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Systematic Determination of TCR–Antigen and Peptide–MHC Binding Kinetics among Field Variants of a *Theileria parva* Polymorphic CTL Epitope

Nicholas Svitek,* Rosemary Saya,* Houshuang Zhang,† Vishvanath Nene,* and Lucilla Steinaa*

CTLs are known to contribute to immunity toward *Theileria parva*, the causative agent of East Coast fever. The Tp967–75 CTL epitope from the Muguga strain of *T. parva* is polymorphic in other parasite strains. Identifying the amino acids important for MHC class I binding, as well as TCR recognition of epitopes, can allow the strategic selection of Ags to induce cellular immunity toward *T. parva*. In this study, we characterized the amino acids important for MHC class I binding and TCR recognition in the Tp967–75 epitope using alanine scanning and a series of variant peptide sequences to probe these interactions. In a peptide–MHC class I binding assay, we found that the amino acids at positions 1, 2, and 3 were critical for binding to its restricting MHC class I molecule BoLA-A*023:01. With IFN-γ ELISPOT and peptide–MHC class I Tet staining assays on two parasite-specific bovine CTL lines, we showed that amino acids at positions 5–8 in the epitope were required for TCR recognition. Only two of eight naturally occurring polymorphic Tp9 epitopes were recognized by both CTLs. Finally, using a TCR avidity assay, we found that a higher TCR avidity was associated with a stronger functional response toward one of two variants recognized by the CTL. These data add to the growing knowledge on the cross-reactivity of epitope-specific CTLs and specificities that may be required in the selection of Ags in the design of a wide-spectrum vaccine for East Coast fever. *The Journal of Immunology*, 2022, 208: 1–13.
appendiculatus, and the natural reservoir is the African buffalo, Syncerus caffer (26, 27). Substantial evidence indicates that CTLs induced by the IVM vaccine that lyses schizont-infected cells are the main actors providing protection to animals infected with the *T. parva* parasite. For example, it has been demonstrated that CD8+ T cells transferred from immune to naïve animals confers passive immunity to disease, providing solid evidence that CTLs are candidate subunit vaccine Ags (28, 29).

Several *T. parva* CTL antigens have been identified, and some of them have been used in different experimental subunit vaccination studies resulting in partial protection toward a lethal infection by the parasite (30–33). For most of these Ags, the minimal epitope sequence was determined experimentally in the context of defined MHC class I molecules (32, 34). Recent studies have demonstrated that many of these CTL Ags vary in their amino acid sequences, which may complicate the induction of broad-spectrum vaccines (35). An example is an epitope in the Tp9 Ag from the Muguga strain of *T. parva*, which is presented by the bovine MHC class I “bovine leukocyte antigen” (BoLA)-A*0203:01 molecule (36), an allele expressed in cattle of the A14 BoLA serotype. This Ag and the Tp9 epitope were shown to be polymorphic in both buffalo- and cattle-allele expressed in cattle of the A14 BoLA serotype. This Ag and the especially because BoLA-1 sequence was determined experimentally in the context of de “T. parva” which may complicate the induction of broad-spectrum vaccines (32, 34). Recent studies have demonstrated that several MHC class I molecules (32, 34). Recent studies have demonstrated that many of these CTL Ags vary in their amino acid sequences, which may complicate the induction of broad-spectrum vaccines (35).

**TP9 ELISPOT**

TP9-BoLA-A*0203:01 peptide–MHC class I tetramer (Tet) staining assays. A set of eight Tp9 peptide epitope variants was then used in BoLA-binding, ELISPOT, and peptide–MHC class I Tet staining assays to determine the level of cross-reactivity of the Tp9-specific CTL lines toward these variants. Finally, a novel TCR avidity assay was developed and used to compare the variation in binding strength of peptide–MHC class I Tet presenting the cross-reacting Tp9 epitope variants to the CTL lines.

**Materials and Methods**

**CTL lines**

Autologous *T. parva* Muguga-infected lymphocyte (TpM) lines from animals BF092, 4003, and 495 and TpM-specific bulk CTL lines after immunization by ITM were established as previously described (37). In brief, PBMCs harvested from immunized animals were stimulated at least three times, at weekly intervals, by coculturing with irradiated autologous TpMs (TpMs:PBMC) in RPMI media containing 10% heat-inactivated FCS for 2 h at 37°C. After washing, the W6/32 mAb (cat # SC-32235; Santa Cruz Biotechnology, TX), which binds to a monomorphic epitope on β2m, but only when it is incorporated in MHC class I complexes, was added for 1 h at 4°C. After washing, anti-mouse IgG1 coupled to peroxidase (cat # A9017-1ML; Sigma-Aldrich) was added to the plate for an hour at room temperature, and further rounds of washes. Colorimetric change was performed by adding the TMB Plus “Ready to Use” Substrate (cat # 4395H; Kern En Tec) for 10 min at room temperature. The reaction was stopped by adding H2SO4 (0.3 M), and OD was measured using a Synergy HT ELISA plate reader (Bio-Tek) at 450 nm. K values were determined by performing a non-linear regression curve fit (One-Site Specific Binding) with GraphPad Prism (version 6).

