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Characterization of lymphocyte subpopulations and major histocompatibility complex haplotypes of mastitis-resistant and susceptible cows

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Bovine mastitis is an infectious disease with a major economic influence on the dairy industry worldwide. Many factors such as environment, pathogen, and host affect susceptibility or resistance of an individual cow to bovine mastitis. Recently, there has been considerable interest in defining genetic and immunological markers that could be used to select for improved disease resistance. In this study we have analyzed the lymphocyte subpopulations of mastitis-resistant and susceptible cows using monoclonal antibodies specific for bovine leukocyte differentiation antigens and flow cytometry. We have also used a microarray typing technique to define the bovine leukocyte antigen (BoLA) class I and class II haplotypes associated with resistance or susceptibility to bovine mastitis. A striking finding of the present study is that susceptibility to mastitis was associated with major histocompatibility complex (MHC) haplotypes that have only a single set of *DQ* genes. The study also revealed that susceptible cows had CD4:CD8 ratios of less than one in both their mammary gland secretions and peripheral blood. These results raise the possibility that the number of *DQ* genes that a cow has and/or a cow's CD4:CD8 ratio could be used as indicators of susceptibility to bovine mastitis.

Key words: Cattle; Mastitis; Major histocompatibility complex; BoLA; Lymphocyte subpopulations; Genetics

Abbreviations: MGS, mammary gland secretions; IMI, intramammary infection; BoLA, bovine leukocyte antigen; SCC, somatic cell count; ACD, acid citrate dextrose; PAE, PBS-ACD-EDTA solution; PBS-FB, first wash buffer; DH, D-region haplotype.

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Introduction

Bovine mastitis is an infectious disease with a major economic influence on dairy production. Prospects for the development of an effective vaccine are limited by the variety of microorganisms causing mastitis and a lack of information on the genetic factors that influence disease resistance. It is evident that resistance to infectious diseases is genetically determined. Consequently, there has been considerable interest in defining genetic and immunological markers that could be used to select for improved disease resistance.

Variations in leukocyte subpopulations at different stages of lactation and in mastitic cows suggest that the defense mechanisms of bovine mammary gland may be governed by cell-mediated immune responses. In a previous study we reported that the number of T lymphocytes in mammary gland secretions (MGS) was decreased during the periparturient period and that the average CD4:CD8 T lymphocyte ratio in MGS was less than 1.0 during the lactation period [30]. The CD4:CD8 ratio was even lower in cows with *Staphylococcus aureus* mastitis [31,46,53]. Several studies have suggested that the composition of T lymphocyte subpopulations in the MGS of cows might correlate with susceptibility to intramammary infection (IMI) [31,46,48]. Although these findings reveal that specific lymphocyte subpopulations may affect the defense of the bovine mammary gland, the functional significance of particular populations has not been completely defined [38,39].

Together with the lymphocyte subpopulations involved in bovine mammary defense against invading pathogens, the antigen presentation capability of antigen-presenting cells is critical for the establishment of effective immunity to IMI. Because of their important role in immune responses, major

histocompatibility complex (MHC) genes are candidate markers for disease resistance. The important role of MHC molecules in the regulation of immune response is attributable to the recognition by T lymphocytes of a complex of foreign peptide antigens and MHC class I or class II molecules. Studies have indicated that certain bovine MHC, also known as the bovine leukocyte antigen (BoLA) complex, class IIa haplotypes are associated with genetic resistance against mastitis [13,19,24,41,42,47]. However, the basis for this association has never been adequately explained. In this study we have analyzed the lymphocyte subpopulations from mastitis-resistant and susceptible cows using monoclonal antibodies specific to bovine leukocyte antigens and flow cytometry. We have also used a microarray typing technique to identify the BoLA class I and class IIa haplotypes associated with resistance or susceptibility to mastitis.

Materials and Methods

Experiment animals

Holstein cows used in this experiment were raised by the National Livestock Research Institute, Rural Development Administration, Korea. Two different groups of animals were selected based on mastitis infection frequency, the frequency of medical treatments and treatment conditions recorded over the past four years. One was termed the resistant group, with no history of medical treatment of mastitis. The other was referred to as the susceptible group with more than two treatments for bovine mastitis. Milk somatic cell counts (SCC) were determined using a Combifoss™ 5000 milk analysis system (Foss Electric Co., Denmark). Over the four-year period, SCC of the resistant cows averaged below 200,000/ml while, with three exceptions, average somatic cell counts of the susceptible cows were higher than 200,000/ml (Table 1).

Isolation of bacteria

Isolation and identification of pathogens from milk of mastitis-susceptible cows was performed by the method of Joo and colleagues [18]. In brief, milk samples from individual quarters of mastitis-susceptible cows were cultured on 5% sheep blood agar (KOMED, Sungnam, Korea) and incubated at 37°C for 48 h. Bacterial colonies presumptively identified by colony characteristics, catalase reaction, hemolytic patterns, coagulase test and biochemical tests were speciated following the National Mastitis Council protocols [17]. Isolates were further analyzed using the VITEK® system (bioMérieux, Inc., Marcy-'Etoile, France).

