived from PBMC of immunized steers, to eliminate T. parva–infected cells. The method enabled differentiation between effector and target cell populations. Its design was adapted from cytotoxicity assays commonly applied in viral immunology, in which infected targets are fluorescently labeled to distinguish them

from effectors [16, 25]. In the current work, T. parva–infected cells were marked with CellTracker Deep Red prior to co-culture. To determine CTL-induced apoptosis and necrosis, the annexin V–PE/7-AAD staining system was employed **(Figure 2)**. Within this framework, annexin V^+/7-AAD^- cells indicate early apoptotic death, annexin V^+/7-AAD^+ cells correspond to late apoptotic or dead cells, and annexin V^-/7-AAD^+ cells denote necrotic death.

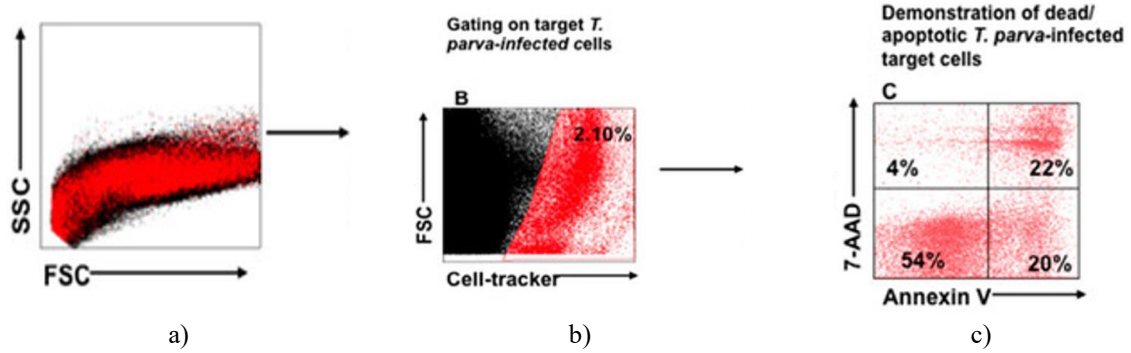


Figure 2. Gating workflow for identifying labeled T. parva–infected cells subjected to CTL-mediated cytotoxicity. (a) Representative population of CTLs and autologous, irradiated T. parva–infected targets stained with CellTracker Deep Red, annexin V, and 7-AAD after 6 h of co-incubation, displayed as SSC vs. FSC. (b) Gate used to isolate the labeled targets (FSC vs. CellTracker). (c) Gated infected cells displayed as 7-AAD vs. annexin V plots, showing live, early apoptotic, late apoptotic, and necrotic subpopulations.

CTLs were obtained after two sequential stimulations. The cytotoxic response was evaluated at 6, 24, and 48 hours of co-culture with irradiated autologous infected targets. A parallel culture of infected cells alone served as a control to estimate spontaneous apoptosis during the same incubation periods. At analysis, gating was performed on the CellTracker Deep Red–positive population to exclude CTLs **(Figures 2a and 2b)**. The gated cells were then examined for annexin V and 7-AAD expression to quantify apoptotic and necrotic fractions **(Figure 2c)**.

A clear time-dependent increase in apoptosis of target cells was observed. At baseline (0 h), about 3% of infected cells were apoptotic **(Figure 3e)**, which rose to 39% after 48 h **(Figures 3f-3h)**, while cultures lacking CTLs **(Figures 3a–3d)** showed minimal change. The total apoptotic fraction was defined as the combined percentage of annexin V^+/7-AAD^- and annexin V^+/7-AAD^+ cells. Cytolytic activity (% specific cell death) was calculated according to the equation:

% specific cell death = (% of dead infected cells—% of spontaneous dead infected cells)/(100% of spontaneous dead infected cells) × 100, as outlined by previous authors [26].

Analysis of samples from all four immunized steers demonstrated a progressive increase in cytotoxicity over time. Mean values of cell death reached 38.29% ± 10.45 at 6 h, 47.21% ± 8.69 at 24 h, and 65.81% ± 16.46 at 48 h, significantly higher than baseline (2.34% ± 2.36) **(Figure 4a)**.

