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Scientific Research

Perforin Expression in *Theileria parva* Specific Cytotoxic T Cells Correlates with Cytotoxicity

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Abstract

Theileria parva **is a protozoan parasite that causes the disease East Coast fever in cattle which results in major economic losses in Eastern, Central and Southern Africa. Efforts to generate vaccines involve measurements of cytotoxic activity since CD8 cells are believed to be responsible for protection of the animals. CTL assays are time consuming, and may require use of radioactive material and they also impose a considerable amount of** ***in vitro*** **work, which may skew the response compared to** ***ex vivo*** **assays. Hence it would be beneficial to identify other markers that correlate with the cytotoxicity. In this** ***in vitro*** **study we examined if the number of tetramer positive CD8 cells and the staining intensity of these correlated with lysis of the target cells. Furthermore, we investigated if the expression of the activation marker perforin correlated with the cytotoxicity. Perforin is involved in permeabilization of the cell membrane of the target cell. Bulk CTL lines and purified CD8 cell lines generated from cattle of the A18 BoLA (MHC) type were analysed for the** ***Theileria parva*** **specific immune responses using a peptide-MHC tetramer and antibodies for perforinin FACS analysis. Thelysis of target cells was determined by a chromium release assay. Results demonstrate that the percentage of tetramer positive cells, in six cell lines generated against the whole parasite, correlate with killing of PBMC pulsed with the peptide. The product of the percentages of perforin and tetramer double positive cells and the net MFI of perforin showed a perfect correlation with the cytotoxicity of the peptide pulsed PBMC. Likewise, the product of percentage perforin positive cells and the staining intensity had the best significant correlation with killing of the pulsed PBMC. The present results suggest that perforin could be a possible biomarker for the cytotoxicity to** ***Theileria parva*** **infections/immunizations.**

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Keywords

Cytotoxic T Cells, Biomarkers, Perforin, *Theileria parva*

1. Introduction

Theileria parva [1] is a tick-borne obligate intracellularprotozoan parasite. It causes an acute and usually fatal disease in cattle, known as East Coast fever leading to major economic losses in Sub-Saharan Africa [2] [3]. The parasite infects bovine lymphocytes which subsequently undergo blast transformation and rapid multiplication [4]. In naive animals, this usually results in overwhelming parasitosis and death within 2 to 4 weeks of infection [5] [6]. Cattle can be vaccinated against the disease by infection with a mixture of live parasite strains, known as the Muguga cocktail and simultaneous treatment with the long-acting antibiotic oxytetracyclin, a method known as the Infection and Treatment Method (ITM) [7]. Although this vaccination procedure is highly effective and can provide life-long immunity, it has some limitations: the live vaccine needs to be stored in liquid nitrogen; the cost to treat an animal is relatively high as one vaccine dose costs 8 - 12 USD [8], and expert veterinary supervision is also required to administer ITM [2]. Besides this, the vaccines may induce a carrier status in the immunized animals as there is evidence of this from both vaccine immunized and naturally infected animals, which means that the lethal parasite can be transmitted to unimmunized animals by ticks [2] [9]. These obstacles have encouraged research towards a subunit vaccine.

It is believed that CTL are crucial for protective immunity [10]-[12]. Several *Theileria parva* CTL antigens have been identified which are presented by specific BoLA Class I molecules to CD8 T-cells, such as Tp1, Tp2, Tp4, Tp5, Tp7 and Tp8 [13] [14]. Therefore, attempts in testing CTL vaccines and antigens will include measurement of the cytotoxic response. However, CTL cytotoxicity assays are time consuming, and may require use of radioactivity and CTL usually require several restimulations when blood is used as source of lymphocytes, so other correlates with CTL cytotoxicity are desirable.

MHC class I tetramers represents a unique way of identifying specific T cells among a population of T cells by binding to the corresponding T cell receptors on the surface of the cells. Tetramer staining has previously been used as direct indicators of CTL avidity [15]-[17]. However, other reports do not find clear relationships between functional avidity and tetramer staining [18] [19]. Hence, further research in this field is wanted.

