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# Identification of Major Histocompatibility Complex Restriction and Anchor Residues of Foot-and-Mouth Disease Virus-Derived Bovine T-Cell Epitopes▽

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**Despite intensive research on the identification of T-cell epitopes in cattle after foot-and-mouth disease virus (FMDV) infection during the last 20 years, knowledge of major histocompatibility complex (MHC) restriction and anchor residues of such epitopes is still sparse. Therefore, as a first step, we tested lymphocytes from two experimentally FMDV serotype A24-vaccinated and -challenged cattle for recognition of FMDV-derived pentadecapeptides in proliferation assays. Two epitopes were identified: amino acid residues 66 to 80 within the structural protein 1D and amino acid residues 22 to 36 within the structural protein 1A. The latter epitope was recognized by lymphocytes from both cattle. Peptide-specific proliferation was caused by a response of $CD4^+$ T helper cells as identified by carboxyfluorescein diacetate succinimidyl ester proliferation assays. Having identified one epitope that was recognized by two cattle, we hypothesized that these animals should have common MHC class II alleles. Cloning and sequencing of DRB3, DQA, and DQB alleles revealed that both animals possessed DQA allele 22021 and DQB allele 1301 but had no common DRB3 allele. A parallel analysis of amino acid residues involved in MHC presentation by peptides with alanine substitutions showed that the amino acid residues in positions 5 and 9 within the pentadecapeptide representing the 1A epitope were important for MHC binding in both cattle. These data indicate that the epitope located on FMDV protein 1A can be presented by MHC class II DQ molecules encoded by DQA allele 22021 and DQB allele 1301 and present the first evidence of the binding motif of this particular DQ molecule.**

Foot-and-mouth disease virus (FMDV) is a member of the family *Picornaviridae*, genus *Aphthovirus.* The virus genome consists of a positive-stranded RNA molecule of about 8,500 nucleotides, which encodes four structural proteins (1A, 1B, 1C, and 1D, also designated VP4, VP2, VP3, and VP1, respectively) and nine nonstructural proteins (NSP) (L/L′, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) (31). Since FMDV is an RNA virus, it shows a high degree of genetic and structural variability in its capsid proteins, illustrated by the existence of seven different serotypes with a large number of subtypes (7, 19). The virus causes a highly contagious disease with high morbidity in cloven-hoofed animals, including important livestock species, such as cattle and swine. FMDV can be controlled by the use of a chemically inactivated whole-virus vaccine; however, a number of disadvantages are associated with the use of this vaccine. For example, the vaccine provides only short-term protection, resulting in the need for periodic revaccination (4), and there is a risk of infectious virus being released during vaccine production. Therefore, a number of countries with large livestock industries have abandoned vaccination, including the United States and the countries of the European Union. However, this policy leaves livestock herds prone to sudden outbreaks of FMD, with dramatic effects on animal welfare and livestock economy, as seen in the United Kingdom in 2001 (20, 33), and in turn has led to intensive research on alternative vaccination strategies.

For the design of novel vaccines, a detailed knowledge of antigenic regions recognized not only by B cells, but also by $CD8^+$ cytolytic T cells and $CD4^+$ T helper cells of the respective host, is crucial. T cells recognize their specific antigen in the context of major histocompatibility complex (MHC) molecules (28), and the genes coding for these molecules are highly polymorphic within an outbred population of a particular species. In cattle, for example, the MHC class II molecules bovine leukocyte antigen (BoLA) DR and DQ are expressed (2). Three of the genetic loci coding for these molecules are polymorphic, and to date, 106 DRB3, 46 DQA, and 52 DQB alleles have been reported (http://www.ebi.ac.uk/ipd/mhc/bola/index.html). Therefore, the identification of T-cell epitopes is influenced by the MHC alleles present in a particular animal, a phenomenon called MHC restriction.

In the past, a number of studies aimed to identify FMDV-specific T-cell epitopes in outbred cattle by the use of overlapping peptides in in vitro restimulation assays (10, 11, 36, 37). Most of these studies focused on the identification of epitopes present in capsid proteins, and a response of MHC class II-restricted $CD4^+$ T helper cells was shown either directly by antibody-mediated blocking experiments (10) or indirectly by the use of cathepsin D-cleaved protein fragments, thereby mimicking in vivo MHC class II antigen processing (36). Some of the epitopes described in these publications were also investigated in experiments with MHC-typed animals (17,

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35–37). However, an identification of binding motifs of particular peptides representing T-cell epitopes was not performed. The role of MHC class I-restricted $CD8^+$ T cells in the immune response to FMDV was addressed only recently (16). In this particular survey with MHC class I-typed animals, for the first time a gamma interferon (IFN-γ) response of sorted $CD8^+$ T cells against whole virus and recombinant protein consisting of P1 and 2A regions was shown. However, single MHC class I presented epitopes were not investigated.

In the study presented here, we aimed to identify T-cell epitopes present in the entire polyprotein of FMDV by using lymphocytes from two MHC class II-typed cattle that were FMDV vaccinated and challenged. Although we were not able to identify epitopes within NSP, two epitopes within capsid proteins were identified and their MHC class II restriction was investigated. In addition, by the use of peptides with alanine substitutions, we defined amino acid residues relevant for MHC binding within the identified epitope sequences.

## MATERIALS AND METHODS

**Animals and experimental vaccination/infection.** Two FMDV-naïve female Brown Swiss cattle (C129 and C813) were vaccinated intramuscularly with a full dose of a monovalent conventional inactivated FMD vaccine, serotype A24, T1003 (Merial, Lyon, France). Twenty-eight days after vaccination, both animals were challenged by intradermal tongue inoculation with homologous FMD A24 virus in accordance with the FMD monograph of the European Pharmacopoeia (12). Both cattle were protected from clinical disease symptoms after challenge infection. However, in FMDV-infected cattle, virus often persists in the pharyngeal region for prolonged periods (beyond day 21 postinfection [p.i.]) (reviewed in reference 1). Therefore, probang samples of both cattle were taken weekly and analyzed by plaque test and PCR for the presence of FMDV as described previously (22). In both animals, FMDV was detected in probang samples from 2 weeks p.i. up to 7 weeks p.i. by both detection methods (data not shown), indicating virus replication in the pharyngeal region. To test whether this virus replication also caused the production of antibodies against epitopes present within NSP, sera from both animals were tested (from week 7 p.i. up to week 68 p.i.) in an FMDV peptide enzyme-linked immunosorbent assay. This enzyme-linked immunosorbent assay detects antibodies against linear B-cell epitopes present within NSP 3B, as described elsewhere (18). In sera from animal C129, no antibodies against epitopes present within NSP 3B were detected. In contrast, in serum from animal C813 taken 7 weeks p.i., antibodies against these NSP epitopes were present (data not shown). However, at later time points, these antibodies were no longer detectable.

Blood sampling for isolation of peripheral blood mononuclear cells (PBMC) started 26 weeks p.i. and was continued until 68 weeks p.i. Thereafter, the animals were slaughtered and blood was collected for isolation and subsequent cryopreservation of PBMC. All animal experiments were approved by the Regional Administrative Authority of Tübingen, Germany (proposal no. BFA167).

**Virus and peptides.** FMDV serotype A24 was propagated overnight on BHK clone Tübingen cells grown to confluence at 37°C. Culture medium (BHK 21 medium supplemented with 1% fetal calf serum [FCS] [Invitrogen, Carlsbad, CA]), and cell debris was collected and centrifuged at $900 \times g$. Thereafter, the supernatants were collected, aliquoted, and stored at −70°C. The virus titer was determined by a cell suspension plaque test as described elsewhere (22).

The sequences of the peptides used in this study were based on FMDV strain A24/Cruzeiro/Brazil/55 (accession no. AJ251476), which was used to vaccinate and challenge the animals under investigation. Each peptide was 15 amino acid residues (aa) long and overlapped by 10 aa, starting from the N-terminal ends of the proteins. This led to a total of 442 peptides covering the entire FMDV polyprotein sequence. In addition, for peptides representing putative T-cell epitopes, peptides with alanine substitutions were synthesized with a single alanine introduced at every consecutive position. Synthesis and analysis of the synthetic peptides used in this study were performed as described elsewhere (3).