Preparation of mononuclear leukocytes from mammary gland secretions and peripheral blood

MGS and peripheral blood were collected in acid citrate dextrose (ACD). Peripheral blood mononuclear leukocytes were separated from erythrocytes and most granulocytes by density gradient centrifugation using Lymphopaque™ (density = 1.086, Nyegaard, Oslo, Norway). Platelets and residual erythrocytes were removed by treatment with Tris-NH_4Cl (0.83% w/v, pH 7.3) followed by two or three washes in phosphate-buffered saline (PBS; pH 7.2) containing 20% ACD. Two hundred ml of MGS were obtained aseptically from each quarter of lactating cows and then pooled. MGS were mixed with an equal volume of PBS-ACD-EDTA solution (PAE; PBS pH 7.2, 20% ACD, 20 mM EDTA) and centrifuged at $\times 400$ g for 30 min at 10°C. Cell pellets were diluted with PAE in 50 ml conical tubes and separated by density gradient centrifugation over Lymphopaque as described above. After several washes in PAE, fluorescence flow cytometry was used to examine the relative proportion of lymphocytes.

Monoclonal antibodies

The panel of monoclonal antibodies (mAb; VMRD, Inc., Pullman, WA) used to examine leukocyte subpopulations is shown in Table 2.

Flow cytometric analysis

Cells were resuspended to 10^7 cells per ml in PBS containing 10 mM EDTA, 0.1% sodium azide, 10% ACD and 2% gamma-globulin free horse serum (first wash buffer; PBS-FB), then distributed in 50 µl aliquots (5×10^5 cells) to wells of V-bottomed, 96 well microtiter plates (Costar®, Corning Inc., Corning, NY) to which 50 µl of PBS-FB or mAb (0.7 µg per 50 µl) had been previously added. Cells were incubated for 30 min at 4°C, then washed three times in PBS-FB. Cells were then mixed with 100 µl of a 1 : 200 dilution of fluorescein-conjugated goat anti-mouse Ig (heavy and light chain specific; Caltag Laboratories, Burlingame, CA). Following incubation for 30 min at 4°C, cells were washed in PBS containing 0.1% sodium azide

Table 1. Average somatic cell counts of bovine mastitis-resistant and susceptible cows (1,000 cells/ml)

Group[a]	No. of cows	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Mean±SD
Susceptible	15	732	446	578	162	219	571	703	444	511	557	138	261	877	117	327	442±234
Resistant	15	95	116	131	41	126	117	129	76	57	103	71	68	79	61	95	91±25

[a]Groups are statistically different with a probability of $P<0.001$.

Table 2. Monoclonal antibodies specific to bovine leukocyte differentiation molecules used to define the distribution of leukocyte subpopulation from peripheral blood and mammary gland secretions

Molecules[a]	Cell type[b]	mAb[c]	Isotype of mAb
CD4	T helper/inducer	CACT138A	IgG_1
CD8	T cytotoxic/suppressor	CACT80C	IgG_1
WC1-N1	γ/δ-T cell subset	B7A1	IgM
SIgM	Naive B cells	Pig45A	IgG_{2b}
ACT2	Activated CD8	CACT26A	IgG_1
ACT3 (CD26)	Activated CD4	CACT114A	IgG_{2b}
MHC-class II	APC[d]	H42A	IgG_{2a}
MHC-DQ	APC	TH81A	IgG_{2a}
MHC-DR	APC	TH14B	IgG_{2a}

[a]Bovine leukocyte differentiation molecules.
[b]Cells expressing molecules.
[c]Monoclonal antibodies that react with specific leukocyte differentiation antigens.
[d]Antigen-presenting cell.

and 10% ACD (second wash buffer) and fixed with 2% formaldehyde in PBS. A Becton-Dickinson FACSCalibur™ flow cytometer and CellQuest™ software were used for data acquisition, and analysis of mAb leukocyte staining patterns, as previously described (Becton Dickinson, San Jose, CA) [9,10].