Two major pathways are involved in CTL cytotoxicity: the perforin granule exocytosis pathway [20] and the Fas-Fas ligand mechanism [21] [22]. However, it has been shown that the FAS-ligand pathway is not important with regards to killing of infected cells by *T. parva* specific CTL [23]. Perforin is an important constituent of cytotoxic granules, which directly contributes to T-cell mediated death via apoptosis or necrosis by permeabilizing target cell membrane [20] [24]. Perforin inserts itself into the target cell's plasma membrane and enables endocytosis of itself and granzyme proteases [25]. The ensuing translocation of pro-apoptotic granzymes into the cytoplasm affords these proteases access to many protein substrates that stimulate apoptosis after cleavage [26]. Perforin has been suggested as a potential biomarker of protection after vaccination against tuberculosis [27].

The aim of this study was to evaluate the correlation of tetramer specific cells and perforin with the cytotoxic capacity of CD8 cells specific for *Theileria parva*. The results demonstrate that perforin and TCR avidity are possible surrogate markers for cytotoxicity, a known correlate with protection against ECF.

2. Material and Methods

2.1. Animals and Immunization

6 months old Friesian-Holstein cattle were immunized (s.c.) by infection with cryopreserved sporozoites (Muguga strain) and simultaneous administration of a long-acting oxytetracycline [7]. FACS analysis and CTL restimulations were carried out approximately 6 months after immunization. Cattle were of the A18 MHC class I haplotype, which consists of a single MHC gene. The haplotype was determined by a combination of serologically typing using class I-specific monoclonal antibodies [28], ELISPOT assays using epitope specific cell lines and class I allele-specific PCR assays. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the International Livestock Research Institute (# 2012.10).

2.2. Parasitized Cell Lines

Cell lines infected with *T. parva* were set up by infection of PBMC *in vitro* with sporozoites harvested from dissected salivary glands from infected ticks as described earlier [29]-[31].

2.3. Generation of CD8 Bulk Cultures and CD8 Enriched Cell Lines

CD8 T cell lines were generated by stimulation of PBMC with irradiated autologous *Theileria parva* infected cells lines essentially as described before [32]-[34]. CTL lines were generated and maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-Glutamine, 50 µM 2-mercaptoethanol, 100 IU of penicillin/ml, 50 µg of gentamicin/ml (all from Sigma-Aldrich, St. Louis, MO, USA). For the purified CD8 cell lines (BV115 and BB007) an additional purification step involved isolation of CD8 cells using MACS magnetic beads. This had been done prior to this study for the BV115 cell line.

2.4. Flow Cytometry Analysis

Monoclonal antibodies used for flow cytometry were: α-bovine CD8 (ILRI-generated ILA51, mouse IgG1), [35]. FITC-conjugated mouse α-human perforin (clone δG9) with an isotype control antibody (clone 27 - 35) both from BD Biosciences., San Diego, CA, USA. The α-human perforin antibody has previously been successfully used on bovine cells [36]. The secondary antibodies were FITC-labelled goat α-mouse (IgG1) or PE-labelled goat α-mouse IgG antibody, (Southern Biotech, Birmingham, AL, USA). The Tp1 tetramer was PE labelled and composed of the A18 MHC I allele (N*01301) and loaded with the $Tp1_{214\text{-}224}$ *T. parva* epitope (provided by Pierre van der Bruggen, Ludwig Institute for Cancer, Brussels Branch, Belgium). Approximately 4×10^5 cells were stained in BD FACS tubes by adding 25 µl Tp1 tetramer to a final concentration of 40 nM followed by incubation for 10 minutes in the dark at RT. 25 µl of the primary antibodies recognizing CD8 or CD25, diluted 1/250 in PBS-0.5% BSA, were added followed by incubation for 30 minutes at 4˚C. Cells were then washed twice in 200 µl PBS-0.5% BSA. Then 25 µl of the secondary antibodies were added, incubated and washed as described above. Cells stained intracellularly were fixed by adding 200 µl of 0.25% paraformaldehyde 7 (PFA) followed by incubation at RT in the dark for 10 minutes. Cells were washed once with 200 µl of ice-cold Dulbecco's PBS and permeabilized by adding 200 µl of PBS-Saponin (Dulbecco's PBS, 0.1% (w/v) saponin, 0.2% Na_3N, 10 mM HEPES, 10% FBS) supplemented with 20% heat inactivated normal goat serum (Fc receptor blocking) to each tube and incubated at RT for 30 minutes or overnight at 4˚C. α-perforin and isotype control antibodies (FITC labelled) were added in FACS medium(RPMI 1640) supplemented with 2% horse serum and 0.02% Na_3N in accordance with the manufacturer's instructions. The plate was incubated for 30 minutes at 4˚C. Cells were pelleted by centrifugation at 1600 rpm for 3 minutes at 4˚C, medium was flicked and pellet broken on a mixer. Finally, cells were re-suspended in 100 µl of FACS medium and analysed with a FACS Canto II flow cytometer (Becton-Dickinson, Mountain View, CA).

