Differential host response to a herpesvirus: Marek's disease virus peptides on chicken MHC class II molecules are derived from only a few genes and illustrate a new mode of peptide binding

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Short title: Only a few MDV peptides bound to MHC class II molecules

30 Abstract

31 Viral diseases pose major threats to humans and other animals, including the billions of
 32 chickens that are an important food source as well as a public health concern due to
 33 zoonotic pathogens. Unlike humans and other typical mammals, the major
 34 histocompatibility complex (MHC) of chickens can confer decisive resistance or
 35 susceptibility to many viral diseases. An iconic example is Marek's disease, caused by an
 36 oncogenic herpesvirus with over 100 genes. Classical MHC class I and class II molecules
 37 present antigenic peptides to T lymphocytes, and it has been hard to understand how such
 38 MHC molecules could be involved in susceptibility to Marek's disease, given the potential
 39 number of peptides from over 100 genes. We used a new *in vitro* infection system and
 40 immunopectidomics to determine peptide motifs for the two class II molecules expressed
 41 by the MHC haplotype B2, which is known to confer resistance to Marek's disease.
 42 Surprisingly, we found that the vast majority of viral peptide epitopes presented by chicken
 43 class II molecules arise from only four viral genes, nearly all having the peptide motif for
 44 BL2, the dominantly-expressed class II molecule in chickens. We expressed BL2 linked to
 45 several MDV peptides, and determined one X-ray crystal structure, showing how a single
 46 small amino acid in the binding site causes a crinkle in the peptide, leading to core binding
 47 peptide of ten amino acids, compared to the nine amino acids in all other reported class II
 48 molecules. The limited number of potential T cell epitopes from such a complex virus can
 49 explain the differential MHC-determined resistance to MDV, but raises questions of
 50 mechanism and opportunities for vaccine targets in this important food species, as well as
 51 providing a basis for understanding class II molecules in other species including humans.

Introduction

The ongoing global pandemic of a coronavirus among humans highlights the enormous challenge of viral disease, and the importance of the appropriate immune responses [1-3]. The classical class I and class II molecules of the major histocompatibility complex (MHC) play crucial roles in resistance to infection and response to vaccines, binding peptides for presentation to thymus-derived (T) lymphocytes of the adaptive immune system as well as natural killer (NK) cells of the innate immune system [4, 5]. The importance of the classical MHC molecules is underscored by their high level of allelic polymorphism, which is mostly driven by molecular arms races with infectious pathogens [5]. However, the MHC of humans and typical mammals is an enormous and complex genetic region encoding a wide variety of molecules, with multigene families encoding class I and class II molecules leading to strong genetic associations with autoimmune disease but relatively weak associations with infectious diseases [4].

In contrast to humans and other typical mammals, the level of resistance to many infectious pathogens in chickens can be strongly determined by the MHC (that is, the BF-BL region of the B locus), at least in part because the chicken MHC is much simpler, with single dominantly-expressed class I and class II loci [6, 7]. As a result of this simplicity, the phenotypes are much clearer: either the dominantly-expressed MHC allele finds a protective peptide to confer resistance or not, leading to strong genetic associations that are easier to discover and dissect [7]. Moreover, the scale of viral challenges in the poultry industry has been clear for decades, with many tens of billions of chickens each year beset by a wide variety of poultry viruses [6], including the first coronavirus ever described as such [8]. On top of the economic importance, zoonotic pathogens (including avian influenza) have been a continuing concern for public health [9, 10]. Despite enormous efforts in biosecurity, vaccination and genetic breeding, condemnation and slaughter of huge numbers of infected chickens are relatively frequent [11].

Among the economically-important diseases for which the chicken MHC is known to determine resistance and susceptibility, Marek's disease (MD) caused by an oncogenic herpesvirus was the first reported, is the best studied and remains an enormous burden for the poultry industry, with continuing outbreaks despite routine vaccination [12-16]. Indeed, current vaccines control disease but not transmission, leading to selection of more virulent strains, which in turn have required more efficacious vaccines [17, 18]. The virus responsible for MD (MDV), in common with other herpesviruses, has a relatively large genome with over 100 genes and a complex life cycle, so it is possible that many genes contribute to resistance at different stages of infection, tumor growth and transmission [14, 16]. Several polymorphic genes located in the MHC have been proposed as candidates to determine MD resistance, including the dominantly-expressed classical class I gene (BF2), an NK receptor gene (B-NK), a gene with some similarities to mammalian butyrophilins (BG1) and the classical class II B genes (BLB1 and BLB2) [19-25]. In comparison to the MHC class I system, very little attention has been focused on chicken class II genes and molecules [7, 26].