**Isolation, freezing, and defrosting of PBMC.** Heparinized blood was collected, and PBMC were isolated by density gradient centrifugation (40 min; $1{,}100 \times g$) using Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). After two washes in phosphate-buffered saline (PBS) (PAA, Pasching, Austria), PBMC were resuspended in cell culture medium consisting of RPMI 1640 with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Invitrogen) and penicillin/streptomycin (100 units/ml and 0.1 mg/ml, respectively; PAA). For some experiments, isolated PBMC were frozen, stored at −150°C, and defrosted for subsequent in vitro cultivation. For the freezing procedure, a medium consisting of 50% RPMI 1640, 40% FCS, and 10% dimethyl sulfoxide (Hybri-Max; Sigma-Aldrich) was used. For defrosting, cells were thawed in a water bath at 37°C and transferred quickly into prewarmed cell culture medium. After a single wash, the cells were used either for proliferation assays or for IFN-γ enzyme-linked immunospot (ELISPOT) assays.

**Lymphocyte proliferation measured by tritium incorporation.** Freshly isolated or defrosted PBMC in cell culture medium were plated at $2 \times 10^5$ cells/well in 96-well round-bottom microtiter plates (Greiner Bio-One, Frickenhausen, Germany). For initial screening, each peptide was tested in four wells, but with a different peptide concentration in each well: 40, 20, 10, and 5 μg/ml, respectively. Cells in medium alone and cells incubated with a 15-mer peptide representing aa 81 to 95 from the Core protein of the classical swine fever virus served as negative controls. For restimulation with homologous FMDV A24, multiplicities of infection (MOIs) were calculated based on PFU derived from plaque tests (see above). Mock controls were used at equal dilutions consisting of cell culture supernatant of uninfected BHK clone Tübingen cells. After 5 days of incubation (37°C and 5% $CO_2$), PBMC cultures were pulsed with 1 μCi of [*methyl*-$^3$H]thymidine (MP Biomedicals, Irvine, CA) for 18 h. Thereafter, cells were collected and incorporation of [$^3$H]thymidine was measured by liquid scintillation counting (Wallac-Microbeta, Waltham, MA).

**Lymphocyte proliferation measured by CFSE dilution.** Peptides causing lymphocyte proliferation in tritium incorporation assays, as well as peptides with alanine substitutions, were tested in carboxyfluorescein diacetate succinimidyl ester (CFSE) assays. For identification of the phenotype of proliferating cells, monoclonal antibodies (MAb) against CD molecules were used, and cells were analyzed by flow cytometry. After isolation or defrosting, PBMC were washed once in cell culture medium containing 5% FCS, adjusted to $2 \times 10^7$ cells/ml, and mixed with an equal volume of CFSE (Invitrogen) labeling solution (PBS with 5 μM CFSE). After incubation at room temperature for 5 min, labeling was stopped by adding an equal volume of pure FCS. Thereafter, the cells were washed twice in cell culture medium (10% FCS) and plated at $2 \times 10^5$ cells/well, together with the respective peptides. After 7 days of incubation (37°C and 5% $CO_2$), PBMC stimulated with the same peptide were fractionated and incubated for two- or three-color staining with the following anti-bovine MAb: CD4 (clone CC8; mouse immunoglobulin G2a [IgG2a]; AbD Serotec, Kidlington, United Kingdom), CD8β (clone CC58; mouse IgG1; AbD Serotec), and T-cell receptor (TCR) δ-chain (clone GB21A; mouse IgG2b,; VMRD, Pullman, WA). In addition, samples with isotype-matched nonspecific MAb (clones NCG01, NCG2A.01, and NCG2B.01; Dianova, Hamburg, Germany) were prepared for evaluation of nonspecific binding. After incubation for 30 min on ice, the cells were washed once in PBS with 2% FCS. Thereafter, fluorescence labeling was performed by the use of anti-mouse isotype-specific secondary antibodies: anti-IgG2a–phycoerythrin (Southern Biotech, Birmingham, AL), anti-IgG1–Alexa Fluor 647 (Invitrogen), and anti-IgG2b–Alexa Fluor 647 (Invitrogen). Samples were incubated again for 30 min, washed twice, and immediately analyzed on a FACSAria (BD Biosciences, San Jose, CA). Per sample, at least $2 \times 10^4$ live cells were acquired, and data were processed by the use of FACSDiva software, version 5.02 (BD Biosciences).

**IFN-γ ELISPOT assays.** Ninety-six-well High Protein Binding Immobilon-P plates (Millipore, Billerica, MA) were coated with anti-bovine IFN-γ MAb (clone 5D10; Biosource, Nivelles, Belgium) at 10 μg/ml in PBS, using 100 μl/well, and stored overnight at 4°C. Subsequently, the plates were blocked for 2 hours at 37°C with cell culture medium. Thereafter, either $5 \times 10^5$ or $2 \times 10^5$ PBMC per well were incubated for 24 h (37°C and 5% $CO_2$) in duplicate with various amounts of peptides causing lymphocyte proliferation in tritium incorporation assays, as well as peptides with alanine substitutions. Subsequently, the plates were washed four times and then incubated for 2 hours at room temperature with a biotinylated anti-bovine IFN-γ MAb (clone 6C3; Biosource) at a concentration of 2.5 μg/ml in PBS using 100 μl/well. This was followed by incubation with streptavidin-alkaline phosphatase (Invitrogen) for 1 h at room temperature, diluted 1:2,000 in PBS (100 μl/well). Subsequently, a further incubation for 5 min with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Sigma Fast; Sigma-Aldrich) was performed. Thereafter, the plates were washed intensively with tap water and dried at room temperature for several days, and the spots were counted on an ELISPOT reader (ELR03; AID GmbH, Straßberg, Germany) using EliSpot Reader software, version 4.0 (AID GmbH).

**Depletion of $CD4^+$ T helper cells by MACS.** Defrosted PBMC were washed once in cell culture medium, followed by incubation with anti-bovine CD4 MAb (clone IL-A11; mouse IgG2a; VMRD) for 20 min on ice. After a single wash in

TABLE 1. Primer sets used for RT-PCR of BoLA class II loci

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | $T_A$ (°C) | Reference | Amplicon size (bp) |
|---|---|---|---|---|---|
| DRB3 | TCCTCTCGCTCTCTATCCTCTGCTGT | AGGAAACCTTTCCATGCTGTGAAGAA | 60 | This study | 915 |
| DQA1[a] | AGGATGATCCTGAACAGAGCCCTGA | TCACAACGGCCCCTGGTGTCT | 60 | 24 | 760 |
| | AGGATGGTCCTGAACAGAGCCCTGA | TCACAACGGCCCCTGGTGTCT | 60 | 24 | 760 |
| DQA2 | AGGATGGTCCTGAACAGAGCTCTGA | CTAGGGTGCAACTCACAAGGGA | 60 | 24 | 760 |
| DQB[a] | ACTGGATCCCCCGCAGAGGATTTCGT | ATAGAATTCACCTWGCCGCTGCCAGGT | 64 | 29 | 300 |
| | ACTGGATCCCCCGCAGAGGATTTCGT | ATAGAATTCACCTCGCCGCTGCAAGGA | 64 | 29 | 300 |

[a] These primer sets were used interchangeably.

PBS plus 2% FCS plus 2 mM EDTA (magnetic cell separation [MACS] buffer), the cells were resuspended and incubated with rat anti-mouse IgG2a and -b MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min on ice. After a further wash in MACS buffer, the cells were transferred to LD columns (Miltenyi Biotec) and placed in a QuadroMACS separator (Miltenyi Biotec), and $CD4^+$ cells were depleted according to the manufacturer's instructions. CD4-depleted PBMC were washed once in cell culture medium, counted, and plated at $2 \times 10^5$ cells/well, together with various concentrations of FMDV-derived peptides. After 6 days of culture, [$^3$H]thymidine incorporation was determined as described above. The purity of CD4-depleted PBMC was analyzed by flow cytometry and compared to that of nondepleted PBMC. To do this, cells of both groups were stained with primary antibodies against CD4 and CD8β (clones IL-A11 and CC58; for staining procedures, see above), followed by incubation with isotype-specific secondary antibodies (anti-IgG2a–Alexa Fluor 647 [Invitrogen] and anti-IgG1–phycoerythrin [Southern Biotech]). Per sample, at least $2 \times 10^4$ live cells were analyzed.