2.5. CTL Cytotoxicity Assay

A standard 4 h-release assay using ^{51}Cr-labeled target cells was used to measure cytotoxicity essentially as described before [31] with a few modifications as described in [37]. ^{51}Cr was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA. For each CTL bulk or cell line, the following targets were included: an autologous *T. parva*-infected PBMC line, an MHC-mismatched *T. parva*-infected cell line, $Tp1_{214\text{-}224}$ peptide-pulsed PBMC and PBMC as a control for the pulsed PBMC. Peptide pulsing was performed before ^{51}Cr-labeling using 1 µM of the Tp1 peptide. The cytotoxicity was calculated as: (experimental release − spontaneous release)/(total release − spontaneous release). CTLs were added in dilutions together with a fixed amount of ^{51}Cr labelled target cells in 96 well plates and incubated for 4 h at 37˚C. The supernatants from the wells were harvested and added to Lumaplates (PerkinElmer, MA, USA) which were allowed to dry. They were analysed in a Top Counter after addition of scintillator (PerkinElmer, MA, USA).

2.6. Statistics

Data was computed using the program GenStat, VSN International (2011). GenStat for Windows 14th Edition.

VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk. The analysis was based on Spearman's Rank Correlation test. P value < 0.05 was recognized as statistically significant.

3. Results

3.1. Cell Cultures from *T. parva* Immunized Cattle Show Avidity Differences for the $Tp1_{214-224}$ CTL Epitope

Four PBMC bulk cell lines (BB007, BA219, BF091, BF092) and two purified CD8 CTL lines (BB007 and BV115) generated from *T. parva* immunized cattle were used for correlation of FACS data and cytotoxic capacity in CTL assays. The FACS analysis included analysis of the presence of $Tp1_{214-224}$ specific CTL and their expression levels of perforin. First, cells were stained with the $Tp1_{214-224}$ tetramer. We used the tetramer single stain to calculate the percentages of tetramer positive cells since all tetramer positive cells were CD8 positive. **Figure 1** shows the staining with the Tp1 tetramer of the six cell cultures after gating on live cells. BF091 had the lowest percentage (0.52%) and the BB007 cell line the highest (43.0%). The tetramer positive cells appeared as single distinct populations except for BF091 bulk, where two overlapping populations were evident. **Figure 2** shows the two tetramer populations from BF091 as a contour diagram. The mean florescence intensities (MFIs) for the tetramer populations (Tet high and Tet low) based on the centres of the two populations reveals that they differ with a factor 3 - 4. We have observed this occasionally in other cell cultures. It may be due to either a difference in affinity for the tetramer or in the number of T cell receptors on the surface. Why this appears in only one of the bulk lines is at present unknown.

Percentages of tetramer positive cells of total cells, of CD8 cells and percentages of CD8 cells are indicated in the first three columns of **Table 1**. The MFIs of the tetramer stained populations varied approximately 3-fold from 8547 (BV115 CTL) as the lowest to 23881 (BF092 bulk) as the highest. The MFI could very likely play a role in correlation with cytotoxicity, since this reflects the avidity of the interaction between the T cell receptor (TCR) and the MHC molecule, *i.e.* the combined effect of TCRs per cell and affinity. Data for the MFIs for the tetramer populations are listed in column 4 in **Table 1**.