Mammalian class II molecules have been intensively studied, so many structural and functional features are known in detail [27-30]. The heterodimer is composed of an α and a β chain, each with two extracellular domains: membrane proximal immunoglobulin C-like (Ig C) domains ($\alpha 2$ and $\beta 2$ domains) and membrane distal domains composed of four β -

strands forming a β -sheet surmounted by predominantly α -helical stretches ($\alpha 1$ and $\beta 1$ domains). The domain organization, including the features of secondary structure and location of intra-domain disulfide bonds, is similar to class I molecules. The membrane distal domains together form a peptide-binding super-domain, with a core sequence from the peptide binding across a groove, the T cell receptor recognizing the top, and the dedicated chaperone DM interacting with key residues on the side for loading of appropriate peptides. A glycosylation site near the end of the $\alpha 1$ domain is likely to be involved in quality control during biosynthesis and peptide loading (as in class I, [31]), while a loop in the $\beta 2$ domain and part of the $\alpha 2$ domain bind the CD4 co-receptor that contributes to T cell signaling (in the same place as CD8 interacts with class I, [30, 32]).

As in human HLA-DR molecules, the chicken α -chain (encoded by the BLA gene) is nearly monomorphic, with most residues on the top of the $\alpha 1$ domain identical with DRA (although there is dimorphic position in the α -helix pointing up towards the T cells, and a four amino acid insertion in one loop of the β -sheet) [33]. Virtually all of the variation responsible for allelic polymorphism and thus for different peptide-binding specificities in different chicken class II molecules is located in the $\beta 1$ domain of the β chain, encoded by either the BLB1 or BLB2 gene [34, 35].

Differential immune responses by classical MHC molecules have typically been understood as MHC molecules either presenting a peptide that confers an effective immune response or not. However, a major concern is how classical MHC molecules could confer susceptibility to a virus such as MDV, since it is hard to imagine how a peptide conferring protection would not be found among 100 viral molecules [19]. This conundrum is particularly an issue for class II molecules for which the peptide motifs are relatively promiscuous compared to class I molecules [28]. A significant barrier to examining this question has been the low frequency of cells infected with MDV within chickens before tumors arise.

In this report, we use a novel culture system for bursal B cells [36], followed by determination of the peptides bound to class II molecules by mass spectrometry (so-called immunopeptidomics or MHC ligandomics, [37, 38]). We identify the peptides bound to class II molecules from the well-characterized MHC haplotype B2 known to confer resistance to MD [13, 39], with and without infection by the very virulent MDV strain RB-1B (also known as RB1B, 40) and the live attenuated virus strain CVI988 (widely used as the Rispens vaccine, [41]). We then express the chicken class II molecule BL2*02 with several dominant pathogen peptides and determine the structure of one such complex by X-ray crystallography to examine whether there are unusual molecular features that might help explain the biology of resistance.

Results

Peptides from two class II molecules were found in bursal B cells from the B2 MHC haplotype

After one day in culture with and without infection, *ex vivo* bursal cells from the M11 chicken line were harvested, MHC molecules were isolated from detergent lysates by affinity chromatography, and the eluted peptides were analyzed by mass spectrometry. In experiments over three years, one uninfected sample, two samples infected with the live attenuated virus strain CVI988 and three samples infected with the very virulent MDV strain RB1B were analyzed.

Several thousand peptides were identified for each infected sample, with between 36% and 84% of a particular sample shared with other samples (Fig S1, Datasets S1-S6). Since rare peptides may not be sampled in repeated analyses, the high level of overlap between analyses gives confidence that most abundant peptides were likely to have been identified.

Overall, the sequences have the features typical of peptides bound by mammalian class II molecules, including an enrichment of proline around position 2 (due to the cleavage preference for aminopeptidases) and a broad distribution of length from 12 to more than 25, with a peak around 16 to 17 amino acids (Fig S2, Datasets S1-S6). Initial analysis by Gibbs clustering identified two nonamer (9mer) core motifs in all samples, but subsequent structural evidence (detailed below) led to a re-analysis that showed one of these motifs to be a decamer (10mer) (Fig 1A). The two motifs turn out to represent the two classical class II molecules in chickens, BL1 and BL2, encoded by a monomorphic DRA-like A gene (BLA) and two polymorphic classical class II B genes (BLB1 and BLB2). At the RNA level, BLB1 is poorly expressed except in the intestine, while BLB2 is well expressed systemically [26]. However, the ratio of peptides from these *ex vivo* bursal B cells was around 40% nonamer and 60% decamer in all samples (Datasets S1-S6).

Fig 1. Two peptide motifs are found for class II molecules from the MHC B2 haplotype, one of which is a decamer for which peptides lead to expressed class II molecules with high thermal stability. (A) peptide motifs determined by Gibbs clustering: upper panel with default length set to 9mer, lower panel with default length set to 10mer. (B) thermal denaturation curve for BL2 molecules expressed with three peptides that have decamer motifs (GVLFYMPTSHVQQMTF from gH with melting temperature 69°C, QIESLSLNGVPNIFLSTKA from gE with 75°C, SSEVLTSIGKPAQFIFA from UL43 tegument protein with 78°C); the peptide TPSDVIEKELMEKLKKK from gI with a nonamer motif did not express.

