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Breadth of the CD4⁺ T-cell response to *Anaplasma marginale* VirB9-1, VirB9-2, and VirB10 and MHC class II DR and DQ restriction elements

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Abstract

MHC class II molecules influence antigen-specific CD4⁺ T-lymphocyte responses primed by immunization and infection. CD4⁺ T-cell responses are important for controlling infection by many bacterial pathogens including *Anaplasma marginale*, and are observed in cattle immunized with the protective *A. marginale* outer membrane (OM) vaccine. Immunogenic proteins that comprise the protective OM vaccine include type IV secretion system (T4SS) proteins VirB9-1, VirB9-2, and VirB10, candidates for inclusion in a multi-epitope vaccine. Our goal was to determine the breadth of the VirB9-1, VirB9-2, and VirB10 T-cell response and MHC class II restriction elements in six cattle with different MHC class II haplotypes, defined by *DRB3*, *DQA*, and *DQB* allele combinations for each animal. Overlapping peptides spanning each T4SS protein were tested in T-cell proliferation assays with autologous antigen presenting cells (APC) and artificial APC expressing combinations of bovine DR and DQ molecules. Twenty immunostimulatory peptides were identified; three representing two or more epitopes in VirB9-1, ten representing eight or more epitopes in VirB9-2, and seven representing seven or more epitopes in VirB10. Of eight DRA/DRB3 molecules, four presented 15 peptides, which was biased as DRA/DRB3*1201 presented ten and DRA/DRB3*1101 presented four peptides. Four DQA/DQB molecules composed of two intrahaplotype and two interhaplotype pairs presented seven peptides, of which five were uniquely presented by DQ molecules. In addition, three functional mixed isotype (DQA/DRB3) restriction elements were identified. The immunogenicity and broad MHC class II presentation of multiple VirB9-1, VirB9-2, and VirB10 peptide epitopes justify their testing as a multi-epitope vaccine against *A. marginale*.

Introduction

Cell to cell interactions of the adaptive immune response are critically important for protection from pathogens. These interactions are orchestrated by the immunological synapse whose primary components are the T-cell receptor (TCR) on CD4⁺ T helper cells and major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APC). The primary role of MHC class II molecules is to display peptides from exogenous proteins on the surface of the APC so they are available to interact with the TCR and initiate an antigen-specific CD4⁺ T-cell response (Germain 1986; Yewdell and Bennink 1990). MHC class II molecules comprise a set of peptide binding proteins of varying specificities

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(Unanue et al. 1989). In cattle, this diversity largely reflects the extensive allelic polymorphism used by the immune system to increase the antigen epitope-binding repertoire (Ellis and Ballingall 1999; Lewin et al. 1999; Park et al. 2004).

Cattle express two MHC class II proteins (DR and DQ), yet approximately one-half of haplotypes also have duplicated DQ regions (Glass et al. 2000; Norimine and Brown 2005). Mice express two class II proteins (H2-A and H2-E), and humans express three (HLA-DR, HLA-DQ, and HLA-DP). Genetic diversity of cattle is permitted through polymorphisms in *DRB3*, *DQA*, and *DQB* genes as well as through pairing of gene products, which can occur for any combination of α - and β -chains. In cattle, antigenic peptides are classically presented by monomorphic DRA paired with polymorphic DRB molecules to T cells (Brown et al. 2002; Glass et al. 2000; Norimine and Brown 2005; Norimine et al. 2006). However, antigenic peptides are also presented by intra- or interhaplotype pairs of DQA and DQB molecules (Brown et al. 2002; Glass et al. 2000; Moreno et al. 1990; Norimine and Brown 2005; Silk et al. 2005). Intrahaplotype pairing refers to the combination of *DQA* and *DQB* gene products encoded by alleles on the same chromosome (within the same haplotype). Interhaplotype pairing refers to the combination of *DQA* and *DQB* gene products encoded by alleles on different chromosomes (between different haplotypes). It has also been noted that isotype-mismatched combinations of *DQ* gene products with *DR* gene products, denoted mixed isotype, can be expressed with human HLA-DR and -DQ (Germain and Quill 1986; Lotteau et al. 1987) and murine H2-A and -E (Sant et al. 1987; Spencer et al. 1993) proteins. However, this has not been shown for cattle or any other species. If some alleles are more active than others for antigen presentation, protective immune responses against a bovine pathogen such as *Anaplasma marginale* will be strongly influenced by certain MHC class II alleles, and their characterization is therefore important for effective vaccine development.

The tick-borne pathogen *A. marginale* causes a persistent infection of cattle characterized by acute and chronic high-load bacteremia. Control measures against anaplasmosis are largely inadequate and the lack of a safe and effective vaccine results in large economic losses (Palmer et al. 2000). Protective immunity against disease is achieved by vaccination with a live, attenuated *A. centrale* vaccine, but this carries the risk of transmitting other blood-borne pathogens, and is not licensed for use in the United States. Protection against disease and, in some cases, infection can be achieved by immunization with purified *A. marginale* outer membranes (OM) (Tebele et al. 1991; Brown et al. 1998). However, the use of purified OM as a commercial vaccine is cost-prohibitive, so that identification of immunogenic and potentially protective proteins within the OM has been a focus of our research (Lopez et al. 2005; 2008). CD4⁺ T lymphocytes are important for inducing protective immune responses following immunization with OM (Brown et al. 1998). Effective vaccines against *A. marginale* will therefore likely require the inclusion of multiple conserved proteins or T-lymphocyte epitopes derived from these, because immunodominant, antigenically variant surface proteins such as major surface protein (MSP)2 have failed to elicit protective immunity (Abbott et al. 2005; Noh and Brown in press). Because MHC class II molecules influence antigen-specific CD4⁺ T-lymphocyte responses, characterization of MHC class II molecules and the identification of immunogenic T-cell epitopes are also important considerations for vaccine development.

Immunogenic proteins comprising the protective OM include the subdominant and conserved type IV secretion system (T4SS) proteins. The T4SS is a membrane protein complex of many bacterial pathogens, which secretes virulence factors and promotes host cell invasion and intracellular survival (Backert and Meyer 2006). It was previously shown that *A. marginale* T4SS proteins VirB9-1, VirB9-2, and VirB10 are strongly immunogenic for CD4⁺ T lymphocytes from *A. marginale* OM-immunized cattle and that these proteins

are highly conserved across *A. marginale* strains and *A. centrale* (Lopez et al. 2007; Morse et al. 2012; Suttén et al. 2010). However, in a recent study, cattle 35160, 35280, and 35287, which are half-matched with respect to *DRB3*1501*, had differential responses to each protein (Morse et al. 2012). To understand the differences in T-cell responses observed for these animals to VirB9-1, VirB9-2, and VirB10 we sought to identify MHC class II-allele specific T-cell epitopes. Determining the breadth of the response to T-cell epitopes on VirB9-1, VirB9-2, and VirB10 in outbred cattle is important to develop a multiple-antigen and peptide-based vaccine construct.

To gain further insight into the antigenic structure of T4SS proteins VirB9-1, VirB9-2, and VirB10, we have investigated the proliferative responses of CD4⁺ T lymphocytes from MHC class II-defined cattle to a series of overlapping peptides spanning the length of each of these proteins. In addition, bovine *cd80* and MHC class II *DR*- and *DQ*-transfected human embryonic kidney 293-F cells were used as artificial APC to determine MHC class II restriction elements. Our study highlights the importance of MHC class II DQ in addition to DR molecules in presenting pathogen-derived peptides to CD4⁺ T cells.