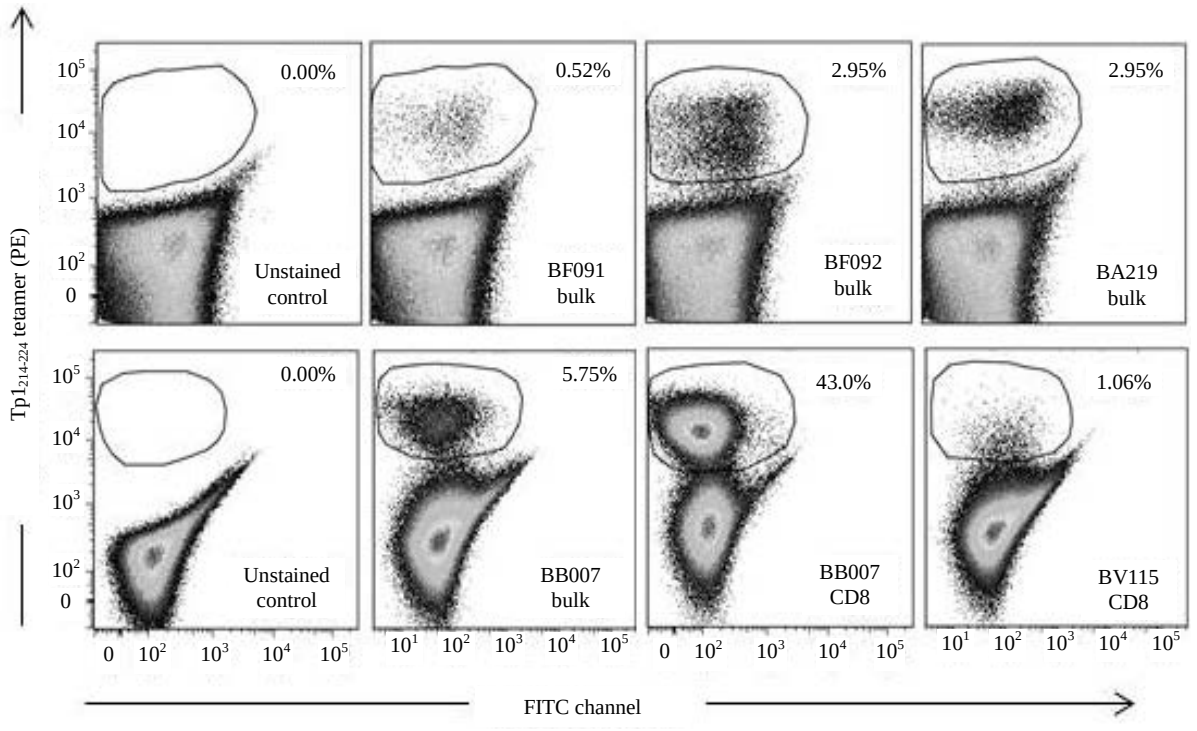

Figure 1. $Tp1_{214-224}$ tetramer positive cells in the six cell lines. Percentages of tetramer positive cell in each of the six cell lines (four bulk lines and two purified CD8 cell lines) are indicated in each diagram. Live cells were gated and tetramers were PE-labelled. All tetramer positive cells were CD8 positive.