**Flow cytometry**

Tp9-BoLA-A*0203:01 peptide–MHC class I Tets containing the Tp9Muguga peptides (purity: 95%; Mimotopes) were APC/FITC (cat #1070-02; Southern Biotech) at a dilution of 1:10. All animals were stained with Fixable Viability Stain 450 (cat # 562247; BD Horizon). Staining was done in PBS–0.5% BSA. Samples were analyzed by BD FACSCanto II flow cytometer, and data were analyzed with FlowJo (version 10). Compensation controls for PE, allophycocyanin, FITC, and Pacific Blue were included for automatic compensation by the FACSDiva software.

**TCR avidity assay**

The 4003 CTL line cells were incubated in PBS 1×0.5% BSA with 50 μM dastatin (cat # S1021; Selleckchem) for 30 min at 37°C to prevent TCR downregulation and intracellular recycling. After this, cells were incubated with 10 μM of Tp9-BoLA-A*0203:01-allophycocyanin Tets containing the Tp9Muguga, Tp9V4, or Tp9V7 peptides (purity: 95%; Mimotopes) and with 25 μM of anti-CD8 (IIA51) at dilutions of 1:250. Primary Abs were labeled with secondary anti-IgG1-FITC (cat # 1070-02; Southern Biotech) at a dilution of 1:500 (25 μL per sample). All samples were stained with Fixable Viability Stain 450 (cat # 562247; BD Horizon). Staining was done in PBS–0.5% BSA. Samples were analyzed on a BD FACSCanto II flow cytometer, and data were analyzed with FlowJo (version 10). Compensation controls for PE, allophycocyanin, FITC, and Pacific Blue were included for automatic compensation by the FACSDiva software. For the analysis of CTL lines, at least 20,000 events in the lymphocytes gate were acquired after gating for live and single cells. Tet+ cells were either gated as a CD8+Tet+ double-positive population or as a single population from the CD8+ lymphocytes population.

**Identification of Anchor Positions of the Tp9 CTL Epitope**

Folding of pMHC class I complexes was assessed with an ELISA assay described by Svitk et al. (34). The BoLA-A*0203:01 molecule (25 nM) and bovine β2m molecule (150 nM) in a 96-well plate with Tp9V7–5 (Muguga, G1 to A9, or V2 to V9) peptides (Mimotopes; purity: 95%) at various peptide concentrations ranging from 0 to 40 μM. After an incubation of 48 h at 18°C, the complexes were transferred to another 96-well plate precoated with streptavidin (catalog number [cat #] 436014; Nunc), which binds to a monomorphic epitope on β2m, but only when it is incorporated in pMHC class I complexes, was added for 1 h at 4°C. After washing, Tp9V7–5 (Muguga, G1 to A9, or V2 to V9) peptides (Mimotopes; purity: 95%) at various peptide concentrations ranging from 0 to 40 μM. After an incubation of 48 h at 18°C, the complexes were transferred to another 96-well plate precoated with streptavidin (catalog number [cat #] 436014; Nunc), which binds to a monomorphic epitope on β2m, but only when it is incorporated in pMHC class I complexes, was added for 1 h at 4°C. After washing, anti-mouse IgG1 coupled to peroxidase (cat # A9017-1ML; Sigma-Aldrich) was added to the plate for an hour at room temperature, and further rounds of washes. Colorimetric change was performed by adding the TMB Plus “Ready to Use” Substrate (cat # 4395H; Kern En Tec) for 10 min at room temperature. The reaction was stopped by adding H2SO4 (0.3 M), and OD was measured using a Synergy HT ELISA plate reader (Bio-Tek) at 450 nm. K values were determined by performing a non-linear regression curve fit (One-Site Specific Binding) with GraphPad Prism (version 6).

**IFN-γ ELISPOT**

IFN-γ ELISPOT assay was performed as previously described (34). In brief, a monoclonal anti-bovine IFN-γ Ab (cat # MCA7183; R&D Systems, St. Louis, MO, U.K.) was incubated overnight at 4°C on ELISPOT plates (cat # MAIPN4550; Millipore, Billerica, MA, USA) and then blocked with RPMI containing 10% heat-inactivated FCS for 2 h at 37°C. Peptides (purity: 95%; Mimotopes) were added at concentrations ranging from 0.1 to 1 μM and washed, the W6/32 mAb (cat # SC-32235; Santa Cruz Biotechnology, TX), which binds to a monomorphic epitope on β2m, but only when it is incorporated in pMHC class I complexes, was added for 1 h at 4°C. After washing, anti-mouse IgG1 coupled to peroxidase (cat # A9017-1ML; Sigma-Aldrich) was added to the plate for an hour at room temperature, and further rounds of washes. Colorimetric change was performed by adding the TMB Plus “Ready to Use” Substrate (cat # 4395H; Kern En Tec) for 10 min at room temperature. The reaction was stopped by adding H2SO4 (0.3 M), and OD was measured using a Synergy HT ELISA plate reader (Bio-Tek) at 450 nm. K values were determined by performing a non-linear regression curve fit (One-Site Specific Binding) with GraphPad Prism (version 6).