Materials and Methods

Antigens and synthetic peptides

Full-length protein sequences for VirB9-1, VirB9-2, and VirB10 of the St. Maries strain of *A. marginale* were used to synthesize peptides generally 30 amino acids in length and overlapping by ten amino acids. This yielded 13 peptides for VirB9-1, 14 peptides for VirB9-2, and 22 peptides for VirB10 (Table 1). The 30-mer VirB10 peptide 2 (P2) was difficult to synthesize, and was divided into two 20-mer peptides with 10 overlapping amino acids and re-named VirB10 P2.1 and P2.2, and an additional peptide representing the overlapping VirB10 P13 and P14 was also synthesized and designated P13-14. VirB9-1 P8-P13 and VirB10 P13-14 were synthesized and purified by Gerhard Munske, Laboratory for Biotechnology and Bioanalysis Unit 1, Washington State University (WSU). The remaining peptides were synthesized and purified by NeoPeptide (Cambridge, MA). All peptides were manufactured with 75% purity. Peptides were solubilized in 10% DMSO in PBS and diluted to a stock concentration of 1 mg/ml.

Cattle

Five age-matched steers and one cow (animal numbers: 35113, 35141, 35160, 35280, 35287, and 583) with varying and heterozygous MHC class II molecules were purchased from local dairies. Bovine lymphocyte antigen (BoLA) MHC class II DRB3 types were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method described previously (Morse et al. 2012; van Eijk et al. 1992) and *DQA1*, *DQA2*, *DQB1*, and *DQB2* alleles were determined by sequencing entire cDNAs using previously described primer sets (Norimine and Brown 2005; Park et al. 2004). The class II haplotypes of all cattle are shown in Table 2. Cattle were immunized with *A. marginale* OM as previously described (Morse et al. 2012).

Two week T-cell lines used for proliferation assays

Peripheral blood mononuclear cells (PBMC) from six *A. marginale* OM-immunized cattle were collected and depleted of CD8⁺ cells and $\gamma\delta$ T cells using monoclonal antibodies (mAb) and complement lysis (Morse et al. 2012). Two-week cell lines from CD4⁺ T cell-enriched PBMC were stimulated with *A. marginale* OM as described (Morse et al. 2012). Proliferation assays were performed with 3×10^4 T cells and 2×10^5 irradiated autologous PBMC, as a source of APC, per well in complete RPMI-1640 medium (cRPMI, Brown et al. 1991) for 3-4 days. Positive control antigens included *A. marginale* OM and full-length

recombinant VirB9-1, VirB9-2, and VirB10, expressed as described (Morse et al. 2012). These were used at a final concentration of 1 µg/ml and peptides were used at 0.1-20 µg/ml. The N-terminal peptide P1 from *B. bovis* rophry associated protein 1 (Rap1 P1) was used as a negative control (Norimine et al. 2002). Lymphocytes were radiolabeled during the last 18 h of culture with ³H-thymidine and the results are reported as stimulation index (SI). The SI was calculated by dividing the mean counts per minute (CPM) of replicate cultures with a test antigen by the mean cpm of cells cultured with medium. The SI for the different peptides were compared to the SI for Rap1 P1 using a one-way ANOVA corrected for multiple comparisons with the Dunnett's test. Statistically significant T-cell stimulation by an antigen was set at $P < 0.05$ and $SI > 3$.

Expression of BoLA-class II and CD80 molecules on artificial APC

Amplification of full-length cDNAs encoding sequences for DRA, DRB3, DQA, DQB, and CD80 was performed by PCR and each PCR product was cloned into the eukaryotic expression vector pCR3.1 (Invitrogen) as previously described (Norimine and Brown 2005). A mixture of 1 µg of each plasmid DNA encoding CD80, one class II α chain, and one class II β-chain, and 200 µl OptiMem (Gibco) plus 6 µl GeneJuice (Novagen) was incubated at room temperature for 10-15 min and then added to human embryonic kidney 293-F cells (Invitrogen) with 90% confluence on a six-well plate. Transfected 293-F cells were incubated and cultured for 2 days in a 37°C incubator as described (Norimine and Brown, 2005). Medium was removed and 50 µg/ml mitomycin C (Sigma-Aldrich) in cRPMI was added to each well and incubated for 2-2.5 h. The transfected cells were then harvested with HBSS containing 2 mM EDTA, washed, and used as APC with the OM-specific two week T-cell cell lines in proliferation assays.

Expression of BoLA-class II and CD80 molecules on transfected 293-F cells was verified by flow cytometry using 15 µg/ml bovine DR-specific mAb TH14B (IgG2a), DQ-specific mAb CC158 (IgG2a), and CD80 specific mAb IL-A159 (IgG1). TH14B was purchased from the WSU mAb center and mAbs CC158 and IL-A159 were kindly provided by the Institute of Animal Health, Compton, UK (Norimine and Brown 2005). For the secondary antibodies, R-phycoerythrin (R-PE)-conjugated goat anti-mouse IgG2a and R-PE-conjugated goat anti-mouse IgG1 (Caltag Laboratories) were used at a 1:200 dilution.

T-cell proliferation assays with transfected 293-F cells as APC

To determine DR and DQ molecules that present immunogenic peptides, transfected 293-F cells treated with mitomycin C were plated in 96-well round-bottomed plates at 5×10^4 cells/well, loaded with 20 µg/ml of an individual peptide in triplicate wells, incubated at 37°C for 1 h, washed three times, and used as APC with T cells from two week cell lines. Proliferation was measured after 3-4 days as previously described (Norimine and Brown 2005). For each peptide, T-cell proliferation to a peptide presented by 293-F cells transfected with CD80 and a pair of class II α and β chains was compared to proliferation using non-transfected 293-F cells, and significance was evaluated by the Student's one-tailed t-test where $P < 0.05$.

Results

Breadth of the CD4⁺ T-cell response to VirB9-1, VirB9-2, and VirB10 peptides

Holstein cattle expressing eight different *DRB3* alleles were used to determine the response to VirB9-1, VirB9-2, and VirB10 peptides. The *DRB3* RFLP haplotypes and corresponding alleles (in parentheses) 3 (*1001), 8 (*1201), 11 (*0902), 16 (*1501), 22 (*1101), 23 (*2703), 24 (*0101), and 27 (*1401) are common among Holstein cattle. Of 878 Holstein calves from Washington and Vermont genotyped for *DRB3* alleles in the past 10 years, the

combined frequencies of these 8 alleles was 90% (see Online Resource 1). To assess the position and minimal number of T-cell epitopes, 30-mer peptides overlapping by 10 amino acids and spanning the complete sequences of VirB9-1, VirB9-2, and VirB10 were synthesized (Table 1). The peptides were tested for T-cell stimulation using OM-specific T-cell lines derived from animals 35113, 35141, 35160, 35280, 35287, and 583 with varying MHC class II haplotypes, specified in Table 2. VirB9-1 peptide-specific responses were detected with T-cell lines derived from animals 35113, 35141, 35280, and 35287, but not with T-cell lines from animals 35160 and 583, as the latter two cattle do not respond to full-length VirB9-1 (Fig. 1a). Animal 35113 (RFLP type 11/22) had significant T-cell responses against peptides VirB9-1 P5, P6, and P10. Animal 35287 (16/22) also responded to VirB9-1 P5, P6, and P10, although the response to P5 was not significant using a multiple comparison correction. T cells from animals 35141 (22/24) and 35280 (16/27) also recognized VirB9-1 P6 and P10.