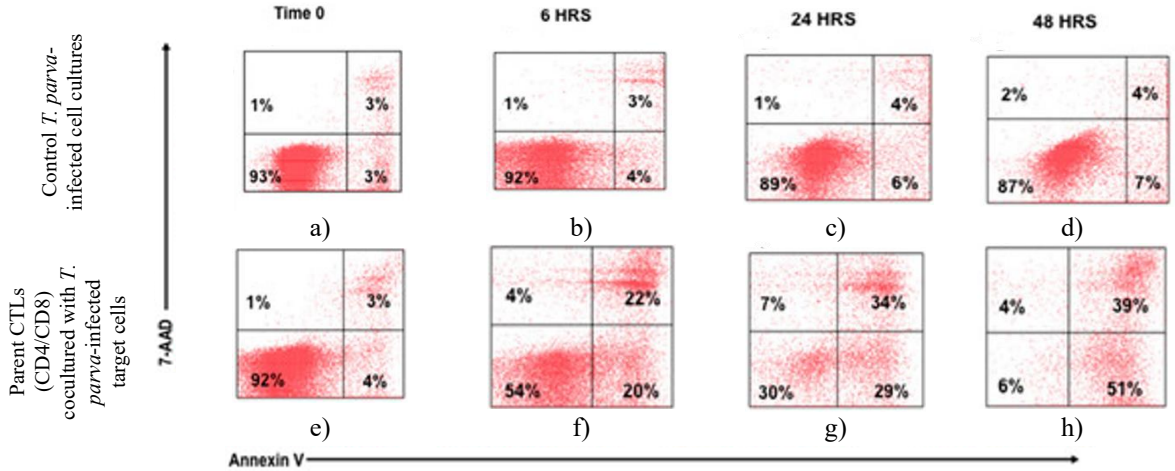
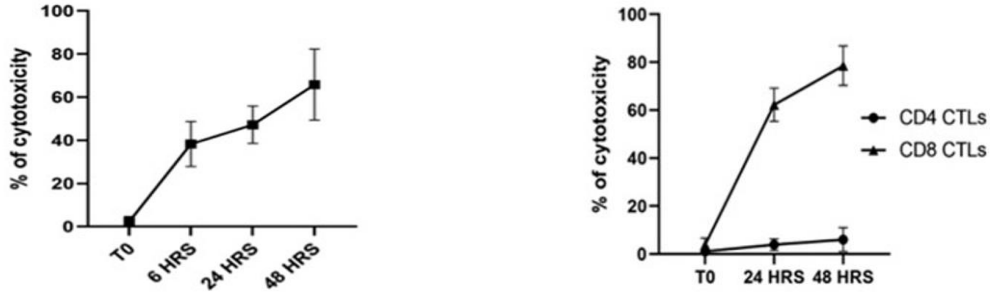


Figure 3. Detection of apoptosis in T. parva–infected targets co-cultured with specific $CD4^+$ and $CD8^+$ T-cell populations. Panels (a–d) show infected targets alone, while (e–h) illustrate co-cultures at 0, 6, 24, and 48 hours. The frequency of apoptotic and dead cells increased progressively in co-cultures, indicating time-

dependent CTL-mediated lysis. Green quadrants highlight viable target populations declining over time. Cultures without CTLs showed negligible loss of viability, confirming that cell death resulted from CTL attack rather than spontaneous apoptosis.



a) b)

Figure 4. Quantification of T. parva–infected cells undergoing apoptosis or death after incubation with effector T cells. (a) Summary of apoptotic/dead infected cells recorded at 0, 6, 24, and 48 h in co-cultures containing both $CD4^+$ and $CD8^+$ T cells. (b) Comparison of infected targets killed by isolated $CD4^+$ (circles) and $CD8^+$ (triangles) T-cell lines at 0, 24, and 48 h following stimulation with autologous irradiated infected cells. Although $CD8^+$ T cells were the predominant mediators of cytotoxicity, $CD4^+$ cells also demonstrated measurable killing capacity.

In later experiments, the initial CTL populations were subdivided into fractions containing only $CD4^+$ or $CD8^+$ cytotoxic T lymphocytes. Each subset was cultured and expanded in the presence of bovine IL-2 before analysis. Two incubation periods, 24 and 48 hours, were selected to evaluate the killing efficiency of the separated T-cell groups. The assessment revealed that elimination of infected target cells was primarily driven by $CD8^+$ T cells in a time-dependent fashion **(Figures 4b, 5a–5c)**, exhibiting mean cytolytic activities of 62.25 (± 6.96) and 78.57 (± 8.21) at 24 h and 48 h, respectively. $CD4^+$ CTLs displayed only minimal cytotoxic responses, averaging 3.96 (± 2.38) and 6.06 (± 4.95) at the corresponding time points **(Figures 4b, Figures 5d–5f)**.