Four MDV genes gave rise to most of the pathogen peptides bound to class II molecules from bursal B cells, with most bound to BL2 molecules

Out of the thousands of peptides characterized, 23 epitopes from 17 MDV genes were identified, but most were found only in one sample and/or as only one species of peptide (Fig 2, Table 1, Table S1). However, multiple species of a single epitope and/or multiple peptide epitopes were found in several infected samples for four MDV proteins: glycoprotein H (gH, encoded by the MDV034 gene), UL43 tegument protein (MDV056), gI (MDV095) and gE (MDV096). Five peptide epitopes were found for gH, two of which had multiple species, of which one was found in all five samples. The UL43 tegument protein had one peptide epitope with 16 species, some of which were found in two to four samples. For gI, one peptide epitope was found with three species, one of which was found in four samples. For gE, two peptides were found, one of which had 18 species, four of which were found in all five samples.

Most of these peptide species had the decamer motif, including those from three of the four MDV proteins with abundant peptides. From the analysis by Gibbs clustering, eight peptide species from five epitopes fit the nonamer BLB1 motif, 45 species from ten epitopes fit the decamer BLB2 motif, nine species from six epitopes fit both motifs (and thus might actually contain two epitopes), and two species each from one epitope didn't fit either motif (Table 1).

Soluble BL2 molecules from the B2 haplotype were expressed in insect cells using Baculovirus, with the peptide attached via a linker to the BLB2 chain [it should be noted that, in contrast to previous reports [34, 42, 43], the N-terminal positions of the BLA and BLB chains are likely identical to those of HLA-DR (Fig S3)]. Confirming the assignments based on motifs, three peptide species with decamer motifs linked to BL2 molecules were successfully expressed, and the complexes had high thermal stabilities (Fig 1B). However, the one peptide with a nonamer motif that was tested did not produce a soluble complex with BL2, and therefore is likely to have been bound to a BL1 molecule on chicken bursal B cells.

Fig 2. Only four MDV proteins give rise to most peptides bound to class II molecules from infected chicken bursal B cells in culture. Thin horizontal line indicates MDV genome of roughly 180 kB, with relevant MDV genes indicated by black boxes labelled with the number of the gene (for instance, MDV022) and with arrowheads indicating class II-bound peptides, above the line for genes and peptides oriented from left to right and below the line for right to left. Each vertical stack of arrowheads indicates a single T cell epitope with multiple peptide species (with the protein name next to the stack), each arrowhead colored according to the number of experimental samples in which the peptide species was found (blue, 1; green, 2; yellow, 3; orange, 4; red, 5).

Table 1. Peptides from class II molecules of bursal B cells infected in vitro proteins and peptides in order of the genomic sequence; blue, green indicate multiple species from same peptide location; soluble BL linked with BLB2: * expressed, ** not expressed; peptide species found in particular experiment indicated by 1.

gene	protein	peptide species	BLB motif	2018	RB-1B 2019	2020	CVI988 2019	2020
MDV022/UL10	gM	KQRGSQSEDERALTQSRSAE	neither					1
MDV025/UL13		IEKTFMDLGKAVVFLNVS	2					1
		IGDFSLALLNTNSTILK	2				1	1
MDV030/UL18	TRX2	GTEPDTLSLLSTFKTRFAAVIT	2		1		1	
MDV031/UL19	MCP	ISAEVLITIGDKLIFLES	2		1		1	
MDV033/UL21		KIGLVLRRGGQS	1					1
MDV034/UL22	gH	SPANGVLFYMPTSHVQQMTF	2					1
		GVLFYMPTSHVQQMTF	2*	1	1	1	1	1
		GVLFYMPTSHVQQMT	2	1				1
		GVLFYMPTSHVQQM	2	1				1
		RPVSKLLASNNLIKFLNTG	2					1
		ELKNLKPIDVVNPEHRFILT	both					1
		ELKNLKPIDVVNPEHRFI	both					1
		NLKPIDVVNPEHRFILT	both					1
		YDFQIAQTHAQLFI	2					1
		TALLVLPISGLGSYVVTRQ	2				1	1
MDV040/UL27	gB	RNQLHELKFDINKVIE	both					1
MDV046/UL32		LKHAIKGGTSAE	both					1
MDV056/UL43		SSEVLTSIGKPAQFIFAL	2		1		1	1
		SSEVLTSIGKPAQFIFA	2*	1	1		1	1
		SSEVLTSIGKPAQFIF	2		1		1	1
		SSEVLTSIGKPAQFI	2	1			1	1
		SSEVLTSIGKPAQF	2					1
		SEVLTSIGKPAQFIFAL	2				1	1
		SEVLTSIGKPAQFIFA	2					1
		SEVLTSIGKPAQFIF	2					1
		SEVLTSIGKPAQFI	2					1
		EVLTSIGKPAQFIFAL	2					1
		EVLTSIGKPAQFIFA	2					1
		EVLTSIGKPAQFIF	2					1
		EVLTSIGKPAQFI	2					1
		EVLTSIGKPAQF	2					1
		VLTSIGKPAQFIFAL	2					1
		VLTSIGKPAQFIFA	2					1
MDV057/UL44	gC	GYmQVImRDHFNRL	1				1	
MDV062/UL49	VP22	TNEELDAFLSRAVIKIT	1					1
MDV066/UL52		VEFCLLNQmASGm	neither					1
MDV070/UL55		EPVPHIGSITSRSYLLR	both					1
MDV078	viL-8	TEIIFALKKNRKV	both					1
MDV082		SDEPHAPGTTmISGPRTQ	1				1	
		TTmISGPRTQ	1				1	
MDV095/US7	gl	TPSDVIEKELMEKLKKKVE	1					1
		TPSDVIEKELMEKLKKK	1**	1		1	1	1
		TPSDVIEKELMEKLKK	1					1
MDV096/US8	gE	IKQIESLSLNGVPNIFLSTKA	2					1
		KQIESLSLNGVPNIFLSTKA	2					1
		QIESLSLNGVPNIFLSTKASN	2			1	1	1
		QIESLSLNGVPNIFLSTKASN	2					1
		QIESLSLNGVPNIFLSTKAS	2					1
		QIESLSLNGVPNIFLSTKA	2*	1	1	1	1	1
		QIESLSLNGVPNIFLSTK	2	1	1	1	1	1
		QIESLSLNGVPNIFLS	2				1	
		QIESLSLNGVPNIFL	2				1	
		IESLSLNGVPNIFLSTKASN	2					1
		IESLSLNGVPNIFLSTKA	2	1	1	1	1	1
		IESLSLNGVPNIFLSTK	2	1	1	1	1	1
		IESLSLNGVPNIFLST	2	1	1		1	1
		IESLSLNGVPNIFLS	2				1	
		IESLSLNGVPNIFL	2				1	
		ESLSLNGVPNIFLSTKA	2					1
		ESLSLNGVPNIFLSTK	2					1
		SLSLNGVPNIFLSTKA	2				1	
		LSLKNFKALVYHVG	both					1
		SLKNFKALVYHVGDTI	both					1