Microarray based MHC class I, *DRB3* and *DQA* typing

MHC typing was performed using bovine class I, *DRB3* and *DQA* microarrays. Arrays were comprised of 15-22 bp oligonucleotide probes spotted on epoxy-silane treated, 12-well, Teflon masked, glass slides (Erie Scientific, Portsmouth, NH) using an Affymetrix 417 arrayer (Affymetrix, Santa Clara, CA) [3]. The class I typing array was based on 118 cDNA or genomic sequences from the BoLA Nomenclature Web Site (http://www.projects.roslin.ac.uk/bola/ bolahome.html) and GenBank. It was comprised of two series of exon 2 probes (25 probes for codons 62-67 and 30 probes for codons 72-77) plus two series of exon 3 probes (27 probes for codons 110-116 and 31 probes for codons 151-157) that define an undetermined number of MHC class I haplotypes. The *DRB3* typing array was based on 66 exon 2 sequences from the BoLA Nomenclature Web Site. It was comprised of 5 series of exon 2 probes (14 for codons 8-15, 13 for codons 27-33, 16 for codons 54-61, 25 for codons 66-72 and 11 for codons 73-79) that define 56 *DRB3* alleles. The *DQA* typing array was based on 47 sequences from the BoLA Nomenclature Web Site, two additional sequences from GenBank and two new sequences derived at Washington State University. This array was comprised of 8 series of exon 2 probes (15 for codons 9-16, 8 for codons 21-30, 11 for codons 32-39, 10 for codons 40-48, 19 for codons 49-58, 13 for codons 59-66, 21 for codons 67-75 and 17 for codons 75-81) that define a minimum of 17 *DQA* haplotypes. Genomic DNA targets for class I and *DRB3* were generated by heminested PCR. First round PCR was performed in a 25 µl reaction volume with unmodified primers. First round class I primers BoC1FP-E2C 5'-GTCGGCTACGTGGACGACACGCAGTTC-3' and BoC1RP-E3C 5'-CCTTCCCGTTCTCCAGGTATCTGCG GAGC-3' span exons 2 and 3, while *DRB3* primers BoDRB3FP-HL030 5'-ATCCTCTCTCTGCAGCACATTT CC-3' and BoDRB3RP-HL031 5'-TTTAAATTCGCGCTC ACCTCGCCGCT-3' only amplify exon 2. The PCR profile for first round amplification was: denaturation at 94°C for 4 min; 10 cycles of 1 min at 94°C, 30 sec at 60°C and 90 sec at 72°C; and a final extension of 5 min at 72°C. For the second round amplification each exon was amplified separately using biotinylated primers and 2 µl of product from the first round in a 50 µl reaction volume for 35 cycles. Primers for second round amplification were: class I exon 2, BoC1FP-E2A 5'-ACGTGGACGACACGCAGTTC-3' and BoC1RP -E2A 5'-CTCGCTCTGGTTGTAGTAGCC-3'; class I exon 3, BoC1FP-E3D 5'-TGGTCGGGGCGGGTCAGGGTCT CAC-3' and BoC1RP-E3C 5'-CCTTCCCGTTCTCCAGG TATCTGCGGAGC-3'; and *DRB3* exon 2, BoDRB3FP-HL030 5'-ATCCTCTCTCTGCAGCACATTTCC-3' and BoDRB3RP-HL032 5'-TCGCCGCTGCACAGTGAAAC TCTC-3'. The *DRB3* primers are identical to those used by van Eijk and coworkers [50]. PCR profiles for second round amplification were: denaturation at 94°C for 1 min; 35 cycles of 30 sec at 94°C, 30 sec at 52°C (class I exon 2) or 60°C (class I exon 3 and *DRB3* exon 2), and 30 sec at 72°C; and a final extension of 5 min at 72°C. Genomic DNA targets for *DQA* typing were produced by multiplex PCR with biotinylated primers in a 50 µl reaction volume. Two sets of *DQA* primers were used: BoDQA1FP-E2A 5'-CTC CGACTCAGCTGACCACATTGG-3' and BoDQA1RP-E2A 5'-TACTGTTGGTAGCAGCAGTAGAGTTGG-3'; and BoDQA2FP-E2B 5'-CCTCAATTATCAGCTGACCACGT TGG-3 and BoDQA2RP-E2B 5'-GGTGGACACTTACCA TTGATAACAGGG-3'. The PCR profile for *DQA* amplification

was: denaturation at 94°C for 4 min; 35 cycles of 1 min at 94°C, 30 sec at 60°C and 90 sec at 72°C; and a final extension of 5 min at 72°C. Following PCR amplification, 10 µl of the reaction mix was diluted to 80 µl in hybridization buffer (5X SSPE, 5x Denhardts; 1X SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 0.9 mM EDTA, pH 7.5), denatured for 5 minutes at 94°C, and 35 µl of diluted PCR product hybridized in duplicate to two wells of a corresponding microarray slide overnight at 50°C. Slides were washed 5 times in diminishing concentrations of SSPE (final concentration 0.1X) at room temperature, incubated for 1 hour at room temperature with 35 µl Streptavidin-Alexa Fluor® 546 conjugate (Molecular Probes, Inc., Eugene, OR) diluted 1 : 500 in hybridization buffer, rinsed 2 times in 0.1X SSPE, dried and scanned on an ArrayWoRx™ scanner (Applied Precision, Issaquah, WA). Spots were scored on a 5-point scale from negative to strongly positive and data were interpreted using Cytofile genotyping software [4,6].