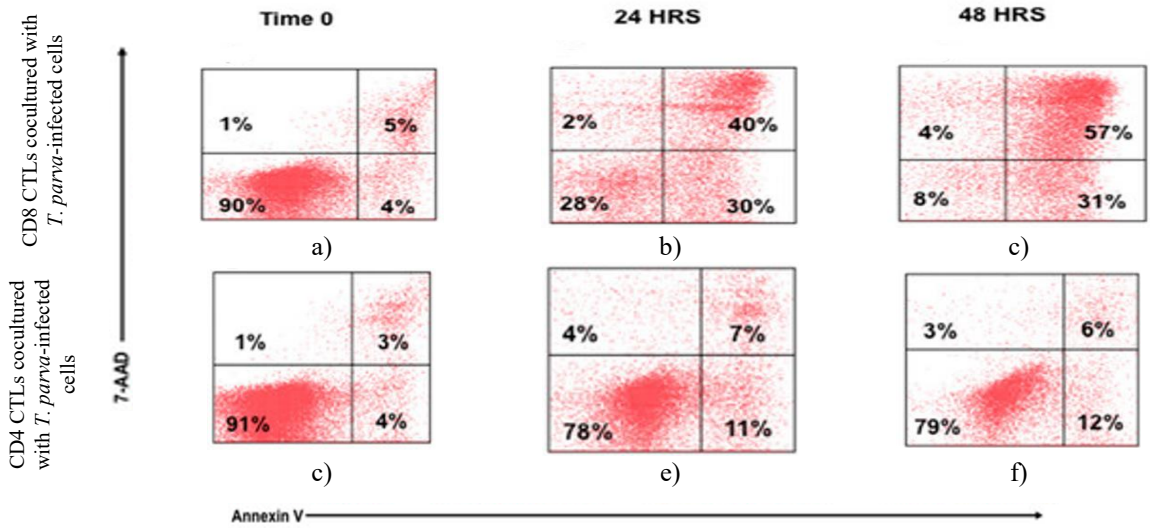


Figure 5. Flow cytometric evaluation of T. parva–specific $CD4^+$ and $CD8^+$ T-cell cytotoxicity. The representative plots illustrate the fractions of apoptotic and dead T. parva–infected targets following 0, 24, or 48 h co-incubation with either $CD8^+$ (a–c) or $CD4^+$ (d–f) T cells. A pronounced decrease in viable targets was visible when $CD8^+$ T cells were present, confirming that the observed target cell death was induced by $CD8^+$ T-cell cytolysis.

Discussion

Research into protective immunity to T. parva and the creation of an effective vaccine has evolved slowly but steadily over the past four decades, in parallel with technological advances (see Morrison *et al*. [27] for an

overview of earlier work). The central immune effectors responsible for controlling T. parva infection are $CD8^+$ CTLs that target schizont-infected lymphocytes [1, 11]. These responses arise naturally during infection and following immunization via the infection-and-treatment method (ITM) [1]. Other than $CD8^+$ CTLs, no immune marker reliably correlating with protection has been confirmed. Some evidence hints that $CD4^+$ T-helper and γδ T cells may contribute [28–31], yet responses from these non-$CD8^+$ subsets remain poorly defined. Standard indicators of T-cell activation—such as IFN-γ release or cell proliferation—do not correspond to protection from clinical disease [12–15].

This limitation has significantly restricted vaccine progress. Although several antigenic constructs have been tested in cattle using different delivery systems, none consistently generated protective immunity [12–15]. Hence, a more complete understanding of protective cellular mechanisms beyond cytotoxicity is needed to guide vaccine development and evaluation. This study sought to refine a flow-cytometry-based cytotoxicity assay, previously applied to investigate CTL responses against PRRS virus in pigs and Mycobacterium species in cattle [16, 17], for use in the T. parva model. This adaptation aimed to enable broader characterization of the T-cell response following natural infection, ITM immunization, or experimental vaccination. Unlike the older chromium-release method [11, 13, 14], which measures only total lysis, the flow-based approach simultaneously captures surface phenotype, MHC–peptide interactions, cell proliferation, and cytokine production, thereby offering a multidimensional view of both cytotoxic and non-cytotoxic subsets.