**RT and locus-specific PCR amplification.** Total RNA was extracted from defrosted PBMC of each animal using Tri Reagent (Sigma) according to the manufacturer's instructions. For subsequent reverse transcription (RT)-PCRs, 200 ng RNA was subjected to the AccessQuick one-tube RT-PCR System (Promega, Madison, WI) for DRB3, DQA, and DQB using the primer sets listed in Table 1. The thermal-cycling parameters were 45°C for 45 min (cDNA synthesis step), followed by 95°C for 5 min, 35 cycles at 95°C for 30 s, the primer-specific annealing temperature ($T_A$) for 30 s, and 72°C for 2 min, with an extension of 72°C for 5 min, and finally 4°C. $T_A$s are given in Table 1. Positive PCR fragments were cloned using the pGEM-T Easy vector system (Promega, Madison, WI), and for each primer combination at each locus, at least 16 clones from each individual were subjected to automated sequencing with M13 standard sequencing primers (Eurofins MWG GmbH, Ebersberg, Germany).

**Sequence analysis.** DRB3 and DQA alleles were assigned by sequencing the entire conserved domain sequence region, whereas DQB alleles were identified by sequencing their exon 2 using the primer combinations listed in Table 1. The DRB3, DQA, and DQB sequences obtained were aligned with the program Clustal X 1.8 (34) with all known reference alleles from the IPD-MHC Database (http://www.ebi.ac.uk/ipd/mhc/bola/index.html; Cattle Sequences, release 1.1.0 18/02/2008). Based on these reference allele alignments, DRB3, DQA, and DQB alleles were determined for the animals studied (see Table 3).

**Statistical analysis.** For tritium incorporation assays and IFN-γ ELISPOT assays, means and standard deviations were calculated by using Microsoft Excel. Data from tritium incorporation assays were further analyzed for normal distribution by a Kolmogorov-Smirnov test. Thereafter, a one-sided Student's *t* test for dependent samples was performed. For both tests, SPSS software, version 14.0, was used.

## RESULTS

**In vitro reactivity to FMDV restimulation.** To investigate the presence of long-lived FMDV-specific memory T cells within PBMC from two FMDV-vaccinated and -infected cattle, proliferation and IFN-γ production against whole virus were analyzed in vitro. PBMC from both animals (C129 and C813) showed a strong dose-dependent proliferative response in tritium incorporation assays after homologous restimulation with FMDV A24 in comparison to mock controls or cells incubated in medium (Fig. 1A). This clear proliferative response against virus was observed over the entire duration

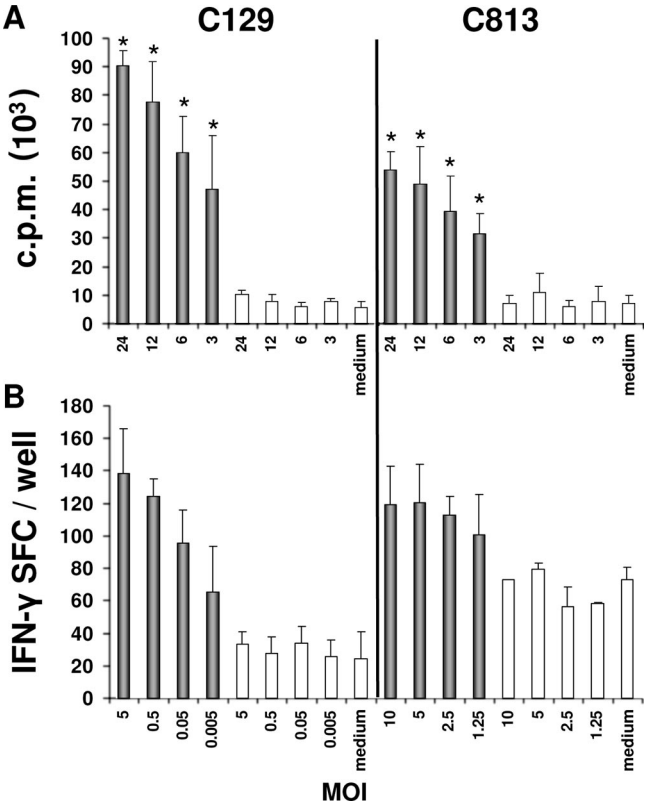



FIG. 1. Proliferative and IFN-γ responses of PBMC after in vitro restimulation with FMDV A24. PBMC from two FMDV-vaccinated and -challenged cattle (C129 and C813) were restimulated in vitro with homologous FMDV serotype A24 (gray bars) or supernatants of mock-infected control cultures serving as negative controls (white bars). Cells cultured only in cell culture medium (medium) also served as negative controls. MOIs were calculated based on the virus titers determined in cell suspension plaque tests and the numbers of cells used in the respective assays. (A) Proliferation of restimulated PBMC measured by [$^3$H]thymidine incorporation expressed as counts per minute. PBMC were seeded at $2 \times 10^5$ per well and incubated for 6 days. The results show mean values plus standard deviations of triplicate cultures and are representative of more than 15 experiments conducted over a period of 10 months. The asterisks indicate statistically significant differences ($P < 0.005$) between the responses of virus-stimulated cultures and the corresponding mock-stimulated cultures. (B) Frequencies of IFN-γ-producing cells in ELISPOT assays. PBMC were seeded at $5 \times 10^5$ per well and incubated for 24 h. The results are expressed as spot-forming cells (SFC) per well and show mean values plus standard deviations of duplicate cultures. The results are representative of five independent experiments. MOIs were selected to show the dose dependency of responses in each readout system (proliferation and IFN-γ production) and for each individual animal.

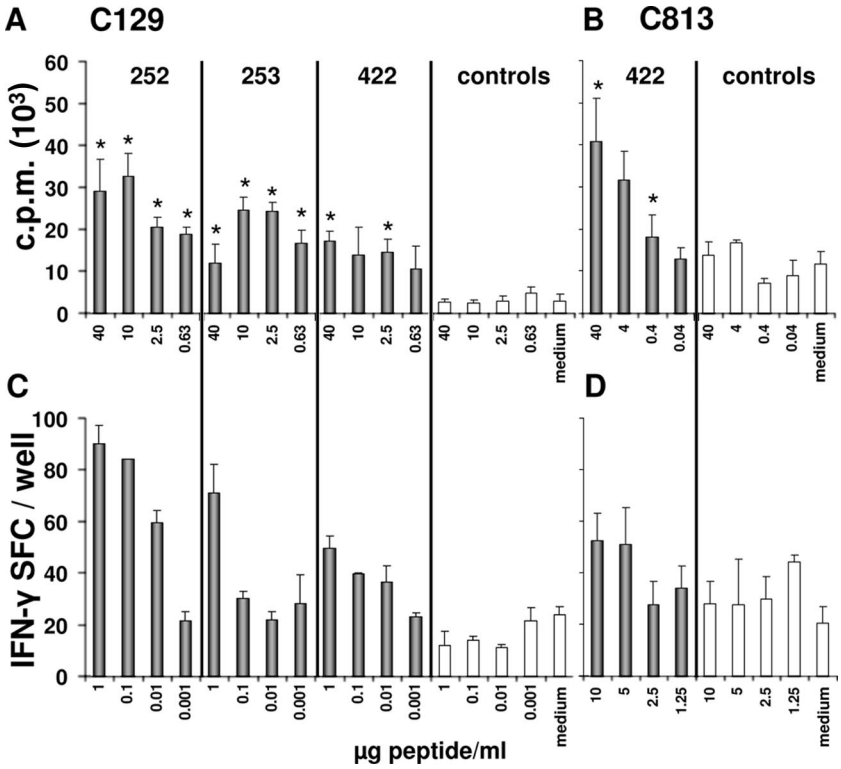



FIG. 2. Proliferative and IFN-γ responses of PBMC after in vitro restimulation with FMDV-derived peptides. (A) [$^3$H]thymidine incorporation of PBMC from animal C129 after stimulation with decreasing amounts of FMDV-derived peptides with internal numbers 252, 253, and 422 (gray bars). Cells incubated with a peptide derived from classical swine fever virus and incubated in medium alone served as negative controls (white bars). PBMC were seeded at $2 \times 10^5$ per well and incubated for 6 days. The results show mean values plus standard deviations of triplicate cultures. (B) Same as panel A, showing results for animal C813. The asterisks indicate statistically significant differences ($P < 0.005$) between responses of FMDV peptide-stimulated cultures and the corresponding cultures stimulated with control peptide. (C) Frequencies of IFN-γ-producing cells in ELISPOT assays. PBMC from animal C129 were seeded at $2 \times 10^5$ per well and stimulated with the same peptides as in panel A for 24 h. The results are expressed as spot-forming cells (SFC) per well and show mean values plus standard deviations of duplicate cultures. (D) Identical to panel C, showing results for animal C813. The results for [$^3$H]thymidine incorporation assays are representative of five experiments, and the results for IFN-γ ELISPOT assays are representative of three experiments. Peptide concentrations were selected to show the dose dependency of responses in each readout system (proliferation and IFN-γ production) and for each individual animal.

of the study, regardless of the time when cells were collected from infected cattle (26 weeks p.i. to 68 weeks p.i.) (data not shown). Also, the response was not influenced by cryopreservation of PBMC (data not shown).