Statistical analysis

Student's T-test was used to compare the differences in the proportions of lymphocytes carrying the various antigens in MGS and peripheral blood between mastitis-resistant and susceptible cows using SAS (version 8.2, SAS Institute, NC, USA). Statistical testing was conducted at $\alpha = 0.05$ and *P* values under 0.05 were considered statistically significant.

Associations between individual BoLA haplotypes, or BoLA class IIa haplotypes, and mastitis susceptibility or resistance were tested using 2×2 contingency tables. Since many of the comparisons had expected frequencies of less than 5, associations were evaluated using Fishers exact test [11]. The number of *DQA* genes carried by cows from the two groups was compared using the Wilcoxon rank sum test (Minitab 10 Xtra, Minitab Inc., State College, PA, USA).

Results

Staphylococcus aureus and *S. epidermidis* were the major pathogens isolated from milk samples from the mastitis-susceptible cows. *S. aureus* was especially common and was isolated from more than one quarter of each dairy cow with a high SCC (>500,000 cells/ml).

The proportions of MGS mononuclear leukocytes from mastitis-susceptible and resistant cows expressing various leukocyte differentiation antigens are given in Table 3. The mastitis-resistant population is free of mastitis and can, therefore, be thought of as a normal population. However, most of the cows in the mastitis-susceptible population had chronic *S. aureus* mastitis (Table 1). Since these cattle had chronic mastitis, variation from the normal, mastitis-resistant population reflects both the effects of infection and genetic susceptibility. The sum of percentages of MGS mononuclear leukocytes stained by antibodies for the primary lymphocyte subpopulations-T helper cells (CD4), cytotoxic/suppressor T cells (CD8), γ/δ-T cells (WC1-N1), and naive B cells (sIgM) - were 56.4% for mastitis-susceptible and 88.3% for mastitis-resistant cows. The proportions of mammary gland mononuclear cells from mastitis-resistant cows that expressed MHC class II *DR+DQ*, *DQ* and *DR* were 78.5%, 59.8% and 68.0%, respectively. The corresponding proportions for mastitis-susceptible cows were 31.2%, 31.2% and 21.6%. The high proportion of mononuclear cells expressing MHC class II in the mastitis-resistant cows indicates a substantial level of lymphocyte and macrophage activation. Conversely, the low proportion of cells expressing MHC class II in the chronically infected, mastitis-susceptible cows suggests a relatively low state of cell activation. The proportions of MGS mononuclear cells expressing CD4 and surface IgM (sIgM) were significantly higher in mastitis-resistant than in

Table 3. Distribution of MGS leukocyte subpopulations from mastitis-resistant and susceptible cows analyzed using monoclonal antibodies specific to bovine leukocyte differentiation antigens and flow cytometry

Bovine leukocyte differentiation antigen	Mean proportion of bovine leukocyte subpopulation in MGS (%)	
	Mastitis-susceptible (n=15)[a]	Mastitis-resistant (n=15)[a]
CD4[b]	7.7±4.5	27.9±6.5
CD8[b]	18.5±8.3	8.6±4.3
WC1-N1 (γ/δ-T cells)[c]	14.5±9.4	20.2±6.7
sIgM (naive B)[b]	15.7±5.3	31.6±9.3
ACT 2[b]	10.8±3.4	5.8±1.3
ACT 3 (CD26)[b]	19.0±5.7	33.3±9.7
MHC-class II[b]	31.2±10.4	78.5±10.5
MHC-DQ[b]	31.2±9.8	59.8±11.4
MHC-DR[b]	21.6±12.5	68.0±9.5
CD4:CD8 ratio[b]	0.42	3.2

[a]Mean±SD.
[b]Groups are significantly different at $P \leq 0.05$.
[c]Groups are not significantly different at $P \leq 0.05$.

Table 4. Distribution of PBMC subpopulations from mastitis-resistant and susceptible cows analyzed using monoclonal antibodies specific to bovine leukocyte differentiation antigens and flow cytometry

Bovine leukocyte differentiation antigen	Mean proportion of bovine lymphocyte subpopulation in PBMC (%)	
	Mastitis-susceptible (n=15)[a]	Mastitis-resistant (n=15)[a]
CD4[b]	2.3±1.6	15.4±3.4
CD8[b]	15.1±3.4	6.1±2.8
WC1-N1 (γ/δ-T cells)[c]	5.8±2.5	6.4±2.5
sIgM (naive B)[b]	34.4±5.8	21.0±3.7
ACT 2[c]	8.7±2.8	7.9±4.8
ACT 3 (CD26)[c]	11.2±3.2	6.6±2.7
MHC-class II[b]	43.0±10.5	35.1±9.7
MHC-DQ[b]	47.4±9.5	38.0±6.8
MHC-DR[c]	42.5±9.8	42.3±10.3
CD4:CD8 ratio[b]	0.15	2.5

[a]Mean±SD.
[b]Groups are significantly different at $P \leq 0.05$.
[c]Groups are not significantly different at $P \leq 0.05$.

mastitis-susceptible cows (Table 3). However, part of the difference between the two populations is a reflection of the increased number of cells expressing lymphocyte differentiation markers in the mastitis-resistant cows. The mastitis-susceptible cows had a significantly greater percentage of CD8+ T cells in their MGS. Furthermore, in this case correcting for the proportion of cells that were lymphocytes would make the difference even more pronounced. The best measure of how CD4 and CD8 lymphocyte populations change in response to chronic *S. aureus* infection is the CD4:CD8 ratio. While in the mastitis-resistant cows the MGS CD4:CD8 ratio was 3.2, in the mastitis-susceptible cows the ratio was inverted and was 0.42.