All cattle except 35160 (3/16), which did not respond to full-length VirB9-2, responded to one or more VirB9-2 peptides (Fig. 1b). VirB9-2 P5 was recognized by T cells from animals 35113 (11/22), 35141 (22/24), 35280 (16/27), and 35287 (16/22). Animal 35113 (11/22) also responded to VirB9-2 P11 and P12. Animal 35287 (16/22) also recognized VirB9-2 P8. Animal 583 (8/23) recognized eight peptides spanning VirB9-2, which were P2, P3, P4, P9, P10, P11, P12, and P13.

All cattle except 35141 responded to VirB10 peptides (Fig. 1c). Animal 35141 weakly responded to recombinant VirB10, however no responses to the peptides were observed. VirB10 P21 was recognized by T cells from cattle 35113 (11/22), 35280 (16/27), and 35287 (16/22). It was previously reported that animal 35280 did not recognize recombinant VirB10 (Morse et al. 2012), but the response to VirB10 is inconsistent for this animal, as we found significant proliferation to VirB10 in two of six assays (Fig. 1c and data not shown). In one of those assays where the response was positive, significant T-cell proliferation to VirB10 P21 by this animal was found. Animal 35287 (16/22) also recognized VirB10 P6. T-cell lines derived from animal 35160, which responded only to VirB10, recognized VirB10 P13 and P14. T cells from animal 583 (8/23) recognized VirB10 P2.1, P3, P13, and P22.

Dose response curves shown in Fig. 2 yielded interesting information about the strength of the T-cell response for specific peptides from each animal. For animals 35113 (11/22), 35141 (22/24), and 35287 (16/22) the strongest T-cell responses were induced by VirB9-1 P6 and VirB9-1 P10, which were significant at all the concentrations tested (Fig. 2a,c,e). Likewise, animal 35280 (16/27) also had a strong T-cell response to VirB9-1 P10 and the next strongest response from this animal was induced by VirB9-2 P5 (Fig. 2j). For animal 35160 (3/16), VirB10 P13 and P14 induced similar T-cell responses and these were still significant at 0.1 $\mu\text{g/ml}$ peptide (Fig. 2d). The fact that these peptides overlap in sequence by ten amino acids suggested the presence of a single epitope in the overlapping region. VirB9-2 P2, P3 and P10-P13 induced the strongest T-cell responses from 583 (8/23), that were significant using as little as 0.1 $\mu\text{g/ml}$ peptide, which represent a minimum of one and three epitopes, respectively (Fig. 2g,h). There was not a single peptide that induced T-cell responses from every animal, yet all four animals that responded to VirB9-1 had strong T-cell responses to P10. Induction of strong T-cell responses at lower peptide concentrations indicates a relatively higher avidity interaction, and peptides with this characteristic would be best for inclusion in a vaccine. For all six MHC class II-diverse animals the peptides with highest avidity were VirB9-1 P6 and P10, VirB9-2 P2, P3, P5 P10, P11, P12, and P13, and VirB10 P13 and P14. Further mapping studies will need to be performed to define the minimal amino acid sequences representing epitopes in these 30-mer peptides, which typically range from 9-12 amino acids.

DR and DQ expression and antigen presentation

To assess the peptide presentation by MHC class II molecules for each animal, artificial APC were loaded with individual peptides, and used in T-cell assays. Artificial APC were generated by transfecting 293-F cells with all possible combinations of *DRA* and *DRB3*, *DQA* and *DQB*, *DRA* and *DQB*, and *DQA* and *DRB3* alleles known for each animal. However, not all of the possible DR and DQ combinations available for each animal were expressed on the surface of transfected 293-F cells. The combinations that did express are listed in Table 3. Each of the expressed MHC class II combinations was tested for the ability to present peptides to T cells from cattle expressing the corresponding alleles (Fig. 3). T cells from animals 35113, 35141, and 35287 were stimulated with *DRA/DRB3**1101 presenting peptides VirB9-1 P6, P10, and VirB9-2 P5 (Fig. 3a,b,e). Also, *DRA/DRB3**1101 presented VirB9-1 P5 to T cells from animals 35113 and 35287 (Fig. 3a,e). T cells from 35280 also responded to VirB9-1 P6, P10, and VirB9-2 P5, but unlike animals 35113, 35141, and 35287, 35280 does not have a *DRA/DRB3**1101 combination (Table 2). The class II molecules presenting these peptides to T cells derived from animal 35280 were identified as *DRA/DRB3**1401 for VirB9-1 P10, and *DQA**2202/*DQB**1301 for VirB9-1 P6 and VirB9-2 P5 (Fig. 3d). Thus, VirB9-1 P6 and P10, and VirB9-2 P5 were presented by multiple MHC class II molecules. The presentation of VirB9-1 P6 by *DQA**2202/*DQB**1301 was also observed using T cells from animal 35287 (Fig. 3e). VirB10 P21 was presented by *DQA**1001/*DQB**1002 to T cells from animals 35113 and 35287 (Fig. 3a,e). Animals 35141, 35160, and 35280 also had this DQ combination, but significant responses to VirB10 P21 were not detected using this DQ pair (Fig. 3b,c,d). For animal 35113, we were unable to identify a restriction element for VirB9-2 P11 and P12 (Fig. 3a), possibly because responses to these peptides were relatively weak, and only detected with 20 µg/ml peptide (Fig. 2b).

T cells from animal 35160 did not respond to VirB9-1 or VirB9-2 peptides, and responded only to P13 and P14 from VirB10, which were presented by *DQA**2202/*DQB**0901 (Fig. 3c). Because the responses to these two overlapping peptides were comparable and both peptides were presented by the same class II molecule, we also tested a 15-mer peptide P13-14 (Table 1) that contains the overlapping 10-mer sequence plus two and three amino acids on the N- and C-termini, respectively, for presentation by artificial APC. The responses to P13, P14, and P13-14 were comparable and all were presented by both irradiated autologous PBMC and *DQA**2202/*DQB**0901 transfected artificial APC (data not shown), suggesting a single epitope, VLRAIVSRDV, is recognized. This *DQA/DQB* combination was clearly an interhaplotype DQ molecule since the *DQA**2202 allele is associated with the DH16A haplotype and the *DQB**0901 allele is associated with the DH03A haplotype (Table 2). Therefore, in order to express *DQA**2202/*DQB**0901, a bovine must be heterozygous. Another interhaplotype combination available only for 35287 was *DQA**2206/*DQB**1301, which presented VirB9-2 P8 and VirB10 P6 (Fig. 3e). This result explains why 35287 was the only animal to respond significantly to these peptides.

As compared to the other five animals, animal 583 has unique DR and DQ combinations (Table 2), and different responses to VirB9-2 and VirB10 peptides were observed. *DRA/DRB3**1201 presented the majority of the peptides to T cells from animal 583: VirB9-2 P2, P3, P9, P10, P11, and P12, as well as VirB10 P2.1, P3, P13, and P22 (Fig. 3f). We were unable to identify a restriction element for VirB9-2 P4, possibly because the response to this peptide was relatively weak, and only detected with 20 µg/ml peptide (Fig. 2g). VirB9-2 P13 was presented by *DRA/DRB3**2703 (Fig. 3f).

MHC class II molecules shown to present immunostimulatory VirB9-1, VirB9-2, and VirB10 peptides to CD4⁺ T cells are summarized in Table 4. We identified four *DRA/DRB3* molecules that presented the majority of peptides. Two intrahaplotype DQ combinations

presented three peptides: DQA*2202/DQB*1301 presented VirB9-1 P6 and VirB9-2 P5 and DQA*1001/DQB*1002 presented VirB10 P21. We also identified two interhaplotype DQ combinations that presented four peptides: DQA*2206/DQB*1301 presented VirB9-2 P8 and VirB10 P6 and DQA*2202/DQB*0901 presented VirB10 P13 and P14 (and the overlapping P13-14). Five of these peptides were not presented by DRA/DRB3 molecules. The MHC class II DQ interhaplotype pair DQA*2202/DQB*0901 was the only functional class II molecule for animal 35160, and it presented the only T-cell epitope(s) identified on VirB10. Thus, interhaplotype pairing was extremely important in this animal for recognition of VirB10.