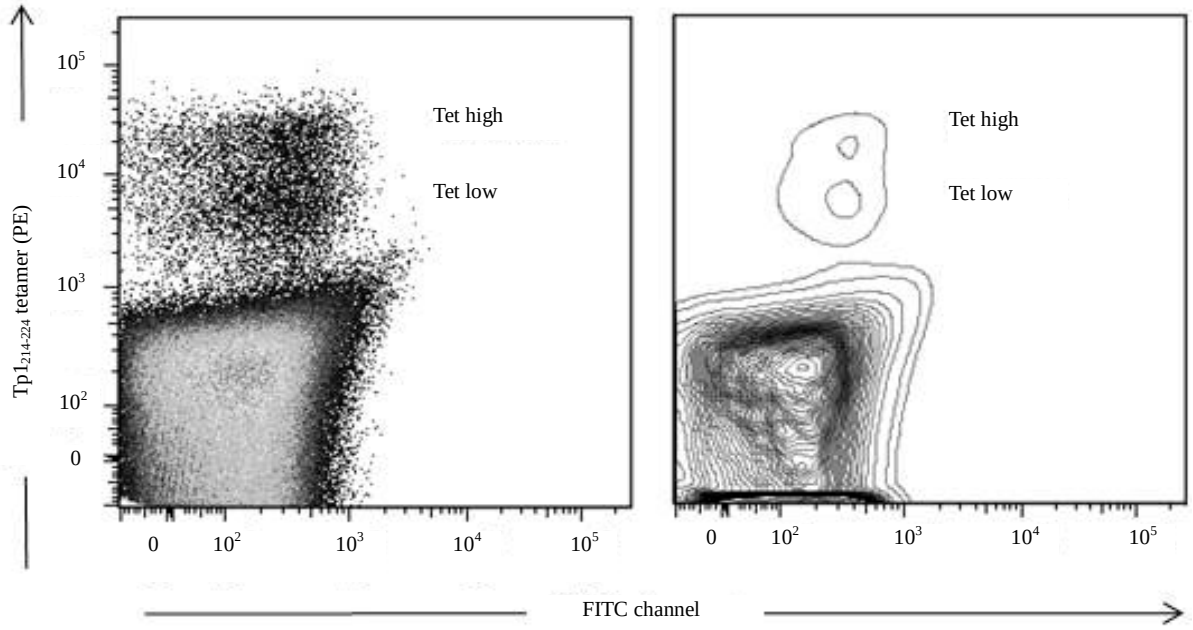

Figure 2. Two populations of $Tp1_{214-224}$ tetramer positive cells in BF091 cell line. The two diagrams show the two overlapping tetramer populations in the BF091 cell line as a dot diagram and as a contour diagram. Cells were stained with PE-labelled $Tp1_{214-224}$ tetramer.

Table 1. Percentages of tetramer positive and perforin positive cells and their MFIs. Percentages of cells stained with $Tp1_{214-224}$ tetramer, α-perforin in the PBMC bulk cell lines and in the cell lines. These populations of cells are gated on the Tp1 tetramer positive (Tp1 tet+) cells. The net MFI for the tetramer staining and the perforin (perf+) staining are also shown. The net MFI's are calculated by subtracting the MFI for the negative control.

Cell line	Tp1 tetramer positive cells (%)		CD8+ cells out of total cells (%)	Net MFI of Tp1 tet + cells	Perf + cells (%)	Net MFI of perf + cells
	of total cells	of CD8 cells				
BA219 bulk	0.52	2.17	24.0	14072	0.51	3309
BF091 bulk	2.95	24.38	12.1	11550	2.90	2920
BF092 bulk	2.63	23.27	11.3	23881	2.57	4324
BB007 bulk	5.75	19.76	29.1	22850	5.62	6733
BB007 CTL	43.00	43.57	98.7	15742	42.90	5542
BV115 CTL	1.06	1.11	95.2	8547	0.81	11384

3.2. Tetramer Specific Cells from Cell Cultures Express Perforin and CD25 at Different Levels

Next, the expression of perforinon the Tp1 tetramer positive cells were examined (**Figure 3**). Staining for perforin revealed that essentially all tetramer positive cells were positive for perforin in BB007 bulk, BB007 CD8 line and for BV115 CD8 line. A very small overlap of the perforin control and the perforin staining were present for BA219 and BF092, showing that the majority of cells were positive for perforin in these cell lines. In BF091 a slightly larger overlap was evident suggesting that some Tp1 specific cells were negative or low inperforin expression. Percent perforin positive cells are shown in column 5 in **Table 1**.