The overall structure of a chicken class II molecule is similar to those of mammals

Of the four soluble BL2 molecules from the B2 haplotype linked to MDV peptides that were expressed, the one with a peptide from gE formed crystals that diffracted to sufficiently high resolution to determine a structure (Fig 3A-C, Table S2). As this work was being written up for publication, a paper was published with the crystal structure of a ribosomal peptide in complex with a soluble BL2 molecule from B19 [43], an MHC haplotype known to confer susceptibility to MD [13, 44].

Overall, the structure of BL2 from B2 (BL2*02) is extremely similar to BL2 from B19 (BL2*19) and to the iconic mammalian class II molecule HLA-DR1 (Fig 3D, E), with root mean square deviations for C α atoms of 0.684 and 0.878 Å, respectively (Table S3). Most of the key residues found in mammalian class II molecules are identical in these three structures and found in nearly identical conformations, including those involved in domain folding (disulfide bonds and glycosylation site) and in inter-domain, DM and CD4 interactions (Fig 3E, Fig S4, Table S4). However, both chicken molecules have a four amino acid extension to the first loop and second β -strand of the α chain compared to HLA-DR1 (Fig 3D, [33, 43]).

Fig 3. The structure of the chicken BL2*02 molecule with peptide QIESLSLNGVNPINFLSTKA from gE is very similar to the BL2*19 molecule and the human DR1*01 molecule. (A) two side views of BL2*02 with peptide in ribbons; α chain and peptide, slate blue; β chain, deep salmon red. (B) two side views of BL2*02 with peptide as solvent-accessible surface calculated by APBS electrostatics (positive charge, blue; negative charge, red). (C) Top view and side view (through β 1 domain omitted for clarity) of BL2*02 in ribbons and peptide in stick as a composite omit ($2F_o - F_c$) map contoured at 1 Å in blue chicken wire. (D) top view of C α backbone of class II molecule with peptides superimposed, α 1 domain above and β 1 domain below. Left panel: BL2*02, slate blue; DR1*01, grey; dotted circle indicates loop with four amino acid insert in chicken class II molecules. Right panel: BL2*02, slate blue; BL2*19, grey with peptide in yellow. (E) top view of a portion of the class II molecules superimposed in ribbons, α 1 domain above and β 1 domain below, with key residues [W α 47(43), E α 59(55), F β 89(89)] in sticks to show differences (including 3₁₀ to α -helix) upon DM binding. Left panel: BL2*02, slate blue; DR1*01, grey. Right panel: DR1*01 (PDB accession 4X5W), white; DR1*01 in DM co-crystal (4GBX), green; red arrows indicate major structural movements.

BL2 from B2 illustrates a new mode of peptide binding for MHC class II molecules

The way in which peptides bind to mammalian class II molecules has been studied in detail, showing that a nonamer core binds as a polyproline II helix, with main chain atoms forming hydrogen bonds (H-bonds) with the MHC molecule and with three or four side chains binding into pockets that determine the specificity of binding between MHC alleles [27, 28]. Typically the bound peptides extend from the N- and C-terminal of the core, permitted by an arginine found in all class II molecules and in the classical class I molecules of non-mammalian vertebrates and with these flanking regions sometimes assuming stable structures that can be recognized by T cells [27, 45-47]. In fact, the P10 side chain of a peptide with a nonamer core has been reported [48] in one structure to bind onto a polymorphic shelf outside of the groove (Fig S5).