Mixed isotype pairs containing DQA and DRB3 alleles were expressed on the transfected 293-F cell surface, so their abilities to present peptides for each animal were also examined. Each combination that was expressed, listed in Table 3, was tested with T cells from animals that had the corresponding alleles. We determined that mixed isotypes DQA*1201/DRB*1201, DQA*2201/DRB3*1201, and DQA*2206/DRB3*1101 presented peptides. Expression of these mixed isotype class II molecules, as determined by flow cytometry analysis using mAb TH14B, is shown in Fig. 4. Fig. 4 also includes the positive controls DRA/DRB3*1201 and DRA/DRB3*1101 and negative controls DQA*1001/DRB3*1101 (which did not express) and non-transfected 293-F cells. VirB9-2 P5 was presented by DQA*2206/DRB3*1101 to T cells derived from cattle 35141 and 35287 (Fig. 5a,b). T-cell lines from animal 583 responded to the mixed isotype combinations DQA*1201/DRB*1201 and DQA*2201/DRB3*1201 presenting VirB9-2 P12 (Fig. 5c). However, a weak T-cell response to this peptide was also still present when non-transfected 293-F cells were used. This can be explained by residual peptide remaining in the well despite repeated washing, and residual autologous APC in the two week cell lines from this animal, but the responses to VirB9-2 P12 presented by DQA*1201/DRB*1201 and DQA*2201/DRB3*1201 were nevertheless significantly greater than the response with 293-F cells alone.

Discussion

A. marginale OM can induce complete protection against disease, and in some animals, infection (Brown et al. 1998; Tebele et al. 1991, Noh and Brown, in press). However, it is unfeasible to use purified OM as a commercial vaccine because the bacteria are difficult to grow in culture and need to be harvested from infected cattle blood. Using blood-derived bacteria poses risks including potential contamination with other blood-borne pathogens and erythrocyte membranes. Use of individual immunodominant surface proteins, such as MSP2, has failed to provide protection against disease (Abbott et al. 2005, Noh and Brown in press). For these reasons, vaccine development for anaplasmosis has focused on identifying subdominant but immunogenic, conserved proteins in the OM (Lopez et al. 2005, 2008). Furthermore, we have evidence that association of proteins within the OM is important for providing increased T-cell help for IgG production (Macmillan et al. 2008; Morse et al. 2012). Therefore a protective vaccine is likely to require multiple subdominant proteins or combinations of individual linked T- and B-cell epitopes derived from the same or associated OM proteins. It would be advantageous to use multiple epitopes derived from multiple proteins rather than multiple proteins if the vaccine were to be delivered by a viral vector. There are limitations on the amount of cDNA that can be expressed as protein in these types of vectors.

A. marginale T4SS proteins VirB9-1, VirB9-2, and VirB10 are strongly immunogenic for CD4⁺ T lymphocytes from OM-immunized cattle (Lopez et al., 2007; Suttén et al., 2010; Morse et al., 2012). Animals 35113, 35141, and 35287, which are half-matched with respect to DRB3*1101 and associated DQ molecules, recognized all three of these proteins. However, animals 35160, 35280, and 35287, which are half-matched with respect to

DRB3*1501 and associated DQ molecules, had differential responses, in that 35160 did not respond to VirB9-1 or VirB9-2, and 35280 responded weakly and inconsistently to VirB10. To understand the differences in T-cell responses observed for these animals to VirB9-1, VirB9-2, and VirB10 we sought to identify MHC class II-allele specific T-cell responses to overlapping peptides. The differential T-cell responses were in part explained by presentation of peptides by other class II molecules not shared between animals or by unique combinations of DQA and DQB molecules. However, when the alleles encoding functional class II molecules were present, CD4⁺ T-cell lines from these three animals did not always respond to the same peptides, as observed for VirB9-1 P6, VirB9-2 P5, and VirB10 P21. VirB9-1 P6 and VirB9-2 P5 were presented by DQA*2202/DQB*1301, an intrahaplotype pair encoded by alleles present in all three cattle, yet neither peptide was recognized by animal 35160. Similarly, VirB10 P21 was presented by intrahaplotype pair DQA*1001/DQB*1002, also encoded by alleles found in all three animals but not recognized by 35160. It is possible that not all class II molecules expressed in 293-F cells are expressed and functional in each individual animal, and haplotype combinations may affect preferential pairing of alpha and beta molecules in vivo. Since expressed BoLA class II molecules are comprised of many functional intra- and interhaplotype DQ molecules and potentially mixed isotypes, the population of antigen-specific CD4⁺ T cells could also differ qualitatively and quantitatively among individual animals.

In summarizing the information for all cattle in this study, 20 immunostimulatory peptides and 11 peptide-presenting elements were identified. These included four DRA/DRB3 pairs, two intrahaplotype-DQA/DQB pairs, two interhaplotype-DQA/DQB pairs, and three DQA/DRB3 mixed isotype pairs. Thus, consistent with previous studies, it was very important to identify DQ-restriction elements that uniquely present pathogen peptide epitopes to CD4⁺ T cells (Brown et al. 2002; Glass et al. 2000; Norimine and Brown 2005). Notably, we now understand the differential T-cell responses observed for these cattle, and can predict T-cell responses to these T4SS proteins and epitopes by cattle with these defined haplotypes. Furthermore, the identification of T-cell epitopes on VirB9-1, VirB9-2, and VirB10 can be used to develop a multiple-antigen, epitope-based vaccine construct.

The four DRA/DRB3 combinations presenting peptides were DRA/DRB3*1101, DRA/DRB3*1401, DRA/DRB3*1201, and DRA/DRB3*2703. The function of DRA/DRB3*1201 in presenting peptides to T cells from animal 583 was important, presenting a minimum of four epitopes from VirB9-2 and four from VirB10, when considering that contiguous overlapping peptides contain a minimum of one epitope. BoLA DRB3 genes have been associated with resistance and susceptibility to several viral, bacterial, and parasitic infections, including bovine leukemia virus infection, (Amills et al. 1998; Xu et al. 1993; Zanotti et al. 1996), foot and mouth disease (FMD) (Alizadeh et al. 2003; Haghparast et al. 2000), *Staphylococcus aureus*-associated mastitis (Park et al. 2004; Rupp et al. 2007), neosporosis (Schwab et al. 2009), and East Coast fever (Ballingall et al. 2004). However, despite the indication of the role that BoLA-DR molecules play in protective immunity, very few studies have performed functional assays with BoLA class II molecules. Among these studies pathogen epitope restriction elements have been identified for DRA/DRB3*1201 presenting *A. marginale* peptides MSP2 P16-7 (Norimine and Brown 2005) as well as MSP1a peptide F2-1-1b (Norimine et al. 2006). DRB3*1101 presented MSP1a F2-B to T cells (Norimine and Brown 2005). Several FMD viral peptides were shown to be presented by DRA/DRB3*1101, DRA/DRB3*1201, and DRA/DRB3*2703 (Alizadeh et al. 2003; Haghparast et al. 2000).