3.3. Cytotoxic Capacity of Cell Cultures Correlates with Tetramer Positive Cells and Expression of Perforin

In order to correlate the presence of tetramer specific cells and the expression of perforin with the cytotoxicity, a

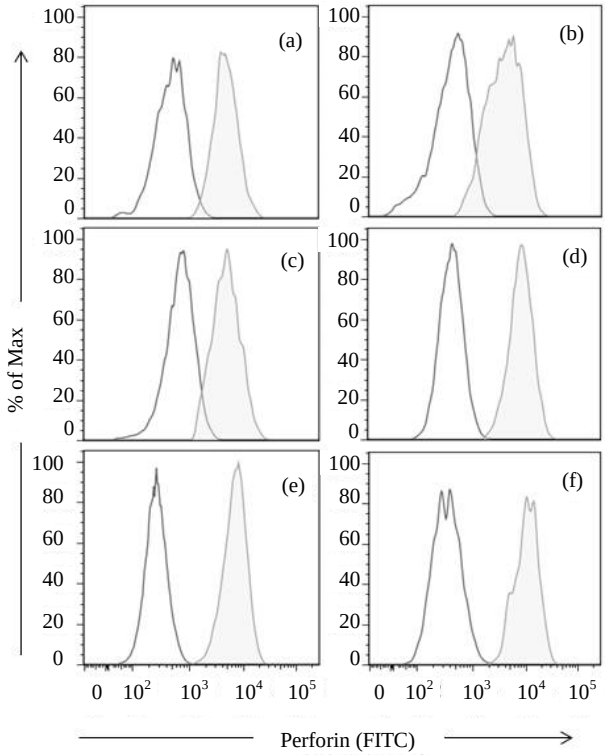

Figure 3. Staining of perforin positive tetramer positive CTL. Overlay histogram of gated tetramer positive cells and their expression of perforin versus the negative controls. Perforin positive tetramer specific cells are represented by (a) BA219 bulk; (b) BF091 bulk; (c) BF092 bulk; (d) BB007 bulk; (e) BB007 CD8 and (f) BV115 CD8. White histograms represent the negative control antibody and shaded histograms represent perforin stained cells.

CTL assay was performed using the same bulks and cell lines as for the FACS analysis (**Figure 4**). *T. parva* specific PBMC bulks and cell lines were tested for the cytotoxic capacity using their respective autologous *T. parva* Muguga infected cell line (TpM) as target as well as an MHC mismatched control cell line, $Tp1_{214\text{-}224}$ pulsed PBMC and PBMC control cells. The highest level of cytotoxicity were obtained using the TpMs except for CTL originating from BB007 that had a very high level of cytotoxicity using $Tp1_{214\text{-}224}$ pulsed PBMC. A higher level of cytotoxicity using TpM than $Tp1_{214\text{-}224}$ pulsed PBMC indicates presence of other CTL specificities. The net cytotoxicity for the autologous TpM and the pulsed PBMCs at three different effectors: target ratios (7.5, 15 and 20) are listed in **Table 2**. These values were used for correlation with the number of stained cells, the MFIs or the product of these two parameters.

Spearman Rank correlation was used for statistical evaluation of the correlation between FACS parameters and the cytotoxicity. **Table 3** shows the parameters that correlated significantly with the cytotoxicity. For simplicity, only the statistics for an effector:target ratio of 20 is shown, since the results were similar for the two other ratios. The number of $Tp1_{214\text{-}224}$ tetramer positive cells correlated with the lysis of PBMC pulsed with the peptide. The correlation was improved if the cytotoxicity was correlated with the product of the number of $Tp1_{214\text{-}224}$ tetramer positive cells and the net MFI, reflecting the importance of both the number of cells and the avidity of interaction. The number of perforin positive cells (gated tetramer positive cells) and the MFIs for perforin also correlated with the cytotoxicity. Again a perfect correlation was seen when the cytotoxicity was correlated with the product of the number of perforin positive cells and the net MFI for perforin.

When cytotoxicity using TpM was correlated with the FACS data, no significant relationships were detected. This could be expected since FACS data were obtained on single tetramer specificity and not on the whole CD8 cell population.