In stark contrast to all reported mammalian class II structures and to BL2*19, the BL2*02 has a decamer core, with the structure showing side chains at peptide positions P1, P4, P5, P8 and P10 of the core binding into pockets, so that the peptides that were first analyzed with a nonamer motif were reanalyzed for this peptide motif (Figs 2A, 4A,B); the decamer sequence predicted by Gibbs clustering corresponded to the core sequence found in the structure. Compared to mammals and to BL2*19, the peptide in BL2*02 shifts the residues pointing up from the core towards the T cell from the typical P2, P3, P4 or P5 and P8 to P2, P3, P6 and P9, shifting register at P4 (Fig 4B). The class II residues that form H-bonds with the main chain atoms of the peptide were originally described for DR1 [27] and are identical in chickens (except for chicken Nα80 and Qβ57 which are R and D in humans, and Iβ71 in BL2*02 which is R in the other two structures), but again BL2*02 shifts register at P4, with subsequent interactions occurring one peptide position after DR1 and BL2*19 (Fig 4D). However, current analyses of such bonding are more refined, with some residues interacting through waters and other polymorphic residues contributing to main chain peptide interactions (Figs S6-S8)

The conformation of the peptide bound to BL2*02 shows a polyproline II helix as in mammals, but deviates downwards just at P4 and then returns to the helical conformation (Fig 4C). Comparing the peptides from the side view confirms that it is peptide position P4 that is located lower in the groove than in other reported class II peptides (Figs 3D, 4B,E,F). This “crinkle” in the peptide is due to a single BLB residue Serβ78 in BL2*02 that leads to a deeper pocket compared to Pheβ78 from BL2*19 and Tyrβ78 from DRB1, and which pulls P4 downwards and pulls the main chain register towards the N-terminus of the peptide, resulting in the decamer core (Fig 4B,E). Comparison of the contacts around P4 shows that this relatively small residue Serβ78 accommodates the peptide in the conformation adopted in BL2*02, while larger residues Tyrβ78 in DR1 and Pheβ78 in BL2*19 would conflict (Fig 4F).

Fig 4. Although similar in overall structure to other class II molecules, BL2*02 binds peptides with a decamer motif due to Serβ78. A-C. top panels, BL2*02 (PDB accession 6T3Y); middle panels, BL2*19 (6KVM); bottom panels, DR1*01 (1DLH). (A) top view of solvent-accessible surface of class II molecules calculated by APBS electrostatics (positive charge, blue; negative charge, red) with P1, P9 and P10 pockets indicated, and with peptides in sticks. (B) side view of peptide in sticks with amino acids indicated and with pockets as surfaces with P1, P9 and P10 pockets indicated, and with length between Cα of P1 and P9 or P10 shown (blue asterisks indicate that the side chains are not resolved). (C)

291 edge-on view of peptide C α backbone, with arrow indicating departure of P4 in BL2*02
 292 from the polyproline II helix. (D) schematic of H-bonds interacting with peptide main chain
 293 atoms (cut-off of 3.2 Å) originally described for DR1*01 (solid lines) compared to BL2*19
 294 (dashed lines) and BL2*02 (dotted lines); additional interactions subsequently found are
 295 shown in Figs. S6-S8). (E) ribbon representation of class II molecule with superimposing
 296 peptide C α backbones for BL2*02 (slate blue), BL2*19 (yellow) and DR1*01 (grey)
 297 showing top view (α 1 domain above, β 1 domain below) and side view (α 1 domain shown,
 298 β 1 domain removed for clarity), and with arrow indicating departure of P4 in BL2*02 from
 299 the polyproline II helix. (F) close-up view of class II molecules in ribbons with peptide C α
 300 backbones, and with the side chains for residues around pocket 4 and peptide P4 as sticks,
 301 superimposing BL2*02 (slate blue) with BL2*19 (grey with yellow peptide) in left hand
 302 panel and with DR1*01 (grey) in right hand panel.

Discussion

The chicken MHC can determine life or death after infection from certain economically-important pathogens, of which MDV is an iconic example. Infection of bursal B cells in culture allowed us to determine the peptides bound to class II molecules, the peptide motifs of the two class II molecules encoded by the B2 haplotype, the MDV genes that give rise to the pathogen peptides that are potential T cell epitopes, and an unexpected mode of peptide binding to the dominantly-expressed class II molecule of this haplotype.

The size distribution, raggedy ends, enrichment of proline in the N-terminal flanking region, and multiple amino acids for anchor positions of the peptides bound to the class II molecules of the chicken MHC B2 haplotype were expected from work on mammalian class II molecules, but there were surprises with the motifs. The BL1 molecule (composed of BLA α chain and BLB1 β chain) has motif with large aliphatic residues at P1 and aromatic and large aliphatic residues at P9, but only a slight enrichment for basic amino acids in between without any obvious anchor residue preferences. The BL2 molecule (BLA α chain and BLB2 β chain), which is the dominantly-expressed class II molecule in most tissues [26], has an unprecedented decamer core motif, with large aliphatic residues at P1 and P4, small and even acidic residues at P5, and large hydrophobic residues at P10.