In cattle, duplication of DQ molecules amplifies the opportunity for intra- and interhaplotype pairing to form functional heterodimers, thereby increasing the complexity of restriction elements and providing more dynamic immune responses (Glass et al. 2000;

Norimine and Brown 2005). We identified the products of two interhaplotype DQ molecules, DQA*2202/DQB*0901 and DQA*2206/DQB*1301. Here, these presented peptides from VirB9-2 and VirB10. This is the first study to report a functional DQA*2202/DQB*0901 molecule, which presented VirB10 P13, P14 and overlapping P13-14. This DQ combination was the only antigen-presenting element identified for animal 35160, explaining its T-cell response to VirB10. Interhaplotype DQA*2206/DQB*1301 was previously reported to present *A. marginale* MSP1a peptide B to T cells (Norimine and Brown 2005) and is apparently not rare because cattle heterozygous for the RFLP type 16/22 are common in Washington State Holstein herds (unpublished observations). The α - and β -chains of this combination may also have some flexibility as to which protein they partner with (Tables 3 and 4 and ref. Norimine and Brown 2005). Furthermore, even though not all possible DQ combinations were expressed on the cell surface, we have observed that there is preferential pairing of DQ molecules and that several DQ molecules observed in this study were noted in previous studies from our lab using different cattle and antigens (Brown et al. 2002; Norimine and Brown 2005; Norimine et al. 2006).

Three mixed isotype combinations that presented peptides were also identified, i.e. DQA*2206/DRB3*1101 presented VirB9-2 P5, and DQA*1201/DRB3*1201 and DQA*2201/DRB3*1201 presented VirB9-2 P12. However, the corresponding DRA/DRB3 combination for these mixed isotypes also presented the same peptide. This suggests that the β -chain may be more important for presenting the two peptides and the α -chain is interchangeable. This is the first documentation that bovine mixed isotypes have the capability to present peptides; however there is precedence for this from studies with mice and humans (Germain and Quill 1986; Lotteau et al. 1987; Sant et al. 1987; Spencer et al. 1993). Despite mixed isotype combinations being a relatively unexplored area, there remains the possibility that this represents a valid mechanism for antigen presentation, but further analyses are required to determine how important this is *in vivo*. We cannot rule out that the bovine mixed isotype peptide presentation is an *in vitro* artifact, as mAb that detect mixed isotypes are not available to confirm that this presentation occurs *in vivo*.

Selecting peptides that contain CD4⁺ T-cell epitopes with high avidity TCR interactions is an essential step for the construction of a multiple-antigen and peptide-based vaccine. Also, vaccine epitopes should be recognized broadly by diverse MHC class II molecules expressed in the target population. For *A. marginale* T4SS proteins, the peptides that have the highest avidity, based on stimulating T-cell proliferation at a low concentration, and are recognized by common MHC class II molecules in cattle are VirB9-1 P6 and P10, VirB9-2 P2, P3, P5, P10, P11, and P12, and VirB10 P13 and P14. VirB9-1, VirB9-2, and VirB10 sequences are highly conserved across *A. marginale* strains and *A. centrale* (Lopez et al., 2007; Morse et al. 2012), and sequences of peptides with the highest avidity (VirB9-1 P5, VirB9-2 P2, P5, P12, and P13, and VirB10 P13 and P14) are completely conserved (100% identical). The peptide sequences for VirB9-1 P10 and VirB9-2 P3 are also completely conserved among *A. marginale* strains and have only one amino acid difference in *A. centrale*. Similarly, VirB9-2 P10 and P11 are completely conserved among all strains of *A. marginale*, but have four and three amino acid differences, respectively, in *A. centrale*. Because both *A. marginale* OM and live *A. centrale* provide protective immunity against disease following *A. marginale* infection, this high degree of conservation in peptide epitopes between these bacteria indicates they may contribute to the protective immune response.

The identification of MHC class II restricted antigenic peptides for inclusion in a vaccine is a high research priority. However, bioinformatic tools have not been developed to accurately predict whether a given peptide will interact with BoLA class II molecules (Jones et al. 2011; Lafuente and Reche 2009). The prediction accuracy is dependent on the experimental

datasets used, and for the bovine system this is severely lacking. In this study, we used six cattle with different haplotypes to identify the MHC class II restriction elements for peptides spanning three immunogenic T4SS proteins. Upon identification of minimal epitopes, the results will be useful to help formulate a prediction model for peptide binding to bovine MHC class II DR, and importantly, DQ molecules.

Different combinations of heterozygous MHC class II molecules in cattle increases the number of pathogen-derived peptide epitopes presented to T cells. We have identified highly antigenic CD4⁺ T-cell epitopes on T4SS proteins VirB9-1, VirB9-2, and VirB10 presented by BoLA class II DR and DQ molecules that are encoded by alleles prevalent among Holstein cattle worldwide. Similar frequencies of the MHC class II alleles studied here were found for Holstein cattle in Japan, where the combined frequencies were 81.2%, 86.9%, 90.3%, and 95.2% in four populations studied (Miyasaka et al., 2011; Takeshima et al., 2003), in Ontario, Canada, where the combined frequency was 89.5% (Sharif et al., 1998), in Iran, where the combined frequencies were 73.2%, 75.5%, 78.1%, and 80.2% in several studies (Nassiry et al., 2008; Pashimi et al., 2009), and in Europe, where the combined frequency was 89.5% (Nassiry et al., 2008). Inclusion of VirB9-1, VirB9-2, and VirB10 peptides linked together in a multiple-antigen and peptide-based vaccine will not only provide more than one immunogenic peptide, but will offer the opportunity for increased T-cell dependent IgG responses upon infection if B cell epitopes are also included (Morse et al., 2012). Furthermore, if protective, such a vaccine would be useful to protect the majority of individuals within Holstein populations across the globe. Thus, a vaccine made up of highly conserved VirB9-1, VirB9-2, and VirB10 peptides will be tested in a future study to protect cattle against *A. marginale*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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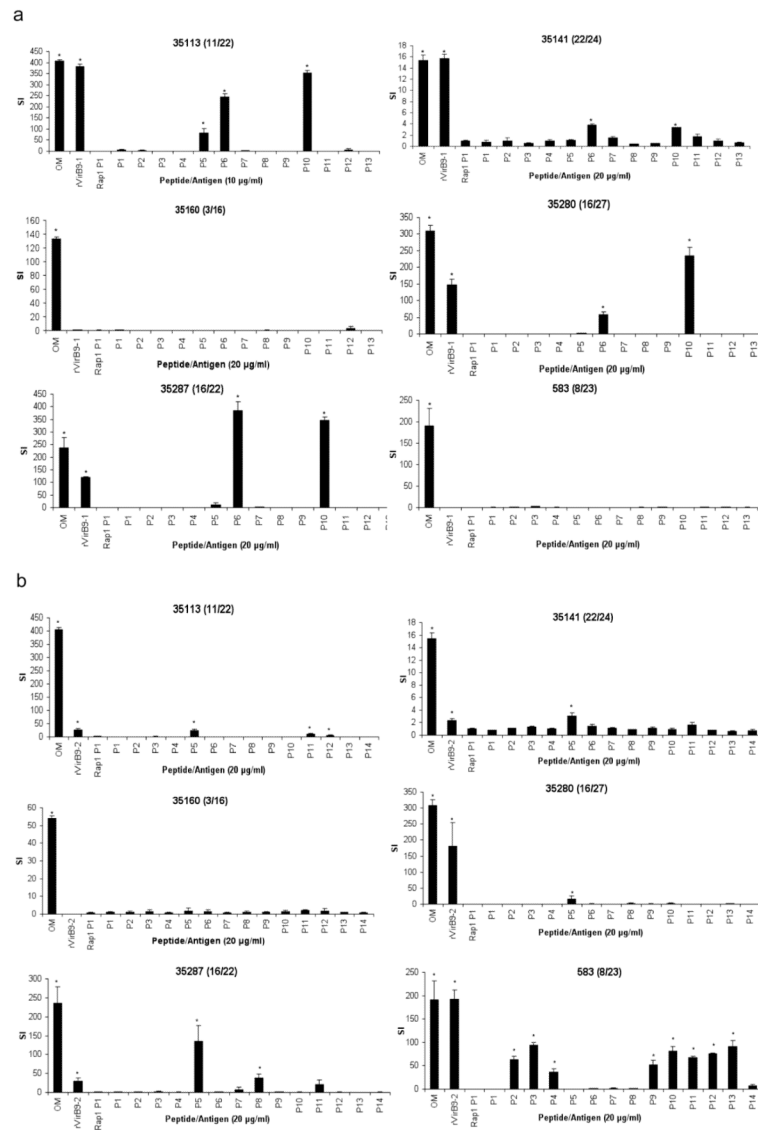
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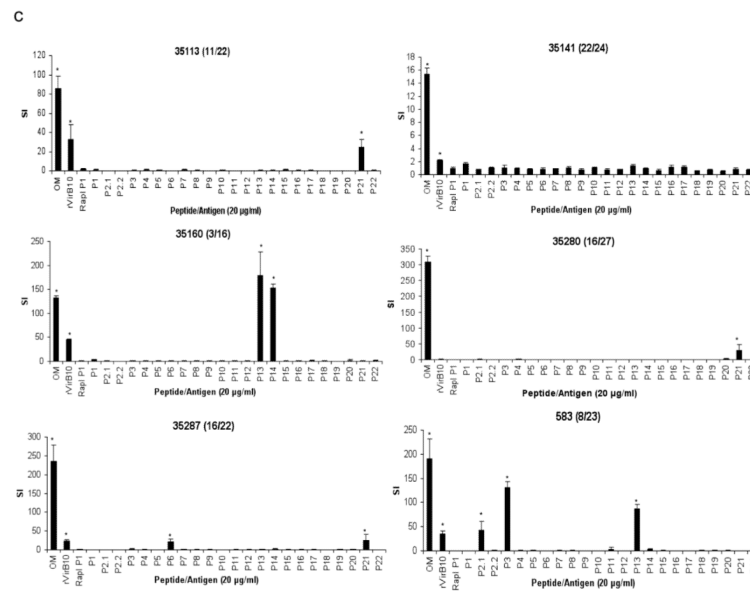