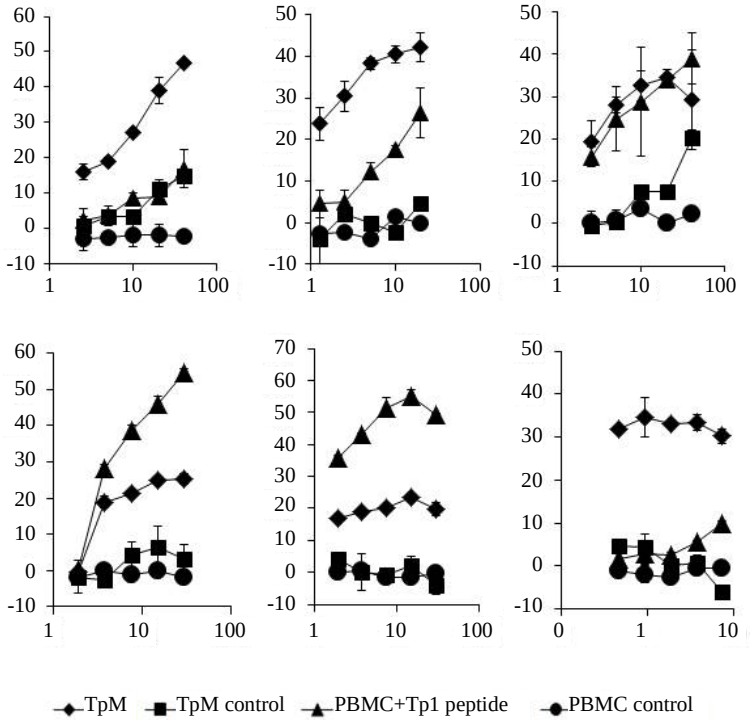

Figure 4. Cytotoxicity by the six cell lines. PBMC bulk lines and purified CD8 T cell lines were tested for cytotoxicity in a ^{51}Cr-release assay. The data are presented as the mean percentlysis at varying E/T ratios (+/−SD). $Tp1_{214-224}$ pulsed autologous PBMC and *T. parva* infected autologous cell lines (TPM) were used as target cells. PBMC (PBMC control) and an MHC mismatched cell line (TPM control) were used as controls.

Table 2. Cytotoxicity of $Tp1_{214-224}$ pulsed PBMC and TpM. Cytotoxicity of the PBMC bulk and purified CD8 cell lines was examined using a ^{51}Cr-release assay at the indicated effector: target ratios (E:T ratio). Target cells consisted of autologous PBMCs pulsed with the $Tp1_{214-224}$ peptide and autologous Muguga-infected lymphocytes (TpM). Unpulsed PBMC and heterologous infected cell lines were used as controls. The net cytotoxicity for the autologous Tp1 peptide pulsed PBMCs and TpM were calculated by subtracting the respective controls.

Cell Line	Effector:target ratio	TpM cytotoxicity (%)	$Tp1_{214-224}$ pulsed PBMC cytotoxicity (%)
BA219 bulk	7.5	20.14	8.28
	15.0	24.70	10.71
	20.0	27.81	10.98
BF091 bulk	7.5	36.62	15.89
	15.0	40.65	22.22
	20.0	35.75	26.58
BF092 bulk	7.5	24.81	24.13
	15.0	26.89	30.58
	20.0	26.96	33.64
BB007 bulk	7.5	16.96	38.77
	15.0	18.32	46.18
	20.0	19.82	46.96
BB007 CTL	7.5	20.89	53.05
	15.0	21.33	56.82
	20.0	23.13	53.86
BV115 CTL	7.5	36.31	10.34

Table 3. Correlation of cytotoxicity and biomarker parameters. Parameters used for correlation with cytotoxicity at E:T ratio of 20 are listed. Spearman Rank correlation was used. Correlation coefficients and P-values are shown.

Parameters correlated with cytotoxicity of $Tp1_{214-224}$ pulsed PBMC at effector: target ratio of 20	Spearman's correlation coefficient	P-Value
% Total Tp1 tetramer positive cells	0.9	0.004
% Tp1 tetramer positive cells times MFI	1	<0.001
% perforin positive cells (gated on Tp1 tetramer positive cells)	0.9	0.004
MFI for perforin (gated on Tp1 tetramer positive cells)	0.8	0.021
% perforin positive cells times MFI for perforin (gated on tetramer positive cells)	1	<0.001

4. Discussion

Investigation of new biomarkers for prediction of disease outcome is an increasingly important topic including the finding of correlates with protection after vaccination. In this series of experiments we investigated the correlation of tetramer positive cells and perforin expression on the tetramer positive cells with the cytotoxicity in six cell lines originating from *T. parva* immunized cattle.