It was also a surprise to find that, in this *in vitro* infection of chicken B cells, only four MDV genes gave rise to 77% (49/64) of the class II-bound peptide species from the pathogen and 44% (10/23) of all the potential T cell epitopes found. In fact, epitopes from these four MDV genes were found in many of the samples, whereas the most peptides were found in only one sample and are likely to be very rare, so the impact of the epitopes from the four MDV genes will be much larger than the numbers suggest. The four genes all encode structural proteins present in the virion, three surface glycoproteins and one polytopic tegument protein (Table S1), but these genes are expressed early after infection of B cells [49]. There are many other proteins present in the virion, and many other proteins expressed early after infection [14, 16], so why these four proteins are the source of most presented peptides is not at all clear yet.

This dominance of just a few class II epitopes from a few MDV proteins stands in contrast to other human and mouse herpesviruses, for which polyclonal (and in some cases polyfunctional) CD4⁺ T cell responses to many codominant epitopes have been reported, with multiple epitopes within some proteins [50-52]. The situation may also be true for vaccinia virus [53, 54], another virus with a similarly large DNA genome. However, this finding of few class II epitopes for MDV could fit with the existence of chicken MHC haplotypes that confer susceptibility, which would be difficult to explain with a larger number of T cell epitopes.

The overall structure of the chicken class II molecule is similar to that of mammals, which is perhaps not such a surprise given the high level of sequence identity, particularly in the BLA α chain [33]. Almost all the key features and residues involved in interactions with DM, CD4 and peptides are conserved. However, the details of the peptide binding to BL2*02 were unexpected, all deriving from a single amino acid polymorphism in the BLB2 β chain. The small MHC residue Ser β 78 leads to a pocket which pulls in the side chain from peptide position P4, resulting in a crinkle in the peptide unlike the completely flat peptide in a polyproline II helix as is normally found. This crinkle pulls the C-terminus of the peptide towards the N-terminus, resulting in a shift of one peptide position along for

the main chain interactions, the anchor residues (P1, P2, P4, P5, P8 and P10) and the side chains pointing up towards the T cell (P3, P6 and P9). It has long been known that differences in the size of a single class II side chain can have strong effects on which peptides are bound and thus on the subsequent functional effects, as in the example of the Val-Ile dimorphism of β 89 in DRB1 [55].

The MHC haplotype B2 is known to confer resistance to MD (as well as other common poultry pathogens, [7]). As mentioned above, a structure was very recently reported for a chicken class II molecule from B19 [43], a haplotype that is known to confer susceptibility to MD [13, 44]. This B19 molecule has a nonamer peptide motif, which leaves open the possibility that such a difference might contribute to the differences in resistance to MD. However, the chicken MHC (the BF-BL region) has low rates of recombination [56-58], meaning that a number of genes are inherited as haplotypes. Thus, much more work is required to determine whether class II molecules are important as suggested [23-25], along with the classical class I genes (BF1 and BF2), the NK receptor/ligand pair (BNK and Blec), and the butyrophilin homolog (BG1) which all have been proposed as candidates to explain the strong genetic associations with infectious pathogens [19-22].

No matter what the contribution of chicken class II molecules to differential resistance to MDV infection, the design and results of this study open the possibility of understanding T cell epitopes to generate improved vaccines for MDV as well as other economically-important and zoonotic poultry pathogens [6, 8-11, 17, 18]. Moreover, the results from this relatively simple system for chicken MHC haplotypes has relevance for human viral disease, given the class I molecules encoded by individual loci in mammals may function like the dominantly-expressed class I molecule of chickens [7].

Methods

MDV infection of bursal B cells in culture

Viruses. MDV reporter viruses expressing the green fluorescent protein (GFP) under the control of the herpes simplex virus 1 (HSV-1) thymidine kinase promoter were generated based on the very virulent RB-1B field strain (GenBank accession number EF523390) and the vaccine strain CVI988 (DQ530348). GFP was inserted into the bacterial artificial chromosome (BAC) backbone replacing the Eco-gpt gene [59]. BACs were confirmed by Illumina MiSeq sequencing to verify sequence integrity.

Chicken embryo cells (CEC) were isolated from 11-day-old VALO specific-pathogen-free (SPF) embryos (VALO Biomedica; Osterholz-Scharmbeck, Germany) as described previously [60]. CEC were maintained in minimal essential medium (MEM, PAN Biotech; Aidenbach, Germany) supplemented with 1-10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (AppliChem; Darmstadt, Germany) at 37°C and 5% CO₂.

All recombinant GFP reporter viruses were reconstituted by transfection of fresh CEC with purified BAC DNA using CaPO₄ transfection [61]. The viruses were propagated on CEC for up to nine passages, and infected cells were stored in liquid nitrogen.

Isolation and culture of bursal B cells. Fertilized eggs of M11 (B2/B2) chickens were kindly provided by Dr. S. Weigend (Federal Research Institute for Animal Health, Mariensee, Germany) and hatched at the Faculty for Veterinary Medicine, Munich. Birds were housed under conventional conditions in aviaries with groups up to 10 birds and received food and water ad libitum. Bursas were isolated from 6-8 week old birds, and cells were obtained by dissociation of the organs in PBS using a stainless-steel sieve. Leukocytes were isolated by density gradient centrifugation on Biocoll (1.077 g/ml, Biochrom, Berlin, Germany).