**Flow cytometry**

Tp9-BoLA-A*0203:01 peptide–MHC class I Tet containing the Tp9Muguga, Tp9V4, or Tp9V7 peptides (purity: 95%; Mimotopes) were APC/FITC (cat #1070-02; Southern Biotech) at a dilution of 1:10. All animals were stained with Fixable Viability Stain 450 (cat # 562247; BD Horizon) at a dilution of 1:250. Primary Abs were labeled with secondary anti-IgG1-FITC (cat # 1070-02; Southern Biotech) at a dilution of 1:500 (25 μL per sample). All samples were stained with Fixable Viability Stain 450 (cat # 562247; BD Horizon). Staining was done in PBS–0.5% BSA. Samples were analyzed on a BD FACSCanto II flow cytometer, and data were analyzed with FlowJo (version 10). Compensation controls for PE, allophycocyanin, FITC, and Pacific Blue were included for automatic compensation by the FACSDiva software. For the analysis of CTL lines, at least 20,000 events in the lymphocytes gate were acquired after gating for live and single cells. Tet+ cells were either gated as a CD8+Tet+ double-positive population or as a single population from the CD8+ lymphocytes population.
well, and cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells were collected every 15 min, washed twice in PBS 1X, and fixed in 1% paraformaldehyde. This competition assay was performed for up to 165 min (total of 12 time points). Samples were analyzed on a BD FACSCanto II flow cytometer, and data were analyzed with FlowJo (version 10). Compensation controls for PE, allophycocyanin, FITC, and Pacific Blue were included for automatic compensation by the FACSDiva software. For the analysis of CTL lines, at least 25,000 events (40,000 on average) in the lymphocytes gate were acquired after gating for live and single cells. Tetramer cells were gated as a single population derived from the CD8+ lymphocytes population. Half-life (minutes) of Tet binding was assessed by measuring the percentage (%) of Tets bound at time (t) × min in the allophycocyanin-positive gate only; binding at t 0 min was set at 100%, and % binding at x min was calculated relative to % bound at t 0 min. Half-life was then determined by a nonlinear curve fit (dissociation – one-phase exponential decay) with GraphPad Prism (version 6). Binding was calculated as follows: % of Tp967–75–Muguga–PE bound at t = 15 to 165 min in the PE-positive gate only minus the % of Tp967–75–Muguga–PE bound on cells in that same gate at t = 15 min to remove cells not stained with the first Tet that could have been stained with the second competing Tet added in excess. Then, the binding (half-binding time, in minutes) needed to achieve a half-maximum binding was determined by a nonlinear curve fit (one site-specific binding) with GraphPad Prism (version 6).

Statistical analyses

Statistical analyses of data were performed with GraphPad Prism version 6 software. To determine the Kd binding, and half-life values, we performed a nonlinear regression curve fit (One-Site, Specific binding). R2 values and confidence intervals were generated to assess curve fit and differences between Kd values. When comparing data generated with the ELISPOT assay with a single peptide concentration, we performed a one-way ANOVA with Dunnett’s correction for multiple comparison. When comparing data generated with the ELISPOT assay with varying peptide concentrations, we performed a two-way ANOVA with Dunnett’s correction for multiple comparison (main column effect). All datasets were compared with the data obtained with the Tp967–75 Muguga epitope. All experiments were repeated twice using duplicate measurements.

Results

Confirmation of the minimal Tp9 epitope for BoLA-1*023:01

In an earlier study, we had defined by ELISPOT and Tet staining a 10-mer peptide from the T. parva (Muguga) Tp9 Ag (67AKFPGMKKSK) that is recognized by a CTL line from animal 495 vaccinated by ITM that kills autologous schizont-infected cells. The animal was heterozygous for BoLA-A10/BoLA-A14 and expressed the BoLA-1*023:01 allele, among other alleles expressed in this cell line. The Tp9 10-mer peptide bound to the BoLA-1*023:01 allele, and peptide–MHC class I Tets stained this CTL (34). However, the BoLA-1*023:01 allele preferentially binds 9-mer and 8-mer peptides (41), and predictions from NetMHCip retrained on a more extensive set of peptide data predicted 67AKFPGMMKSK as a better peptide binder to BoLA-1*023:01 (data not shown).

We therefore decided to clarify experimentally a minimal size of the Tp9 epitope. The 10-mer and peptides with 1 or 2 aa deletions at the N- and C-terminal ends (Fig. 1A) were tested in a peptide-BoLA-1*023:01 class I molecule ELISA binding assay (Fig. 1B). An epitope from the Tp5 Ag, which was known to bind BoLA-1*023:01 class I molecule (34), was also included together with control epitopes (BoLA-T5control [KMFNRTLSTY] and BoLA-1*023:01control [WMYEGKHL]) not related to T. parva, but known to bind with high affinity to the BoLA-1*023:01 molecule. The binding assay clearly shows that deletion of 1 or 2 aa at the N-terminal end (Tp968–76 and Tp969–76) completely prevents peptide binding to this BoLA molecule because no folding can be measured (null OD in the ELISA), but that deletion of 1 or 2 aa at the C-terminal end does not (Tp967–74 and Tp967–75). These data establish the identity of the N-terminal end of the epitope, and that 10-mer, 9-mer, and 8-mer peptides can bind to BoLA-1*023:01. Similar results were obtained in IFN-γ ELISPOT using CTL 495 (Supplemental Fig. 1). An ELISPOT assay in fact confirmed that all three Tp9 peptides, which showed binding to the BoLA molecule (Tp967–76, Tp967–75, and Tp967–74), demonstrated the capacity to stimulate a Tp9-specific CTL line (495) to similar levels (Supplemental Fig. 1). The reason that the ELISPOT data in Supplemental Fig. 1 did not exclude the 8-mer peptide as the minimal epitope is most likely due to saturation reached in the assay because relatively high peptide concentration was used in that experiment. Because it was difficult to determine which was the authentic minimal epitope recognized by this cell line, a peptide–MHC class I staining was done using combinations of Tets harboring the different 8-, 9-, and 10-mer Tp9 peptides, which were positive in the IFN-γ ELISPOT assay. This assay undoubtedly demonstrated that the most dominant minimal Tp9 epitope was in fact Tp967–75 because the Tet generated with this epitope stained the majority of cells as a clearly distinct population, whereas cells stained with the other Tets were constituting only a very small fraction of this CTL line (Fig. 1C). Moreover, this exact dominant minimal 9-mer epitope sequence of the T. parva Muguga Tp9 Ag was independently confirmed by another group (sequence mentioned in Ref. 42).