The present data confirm successful implementation of the flow cytometry protocol for T. parva. Comparable to findings from chromium-release experiments [32], strong cytotoxicity was detected at each time point when T. parva–specific parent or $CD8^+$ T-cell lines served as effectors. In addition to avoiding radioactive tracers, this approach distinguishes between early apoptosis (annexin-V staining) and late membrane disruption (7-AAD uptake) [33]. Because the chromium assay quantifies only the latter phase, it underestimates early apoptotic events and is therefore less sensitive.

Moreover, the revised assay permitted parallel phenotypic profiling of active T-cell populations. The majority of PBMCs stimulated with autologous irradiated T. parva–infected cells displayed a $CD45R0^+$ $CCR7^-$ effector-memory phenotype and expressed CD25, a marker of T-cell activation. Future analyses could investigate expression of markers associated with bovine γδ T cells, regulatory subsets, and helper T-cell lineages, as well as intracellular molecules such as perforin, granzyme B, and cytokines including IL-17, IFN-γ, and IL-10. Combining these readouts with cytotoxic and proliferative measurements would enable a more comprehensive view of the immune mechanisms acting against T. parva.

A notable observation from this investigation was the detection of a modest yet statistically significant $CD4^+$ cytotoxic T-lymphocyte (CTL) response against *T. parva*-infected cells. To the best of current knowledge, this represents the first evidence of cytolytic $CD4^+$ T-cell activity specific to *T. parva*. Although such responses are considerably rarer than those mediated by $CD8^+$ CTLs, comparable $CD4^+$ CTL activity has previously been described in cattle infected with *Neospora caninum* [34, 35] and in humans infected with *Toxoplasma gondii* [36, 37].

Given the extensive cell-sorting and validation steps performed—including multipoint phenotyping of $CD4^+$ and $CD8^+$ subsets before and after magnetic separation using anti-bovine CD4 and CD8 monoclonal antibodies—the possibility of cross-contamination between subsets is minimal. However, it remains theoretically possible that a minor proportion of $CD4^+$ CTLs expressed low levels of CD8, forming a double-positive (DP) T-cell population. Such DP T cells occur naturally in healthy humans, pigs, and rodents, where antigenic stimulation tends to increase their frequency in peripheral blood [38]. To date, this phenomenon has not been reported in cattle [38].

The functional contribution of cytotoxic $CD4^+$ T cells to immune control of *T. parva* remains uncertain. Prior work has shown that adoptive transfer of purified $CD8^+$ CTLs, devoid of $CD4^+$ T cells, from immune to naïve twin calves was sufficient to confer resistance [11]. Whether $CD4^+$ CTLs enhance this protection in vivo still requires clarification. Alongside better definition of *T. parva*-specific CTLs, it is essential to delineate additional cellular components necessary for CTL induction to inform vaccine design. The present findings reveal that both $CD4^+$ and $CD8^+$ T cells proliferate upon exposure to infected cells, with activated effector-memory $CD4^+$ T cells being numerically dominant. While a few earlier studies alluded to a supportive role of $CD4^+$ T-helper cells in promoting protective CTL responses against *T. parva* [28, 29, 39], comprehensive profiling of these helper subsets and their functional dynamics is still lacking.

Intercellular cooperation in CTL Induction

A foundational study by Taracha *et al*. demonstrated that CTL development against *T. parva*-infected targets occurred only when both infected antigen-presenting cells and T cells were present within the same culture system [39]. When $CD4^+$ T cells were physically separated from infected cells and $CD8^+$ T cells by a semipermeable barrier, functional CTL maturation was completely abrogated. Comparable outcomes have been documented in *Mycobacterium avium* subsp. *paratuberculosis* (Map) models, where the simultaneous recognition of antigenic peptides by both $CD4^+$ and $CD8^+$ T cells was required for CTL activation [17, 40, 41]. The use of monoclonal antibodies against MHC class I and II molecules further confirmed this co-recognition dependency, as blockade of either MHC I or II alone disrupted CTL induction [41].