Table 4 shows the percent of peripheral blood mononuclear cells (PBMC) from mastitis-susceptible and resistant cows stained by antibodies for leukocyte differentiation antigens. The sum of percentages of PBMC stained by antibodies for the primary lymphocyte subpopulations-T helper cells (CD4), cytotoxic/suppressor T cells (CD8), γ/δ-T cells (WC1-N1), and naive B cells (sIgM)- were 57.6% for mastitis-susceptible and 48.9% for mastitis-resistant cows. The remaining cells were presumably monocytes, memory B cells, WC1-N1 negative γ/δ-T cells, and lymphocytes expressing low levels of differentiation markers. Since lymphocytes comprised similar proportions of the PBMC in the two populations the percentages can be directly compared. In comparison to resistant cattle, susceptible cattle had a relative increase in the proportions of lymphocytes that were CD8+ T lymphocytes and naive B lymphocytes and a relative decrease in the proportion that was CD4+ lymphocytes. Furthermore, the CD4:CD8 ratio was inverted; the resistant cows had a CD4:CD8 ratio of 2.5 while the susceptible cows had a ratio of 0.15.

The proportion of activated, ACT2-expressing, γ/δ-T cells and CD8+ lymphocytes was significantly higher in MGS from susceptible cows (Table 3). However, in peripheral blood this proportion did not differ between the two groups (Table 4). The proportion of activated, ACT3-expressing T lymphocytes was significantly higher in MGS of resistant cows than susceptible cows (Table 3). Under most conditions ACT3 is a marker for activated CD4+ T lymphocytes [30]. However, recently it has been shown that bovine CD8+ lymphocytes express ACT3 in response to stimulation by staphylococcal enterotoxin C [15,20,21]. The high proportion of ACT3+ lymphocytes in the MGS of mastitis-resistant cows can, to a large degree, be explained by the high proportion of CD4+ lymphocytes in these cattle. In susceptible cattle, however, there were considerably more ACT3+ lymphocytes than CD4+ lymphocytes in both the MGS and peripheral blood (Tables 3 and 4). Consequently, it is likely that in the mastitis-susceptible cattle there was significant expression of ACT3 on CD8+ lymphocytes.

The 30 cattle in this study had 17 BoLA haplotypes comprised of 11 class I haplotypes, including a "Blank" class I haplotype, associated with 11 class IIa haplotypes (Table 5). Although class I typing was done using microarrays, the serological names have been used for class I haplotypes [7]. Sequence based, D-region haplotype (DH) nomenclature is used for class IIa haplotypes [8,34]. The "Blank" class I haplotype represents a class I haplotype that cannot be defined with our current panel of probes. There is, nevertheless, strong evidence for the existence of a Blank-DH22H haplotype. It is also possible that the A14(A8)-DH26B haplotype is really a Blank-DH26B haplotype. This haplotype has not been identified in other Holstein cattle and was carried by a cow that typed as an A14(A8)-DH11A/A14(A8)-DH26B class I homozygote. The sequences of all *DRB3* and *DQA* alleles detected in the study population

Table 5. Association between mastitis susceptibility and BoLA haplotypes

BoLA Haplotype[a]	Susceptible	Resistant	P[b]
A10-DH03A	1	1	
A10-DH26B	1	0	
A11-DH24A	7	3	0.098
A12(A30)-DH07A	1	0	
A12(A30)-DH16A	1	5	0.076
A13-DH23A	0	1	
A14(A8)-DH11A	4	3	
A14(A8)-DH26B	1	0	
A14(A8)-DH27A	0	2	
A15(A8)-DH22H	1	1	
A19(A6)-DH24A	5	1	0.076
A20-DH08A	1	4	0.144
A31(A30)-DH12C	0	3	0.112
w44-DH07A	1	2	
w44-DH08A	1	0	
w44-DH27A	2	0	
Blank-DH22H	1	3	

[a]BoLA haplotypes are identified by class I serotype and class IIa haplotype (DH) [6,7].
[b]Probability determined using Fishers Exact Test.

were confirmed by cloning and sequencing of exon 2 from at least one representative American or Korean Holstein (data not shown). Each sequence that was obtained, except for two new *DQA* sequences, exactly matched a previously described sequence from one of the cows haplotypes and corresponded to a sequence predicted on the basis of our microarray typing. Consequently, we are confident that our allele assignments correspond to the alleles officially named by the BoLA Nomenclature Committee [8,34].