Determination of Tp9 epitope anchor positions using alanine scanning and glycine-substituted Tp9 peptides in peptide binding assays with BoLA-1*023:01

A recent study demonstrated sequence diversity of the Tp9 Ag in buffal- and cattle-derived parasites (36, 43), including in the Tp9 Muguga epitope sequence restricted by BoLA-1*023:01. To identify the key amino acids in the Tp9 epitope important for binding to the BoLA-1*023:01 MHC class I molecule, we designed a series of peptides with alanine substitutions in the Tp967–75 Muguga epitope as performed with the Tp1 T. parva CTL epitope in a previous study (44), except for the amino acid at position 1, which was already alanine. This was replaced by a glycine instead. The ELISA-based peptide–BoLA class I binding assay was used to determine the binding affinities of the substituted peptides. Peptides with alanine at position 2 or 3 demonstrated a substantially lower binding affinity to BoLA-1*023:01 molecule as measured by a higher Kd value (192.2 and 86.2 nM, respectively). The other substituted peptides showed binding affinities similar to the Tp967–75 Muguga epitope: 13.40 nM for Tp9G1, 27.97 nM for Tp9A1, 23.04 nM for Tp9A2, 16.63 nM for Tp9A3, 10.08 nM for Tp9A4, 2.58 nM for Tp9A5, and 15.00 nM for Tp9A6 as compared with 13.93 nM for Tp967–75 Muguga (average between experiments) and 6.94 nM for the BoLA-1*023:01–positive binding control (Fig. 2). This shows that positions 2 and 3 are key anchor residues because these alanine substitutions render them as weak binders (Kd values of at least 100% increase as compared with the control Tp967–75 used in the assay and up to 500 nM are considered weak binders).

Determination of Tp9 epitope TCR contact residues using alanine scanning and glycine-substituted Tp9 peptides in IFN-γ ELISPOT assays

The next step was to identify the key amino acids in the Tp9 epitope that influence recognition by Tp9-specific CTLs originating from two different cattle immunized by ITM. As expected, the two peptides with alanine at positions 2 (Tp9A2) and 3 (Tp9A3) showed a decreased capacity to stimulate CTL 495 and CTL 4003 for the second peptide and CTL 4003 for the first peptide (Fig. 3A). Additional variations were observed between the two CTL lines in terms of their reactivity toward the different alanine-substituted peptides. This was the case for Tp9A6, which reacts to lower levels with CTL 4003 but reacted with CTL 495 to the same level as the Tp967–75 Muguga epitope. This experiment identified position 5, 7, and 8 to be important for TCR recognition by both Tp9-specific CTL lines and...
position 6 as important for CTL 4003 only (Fig. 3A). Because the cell line CTL 495 is heterozygous (A10/A14), it is theoretically possible that the observed difference for Tp9A2 and Tp9A6 between the two cell lines could be because of additional MHC alleles from the A10 haplotype of CTL 495 that bind the peptide. However, this was ruled out by using the Net MHC pan 4.1 version (https://services.healthtech.dtu.dk/service.php?NetMHCpan-4.1) to predict binding of the peptide for the A10 haplotype (BoLA-2*012:01 and BoLA-3*002:01).

To confirm this pattern, we generated peptide–MHC class I Tets using all peptides that could bind to the BoLA-1*023:01 class I molecule, to assess their binding capacity by flow cytometry (Fig. 3B). We used CTL 4003 for this evaluation and observed that the Tets containing Tp9A5 to Tp9A8 peptides were not able to stain the CD8+ CTL, confirming that each of the amino acids in these positions is required for TCR recognition by this CTL.

Determination of the ability of naturally occurring variants of the Tp9 epitope to bind BoLA-1*023:01

The results using the alanine-substituted peptides raise the question of how field variants of the Tp9 epitope (Fig. 4A) would perform in binding to BoLA-1*023:01. Using the ELISA-based MHC class I binding assay, it was observed that field variants V4 and V9 retain their binding affinities to this BoLA class I molecule because their Kd values are similar to the Tp9Muguga Kd value (25.00 and 33.68 nM, respectively, compared with 29.74 nM on average for Tp9Muguga in these assays). In the case of the field variants V3, V5, V6, V7, and V8, their binding affinity was substantially reduced, indicating that they can be considered as weak binders (46.52 nM for Tp9V3, 229.3 nM for Tp9V5, 72.14 nM for Tp9V6, 466.7 nM for Tp9V7, 262.9 nM for Tp9V8). On the other end of the spectrum, the Tp9 field variant V2 had an extremely higher Kd value, indicating a complete loss in binding affinity to BoLA-1*023:01 class I molecule (23,809.00 nM).