The present work indicates that methodologies initially optimized for investigating immune responses to Map peptide vaccines can be effectively adapted to analyze *T. parva* immunity. Utilizing the flow cytometric assay established here, future in vitro and ex vivo studies can systematically characterize:

1. The phenotypic and functional profiles of $CD4^+$ helper subsets facilitating CTL maturation;
2. The temporal dynamics and signaling interactions among $CD4^+$ T cells, $CD8^+$ T cells, and antigen-presenting cells during CTL induction; and
3. The effects of modulating $CD4^+$ T-cell or APC phenotypes—including cytokine output and surface marker expression—on CTL development against *T. parva*.

The resulting data will guide rational vaccine formulation by identifying optimal adjuvants and delivery strategies that steer immune activation toward protective outcomes. Moreover, this assay provides a reproducible system to quantitatively evaluate vaccine-induced cellular immunity to *T. parva* under controlled conditions.

Conclusion

In conclusion, the current study successfully adapted a flow cytometry–based cytotoxicity platform, previously used to analyze $CD8^+$ CTL responses against *Mycobacterium* species and PRRS virus, to overcome a critical technical barrier in *T. parva* immunology. This platform enables ex vivo assessment of both primary and memory CTL responses, thereby facilitating detailed examination of antigen processing, presentation by APCs, and the bidirectional signaling between $CD4^+$ and $CD8^+$ T cells necessary for CTL differentiation.

Importantly, the method allows retention and analysis of $CD8^+$ CTLs before and after target-cell killing**,** permitting deeper exploration of their activation signatures and regulatory determinants. Consistent with earlier observations by Taracha *et al*. [39], our findings support the hypothesis that $CD4^+$ T-cell help is crucial for optimal $CD8^+$ CTL generation against *T. parva*. Stimulation of PBMCs from immune cattle with autologous infected cells induced robust proliferation of both $CD4^+$ and $CD8^+$ subsets, which exhibited markers characteristic of activated effector-memory phenotypes.

This refined assay therefore provides a robust experimental framework to identify immune correlates of protection and dissect mechanisms of disease susceptibility in *T. parva* infection. Ultimately, these insights will underpin the development of next-generation vaccines designed to mitigate the significant economic burden of this parasitic disease in cattle.

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Conflict of Interest: None

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Ethics Statement: None

References

1. Nene V, Kiara H, Lacasta A, Pelle R, Svitek N, Steinaa L. The biology of Theileria parva and control of East Coast fever—Current status and future trends. Ticks Tick Borne Dis. 2016;7(4):549–64.
2. Shaw MK, Tilney LG. How individual cells develop from a syncytium: Merogony in Theileria parva (Apicomplexa). J Cell Sci. 1992;101(Pt 1):109–23.