In addition, IFN-γ production was investigated by ELISPOT assays after FMDV restimulation. Again, PBMC of animal C129 showed a dose-dependent response to virus in comparison to cells incubated in mock controls or medium alone (Fig. 1B). However, this response was less obvious for the second animal (C813), in which the frequency of IFN-γ-producing cells was already quite high after incubation in cell culture medium or mock controls and there was only a moderate increase when the cells were stimulated with FMDV (Fig. 1B).

**Identification of T-cell epitopes by pentadecapeptides.** Having identified a proliferative response of PBMC from both animals against whole virus, in the next step, 442 overlapping pentadecapeptides, representing the entire FMDV polyprotein, were tested in tritium incorporation assays with PBMC from both cattle. Among all the peptides tested, three peptides with the internal numbers 252, 253, and 422 caused a dose-dependent proliferation in PBMC from animal C129 (Fig. 2A). For animal C813, only one reactive peptide, number 422, was identified (Fig. 2B). FMDV-derived peptides that were classified as negative caused proliferation rates that were on the same level as those of control cultures (data not shown). To confirm the data achieved in tritium incorporation assays, peptides 252, 253, and 422 were also tested in IFN-γ ELISPOT assays (Fig. 2C and D). For animal C129, again, all three peptides caused an increase in the frequency of IFN-γ-producing cells compared to PBMC cultured in the presence of a control peptide (classical swine fever virus Core protein) (see Materials and Methods) or medium alone (Fig. 2C). Also, the magnitudes of the responses in the two readout systems (proliferation versus IFN-γ production) were similar, with peptide 252 causing the strongest proliferation/IFN-γ production whereas the neighboring peptide, 253, generated a somewhat lower response. The third peptide (422) induced lower responses in both assays than peptide 252. Furthermore, in animal C813, peptide 422 caused only a weak increase in the frequency of IFN-γ-producing cells (Fig. 2D), since spontaneous production of the cytokine in control cultures was relatively high, which is in accord with the results in Fig. 1B for this animal.

Table 2 provides more detailed information on the peptides

TABLE 2. Amino acid sequences and positions of peptides in FMDV proteins inducing IFN-γ response and lymphocyte proliferation in FMDV-vaccinated and -infected cattle

| FMDV protein | Peptide no. | Sequence | Residues | Recognized by animal no.: |
|---|---|---|---|---|
| 1A | 422 | INNYYMQQYQNSMDT | 22–36 | C129, C813 |
| 1D | 252 | LRAATYYFSDLEIVV | 66–80 | C129 |
| | 253 | YYFSDLEIVVRHEGN | 71–85 | C129 |
| C[a] | Negative control | KLEKALLAWAVIAIV | 81–95 | |

[a] Core protein of the classical swine fever virus.

that were recognized in the assays performed, including the amino acid sequences. Peptide 422, which was recognized by lymphocytes from both animals, represented aa 22 to 36 of the FMDV protein 1A (VP4). Peptides 252 and 253 overlapped by 10 aa and spanned residues 66 to 85 within the 1D (VP1) protein.

**Phenotype of cells proliferating in response to peptides.** Since pentadecapeptides were used for the identification of T-cell epitopes, one could assume that primarily $CD4^+$ T helper cells would recognize such peptides in the context of MHC class II molecules. However, in the past, 15-mer peptides were used successfully for the identification of $CD8^+$ T-cell epitopes (30, 32). In addition, cattle have a high frequency of TCR γδ T cells in blood circulation, and information about which antigenic structures are recognized by these cells is still rudimentary. Therefore, we performed CFSE proliferation assays with PBMC from both cattle in combination with staining of cell surface markers for T helper cells, cytolytic T cells, and TCR γδ T cells. This approach provided the possibility to investigate the phenotype of proliferating cells. For animal C129, peptides 252 and 422 were used for stimulation, representing the 1D and the 1A epitopes, respectively. PBMC from animal C813 were stimulated only with peptide 422, since no other stimulatory peptides had been identified for this animal (Fig. 2B). For both animals, the classical swine fever virus-derived peptide was used as a negative control. The results are shown in Fig. 3A, with gates on proliferating ($CFSE^{low}$), as well as nonproliferating ($CFSE^{high}$) cells of each T-cell subpopulation, together with the percentages of cells located in the respective gates.

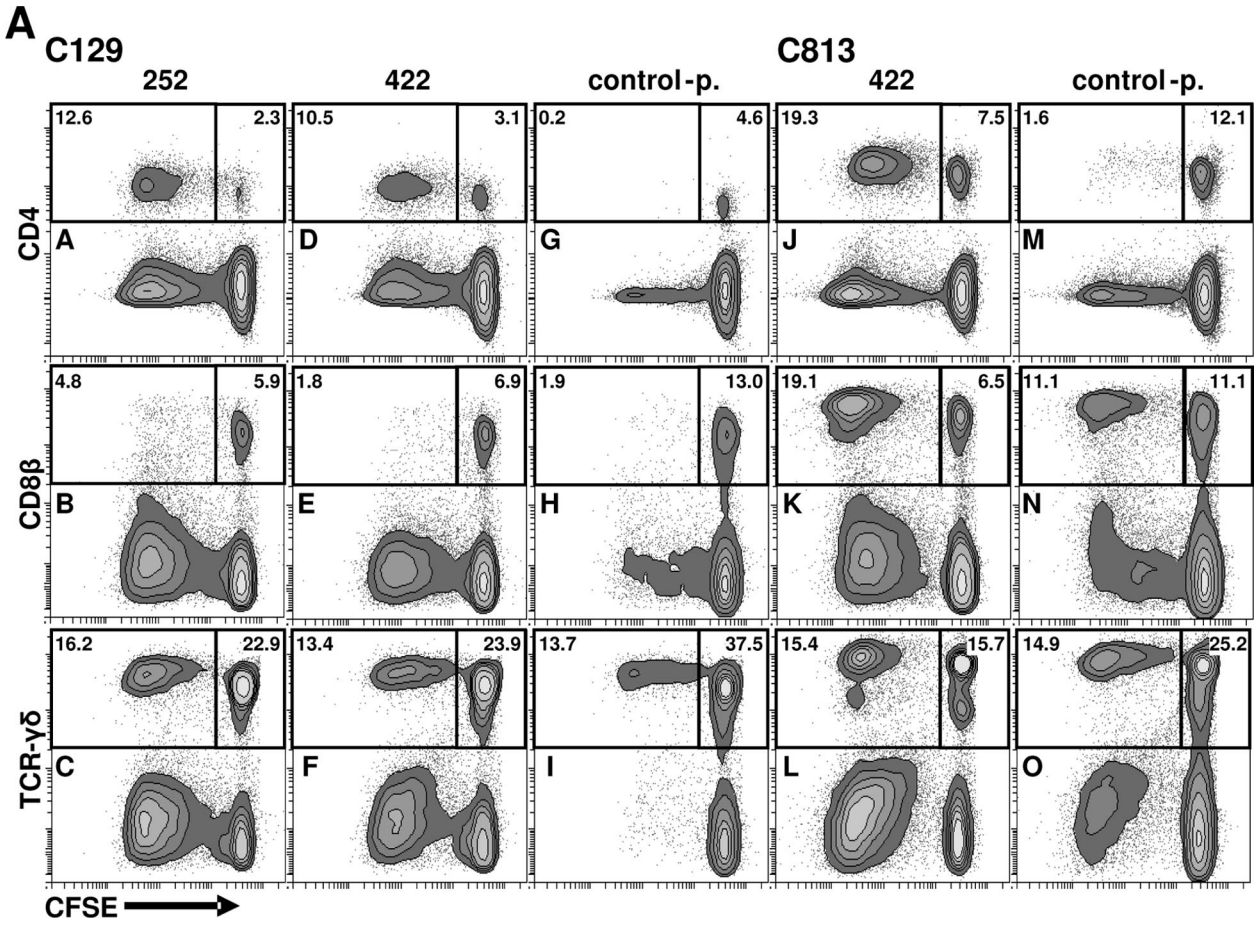
As shown in Fig. 3A, contour plots A and D, in PBMC from animal C129, the two FMDV peptides induced proliferation of 12.6% and 10.5% of $CD4^+$ T helper cells, respectively, compared to only 0.2% $CD4^+$ proliferating cells in samples incubated with control peptide (contour plot G). In contrast, samples incubated with MAb against CD8β for the identification of cytolytic T cells (contour plots B and E) showed that stimulation with peptide 252 led to only a slight increase in proliferating cells with this phenotype (4.8%) (contour plot B) compared to 1.9% in control samples (contour plot H). Stimulation with peptide 422 did not induce more $CD8\beta^+$ proliferating cells than in control samples (contour plot E versus H). Similar results were obtained for TCR γδ T cells, with the majority of cells with this phenotype being responsible for the observed background proliferation (13.7%) (contour plot I), whereas incubation with FMDV-derived peptides increased the proliferation of TCR γδ T cells only moderately (contour plot C) or not at all (contour plot F).