Determination of the ability of naturally occurring variants of the Tp9 epitope to stimulate Tp9-specific CTLs

To evaluate whether the CTL lines were able to cross-react with variants of the Tp9 epitope, we carried out an ELISPOT assay (Fig. 5).
Muguga Tet (Fig. 7A). The half-life rate for the Tp9V4 was closer, but not equal to, what was observed for the Tp967/C159 Muguga Tet. This translates to a faster binding rate for the Tp967/C159 Muguga Tet in the presence of the Tp9V7 Tet, and to a lesser extent in the presence of the Tp9v4 Tet, but in a much slower binding rate when Tp967/C159 competes with itself (Fig. 7B).

IFN-γ ELISPOT activity in the presence of varying concentrations of the Tp9 epitope and naturally occurring variants V4 and V7 of the epitope

To underpin the observed variation in binding kinetics between the Tet’s, we conducted an ELISPOT assay with the two Tp9-specific CTLs and a clone from CTL 495 (clone 8) by exposing them to Tp967/C159 Muguga, Tp9V4, and Tp9V7 at varying concentrations. CTL 4003 was stimulated equally well by Tp967/C159 Muguga and Tp9V4 epitopes (Fig. 8A), whereas CTL 495 showed a higher sensitivity to the Tp967/C159 Muguga epitope compared with Tp9V4, for which the response was slightly decreased (Fig. 8B). However, using a clone of CTL 495, a much higher sensitivity was observed toward the Tp9V4 epitope as compared with the Tp967/C159 Muguga epitope, which was the original epitope, used for generation of this CTL line (Fig. 8C). In all assays using the two CTL lines and the clone, cells showed very little reactivity to Tp9V7, except for CTL 4003 at higher peptide concentrations (Fig. 8), confirming our observation using the TCR avidity assay. These observations indicate that the V4 and V7 variants are the only ones, among the variants tested, to have
the capacity to bind to BoLA-1*023:01 and to stimulate Tp9MUGUGA-specific CTLs (Fig. 9).

Discussion
The development of an effective subunit vaccine against *T. parva* will require a thorough understanding of the protective cellular immune response to the parasite and identification of the correct panel of Ags that can provide broad protection against different field strains. A detailed characterization of the ability of *T. parva*-specific cytotoxic CD8+ T lymphocytes to recognize variants of these Ags from parasite strains in different endemic areas is needed to achieve this. This study was undertaken to investigate the ability of Tp9 epitope–specific CTLs restricted by BoLA-1*023:01 to cross-react with antigenic variants from other parasite strains.
Previous studies have demonstrated that partial protection can be achieved against challenge with a homologous strain of the parasite (33). Furthermore, early studies showed some level of interstrain cross-protection, and more recent studies indicated that immunizing cattle with the Muguga strain of *T. parva* resulted in substantial cross-reactivity of the CTLs with the parasite strains included in the Muguga cocktail (Serengeti-transformed and Kiambu 5) and with other selected strains (45). This indicated that there are epitopes in *T. parva* leading to CTL cross-reaction. Nonetheless, recent studies have shown that differential specificities toward CTL Ags can emerge from immunization with the Muguga cocktail ITM vaccine, even in identical MHC background (46), and breakthroughs have been observed in the field with cattle immunized by ITM in a buffalo-endemic area (47). Moreover, partial cross-reaction of a Tp2 CTL line toward certain natural variants of the Tp249/C159 *T. parva* CTL epitope has been demonstrated in vitro (48). However, the majority of these variants were escape mutants. An immune response toward Tp9 in ITM-immunized cattle has been demonstrated (49), and its equivalent in *T. annulata*, Ta9, has been shown to be an immunodominant Ag for the CTL response in BoLA-A14 cattle (50, 51). Remarkably, the CTL response in such cattle is directed to a single epitope on Tp9, which is restricted by the BoLA-1*023:01 allele and is one of the most prevalent BoLA
However, the *T. parva* Tp9 Ag and Tp9 epitope have recently been shown to be polymorphic in field strains, which could potentially reduce the efficacy of Tp9 in the design of subunit vaccines. We found that CTLs with specificity to Tp9Muguga recognized only two of the eight-field variant Tp9 epitopes, suggesting that this would in fact be problematic if Tp9 is used as a CD8+ T cell antigen in the context of the BoLA-1*023:01 class I molecule. The cross-reactivity in our study is done from the perspective of the *T. parva* Muguga strain, which is a limitation. However, it is the only strain for which CTL epitopes have been discovered, and it is likely that only the strains behind Tp9V4 and Tp9V7 could elicit CTL to the same epitope from *T. parva*. Because we did not have these strains isolated and produced as stabilates for immunization, this was not possible to investigate. However, this Ag may still be used as a promising cellular immune Ag because it induces CD4+ T cell responses in several MHC class II haplotype backgrounds (49, 52). Alternatively, several identified *T. parva* CTL Ags, such as Tp5, which are not polymorphic or show very little polymorphism, could be combined with Tp9 to induce broad CD4+ and CD8+ protective T cell responses.