3. Tretina K, Gotia HT, Mann DJ, Silva JC. Theileria-transformed bovine leukocytes have cancer hallmarks. Trends Parasitol. 2015;31(7):306–14.
4. Houston EF, Taracha EL, Brackenbury L, MacHugh ND, McKeever DJ, Charleston B, et al. Infection of cattle with Theileria parva induces an early CD8 T cell response lacking appropriate effector function. Int J Parasitol. 2008;38(14):1693–704.
5. Bastos RG, Sears K, Dinkel KD, Knowles DP, Fry LM. Changes in the molecular and functional phenotype of bovine monocytes during Theileria parva infection. Infect Immun. 2019;87(12):e00703-19.
6. Fry LM, Schneider DA, Frevert CW, Nelson DD, Morrison WI, Knowles DP. East Coast fever caused by Theileria parva is characterized by macrophage activation associated with vasculitis and respiratory failure. PLoS One. 2016;11(5):e0156004.
7. Bastos R, Franceschi V, Tebaldi G, Connelley T, Morrison WI, Knowles DP, et al. Molecular and antigenic properties of mammalian cell-expressed Theileria parva antigen Tp9. Front Immunol. 2019;10:897.
8. Bishop RP, Odongo D, Ahmed J, Mwamuye M, Fry LM, Knowles DP, et al. A review of recent research on Theileria parva: Implications for the infection and treatment vaccination method for control of East Coast fever. Transbound Emerg Dis. 2020;67(Suppl 1):56–67.
9. Patel E, Mwaura S, Kiara H, Morzaria S, Peters A, Toye P. Production and dose determination of the Infection and Treatment Method (ITM) Muguga cocktail vaccine used to control East Coast fever in cattle. Ticks Tick Borne Dis. 2016;7(2):306–14.
10. Di Giulio G, Lynen G, Morzaria S, Oura C, Bishop R. Live immunization against East Coast fever—Current status. Trends Parasitol. 2009;25(2):85–92.
11. McKeever DJ, Taracha ELN, Innes EL, MacHugh ND, Awino E, Goddeeris BM, et al. Adoptive transfer of immunity to Theileria parva in the CD8+ fraction of responding efferent lymph. Proc Natl Acad Sci U S A. 1994;91(5):1959–63.
12. Mwangi DM, Honda Y, Graham SP, Pelle R, Taracha EL, Gachanja J, et al. Treatment of cattle with DNA-encoded Flt3L and GM-CSF prior to immunization with Theileria parva candidate vaccine antigens induces CD4 and CD8 T cell IFN-γ responses but not CTL responses. Vet Immunol Immunopathol. 2011;140(3–4):244–51.
13. Graham SP, Pelle R, Honda Y, Mwangi DM, Tonukari NJ, Yamage M, et al. Theileria parva candidate vaccine antigens recognized by immune bovine cytotoxic T lymphocytes. Proc Natl Acad Sci U S A. 2006;103(9):3286–91.
14. Svitek N, Saya R, Awino E, Munyao S, Muriuki R, Njoroge T, et al. An Ad/MVA vectored Theileria parva antigen induces schizont-specific CD8+ central memory T cells and confers partial protection against a lethal challenge. NPJ Vaccines. 2018;3:35.
15. Fry LM, Bastos RG, Stone BC, Williams LB, Knowles DP, Murphy SC. Gene gun DNA immunization of cattle induces humoral and CD4 T-cell-mediated immune responses against the Theileria parva polymorphic immunodominant molecule. Vaccine. 2019;37(12):1546–53.
16. Chung CJ, Cha SH, Grimm AL, Ajithdoss D, Rzepka J, Chung G, et al. Pigs that recover from porcine reproduction and respiratory syndrome virus infection develop cytotoxic CD4+CD8+ and CD4+CD8- T-cells that kill virus infected cells. PLoS One. 2018;13:e0203482.
17. Abdellrazeq GS, Elnaggar MM, Bannantine JP, Park KT, Souza CD, Backer B, et al. A Mycobacterium avium subsp. paratuberculosis relA deletion mutant and a 35 kDa major membrane protein elicit development of cytotoxic T lymphocytes with ability to kill intracellular bacteria. Vet Res. 2018;49:53.
18. Radley DE, Brown CG, Cunningham MP, Kimber CD, Musisi FL, Purnell RE, et al. East Coast fever: challenge of immunised cattle by prolonged exposure to infected ticks. Vet Rec. 1975;96:525–7.
19. Brown CG, Radley DE, Cunningham MP, Kirimi IM, Morzaria SP, Musoke AJ. Immunization against East Coast fever (Theileria parva infection of cattle) by infection and treatment: Chemoprophylaxis with N-pyrrolidinomethyl tetracycline. Tropenmed Parasitol. 1977;28(3):342–8.
20. Young AS, Leitch BL, Dolan TT, Mbogo SK, Ndungu SG, Grootenhuis JG, et al. Evaluation of infection and treatment methods in immunization of improved cattle against theileriosis in an endemic area of Kenya. Vet Parasitol. 1990;35(3):239-57.
21. Baldwin CL, Black SJ, Brown WC, Conrad PA, Goddeeris BM, Kinuthia SW, et al. Bovine T cells, B cells, and null cells are transformed by the protozoan parasite Theileria parva. Infect Immun. 1988;56(2):462–7.