Fisher's Exact Test was used to evaluate associations between individual BoLA or class IIa haplotypes and mastitis susceptibility or resistance (Tables 5 and 6). None of the BoLA haplotypes were associated with mastitis susceptibility or resistance with a statistically significant probability of $P \leq 0.05$ (Table 5). However, the data suggested that the A11-DH24A and A19(A6)-DH24A haplotypes might be associated with susceptibility ($P = 0.098$ and $P = 0.076$, respectively) and that the A12(A30)-DH16A, A31(A30)-DH12C and A20-DH08A haplotypes might be associated with resistance ($P = 0.076$, $P = 0.112$ and $P = 0.144$, respectively). Analysis of associations between class IIa haplotypes and susceptibility or resistance revealed a statistically significant association between DH24A and susceptibility ($P = 0.012$). It is noteworthy that

Table 6. Association between mastitis susceptibility and class IIa haplotypes

DH[a]	*DRB3* allele	*DQA* alleles	*DQB* alleles	Phenotypic Frequency (%)	Susceptible	Resistant	P[b]
03A[d]	**1001*[g]	**10012*[g] **2101*[g]	**1003* **0902*	6.7	1	1	
07A[e]	**0201*[g]	**0203*[g]	**0201*	13.3	2	2	
08A[d]	**1201*[g]	**12011*[g] **2201*[g]	**1005* **1201*	20.0	2	4	0.221
11A[e]	**0902*[g]	**0204*[g]	**0301*	23.3	4	3	
12C[c,f]	**1701*[g]	**wsu2-1*[h] ND[i]	ND	10.0	0	3	0.112
16A[d]	**1501*[g]	**10011*[g] **22021*[g]	**0102* **1101*	20.0	1	5	0.076
22H[c,d]	**1101*[g]	**10011*[g] **wsu2-2*[h]	ND ND	16.7	2	3	
23A[d]	**2703*[g]	**0101*[g] **22031*[g]	ND ND	3.3	0	1	
24A[e]	**0101*[g]	**0101*[g]	**0101*	50.0	11	4	0.012
26B[c,d]	**0601*[g]	**10011*[g] **25012*[g]	ND ND	6.7	2	0	
27A[e]	**14011*[g]	**1401*[g]	**1401*	13.3	2	2	

[a]Class IIa (D-region) haplotypes [6,23,35].
[b]Probability determined using Fishers Exact Test.
[c]New class IIa (DH) haplotype.
[d]Haplotype has duplicated *DQA* and *DQB* genes with *DQA* genes of the *W1* and *A5* subtypes [43].
[e]Haplotype has single *DQA* and *DQB* genes with a *DQA* gene of the *W1* subtype [43].
[f]Haplotype probably has 2 *DQA* genes of the *A5* subtype and a single *DQB* gene [43].
[g]Exon 2 sequence confirmed at Washington State University.
[h]New *DQA* allele sequenced at Washington State University.
[i]Not determined.

Table 7. Total number of *DQA* alleles and number of *DQA* alleles of the two major subtypes (*DQA-W1* and *DQA-A5*) carried by mastitis-susceptible and resistant cows

Number of alleles[a]	Number of cows in each group with specified number of alleles					
	DQA alleles[b]		*DQA-W1* alleles[b]		*DQA-A5* alleles[c]	
	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
0	0	0	0	0	7	1
1	2	0	3	5	8	11
2	6	5	12	10	0	3
3	7	8	0	0	0	0
4	0	2	0	0	0	0

[a]The number of unique *DQA* alleles is shown. For homozygous cows each allele was only counted once.
[b]All cows have at least one *DQA* allele of the *DQA-W1* subtype. The susceptible and resistant groups were not significantly different at $P \leq 0.05$.
[c]The two groups were significantly different, Wilcoxon rank sum test $P=0.006$.

this is the class IIa haplotype with the highest phenotypic frequency (50%). Other associations would be substantially harder to detect due to low haplotype phenotypic frequencies (see Table 6).