For animal C813 also, a strong increase in proliferation of $CD4^+$ T helper cells was observed after stimulation with peptide 422 (19.3%) (contour plot J) compared to cells stimulated with control peptide (1.6%) (contour plot M). Furthermore, a considerable fraction of $CD8\beta^+$ T cells proliferated in response to peptide 422 (19.1%) (contour plot K), but cells with this phenotype also proliferated strongly in control cultures (11.0%) (contour plot N). Equal amounts of proliferating TCR γδ T cells were observed in cultures stimulated with FMDV-derived peptide 422 and in control cultures (contour plot L versus O), which is similar to results from animal C129.

For a further identification of T-cell subpopulations showing high proliferation rates in response to a particular peptide, the quotients of proliferating divided by nonproliferating T-cell subpopulations were calculated (Fig. 3B). Quotients of >1 were considered indicators of a strong proliferation rate of the respective T-cell subset. The results in Fig. 3B show that $CD4^+$ T helper cells of both cattle responded strongly to stimulation with the FMDV-derived peptides. However, as mentioned above, $CD8\beta^+$ T cells of animal C813 also seemed to respond to peptide 422, as indicated by a proliferation quotient of 2.9. To address the question of whether this was a specific response or a bystander effect, we performed a MACS depletion of $CD4^+$ T helper cells with PBMC from animal C813. These CD4-depleted PBMC were stimulated with peptide 422, and their response was compared to that of nondepleted PBMC in tritium incorporation assays. The results, together with the purity of the CD4-depleted PBMC, are shown in Fig. 3C. As in previous experiments, PBMC from animal C813 showed a strong dose-dependent proliferative response after stimulation with peptide 422. In contrast, CD4-depleted PBMC (purity, >98%) had lost their responsiveness to peptide 422, with counts per minute reduced to the level of control cultures.

To further rule out a general immune-stimulatory capacity of peptide 422 that might have caused the collective response of $CD4^+$ T helper cells and $CD8\beta^+$ cytolytic T cells, we tested peptide 422 with PBMC derived from four FMDV-naïve cattle in tritium incorporation assays. In none of these experiments did peptide 422 cause a proliferation above the level of control cultures (data not shown). Therefore, in summary, these data suggested that $CD4^+$ T helper cells specifically responded to FMDV-derived peptides 252 and 422, whereas cytolytic T cells and TCR γδ T cells seemed to perform bystander or background proliferation.

**Identification of MHC anchor residues.** Having identified a strong response of $CD4^+$ T helper cells to the FMDV-derived peptides 252 and 422, we moved on to investigate which amino acids within the respective peptide sequences were important for binding in MHC class II molecules and, in turn, the observed T-cell responses. For this purpose, we synthesized peptides with alanine substitutions, with a single alanine residue introduced at every consecutive position (Ala-scan). Depending on the alanine residues already present in the original peptide sequence, this resulted in 13 peptides with alanine substitutions for peptide 252, 15 peptides with alanine substitutions for peptide 253, and 15 peptides with alanine substitutions for peptide 422. For animal C129, peptides with alanine substitutions were tested in IFN-γ ELISPOT assays and CFSE

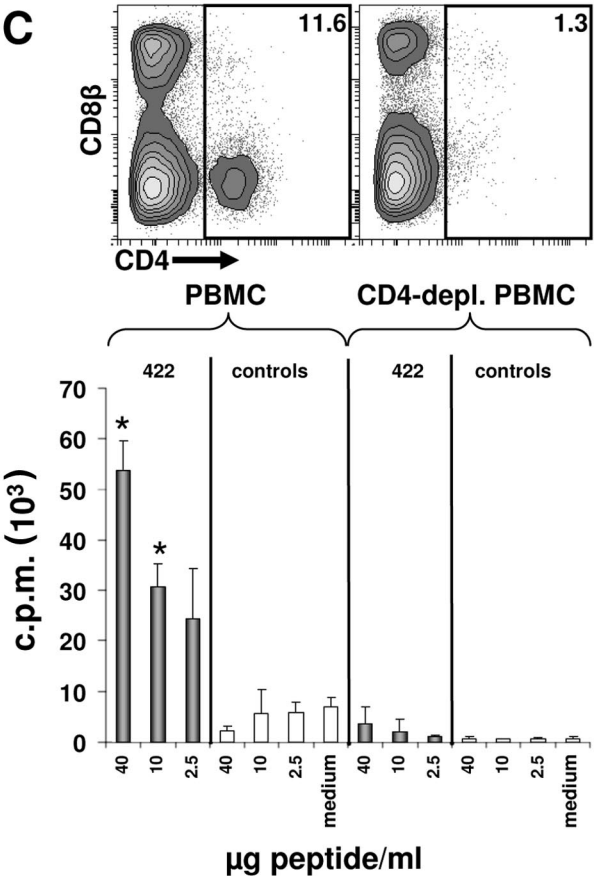



## B

Phenotype-specific proliferating/resting ratio

| C129 | | | | C813 | |
|---|---|---|---|---|---|
| | 252 | 422 | Control-p. | 422 | Control-p. |
| CD4 | 5.478 | 3.387 | 0.043 | 2.573 | 0.132 |
| CD8β | 0.814 | 0.261 | 0.146 | 2.938 | 1.000 |
| TCR-γδ | 0.707 | 0.561 | 0.365 | 0.981 | 0.591 |

## C



proliferation assays. The performances of these two readout systems are illustrated in Fig. 4A, showing representative wells of IFN-γ ELISPOT assays after stimulation with the unmodified peptide 252 and two peptides with alanine substitutions, with the inserted alanine residue at either position 13 (252-13) or position 11 (252-11). In addition, the results for these peptides in CFSE proliferation assays are shown, depicting either total CFSE proliferation (Fig. 4A, histograms) or proliferation of $CD4^+$ T cells (Fig. 4A, contour plots). Both methods showed that the peptide with alanine substitution 252-11 caused a strong decrease in the frequency of IFN-γ-producing cells, as well as a reduction in the overall proliferation rate, and especially proliferation of $CD4^+$ T helper cells. In contrast, for peptide 252-13, only a slight reduction in the frequency of IFN-γ-producing cells and no decrease in proliferation of total or $CD4^+$ T cells were observed. The entire set of results obtained with peptides with alanine substitutions for peptide 252 is shown in Fig. 4B. Both methodological approaches indicated that the amino acid residues at positions 6, 7, 8, 10, 11, and 12 were important for MHC binding, since their replacement by alanine residues caused a considerable reduction in IFN-γ-producing cells or proliferating $CD4^+$ T cells. However, this was not the case when the serine residue at position 9 was replaced by an alanine residue. These results were supported by data obtained by the use of peptides with alanine substitutions for the neighboring peptide 253. Figure 4C shows that the frequency of IFN-γ-producing cells was reduced when peptides with alanine residues at positions 1, 2, 3, 5, 6, and 7, which are equivalent to positions 6, 7, 8, 10, 11, and 12 within peptide 252, were used for stimulation. In contrast, the frequency of IFN-γ-producing cells was relatively unaffected when alanine residues were introduced at the remaining positions of peptide 253.