Binding of peptides to MHC class I molecules is a selective process in the presentation of epitopes to T lymphocytes. Insights into the mechanisms that influence peptide binding to MHC class I molecules can provide a better understanding of factors important for triggering an optimal cellular immune response and of the initial events that govern T cell function. In this study, positions 2 and 3 in the Tp9 epitope were identified as anchor positions from the alanine scanning experiments. Interestingly, the reduction in binding of the peptides harboring an alanine substitution in positions 2 and 3 is consistent with the elution data obtained from a recent mass spectrometry study of peptides eluted from BoLA molecules of a A14-haplotype TpM line, which indicated that these two positions are favored by BoLA-1*023:01 (41). Moreover, even though position 9 is of importance for BoLA-1*023:01, based on the published mass spectrometry data (41), the serine at this position in the Tp9 epitope is not among the amino acids that are preferred by BoLA-1*023:01, which include leucine (L), phenylalanine (F), tyrosine (Y), and methionine (M) (Supplemental Fig. 3A). Therefore, the data obtained with the alanine substitution at position 9 of Tp9 is again consistent with the elution data. Moreover, replacing the serine with an alanine at position 9, which should have a slight detrimental effect on binding of the epitope according to the elution data, does not affect binding of Tp967/C15975 and confirms that this position is not required in the context of the Tp9Muguga epitope. In addition, a previous study was performed to determine the anchor residues in 9-mer peptides binding to BoLA-1*023:01 using positional scanning combinatorial peptide libraries (PSCPLs) (53). The PSCPLs found a binding motif with the anchor position 9 as the most preferred by this MHC class I molecule, with methionine (M), phenylalanine (F), tyrosine (Y), and histidine (H) or leucine (L) being again the amino acids of choice at this position (Supplemental Fig. 3B). According to this study, the binding motif of BoLA-1*023:01 had other anchor positions of lesser importance, which appeared to be present at
positions 1, 2, and 6. Interestingly, the two residues of Tp9 that were required for binding to BoLA-1*023:01 based on the alanine scanning were K and F at positions 2 and 3 (Fig. 9A). Corroborating this observation, in the mass spectrometry study, a lysine has been shown to be highly prevalent at position 2 of 9-mer peptides (mostly self-peptides) eluted and analyzed by mass spectrometry from BoLA molecules of a A14-haplotype TpM line (41). Most of the field variants had reduced binding affinities for BoLA-1*023:01, even though they had the K and F intact at positions 2 and 3, indicating that other positions influenced the binding. However, variant 2 (Tp9V2) completely lost the binding capacity (23,809.00 nM) (Fig. 9B). The reduction in binding of field variants is most probably due to the presence of several amino acid substitutions that may cause overall conformational changes of the peptide and possibly distort the orientation of the lateral chains of the amino acids at key anchor positions. This could be the result of polar amino acids that contain aromatic rings, such as the histidine, or positively charged amino acids with long lateral chains, such as the arginine or the tyrosine, at positions 4 and 5, which are neighboring the anchor positions 2 and 3, indicating that other positions influenced the binding. Furthermore, the difference in binding between variants 4 (25.00 nM) and 7 (466.7 nM) is most probably due to the unique difference between the two variants at position 1, where in the former the alanine is intact as compared with Tp9Muguga and in the latter the alanine is substituted with an asparagine. This seems to have an impact in the binding of variant 7 because it reduces the binding affinity to the BoLA molecule to similar levels as measured with the Tp9A2 and Tp9A3 peptides and the TCR avidity of the peptide–BoLA complex. This indicates that the asparagine at this position has a detrimental effect on the binding of this variant. This is in agreement with data generated by the PSCPL experiments using random 9-mer peptides, which indicated that the asparagine in position 1 had a detrimental effect on peptide binding in the peptide binding groove of the BoLA-1*023:01 molecule. This suggests that position 1 of this Tp9 epitope also plays a role in binding and can be considered an anchor position in the context of the Tp9Muguga epitope (Fig. 9A).

Recent reports have shown a better correlation of peptide–MHC class I stability assay with the antigenic quality of an epitope (54), which measures the rate at which the MHC class I molecule unfolds in the presence of an immunodominant epitope, compared with the binding assay that measures the lowest concentration needed to achieve proper folding of the MHC class I molecule on binding by an immunodominant epitope. The stability assay could therefore be included in future studies to characterize epitope binding. However, the requirement of using radioactive isotopes impairs the accessibility and practicality of these assays.

In the case of the TCR recognition of the Tp9 epitope, the amino acids at positions 5–8 (GMKK) all seem to be important because only variants 4 and 7, which have a GMKK sequence intact in this region, retain their capacity to stimulate Tp9Muguga*
specific CTL lines (Fig. 9B). An interesting observation was that some alanine-substituted Tp9_Muguga peptides had an increased binding affinity to the BoLA molecule. This resulted in an increased reactivity by the Tp9-specific CTL lines toward these mutant peptides. For instance, the Tp9G1 and Tp9A9 peptides had a higher binding affinity to BoLA-1*023:01 class I molecule, which resulted in an enhanced stimulation of CTL 4003 and 495 in the ELISPOT assay (one-way ANOVA with Dunnett’s correction for multiple comparison, p ≤ 0.01 and p ≤ 0.001, respectively). Some studies have shown the impact of replacing amino acids at key positions in a T cell epitope for the improvement of MHC class I stability, kinetics, and immunogenicity (55, 56). This observation indicates that epitope or Ag engineering of the Tp9 epitope region could potentially lead to a stronger Ag and potentially increase cross-reactivity with other field strains. The difference in response between the CTL lines toward the different Tp9 alanine mutant peptides and field variants is not an unusual phenomenon. A previous study from our group had observed that CTLs from cattle expressing the same BoLA class I molecules react differently toward the same T. parva strain (46). These differences can be caused by several factors, including differences in TCR repertoire, single-nucleotide polymorphism in innate or adaptive immunity genes, or difference in epigenetic factors.