Inspection of the data revealed that haplotypes with non-duplicated *DQ* genes were more prevalent in the mastitis-susceptible group. Consequently, a comparison of the number of *DQA* alleles carried by cows in the two groups was conducted. There were 11 class IIa haplotypes present in the study population: four haplotypes with a single *DQA* gene of the *DQA-W1* subtype (DH07A, DH11A, DH24A and DH27A); one haplotype that probably has two *DQA* genes of the *DQA-A5* subtype but only a single *DQB* gene (DH12C); and six haplotypes with duplicated *DQA* genes with one *DQA-W1* and one *DQA-A5* subtype gene (DH03A, DH08A, DH16A, DH22H, DH23A and DH26B). It is unclear whether the DH12C haplotype, which was present in 3 mastitis-resistant but no mastitis-susceptible cows, has one or two functional *DQA* genes of the *DQA-A5* subtype. This haplotype has a *DQA*13C* RFLP pattern which has two *DQA-A5* exon 2 fragments, however, thus far only a single *DQA* gene has been identified by exon 2 cloning and sequencing [5,6,43]. We were, therefore, conservative and assigned this haplotype only a single *DQA-A5* subtype gene. The Wilcoxon rank sum test was used to compare the total number of unique *DQA* alleles, *DQA-W1* subtype alleles, and *DQA-A5* subtype alleles carried by cows in the two groups (Table 7). For homozygous cows each allele was only counted once. The total number of *DQA* alleles and the number of *DQA-W1* subtype alleles were not significantly different between the two groups ($P = 0.12$ and $P = 0.42$, respectively). However, the probability that cows in the two groups carried the same number of *DQA-A5* subtype alleles was only $P = 0.006$. Since the susceptible cows had significantly fewer *DQA-A5* subtype alleles than the resistant cows, the data suggest that *DQA-A5* subtype alleles play an important role in immunity to mastitis causing bacteria such as *S. aureus*.

Discussion

A critical component of any disease association study is accurate definition of disease susceptibility or resistance. Our classification of cows as mastitis-resistant or susceptible was based on a four-year history of treatment for clinical mastitis. Cows classified as resistant were never treated for mastitis while cows classified as susceptible were treated at least twice. The average somatic cell count data for the four-year period (Table 1) suggest that most of the susceptible cows had chronic, subclinical intramammary infections. This is consistent with the culture data that showed that most of these cattle were infected with *S. aureus*. It is thus possible that our results pertain to susceptibility to chronic *S. aureus* mastitis rather than mastitis in general. It is important to appreciate that some genetically susceptible cows may not have gotten mastitis during the four-year period because they were not exposed to *S. aureus* at a high enough dose. Conversely, cows resistant to *S. aureus* could have been classified as susceptible because they had two episodes of mastitis caused by some other pathogen. It is interesting that two of three cows classified as susceptible despite having average somatic cell counts below 200,000/ml (Table 1; cows S4 and S14) were the only cows in the study with the DH26B class IIa haplotype. It is possible that these two cows were genetically susceptible to a pathogen other than *S. aureus*.

Previously it was found that the relative proportions of lymphocytes and macrophages in MGS varied during lactation [30]. Furthermore, a substantial number of studies have shown that in MGS and mammary gland parenchyma of uninfected cows, CD8+ T lymphocytes outnumbered CD4+ lymphocytes [22,30,33,38,45,46,48,53]. The inverse was found in peripheral blood from uninfected cows where CD4+ T lymphocytes were more numerous [30,33,38,40, 48]. Our study differed from earlier studies in that MGS from our mastitis-resistant cows had substantially more CD4+ than CD8+ lymphocytes (Table 3). Since this finding

differs from the earlier studies it needs to be confirmed. Another novel finding was that in comparison to our mastitis-resistant cows, our susceptible cows had inverted peripheral blood CD4:CD8 ratios with more CD8+ than CD4+ lymphocytes (Tables 4). Our observations for both MGS and peripheral blood suggest that CD4+ lymphocytes may be protective.

It has been shown that activated, ACT2-expressing, CD8+ T lymphocytes from MGS of *S. aureus* infected cows can suppress CD4+ T lymphocyte proliferation [31,48]. Suppression of CD4+ T lymphocyte proliferation may be attributable to release by CD8+ lymphocytes of IL-10, a regulatory cytokine that suppresses antigen presentation by macrophages [32]. In our study, mastitis-susceptible cows had a reduced frequency of MHC class II positive leukocytes in their MGS (Table 3). Inhibition of macrophage activation would be one explanation for this observation. The ACT3 activation marker was recently shown to be the bovine orthologue of CD26 [20,21]. A decreased proportion of CD4+ T lymphocytes in MGS from mastitis-susceptible cows was correlated with a lower proportion of cells expressing ACT3, traditionally thought of as an activation marker for CD4+ lymphocytes [30]. Nevertheless, our mastitis-susceptible cows had a higher proportion of ACT3+ lymphocytes than CD4+ lymphocytes in both their MGS and peripheral blood (Tables 3 and 4). This is inconsistent with expression of ACT3 solely on CD4+ lymphocytes. Fortunately, an explanation for this paradox is provided by recent studies that have demonstrated that staphylococcal enterotoxin C induces ACT3 expression by CD8+ lymphocytes [15,20,21].

The proportion of naive B lymphocytes (sIgM+) in peripheral blood was significantly elevated in susceptible cows. We do not know if the higher percentage of naive B lymphocytes was associated with production of *S. aureus* specific antibody. It is likely, however, that our chronically infected cows were producing antibody against *S. aureus.* A critical question is the relative proportions of different isotypes of antibody produced by mastitis-susceptible and resistant cows. Antibody responses in mastitis-susceptible cattle may be skewed toward production of IgG1, associated with a Th2 response, rather than IgG2, associated with a Th1 response [14].