22. Goddeeris BM, Morrison WI. Techniques for the generation, cloning, and characterization of bovine cytotoxic T cells specific for the protozoan Theileria parva. J Tissue Cult Methods. 1988;11(2):101–10.
23. Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur J Immunol. 2004;34(3):669–76.
24. Allen AJ, Park KT, Barrington GM, Hamilton MJ, Davis WC. Development of a bovine ileal cannulation model to study the immune response and mechanisms of pathogenesis paratuberculosis. Clin Vaccine Immunol. 2009;16(3):453–63.
25. Packard BZ, Telford WG, Komoriya A, Henkart PA. Granzyme B activity in target cells detects attack by cytotoxic lymphocytes. J Immunol. 2007;179(6):3812–20.
26. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: Comparisons to a 4 h 51Cr-release assay. J Immunol Methods. 2007;325(1–2):51–66.
27. Morrison WI, Goddeeris BM, Brown WC, Baldwin CL, Teale AJ. Theileria parva in cattle: Characterization of infected lymphocytes and the immune responses they provoke. Vet Immunol Immunopathol. 1989;20(3–4):213–37.
28. Baldwin C, Goddeeris BM, Morrison WI. Bovine helper T-cell clones specific for lymphocytes infected with Theileria parva (Muguga). Parasite Immunol. 1987;9(5):499–513.
29. Baldwin C, Iams KP, Brown WC, Grab DJ. Theileria parva: CD4+ helper and cytotoxic T-cell clones react with a schizont-derived antigen associated with the surface of Theileria parva-infected lymphocytes. Exp Parasitol. 1992;75(1):19–30.
30. Brown WC, Lonsdale-Eccles JD, Demartini JC, Grab DJ. Recognition of soluble Theileria parva antigen by bovine helper T cell clones: Characterization and partial purification of the antigen. J Immunol. 1990;144(1):271–7.
31. Daubenberger CA, Taracha EL, Gaidulis L, Davis WC, McKeever DJ. Bovine γδ T-cell responses to the intracellular protozoan parasite Theileria parva. Infect Immun. 1999;67(5):2241–9.
32. Morrison WI, Goddeeris BM, Teale AJ, Groocock CM, Kemp SJ, Stagg DA. Cytotoxic T cells elicited in cattle challenged with Theileria parva (Muguga): Evidence for restriction by class I MHC determinants and parasite strain specificity. Parasite Immunol. 1987;9(6):563–78.
33. Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. Expert Rev Vaccines. 2010;9(6):601–16.
34. Staska LM, Davies CJ, Brown WC, McGuire TC, Suarez CE, Park JY, et al. Identification of vaccine candidate peptides in the NcSRS2 surface protein of Neospora caninum by using CD4+ cytotoxic T lymphocytes and gamma interferon-secreting T lymphocytes of infected holstein cattle. Infect Immun. 2005;73(2):1321–9.
35. Staska LM, McGuire TC, Davies CJ, Lewin HA, Baszler TV. Neospora caninum-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. Infect Immun. 2003;71(6):3272–9.
36. Curiel TJ, Krug EC, Purner MB, Poignard P, Berens RL. Cloned human CD4+ cytotoxic T lymphocytes specific for Toxoplasma gondii lyse tachyzoite-infected target cells. J Immunol. 1993;151(4):2024–31.
37. Purner MB, Berens RL, Nash PB, van Linden A, Ross E, Kruse C, Krug EC, Curiel TJ. CD4-mediated and CD8-mediated cytotoxic and proliferative immune responses to Toxoplasma gondii in seropositive humans. Infect Immun. 1996;64(10):4330–8.
38. Overgaard NH, Jung J, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: More than just a developmental stage? J Leukoc Biol. 2015;97(1):31–48.
39. Taracha ELN, Awino E, McKeever DJ. Distinct CD4+ T cell helper requirements in Theileria parva-immune and -naive bovine CTL precursors. J Immunol. 1997;159(9):4539–45.
40. Franceschi V, Mahmoud AH, Abdellrazeq GS, Tebaldi G, Macchi F, Russo L, et al. Capacity to elicit cytotoxic CD8 T cell activity against Mycobacterium avium subsp. paratuberculosis is retained in a vaccine candidate 35 kDa peptide modified for expression in mammalian cells. Front Immunol. 2019;10:2859.
41. Abdellrazeq GS, Fry LM, Elnaggar MM, Bannantine JP, Schneider DA, Chamberlin WM, Mahmoud AHA, Park KT, Hulubei V, Davis WC. Simultaneous cognate epitope recognition by bovine CD4 and CD8 T cells is essential for primary expansion of antigen-specific cytotoxic T-cells following ex vivo stimulation with a candidate Mycobacterium avium subsp. paratuberculosis peptide vaccine. Vaccine. 2020;38(8):2016–25.