Fig. 1. Stimulation of CD4⁺ T-cell lines from *A. marginale* OM-immunized cattle with synthetic peptides from VirB9-1, VirB9-2, and VirB10. Short term T-cell lines from six animals (animal number and RFLP type in parenthesis is indicated on the top of each panel) were stimulated with 10 or 20 µg/ml of peptides from (a) VirB9-1 peptides P1-P13, (b) VirB9-2 peptides P1-P14, and (c) VirB10 peptides P1, P2.1, P2.2, and P3-P22. As a negative control, a 30-mer peptide derived from *B. bovis* Rap1 was used at an equal concentration. Additional controls were *A. marginale* OM and the full length recombinant protein used at 1 µg/ml. Results are presented as SI, and responses significantly higher than those for *B. bovis* Rap1 P1 are indicated with asterisks, where $P < 0.05$

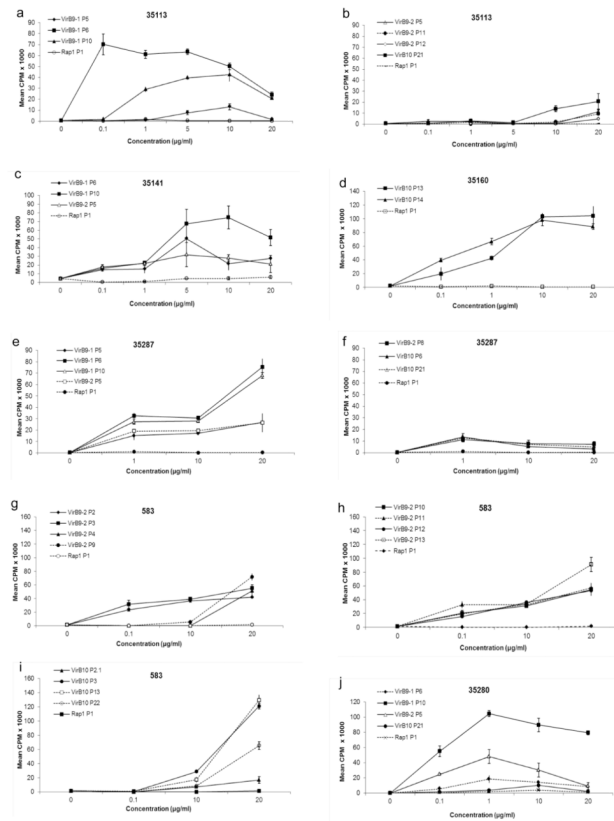


Fig. 2. Dose-dependent proliferative responses to stimulatory peptides. Short-term T-cell lines from six animals (a) 35113, (b) 35113, (c) 35141, (d) 35160, (e) 35287, (f) 35287, (g) 583, (h) 583, (i) 583, and (j) 35280, were stimulated with the 0.1 to 20 $\mu\text{g}/\text{ml}$ of the indicated peptides and cultured for three days, radiolabeled, and harvested. As a negative control, *B. bovis* Rap1 P1 was used at an equal concentration. Proliferation is presented as the mean CPM