A substantial number of studies have attempted to associate bovine MHC class I or class II alleles with resistance or susceptibility to mastitis [1,13,19,24-26,28,29, 36,37,41,42,47,49,52]. The results of the class I association studies are inconsistent with many different class I alleles (haplotypes) appearing to confer susceptibility or resistance. A likely explanation for this is that resistance is controlled by a linked class II gene rather than by a class I gene. Since the studies were done in a variety of breeds and the predominant class I-class IIa haplotypes vary between breeds, one would expect variable results. In contrast to the class I studies, there is considerable agreement between the class II association studies. The strongest association found in the present study was between the class IIa haplotype DH24A and susceptibility to mastitis ($P = 0.012$). DH24A has a *DRB3* allele with PCR-RFLP pattern *DRB3.2*24* and *DQ* genes with the *DQ*-RFLP type *DQA*1A,DQB*1* [6,8]. These markers for DH24A were associated with mastitis susceptibility in 3 previous studies [19,24,47]. Since these studies used different definitions of mastitis susceptibility and different analysis methods it is impressive that they all identified the same class IIa haplotype. It is also fascinating that the DH16A and DH08A class IIa haplotypes (*DRB3* alleles **1501* and **1201,* respectively) associated with resistance to mastitis in our study, with respective *P* values of $P = 0.076$ and $P = 0.221$, were also associated with resistance in two other studies [41,47]. DH07A, which includes *DRB3* allele **0201,* is another class IIa haplotype of interest. This haplotype was fairly rare in our cattle and was not associated with either susceptibility or resistance. However, it was associated with susceptibility to mastitis in two previous studies [41,47].

An interesting feature of bovine MHC class IIa haplotypes is that some haplotypes have a single set of *DQA* and *DQB* genes while other haplotypes have two sets of *DQ* genes [2,6,43,44]. The DH24A and DH07A haplotypes, which have been associated with susceptibility to mastitis, have previously been shown to have a single set of *DQ* genes. In contrast, the DH16A and DH08A haplotypes, which appear to be associated with resistance to mastitis, have previously been shown to have duplicated *DQ* genes. The apparent association of haplotypes with a single set of *DQ* genes with susceptibility to mastitis and haplotypes with two sets of *DQ* genes with resistance has led us to hypothesize that cows expressing a wider range of distinct *DQ* alleles mount stronger Th1 responses to *S. aureus* and are more resistant to mastitis. We plan to test this hypothesis by doing a controlled challenge study using putative mastitis-susceptible and resistant cattle selected on the basis of the MHC definition of genetic susceptibility and resistance described in this paper.

Glass and colleagues have performed extensive analysis of foot-and-mouth disease virus (FMDV) peptide presentation by bovine class II molecules [16,23]. Their studies have found that: (1) both *DR* and *DQ* molecules present FMDV peptides, (2) the number of distinct *DQ* molecules expressed by a cow can be increased by interhaplotype pairing of *DQA* and *DQB* molecules, and (3) there were no FMDV-specific clones restricted by the *DQA*0101/DQB*0101* heterodimer encoded by both DH24A and DH15B [16]. In relationship to our mastitis data, it is interesting that DH24A and DH15B have non-duplicated *DQ* genes and that DH24A is the haplotype that shows the strongest association with mastitis susceptibility.

A striking finding of the present study is that susceptibility

to mastitis was associated with MHC haplotypes that have only a single set of *DQ* genes. Furthermore, this study suggests that susceptible cows have an inverted CD4:CD8 ratio in their peripheral blood as well as MGS. It is possible that the number of *DQ* genes that a cow has, the number of CD4+ helper T cells in the cows blood and susceptibility to mastitis are directly linked. Cattle expressing fewer *DQ* isoforms would have lower rates of positive selection of CD4+ helper T cells in their thymuses. However, the number of class II isoforms also influences negative selection. Models of positive and negative T cell selection, and recent experimental data, suggest that the optimal number of unique class II molecules for achieving the largest possible helper T cell repertoire is between five and seven [12,27,51]. Depending on the frequency by which bovine T cell clones positively selected to recognize *DR* molecules get negatively selected by *DQ* molecules, and vice versa, the optimal number may actually be somewhat larger than this. Hence, cattle carrying two haplotypes with non-duplicated *DQ* genes may have smaller helper T cell repertoires than cattle with one or two haplotypes with duplicated *DQ* genes. Presentation of fewer peptides and a smaller helper T cell repertoire would result in reduced activation and expansion of helper T cell clones. In addition, production and activation of fewer CD4+ helper T cells and more CD8+ cytotoxic/suppressor T cells could cause an inversion of the CD4:CD8 ratio. Furthermore, a suboptimal helper T cell response would probably lead to poor antibody production and susceptibility to mastitis.

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