It is largely accepted that a stronger TCR avidity correlates with a better recognition of infected cells (57, 58), and the relationship between avidity and TCR functionality has been the
subject of intense research to improve T cell–based immunotherapy against cancer (59). Furthermore, the TCR of memory Th1 cells has a reduced requirement for the involvement of stimulation by costimulatory molecules because they become more sensitive on engagement of their TCR by an infected cell (60), suggesting a direct correlation of TCR avidity with a rapid immune response. Moreover, a stronger TCR affinity, which measures the strength of the interaction between a single TCR and a peptide–MHC molecule and contributes to the overall avidity of several TCRs toward p-MHC complexes during the formation of an immunological synapse, has been correlated with the polyfunctionality of CD8+ T cells (61) and therefore with a better functional response to pathogens. A stronger TCR affinity has also been correlated with a swifter response to an infected cell, while a lower-affinity TCR–antigen interaction requires more time to elicit the same response (62). The high sensitivity of a TCR molecule is of particular value for the detection of low-intensity Ags, such as those derived from intracellular pathogens, which can downregulate p-MHC complexes on the surface of infected cells. However, above a specific TCR–p-MHC threshold, T cell function cannot be enhanced (57), and in some cases, it can be detrimental to T cell functionality (63). The ability to measure TCR avidity can help in selecting the best epitope, and several assays have been developed to measure the avidity of TCRs toward their epitopes, such as Tet dilution assay (64), the use of MHC class I–specific Abs that compete with Tet binding (65), or the use of functional cellular assays such as ELISPOT or cytotoxicity assays. In this study, we developed and optimized an assay to measure both half-life and binding rates of peptide MHC class I to TCR. One of the advantages of the assay is that inhibitory mAbs are not needed. All that is needed is the same Tet generated with two different fluorochromes.

In summary, we have identified key amino acids that are required for BoLA class I and TCR recognition by the polymorphic Tp967–75 epitope. To our knowledge, this is the first report

FIGURE 8. Reactogenicity determination of Tp9-specific CTL lines toward different concentration of Tp9 field variant epitopes. (A) ELISPOT assay with CTL 4003. (B) ELISPOT assay with CTL 495. (C) ELISPOT assay with a clone (clone 8) of CTL 495 with peptides ranging from 0.1 to 1000 nM. Mean and SD of a representative experiment are shown. **p > 0.05, ***p ≤ 0.01, ****p ≤ 0.0001. The experiment was repeated twice with duplicate measurements.
to study the impact of Tp9 polymorphism on binding to BoLA class I molecules and recognition by a Tp9-Muguga-specific T cell line and stress the importance of understanding the impact of CTL Ag polymorphism in the immunobiology of T. parva. This study also describes an easy-to-use TCR avidity assay for the characterization of the cross-reactivity by T cells toward variant epitopes from T. parva field strains. These results suggest that Tp9 alone would not be sufficient for covering BoLA A14–positive cattle in a subunit vaccine because of polymorphisms, and that other Ags would be needed to provide broad-spectrum immunity toward T. parva strains circulating in the region.