Since peptide 422 was recognized by T cells from both cattle, alanine substitutions in this peptide were tested with PBMC from both individuals. The results are summarized in Fig. 5. For animal C129, CFSE proliferation and IFN-γ ELISPOT assays were performed in parallel. For animal C813, only CFSE proliferation experiments were carried out due to high spontaneous production of IFN-γ in ELISPOT assays (see Fig. 1B and 2D). For animal C129, two peptides with alanine substitutions (422-05 and 422-09) caused a reduction in IFN-γ-producing cells, as well as proliferation of $CD4^+$ T cells, indicating that the tyrosine residues at positions 5 and 9 were important for MHC binding. The same peptides caused a strong drop in overall proliferation, as well as proliferation of $CD4^+$ T cells, in animal C813. However, for this animal, positions 3, 4, 7, and 9 also seemed to be important for MHC class II binding, since peptides bearing alanine residues at these positions also led to strong decreases in the proliferation of T helper cells.

**Identification of MHC class II alleles.** Having identified an epitope that was recognized by T cells from both cattle (peptide 422) and that also appeared to have a common anchor motif for MHC class II molecules (aa 5 and 9), we hypothesized that the two animals should have common MHC class II alleles. Therefore, we isolated mRNA from PBMC of both cattle; performed RT-PCR with primers specific for BoLA loci DRB3, DQA, and DQB; and cloned and sequenced the PCR products obtained. Alignment of the sequenced transcripts for BoLA DRB3 revealed the existence of two different alleles in animal C129 and one in C813, but the two cattle had no common allele for the locus (Table 3). For DQA, three different alleles were identified, with one of them being present in both animals (DQA*22021) (Table 3). Finally, sequence analyses of DQB transcripts led to the identification of six alleles, and again, one of them, DQB*1301, was shared by the two cattle (Table 3). Therefore, we concluded that both animals expressed one set of identical MHC class II DQ molecules that were encoded by DQA allele 22021 and DQB allele 1301 and that these DQ molecules were responsible for presentation of peptide 422.

## DISCUSSION

In the present study, we aimed to identify novel T-cell epitopes present within the entire polyprotein encoded by FMDV. Studies in the past used mainly synthetic peptides or protein fragments representing sequences of the structural proteins (1A, 1B, 1C, and 1D) (10, 11, 36, 37). With the exception of the 1A protein, which is relatively conserved, the disadvantage of T-cell epitopes identified in these regions in regard to vaccine improvement is the high sequence variability encountered between different FMDV serotypes (7). There-

FIG. 3. Identification of the phenotype of proliferating PBMC after stimulation with FMDV-derived peptides. (A) PBMC from animal C129 (contour plots A to I) were labeled with CFSE and stimulated with FMDV-derived peptides with internal numbers 252 and 422 (contour plots A to F). Cells incubated with a peptide derived from classical swine fever virus served as negative controls (control-p.) (contour plots G to I). After 6 days of culture, cells were collected and stained with MAb against CD4 (contour plots A, D, and G), CD8β (contour plots B, E, and H), and TCR γδ (contour plots C, F, and I). The same experimental setup was performed with PBMC from animal C813 (contour plots J to O) using peptide 422 (contour plots J, K, and L) and the negative control peptide (contour plots M, N, and O). The results are displayed in contour plots showing $2 \times 10^4$ analyzed cells. Marker-positive (CD4, CD8β, and TCR γδ) proliferating ($CFSE^{low}$) and nonproliferating ($CFSE^{high}$) cells were quantified; the percentages of the respective populations are displayed in the upper left and upper right corner of the contour plots. The results are representative of three experiments. (B) Quotients of the percentage of proliferating divided by the percentage nonproliferating T-cell subpopulations were calculated, and values of >1 are highlighted in gray boxes. (C) PBMC from animal C813 were depleted of $CD4^+$ T helper cells by MACS and tested in tritium incorporation assays in comparison to nondepleted PBMC after stimulation with FMDV-derived peptide 422 (gray bars). Cells incubated with a peptide derived from classical swine fever virus and incubated in medium alone served as negative controls (white bars). PBMC and CD4-depleted PBMC were seeded at $2 \times 10^5$ per well and incubated for 6 days. The results show mean values plus standard deviations of triplicate cultures. The asterisks indicate statistically significant differences ($P < 0.005$) between the responses of cultures containing PBMC stimulated with peptide 422 and the corresponding cultures containing CD4-depleted PBMC. Expression profiles of CD4 and CD8β staining of PBMC and CD4-depleted (depl.) PBMC are shown in contour plots to illustrate sorting efficiencies. The percentage of $CD4^+$ cells is indicated in the upper right corner of each contour plot.