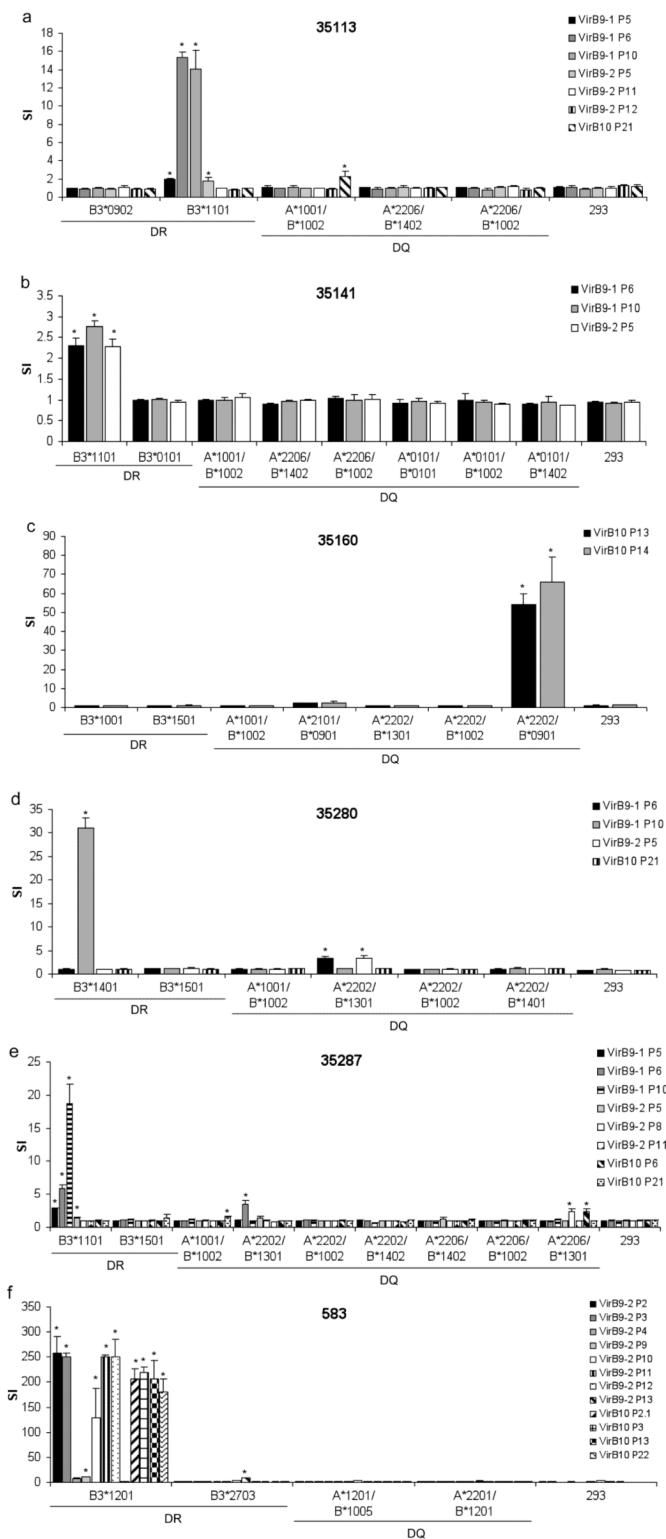


Fig. 3. Identification of BoLA class II restriction elements for VirB9-1, VirB9-2, and VirB10 epitopes using 293-F cells expressing bovine DR and DQ molecules. Short-term T-cell lines

from six animals (a) 35113, (b) 35141, (c) 35160, (d) 35280, (e) 35287, and (f) 583 were cultured for three days with human embryonic kidney 293-F cells expressing the indicated CD80 and DR or DQ molecules that were loaded with 10 $\mu\text{g/ml}$ of the indicated peptide. Results are presented as SI and responses T-cell responses using each artificial APC pulsed with an individual peptide were compared to those using non-transfected 293-F cells incubated with the same peptide. Significant responses were determined using a Student's one-tailed t-test, where $P < 0.05$, and are indicated by asterisks

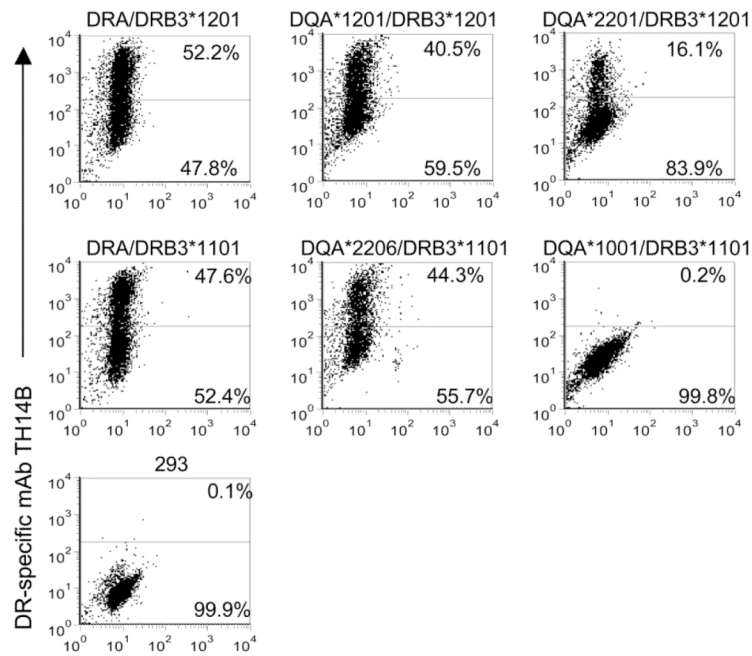


Fig. 4. Expression of peptide-presenting mixed isotype pairs. Human embryonic kidney 293-F cells expressing CD80 and the indicated mixed isotype BoLA class II molecules were stained with DR-specific mAb TH14B and secondary antibody R-PE conjugated goat anti-mouse IgG2a, and analyzed by flow cytometry. Positive controls were mAb TH14B labeled DRA/DRB3 molecules and negative controls were mAb TH14B stained non-transfected 293-F cells and DQA*1001/DRB3*1101, which failed to express

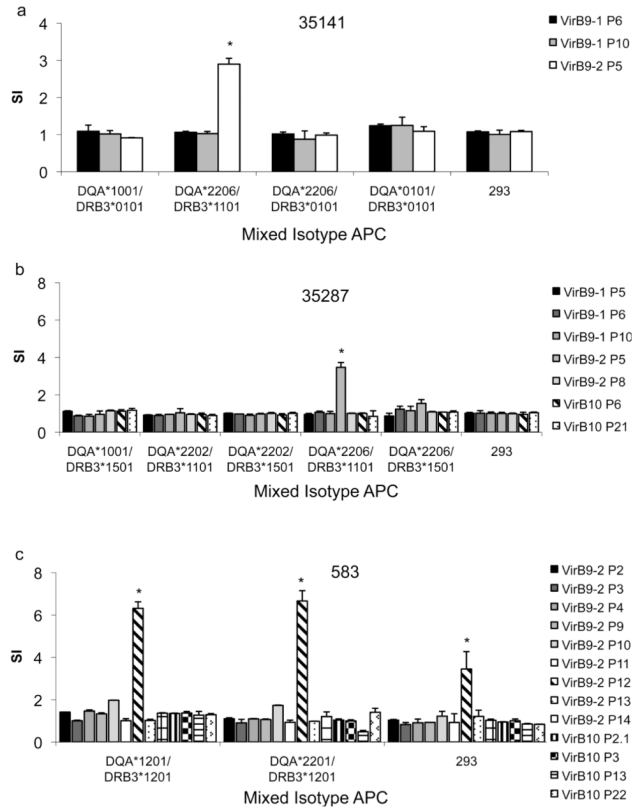


Fig. 5. Identification of BoLA class II restriction elements for VirB9-1, VirB9-2, and VirB10 epitopes using 293-F cells expressing bovine class II mixed isotypes. Short term T-cell lines from six animals (a) 35141, (b) 35287), and (c) 583 were cultured for three days with human embryonic kidney 293-F cells expressing CD80 and the indicated mixed isotype BoLA class II molecules that were loaded with 10 μ g/ml of the indicated peptide. Results are presented as SI and T-cell responses using artificial APC pulsed with an individual peptide were compared to those of non-transfected 293-F cells incubated with the same peptide. Significant responses were determined using a Student's one-tailed t-test, where $P < 0.05$, and are indicated by asterisks