B cells were cultured at 40 °C in Iscoves modified Dulbeccos's medium (IMDM), 100 U/ml penicillin, 100 µg/ml streptomycin, 8% (vol/vol) fetal bovine serum (all Bio&Sell, Nürnberg, Germany) and 2% (vol/vol) chicken serum (ThermoFisher Scientific, Waltham, USA), with the addition of recombinant soluble chicken BAFF and chicken CD40L, as described [36, 62, 63].

Infection of bursal B cells. For MDV infection, 1×10^7 B cells were co-cultured with 3×10^5 freshly-thawed MDV-infected CECs (representing an infectious dose between 0.5 to 1×10^5 plaque-forming units) in 1 ml in a 24 well plate. Up to 10^9 cells were harvested 24 hours after infection, washed with PBS three times and frozen at -80°C. At this time point cultures contained 25-35% viable B cells and 10-15% of viable B cells were GFP positive (infected with MDV), as assessed by flow cytometry with the Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific) using a FACSCanto II flow cytometer and FACS DIVA and FlowJo software (BD, Heidelberg, Germany).

Ethical permission. Permission for the procedures involving animals was given by Landesamt für Gesundheit und Soziales (LAGeSo) in Berlin (approval number T0245/14)

and in Munich (file number KVR-I/221-TA 160/13-He, Inst.-Nr. 01-17a) with organ sampling in accordance with the German Animal Welfare Act.

Isolation of class II molecules and immuno-peptidomics

Isolation of peptides from class II molecules. As described (37, 64), frozen cells were thawed into 10 mM CHAPS (AppliChem, St. Louis, MO) in PBS (Gibco, Carlsbad, CA) with complete protease inhibitor (Roche, Basel, Switzerland), and class II molecules were isolated by standard immunoaffinity purification using purified monoclonal antibody 2G11 [65] produced in-house and covalently linked to CNBr-activated Sepharose (GE Healthcare, Chalfont St Giles, UK). MHC-peptide complexes were eluted by repeated addition of 0.2% trifluoroacetic acid (TFA, Merck, Whitehouse Station, NJ). Peptides were purified by ultrafiltration using centrifugal filter units (Amicon; Millipore, Billerica, MA), desalted using ZipTip C18 pipette tips (Millipore) and eluted in 35 µl 80% acetonitrile (Merck), 0.2% TFA, then vacuum-centrifuged and resuspended in 25 µl 1% acetonitrile, 0.05% TFA and stored at -20°C.

Analysis of peptides by LC- and below MS/MS. Peptides were separated by reverse-phase liquid chromatography and analyzed from 2-3 technical replicates, with sample shares of 33% or 50% trapped on a 75 µm × 2 cm trapping column (Acclaim PepMap RSLC; Thermo Fisher) at 4 µl/min for 5.75 min. Peptide separation was performed at 50°C and a flow rate of 175 nl/min on a 50 µm × 25 cm separation column (nano-UHPLC, UltiMate 3000 RSLCnano; Thermo Fisher, Waltham, MA) applying a gradient ranging from 2.4 to 32.0% of acetonitrile over the course of 90 min. Samples were analyzed as previously described [66] using an online-coupled Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher, Waltham, MA), implementing a top-speed CID method with survey scans at 120k resolution and fragment detection in the Orbitrap (OTMS2) at 60k resolution. The mass range was limited to 400–650 m/z with precursors of charge states 2+ and 3+ eligible for fragmentation.

Database search and spectral annotation. LC-MS/MS results were processed using Proteome Discoverer (v.1.3 and 1.4; Thermo Fisher) to perform database search using the Sequest search engine (Thermo Fisher) with the chicken and appropriate MDV proteomes as reference database annotated by the UniProtKB/Swiss-Prot (www.uniprot.org), status April 2018. The search combined data of three technical replicates, was not restricted by enzymatic specificity, and oxidation of methionine residues was allowed as dynamic modification. Precursor mass tolerance was set to 5 ppm, and fragment mass tolerance to 0.02 Da. False discovery rate (FDR) was estimated using the Percolator node (67) and was limited to 5%. Length of peptides was limited to 12–25 amino acids. Possible peptide motifs of peptides were grouped using GibbsCluster-2.0 (68), and Venn diagrams were done by BioVenn (69).

Expression of class II molecules and structural determination

Cell lines. Sf9 insect suspension cells (*Spodoptera frugiperda* female ovarian cell line, ATCC CRL-1711) were used for production of baculovirus and High Five insect suspension cells (*Trichoplusia ni* female ovarian cell line, GIBCO B85502) streptomycin

were used for protein production, both grown in Insect-XPRESS Protein-free Insect Cell Medium (Lonza, BE12-730Q) supplemented with L-glutamine (to 1%), 50 U/ml penicillin and 50µg/ml streptomycin at 27°C with 135 rpm shaking.