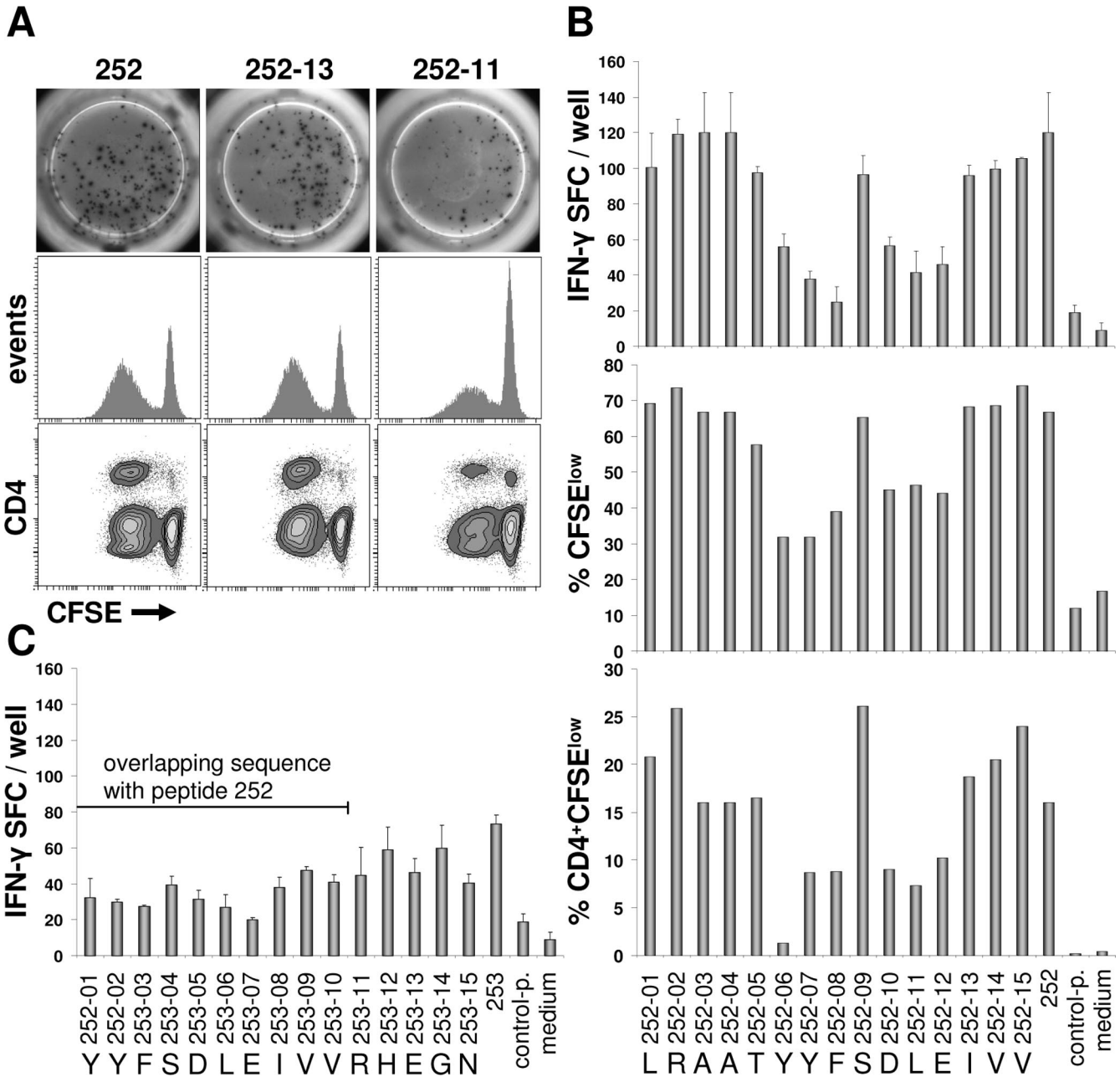



FIG. 4. Identification of MHC class II anchor motifs of FMDV-derived peptide 252. PBMC from animal C129 were incubated with peptides with alanine substitutions, with a single alanine residue introduced at every consecutive position of the original peptide 252. The response to these peptides was analyzed in IFN-γ ELISPOT and CFSE proliferation assays. (A) Assortment of raw data showing results for the original peptide 252 and peptides with an alanine introduced at position 13 (252-13) or position 11 (252-11). Representative wells of IFN-γ ELISPOT assays are shown, as well as histograms or contour plots from CFSE proliferation assays. (B) The number of IFN-γ-producing cells (spot-forming cells [SFC] per well) and the percentage of $CFSE^{low}$, as well as $CD4^{+}CFSE^{low}$, cells were analyzed and illustrated in bar diagrams showing the results for all peptides with alanine substitutions. The peptide concentrations in IFN-γ ELISPOT assays were 50 ng/ml; for CFSE proliferation assays, 500 ng/ml was used. The unmodified peptide 252, a peptide derived from classical swine fever virus (control-p.), and cells in culture medium alone (medium) served as controls and are depicted at the end of each bar diagram. The amino acid residues replaced by alanine are indicated below the peptide numbers. Since peptide 252 already contained in its original sequence two alanine residues at positions 3 and 4, for these positions, results from the original peptide 252 are shown. (C) Identical to panel B, but results for peptides with alanine substitutions of peptide 253 are shown. For these peptides, only IFN-γ ELISPOT assays were performed. The peptide concentrations were 500 ng/ml. The results for both sets of peptides are representative of two experiments.

fore, with the use of 305 overlapping peptides representing NSP, we tried to identify T-cell epitopes located in these conserved regions, but failed to do so.

For the identification of T-cell epitopes within NSP, the use of PBMC from cattle that had been vaccinated prior to infection may have been disadvantageous, since vaccination with inactivated virus initially primed the immune response to epitopes present within structural proteins. However, as out-

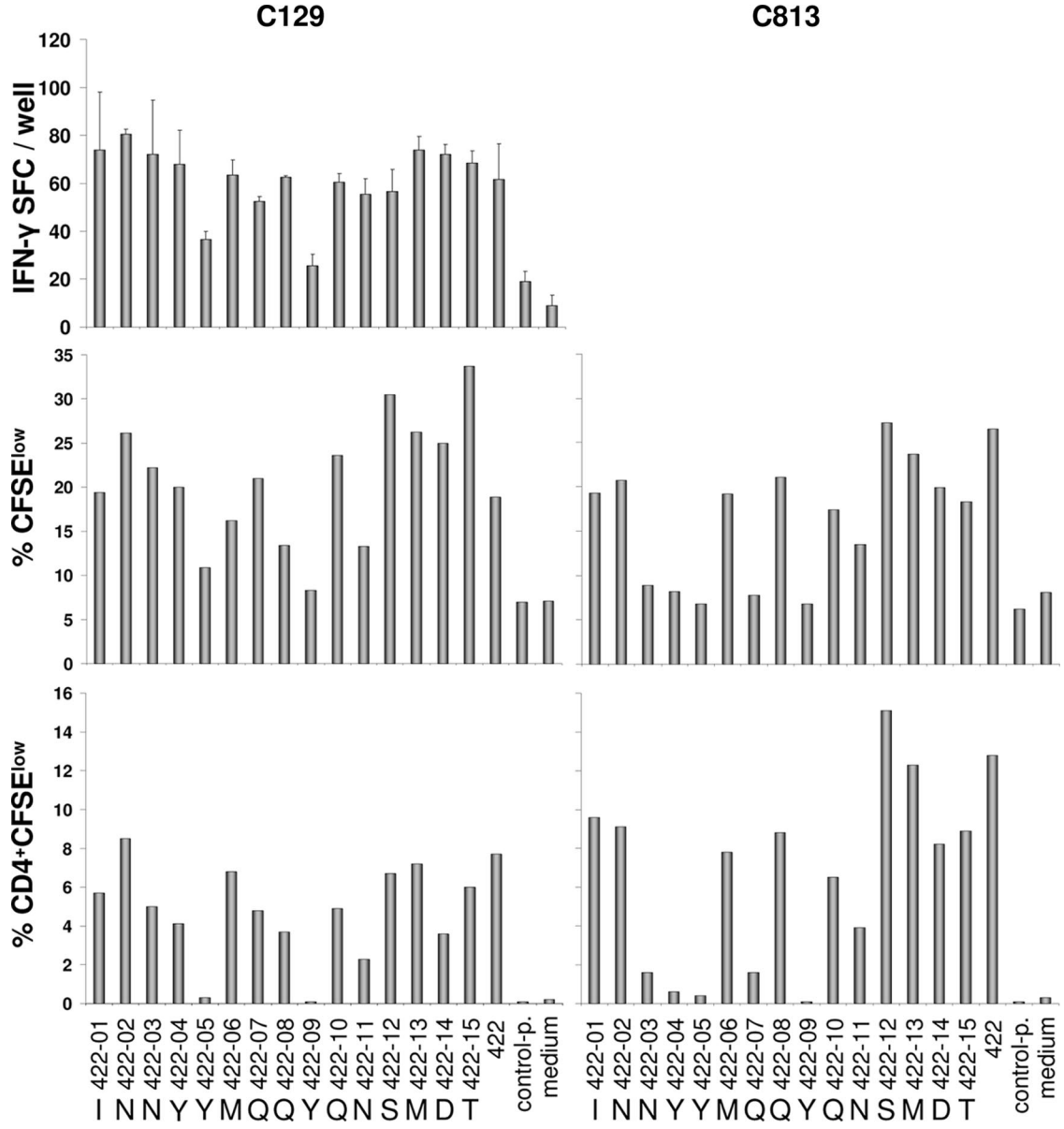



FIG. 5. Identification of MHC class II anchor motifs of FMDV-derived peptide 422. PBMC from cattle C129 and C813 were incubated with peptides with alanine substitutions, with a single alanine residue introduced at every consecutive position of the original peptide 422. The responses to these peptides were analyzed in IFN-γ ELISPOT and CFSE proliferation assays (for C813, only CFSE proliferation assays were performed). The number of IFN-γ-producing cells (spot-forming cells [SFC] per well) and the percentage of $CFSE^{low}$, as well as $CD4^{+}CFSE^{low}$, cells were analyzed and illustrated in bar diagrams showing the results for all peptides with alanine substitutions. The peptide concentrations in IFN-γ ELISPOT assays were 50 ng/ml, and for CFSE proliferation assays, 500 ng/ml was used (C813, 2 μg/ml). The unmodified peptide 422, a peptide derived from classical swine fever virus (control-p.), and cells in culture medium alone (medium) served as controls and are depicted at the end of each bar diagram. The amino acid residues replaced by alanine are indicated below the peptide numbers. The results are representative of two experiments for each animal.

lined in Materials and Methods, in both animals, FMDV replicated in the pharynx for 7 weeks p.i., which may have led to priming of new NSP-specific T-cell clones, at least in the local mucosal immune compartment. Furthermore, one of the animals (C813) (see Materials and Methods) transiently developed antibodies against linear B-cell epitopes present within NSP 3B. Since these antibodies were identified in serum, one could speculate that a systemic immune response of T cells against epitopes within NSP had occurred, at least in animal C813. In addition, experiments by Collen et al. with recombinant FMDV proteins identified NSP 3D as an immunodominant region for T cells derived from cattle that were either infected, vaccinated and challenged, or repeatedly vaccinated (8). An explanation for this could be the reported association of the 3D protein with FMDV particles (23). Furthermore, in studies by our group with pigs that were vaccinated and later infected with FMDV, T-cell epitopes within 3D could be readily detected (14).