Table 1

Sequences of overlapping peptides used to measure T-cell responses

Protein	Peptide	Sequence
VirB9-1	P1	MKKAFMVCAVALLCSSAAFQKQEPRESIAAD
	P2	KQEPRESIAADDDHIKIIINFNPQSIHRYTGFY
	P3	QSIHRYTGFYGYQSSILFESGEVIDTVSMG
	P4	GEVIDTVSMGDSTGWQLVPKGNRLFIPVVG
	P5	GNRLFIPVVDNADTNVTIITNRRVYYFEL
	P6	TNRRVYYFELHAEASGLDDPRLAYEVRFV
	P7	PRLAYEVRFVYPAASSVDAASSSDLGGGVS
	P8	SSSDLGGGVSFPTYQNDVPDLSPEVAKKG
	P9	LSDPEVAKKGLNFDYSVSHTAGSANIVPIR
	P10	AGSANIVPIRVFDDRKFYMQFSNVNGDLP
	P11	QFSNVNGDLPISIFNVDAEGYESLVNFRIVG
	P12	ESLVNFRIVGDYVVVERVSPAFTLRYGSST
	P13	AFTLRYGSSTACVFNEKLYRTSSTSRRGRG
VirB9-2	P1	MNFYKNLLACALLTVVFTGGVAQSAVSGG
	P2	GVAQSAVSGGAPVSVDSRIKTFVYSPNEIF
	P3	TFVYSPNEIFTVVFNHGYHSFIEFSKGETI
	P4	FIEFSKGETIKVMAMGDSVHWKVKPVDNKL
	P5	WKVKPVDNKLFIPLEREGKTNMLVETNKG
	P6	TNMLVETNKGSRSYAFDLVSKSAGPDAAGYK
	P7	SAGPDAAGYKEVADELGRVDSPLLDMAVYV
	P8	SPLLDMAVYVRFYYPDNNREFDLKGAGLAD
	P9	FDLKGAGLADLSAPSLAKNPNSGEVTVRPN
	P10	NSGEVTVRPNATGKNYVYSASSADATIVPV
	P11	SSADATIVPVKTFDDGALTYFQFYDNNKVI
	P12	FQFYDNNKVIPKVFVSVGRHGKVKPCRMLLL
	P13	KKVPCRMLLLKGYVIIIEGVHKRLYLDYGKS
	P14	KRLYLDYGKSGVEVVNTVL
VirB10	P1	MSLGMSDETKDNNYGDGVEESVNVVGVHKS
	P2.1	SVNVVGVHKS KKL FVVLVVC
	P2.2	KKL FVVLVVC AITGMAYYMF
	P3	AITGMAYYMFFRGS GTTETSEEPQQVIEKQ
	P4	EEPQQVIEKQDV DKLK ESEAPA QETAPRI
	P5	APAQETAPRI LTPPKLPDL PPLVMPTAPE
	P6	PPLVMPTAPE LPTLARIA KKKKEEPVVEET
	P7	KKEEPVVEET KEILPPAAES FFEPELQRRP
	P8	FFEPELQRRP MEDD GPPQHIPMPYRPGGGA
	P9	PMPYRPGGGA IPEPVPSFLGYDREKRGTPM
	P10	YDREKRGTPM IVLGGGGDGGPSEDGGGQGT
P11	PSEDGGGQGT DSRFSTWSTLDGTSSPSVKA	

Protein	Peptide	Sequence
	P12	DGTSSPSVKATRVGDPGYVILQGHMIDAVL
	P13	LQGHMIDAVLETAINSDIPGVLRAIVSRDV
	P13-14	PGVLRAIVSRDVYAE
	P14	VLRAIVSRDVYAEAGNMVMIPKGSRLIGSY
	P15	PKGSRLIGSYFFDASGNTRVTVSWSRVIL
	P16	VTVSWSRVILPHGIDIQINSAGTDELGRNG
	P17	AGTDELGRNGSAGFIDTKMGNVLTSTILLA
	P18	NVLTSTILLAGVSMGTAFVTSKIPALQSEI
	P19	SKIPALQSEIKDTTEEKGEKKKEKSSTLP
	P20	KKEKSSTLPVKIVSDAVKDFSESMKALIK
	P21	FSESMKALIKKYVDTSKPTIYVDQGTVMKV
	P22	YVDQGTVMKVFVNQDIVFPREAVRR

Table 2

DRB3, *DQA*, and *DQB* alleles identified in the cattle used in this study

Animal	Haplotype	<i>DRB3</i>		<i>DQA</i>		<i>DQB</i>	
		RFLP	Allele	Allele(s)	Allele(s)	Allele(s)	Allele(s)
35113	DH11A	11	*0902	*0204	*1803		
	DH22H	22	*1101	*1001	*1002		
35141	DH22H	22	*1101	*1001	*1002		
				*2206	*1402		
35160	DH24A	24	*0101	*0101	*0101		
	DH03A	3	*1001	*1001	*1002		
35280	DH16A	16	*1501	*1001	*0901		
				*2101	*1002		
35287	DH27A	27	*1401	*ND ^a	*1401		
	DH16A	16	*1501	*1001	*1002		
583	DH22H	22	*1101	*1001	*1002		
	DH08A	8	*1201	*1201	*1005		
DH23A		23	*2703	*ND	*ND		
				*ND	*ND		

^aND, not determined

Table 3

Expressed BoLA class II α/β combinations

BoLA class II molecule			Percentage of cells expressing class II
Isotype	A chain	B chain	
DR^a			
	*0101	*0101	49.6
		*0902	56.5
		*1001	53.0
		*1101	61.8
		*1201	68.0
		*1401	60.8
		*1501	51.7
		*2703	60.0
DQ^b-Intrahaplotype			
	*0101	*0101	34.5
	*1001	*1002	59.7
	*1201	*1005	45.7
	*2101	*0901	42.0
	*2201	*1201	49.5
	*2202	*1301	52.4
		*1002	30.6
	*2206	*1402	59.9
		*1002	37.1
Interhaplotype			
	*0101	*1002	45.6
		*1402	56.8
	*2202	*0901	57.5
		*1401	16.8
		*1402	57.0
	*2206	*1301	17.6
Mixed Isotype^{a,c}			
	*0101	*0101	60.0
	*1001	*0101	55.7
		*1001	39.4
		*1501	29.2
	*1201	*1201	44.8
	*2101	*1001	22.2
		*1501	33.4
	*2201	*1201	31.4
	*2202	*1001	38.7

BoLA class II molecule			Percentage of cells expressing class II
Isotype	A chain	B chain	
		*1501	40.0
		*1401	19.4
		*1101	23.9
	*2206	*0101	52.4
		*1101	44.3
		*1501	33.0

^aExpression of all combinations containing DR was detected using Th14B mAb.

^bExpression of all intra- and interhaplotype DQ combinations was detected using CC158 mAb.

^cCombinations of DRA and DQB were not detected, and only DQA and DRB combinations that were detected are shown.

Table 4

Summary of BoLA-class II restriction elements presenting peptides to T cells

BoLA-class II restriction element		Presented peptide	Animal(s) #
Isotype	Allele		
DR			
	DRA/DRB3*1101	VirB9-1 P5	35113, 35287
		VirB9-1 P6	35113, 35141, 35287
		VirB9-1 P10	35113, 35141, 35287
		VirB9-2 P5	35113, 35141, 35287
	DRA/DRB3*1401	VirB9-1 P10	35280
	DRA/DRB3*1201	VirB9-2 P2	583
		VirB9-2 P3	583
		VirB9-2 P9	583
		VirB9-2 P10	583
		VirB9-2 P11	583
		VirB9-2 P12	583
		VirB10 P2.1	583
		VirB10 P3	583
		VirB10 P13	583
		VirB10 P22	583
	DRA/DRB3*2703	VirB9-2 P13	583
DQ			
Intrahaplotype			
	DQA*1001/DQB*1002	VirB10 P21	35113, 35287
	DQA*2202/DQB*1301	VirB9-1 P6	35280, 35287
		VirB9-2 P5	35280
Interhaplotype			
	DQA*2202/DQB*0901	VirB10 P13	35160
		VirB10 P14	35160
	DQA*2206/DQB*1301	VirB9-2 P8	35287
		VirB10 P6	35287
Mixed isotype			
	DQA*2206/DRB3*1101	VirB9-2 P5	35113, 35141, 35287
	DQA*1201/DRB3*1201	VirB9-2 P12	583
	DQA*2201/DRB3*1201	VirB9-2 P12	583