Cloning, protein expression and purification. For protein production and crystallography, the different constructs were cloned separately into the baculovirus insect cell expression vector, pFastBac1 (GIBCO 10360014), modified to contain an N-terminal GP67 secretion signal sequence (for amino acid sequences of each portion, see Fig. S9). For the BLA construct, the GP67 signal sequence was followed by the ectodomain of the α -chain of BLA (Ha5 to Ea188; GenBank accession number AY357253, but with S79 due to dimorphism noted in [33]), then a short Gly/Ser-linker (G4S), a c-fos dimerization domain [70], a two amino acid linker (GT), and an Avi-tag sequence for biotinylation. For the BLB2 constructs, the GP67 signal sequence was followed by a peptide sequence (described in Fig. S9), a 15 amino acid Gly/Ser-linker [3(G4S)], the ectodomain of the β -chain of BLB2 (S β 4 to K β 198; AB426141), a TEV-protease cleavage site, a c-jun dimerization domain [70], a Gly/Ser-linker [2(G4S)], a V5-tag and a 6x His-tag. Correct cloning was confirmed by DNA sequencing.

To generate bacmids, the constructs were transformed DH10Bac cells (GIBCO 10361012), plated out and clones grown up overnight. Bacmid DNA was subjected to PCR using M13 primers to confirm transposition of inserts, and then transfected into Sf9 cells as follows to generate P0 viral stocks. First, 20 µl bacmid DNA and 15 µl Fugene (Promega E2311) were each diluted into 600 µl of Lonza Insect Xpress medium, mixed together and incubated at room temperature for 20 minutes and then 200 µl of the mixture were transferred to a well of a 6 well plate containing 9 x10⁵ Sf9 cells in 2 ml antibiotics-free medium and incubated for 4-6 days at 27°C. The cell medium containing baculovirus was collected, passed through a 0.2 µm filter (Sartorius 16532-K), and then this P0 stock was used to amplify the number of viruses to give the P1 stock as follows. First, 1 ml P0 stock was added to 50 ml of 2 x10⁶ Sf9 cells/ml in suspension and cultured at 27°C for 48 hours with shaking at 135 rpm, then the cells were pelleted, and the culture media was filtered as above. Both P0 and P1 viral stocks were stored at 4°C until needed.

High Five cells were grown in 0.6 L medium in 2 L flasks and transduced with 6-10 ml P1 stock. 48-72 hours after infection, culture media was collected and incubated overnight at 4°C with nickel-Sepharose Excel (GE Healthcare 17-3712-01). His-tagged proteins were eluted from the nickel-Sepharose with 1 M imidazole in TrisCl pH 8.5, then purified by FPLC size-exclusion chromatography using Superdex S200 in 100 mM TrisCl pH 8.5, and subjected to endoproteinase Glu-C (V8 Protease, Sigma) cleavage at 37°C overnight to remove C-terminal tags and dimerization domains, with successful proteolysis confirmed by size shift in SDS-PAGE. The proteins were again purified as above using Superdex S200 in 25 mM TrisCl pH 8.5, and then concentrated using Amicon Ultra (with 3,000 Da molecular weight cut-off, Merck UFC8003) to 3-12 mg/ml as determined using a nanodrop spectrophotometer.

Crystallization and structure determination. Crystallization conditions were screened using the PEGs II suite (Qiagen) at 20°C, with 10.45 mg/mL protein in 25 mM TrisCl pH 8.5 mixed at 1:1 or 1:2 with mother liquor to give 0.6-0.9 µl sitting drops. The protein complex BL2*02 with the peptide QIESLSLNGVNPINFLSTKA from MDV protein gE crystalized in 100 mM TrisCl pH 8.5, 200 mM sodium acetate, and 30 % w/v PEG 4000. Crystals were

cryo-cooled in mother liquor supplemented with 10-35% (v/v) glycerol before data collection.

Diffraction data was collected remotely on the I04-1 beamline (Diamond Light Source, Oxford UK) at a wavelength of 0.978 Å. Data reduction and scaling were performed using XDS (71) and SCALA (72). The crystal of the BL2*02 belongs to the C 1 2 1 space group, and the structure was solved by basic molecular replacement deploying Phaser from the CCP4i package (73) using HLA-DR1 (4X5W) as the search model (74). Further rounds of manual model building and refinement were done using Coot (75) and Phenix (76). Further details about collection and refinement are shown in Table S2. The structure was deposited in the Protein Database (PDB) on 14 October 2019 and assigned accession number 6T3Y.

Interaction analysis. LigPlot+ (77) was used to calculate the potential interactions from the crystal structure. Unless stated otherwise, the maximum hydrogen-acceptor distance was set to 2.70 Å and the maximum donor-acceptor distance was set to 3.35 Å. The minimum and the maximum contact distances of hydrophobic residue to any contact were set to 2.90 Å and 3.90 Å, respectively. PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was used to display and analyze the structural data visually.

Differential scanning fluorimetry. Differential scanning fluorimetry was performed using a CFX Connect real time PCR thermal cycler (Biorad) set to Fluorescence Resonance Energy Transfer (FRET) scan mode. SYPRO orange (Invitrogen S6651) was diluted to 1:500 in 25 mM TrisCl pH 8 and 12.5 µL mixed with 12.5 µL 10 µM protein in 25 mM TrisCl pH 8 in each well of a 96 well plate, with buffer alone serving as negative control. The samples were scanned every 0.5 °C from 25 to 95°C. Data analysis was performed in Prism (GraphPad).

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Figure

A

Cluster 1

Cluster 2

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B

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