One possible reason for the failure to identify T-cell epitopes within NSP could be the immunodominance of particular mem-

TABLE 3. Identified BoLA class II alleles from expressed loci

| Locus | Allele | Presence in animal no.[a]: | |
|---|---|---|---|
| | | C129 | C813 |
| DRB3 | 0501 | | + |
| | 0701 | + | |
| | 1601 | + | |
| DQA | 22021 | + | + |
| | 2801 | + | |
| | 10012 | | + |
| DQB | 0103 | + | |
| | 0601 | | + |
| | 1301 | + | + |
| | 2901 | | + |
| | 3601 | + | |
| | 10021 | | + |

[a] +, present.

ory T-cell clones remaining in vivo, which recognized the two epitopes from structural proteins that we identified. Indeed, an independent study by our group, using lymphocytes from cattle that were singly infected, failed to identify T-cell epitopes within NSP of FMDV (13). However, PBMC from one animal in this study responded by proliferation and IFN-γ production against two peptides derived from NSP 2C (representing aa 81 to 95 and 176 to 190, respectively) at 15 and 16 weeks p.i. Of note, this response was no longer detectable at 21 weeks p.i. Since we started in the present study with screening for peptide-recognizing T-cell clones 6 months postchallenge, effector T-cell clones recognizing T-cell epitopes within NSP may have been missed due to their inability (possibly due to insufficient TCR stimulation) to convert into the pool of long-lived memory cells.

Instead, two T-cell epitopes located within structural proteins were identified: peptide 252, representing aa 66 to 80 within protein 1D, and peptide 422, which represents aa 22 to 36 within protein 1A. The response to both peptides seemed to be MHC class II restricted, since a specific increase in proliferation was observed only for $CD4^+$ T helper cells (Fig. 3). The T-cell epitope located on protein 1D (66 to 80) was described recently by our group for cattle that had been infected with FMDV strain O/UKG/35/2001 (13). Also, in FMDV-vaccinated swine, an epitope with similar positions (aa 62 to 76; two out of seven investigated animals responded) was reported (27). In our study of cattle, we had investigated four inbred animals that were either heterozygous or homozygous for MHC class I serotype A31, and this class I haplotype has a linkage with the BoLA DRB3*0701 allele (9). Therefore, it is interesting that animal C129, which recognized the 1D (66 to 80) epitope also expressed allele DRB3*0701 (Table 3). Furthermore, as shown in Fig. 4, we investigated amino acid residues that were important for MHC binding and identified positions 6, 7, 8, 10, 11, and 12 as putative anchor residues. In our recent study (13), we used overlapping peptides based on the FMDV strain O/UKG/35/2001 (accession no. AJ539141) (21), since this strain had been used for infection of the animals. A comparison of the sequences of aa 66 to 80 within 1D of FMDV strain O/UKG/35/2001 and FMDV A24/Cruzeiro/Brazil/55 (used in the present study) revealed that the amino acid residues at positions 6, 7, 8, 10, 11, and 12 were conserved in both strains. Therefore, although no direct proof can be provided, our data suggest that the 1D (66 to 80) epitope is recognized in the context of the BoLA-DRB3*0701 allele.

The second epitope located on protein 1A (22 to 36) has also been described in earlier publications studying PBMC from FMDV-vaccinated cattle (35–37). Interestingly, this region was even recognized by PBMC from repeatedly FMDV-vaccinated pigs, and the study by Blanco et al. also demonstrated a specific response of MHC class II-restricted $CD4^+$ T helper cells (5). In reference 35, three BoLA MHC class II haplotypes were described as being associated with a response to the 1A (22 to 36) epitope: DH7A, DH8A, and DH18A. In that study, the authors did not investigate whether DR or DQ molecules encoded by the respective haplotypes were responsible for MHC class II presentation of the 1A epitope. However, in a subsequent study by Haghparast et al. (17), MHC class II peptide-binding assays were performed to identify single DRB molecules that bind peptides representing the epitope. These assays revealed that BoLA DR molecules encoded by the alleles DRB3*0201 and DRB3*1201 bind peptides representing sequences of 1A (20 to 34) and 1A (22 to 36), respectively. These two DRB3 alleles are represented within haplotypes DH7A and DH8A (17); the third haplotype (DH18A) (35) was not investigated.

The BoLA class II alleles identified in the cattle used in this study did not indicate that the 1A (22 to 36) epitope was recognized in the context of DR molecules, since no common DRB3 alleles were identified (Table 3). Instead, we found two common DQA alleles and DQB alleles. Together with the mutual binding motif (tyrosine residues at positions 5 and 9) (Fig. 5), this strongly suggests that peptide 422 was recognized in the context of MHC class II molecules encoded by DQA allele 22021 and DQB allele 1301 (summarized in Fig. 6). This is supported by recent publications showing that DQ molecules in cattle are relevant for peptide presentation to T helper cells in immune responses against FMDV-derived peptides (15), *Babesia bovis* (26), and *Anaplasma marginale* (6). Furthermore,

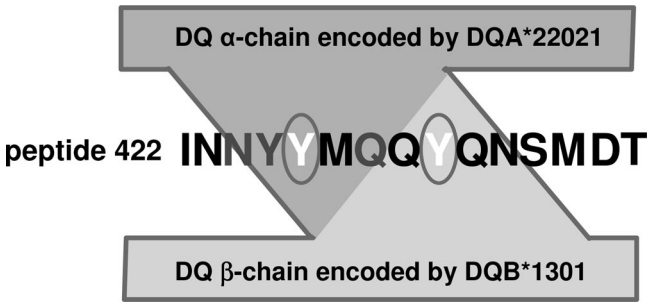



FIG. 6. Model of an MHC class II DQ molecule presenting peptide 422 from FMDV protein 1A. Analyses performed in this study revealed that PBMC from both cattle responded to peptide 422, expressed the DQA allele 22021 and the DQB allele 1301, and showed decreased proliferation when the tyrosine residues at positions 5 and 9 were replaced by alanine. Therefore, a model is presented with an MHC class II molecule encoded by DQA*22021 for the α-chain and DQB*1301 for the β-chain. Within this class II molecule, the sequence of peptide 422 is shown with the two tyrosine residues that were important for MHC binding in both cattle highlighted. Further amino acid residues that were important for binding in animal 813 (N at position 3, Y at position 4, and Q at position 7) are indicated in gray.

it was demonstrated that interhaplotype and intrahaplotype pairing of DQA and DQB molecules can occur in the priming of bovine $CD4^+$ T helper cells (24). The existence of three further amino acid residues with putative anchor functions, identified when peptide 422 was tested with PBMC from animal C813 (positions 3, 4, and 7) (Fig. 5), might indicate that for this animal, a second MHC class II molecule was able to present the peptide. However, none of the 1A (22 to 36) epitope-presenting DRB3 alleles identified by Haghparast et al. (17) was found in animal C813.

Nevertheless, our data provide knowledge of sequences and antigenic peptides required for the development of class II tetramers, as was done recently for *A. marginale*-derived epitopes (25). The development and use of such tetramers should enhance our knowledge of the quantity of T cells recognizing the 1A (22 to 36) epitope within cattle of particular haplotypes and could lead to the identification of further correlates of protection against FMDV infection.

In summary, our data expand the still-fragmentary knowledge of the cellular immune responses following FMDV vaccination and infection. Obviously, of the T helper cell epitopes recognized during an immune response in cattle, only a few lead to the establishment of long-lived memory T-cell clones, and for unknown reasons, these clones seem to recognize epitopes within structural proteins rather than NSP of the virus. Also, by comparing our data with earlier publications (17, 35–37), it becomes obvious that the epitope 1A (22 to 36) is recognized in a wide variety of BoLA class II haplotypes and should therefore be considered a promising candidate for novel subunit vaccines in cattle.

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