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Disclosures

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References

1. Wong, P., and E. G. Pamer. 2003. CD8 T cell responses to infectious pathogens. Annu. Rev. Immunol. 21: 29–70.
2. Alcover, A., B. Alarcón, and V. Di Bartolo. 2018. Cell biology of T cell receptor expression and regulation. Annu. Rev. Immunol. 36: 103–125.
3. La Gruta, N. L., S. Gras, S. R. Daley, P. G. Thomas, and J. Rossjohn. 2018. Understanding the drivers of MHC restriction of T cell receptors. Nat. Rev. Immunol. 18: 467–478.
4. Zhang, W., A. C. Young, S. I. Marai, S. G. Nathenson, and J. C. Sacchettini. 1996. Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. Proc. Natl. Acad. Sci. USA 89: 8403–8407.
5. Rammensee, H. G. 1995. Chemistry of peptides associated with MHC class I and class II molecules. Curr. Opin. Immunol. 7: 85–96.
6. Busch, E., K. D. Kubon, J. K. M. Mayer, G. Pidelaserra-Marti, J. Albert, B. Hoyler, J. P. W. Heidbuchel, K. B. Stephenson, B. D. Lighty, W. Owen, et al. 2020. Mea-
sles vaccines designed for enhanced CD8+ T cell activation. Viruses 12: 242.
7. Lin, W. H., C. H. Pan, R. J. Adams, B. L. Laube, and D. E. Griffin. 2014. Vac-
cine-induced measles virus-specific T cells do not prevent infection or disease but facilitate subsequent clearance of viral RNA. mBio 5: e01047.
8. Bassi, M. R., M. Kongsgaard, M. A. Steffensen, C. Fenger, M. Rasmussen, K. Skjodt, B. Finsen, A. Stryhn, S. Buus, J. P. Christensen, and A. R. Thomsen. 2015. CD8+ T cells complement antibodies in protecting against yellow fever virus. J. Immunol. 194: 1141–1153.
9. Fuertes Marraco, S. A., C. Sonesson, M. Delorenzi, and D. E. Speiser. 2015. Genome-wide RNA profiling of long-lasting stem cell–like memory CD8 T cells induced by Yellow Fever Vaccination. Sci. Rep. 5: 10903.
10. Hodgson, S., K. Moffat, H. Hill, J. T. Flannery, S. P. Graham, M. D. Baron, and K. E. Darpel. 2018. Comparison of the immunogenecities and cross-lineage effi-
cacies of live attenuated pestes des petits ruminants virus vaccines PPRV/Nigeria/ 75/1 and PPRV/Sungri/96. J. Vet. 92: e01146.
11. Mohn, K. G., K. A. Brokstad, S. Islam, F. Oftung, C. Tøndel, H. J. Aarstad, and R. C. Cox. 2020. Early induction of cross-reactive CD8+ T-cell responses in tons-
ils after live-attenuated influenza vaccination in children. J. Infect. Dis. 221: 1526–1537.
12. Kaganavelu, S., J. M. Termini, S. Gupta, F. N. Raffa, K. A. Fulller, Y. Rivas, S. Philip, R. Kornbluth, and G. W. Stone. 2014. HIV-1 adeno viral vector vac-
cines expressing multi-meric BAFF and 4-1BBL enhance T cell mediated anti-
 viral immunity. PLoS One 9: e90100.
13. Jaworski, J. P., S. J. Krebs, M. Trivoto, D. N. Kovalar, Z. Brower, W. F. Sutton, G. Waagmeester, R. Sartorius, L. D’Apice, A. Caivano, et al. 2012. Co-immuni-
ization with multimeric scaffolds and DNA rapidly induces potent autologous HIV-1 neutralizing antibodies and CD8+ T cells. PLoS One 7: e31469.
14. Ura, T., A. Yoshida, K. X. Qin, S. Yoshizaki, S. Yashima, S. Abe, H. Mizuguchi, and K. Okuda. 2009. Designed recombinant adenovirus type 5 vector induced en
dlve-speciﬁc CD8(+) cytotkiotic T lymphocytes and cross-reactive neutraliz-
ing antibodies against human immunodeficiency virus type 1. J. Gene Med. 11: 139–149.
15. Ewer, K., J. G. A. O’Hara, C. J. Duncan, K. A. Collins, S. H. Sheehy, A. Reyes-Sandoval, A. L. Goodman, N. J. Edwards, S. C. Elias, F. D. Halstead, et al. 2013. Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. Nat. Commun. 4: 2836.
16. Pearson, F. E., C. O’Mahony, A. C. Moore, and A. V. Hill. 2015. Induction of CD8+ T cell responses and protective efficacy following microneedle-mediated delivery of a live adenovirus-vectorised malaria vaccine. Vaccine 33: 3248–3255.
17. Sedegah, M., M. R. Hollingdale, F. Farooq, H. Ganesan, M. Belmonte, Y. Kim, B. Peters, A. Sette, J. Huang, M. D. Baron, and S. P. Graham. 2014. Transcriptomic analysis reveals a previously unknown role for CD8+ memory CD8+ T cells targeting AMA1 class I epitopes. Infect. Immun. 82: 2947–2955.
18. Menescuuri, A. R., S. Sureshchandra, A. Mazzi, H. Feldmann, and I. Messaoudi. 2017. Transcriptomic analysis reveals a previously unknown role for CD8+ T-
cells in VSVV-EBOV mediated protection. Sci. Rep. 7: 919.
19. Feng, Y., C. Li, P. Hu, Q. Wang, X. Zheng, Y. Zhao, Y. Shi, S. Yang, C. Yi, Y. Feng, et al. 2018. An adenovirus serotype 2 vectored ebolavirus vaccine gener-
ates robust antibody and cell-mediated immune responses in mice and rhesus macaques. Emerg. Microbes Infect. 7: 101.
20. Kobayashi, S., S. Ohno, T. Sada, M. Taniechi, S. Yokoyama, M. Morii, A. Kobayashi, H. Hayashi, T. Uchida, and M. Matsui. 2008. Efficient induction of cytotoxic-T lym-
phocytes specific for severe acute respiratory syndrome (SARS)-associated corona-
Virus by immunization with surface-linked liposomal peptides derived from a non-
structural polyprotein 1a. Antiviral Res. 84: 168–177.
21. Bodmer, B. S., A. H. Fiedler, J. R. H. Hansauer, S. Prüfer, and M. D. Mühlebach. 2018. Live-attenuated bivalent measles virus-derived vaccines targeting Middle East respiratory syndrome coronavirus induce robust and multifunctional T cell responses against both viruses in an appropriate mouse model. Virology 521: 99–107.
22. Channappanavar, R., C. Fet, J. Zhao, D. K. Meyerholz, and S. Perlman. 2014. Virus-specific memory CD8 T cells provide substantial protection from lethal

FIGURE 9. Binding affinity and TCR recognition of Tp9 in relation to cross-protection. (A) Summary schematic presentation of anchor positions and TCR recognition sites on the Tp967–75 Muguga epitope. The green color indicates the amino acids in the Tp9Muguga epitope important for binding to the BoLA-1*023101 molecule, and the blue color indicates the amino acids important for TCR recognition. (B) Summary of BoLA-1*023103 binding and Tp9-specific TCR recognition by the field var-
iant of Tp9.