We found that the percentage of tetramer positive cells correlated with the cytotoxicity when the target cells were pulsed with the peptide used in the tetramer. An even better correlate with the cytotoxicity was the product of the percentage of tetramer positive cells and the net MFI of the staining. The MFI is a result of the number of T cell receptors (TCRs) per cell and their affinity for the tetramer, which obviously is of importance during the interaction of the CTL and the target cell. This suggests that tetramer staining (the product of the percentage of positive cells times the MFI of the tetramer population) can be used to predict the level of cytotoxicity, which in the case of *Theileria parva* correlates with protection. Such correlations may be elaborated further to include a mix of tetramers if several specificities are known or investigated at the same time. The tetramer staining did not correlate with the cytotoxicity to the *Theileria parva* infected target cells, indicating that there are other CTL specificities in the cell lines which contribute to the cytotoxicity.

Cytotoxic CD8 T cells primarily act through secretion of cytotoxic granules containing perforin and several granule associated proteases, including granzymes [38]. A mechanism of perforin-dependent granule independent CTL cytotoxicity has also been shown [39]. All cell lines in the present study expressed high levels of perforin. Especially the BV115 CTL line had a high MFI for perforin. Interestingly, this cell line had the lowest MFI for the tetramer staining. Considering data from all cell lines, both the number of perforin positive cells and the MFI correlated with the cytotoxicity, independently. The product of the percentages of perforin and tetramer double positive cells and the net MFI of perforin showed a perfect correlation with the cytotoxicity of the peptide pulsed PBMC. Perforin has previously been suggested as a marker for protection in tuberculosis based on the presence of perforin in immune cells compared to non-immune cells after restimulation. In this report, we evaluated if the level of perforin expression correlated with the level of cytotoxicity rather than the mere presence of perforin which gives a more detailed picture of the functional relationship.

While in this report we examined perforin, other potential cytotoxicity markers could be molecules such as, e.g., granzyme B and granulysin. Granzyme B is also a mediator of cytotoxicity and has been shown to correlate with protection and enhanced CTL responses in virus-stimulated peripheral blood mononuclear cells after influenza vaccination [40]. Granulysin has been proposed as a prospective candidate biomarker for protection after immunization against *Mycobacterium bovis* [27]. These markers will be considered in future analyses.

In this study we examined the correlation of various parameters on tetramer specific T cells with the lysis of target cells pulsed with the same peptide. This is a very clean setup where "noise" from other T cell specificities is avoided and initially this is an ideal way of studying such correlations. Subsequently, studies can be elaborated to include whole CD8 populations, to include a mix of tetramers and it can be extended to *ex vivo* studies with additional markers. Such a study is currently on going at ILRI. This may allow prediction of cytotoxicity from FACS studies using PBMC directly from immunized/infected animals which would ease the read outs from animal experiments tremendously.

5. Conclusion

The results demonstrate that the percentage of tetramer positive cells in six cell lines correlate with lysis of

PBMC pulsed with the peptide. A better correlate was obtained if the cytotoxicity was correlated with the product of the percentage tetramer positive cells and the MFI. Likewise, the product of percentage perforin positive cells and the corresponding MFI had the best significant correlation with killing of the pulsed PBMC. In conclusion, perforin and TCR avidity can be considered as potential biomarkers for cytotoxicity.

Competing Interests

The authors declare that they have no competing interest.

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Abbreviations

CTL: cytotoxic T cells;
MFI: mean fluorescence intensity;
BoLA: bovine leucocyte antigen;
ECF: East Coast fever;
Theileria parva: *T. parva*;
PBMC: peripheral blood mononuclear cells;
TpM: *T. parva* Muguga infected PMBC (cell line);
IL2: interleukin 2;
FACS: fluorescence activated cell sorting;
ELISPOT: enzyme-linked immuno-sorbent assay;
TCR: T cell receptor.

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