Bovine materno-fetal alloimmune mediated disorders

MHC class I (in)compatibility in Retained Fetal Membranes and Bovine Neonatal Pancytopenia

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Materno-foetaal alloimmuun gemedieerde aandoeningen in het rund

MHC klasse I (in)compatibiliteit in Retentio Secundinarum en Boviene Neonatale Pancytopenie
(met een samenvatting in het Nederlands)

Proefschrift

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"… to boldly go where no one has gone before."

Captain Jean-Luc Picard

Contents

1

General Introduction

Part of this chapter is submitted as:

The role of placental MHC class I expression in immune assisted separation of the fetal membranes in cattle

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Manuscript submitted

Pregnancy: an immunological conundrum

The fetus inherits and expresses paternal alloantigens and can therefore be considered as 'foreign' to the maternal immune system. Preventing immunological rejection of the fetus is critical for a successful pregnancy and this presents a paradox to the maternal immune system; tolerate the semi-allogeneic fetus, while maintaining immunity to infections. Following parturition the maternal immune system is in contact with the neonatal calf indirectly through the transfer of maternal antibodies via the colostrum. Regulation of materno-fetal alloimmunity is pivotal to successful pregnancy and to avoid the transfer of pathogenic maternal alloantibodies. This thesis explores the adverse effects of materno-fetal alloimmunity in cattle on pregnancy and on the neonatal calf, with an emphasis on the role of MHC class I (in)compatibility between dam and calf on materno-fetal alloimmunity. Two disorders, Retained Fetal Membranes and Bovine Neonatal Pancytopenia, associated with hypo- and hyper materno-fetal alloimmune responsiveness, respectively, were studied.

Bovine Major Histocompatibility Complex class I

The Major Histocompatibility Complex (MHC), a gene complex on chromosome 23, encodes molecules that play an important role in the immune system. The MHC gene family is divided in three subgroups; MHC class I, II and III. Central to adaptive immunity is the presentation of antigens to T cells by MHC class I and II (box 1).

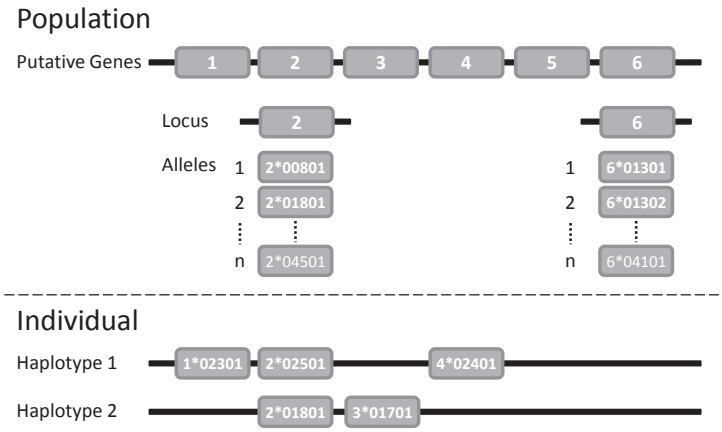


Figure 1. A schematic view of the genomic organization of the Bovine classical major histocompatibility complex class I at population level and at the individual level. Six putative genes have been defined in Cattle and several alleles have been discovered for each of these 6 loci. An individual inherits a maternal and a paternal MHC class I haplotype. On each haplotype alleles of one to three loci are present and alleles of both haplotypes are codominantly expressed.
Order and distance of genes are not representative.

The bovine MHC class I genetic region may contain up to 15 (partial) genes and includes classical, non-classical, pseudo and partial MHC genes (1, 2). There are six putative classical MHC class I genes, based on the segregation of alleles in a phylogenetic analysis of the coding sequence from exon 4 to 8 (alpha-3 domain to stop codon) (1, 3, 4) (Fig. 1). MHC class I is the most polymorphic gene known and to date the full length cDNA sequences of 97 classical MHC class I alleles have been submitted to the cattle MHC section of the Immuno Polymorphism Database (IPD; www.ebi.ac.uk/ipd/mhc/bola). The highest variation between allele sequences are in the regions encoding the antigen binding cleft (5), reflecting the selection pressure for the peptide repertoire that MHC class I alleles can present (Box 2). Based on the bovine whole genome assembly (3) and the mapping study of the A14 haplotype by Di Palma et al (2) four genes can be mapped and are therefore known to represent separate loci. Different combinations of one to three genes are functionally present on a haplotype (1, 3, 6) (Fig. 1). Although some genes are never present together on the same haplotype, there does not seem to be a functional difference between MHC class I molecules and alleles from all MHC class I genes have been shown to be expressed and able to present peptides to CD8 T cells (3). Although many different MHC class alleles and haplotypes have been defined, within a given cattle population haplotype diversity is usually limited and characterized by several dominant haplotypes (7).

Five putative non-classical MHC class I (NC-MHC class I) genes have been defined based on phylogenetic analysis of allele sequences similar to the method described for classical MHC class I (4, 8). Four genes have been mapped using the bovine whole genome assembly (9). As for classical MHC class I genes, a variable number of NC-MHC class I genes are present per haplotype, with gene 1 apparently ubiquitously present (9). Bovine NC-MHC class I genes are mono or oligomorphic, have a restricted cellular expression pattern and may be secreted (8). Although no studies into the exact functions of bovine NC-MHC class I genes have been performed, they are likely comparable to human and rodent NC-MHC class I (Box 3).

The central role of MHC in alloimmunity

Antigens that are disparate between members of the same species are called alloantigens and alloimmunity is the immune response following recognition of an alloantigen as non-self. There are three different pathways of allorecognition (reviewed by Afzali et al. (10)): i) the direct pathway is the recognition of peptide-MHC complexes on 'donor' cells by self T cells without intervention of self-antigen presenting cells (APC) ii) the indirect pathway is the presentation of processed alloantigens to CD4 T cells via self MHC class II on APC's iii) in the semi-direct pathway self-APC's acquire intact 'donor' peptide-MHC class I complexes and concurrently present processed alloantigens in the context of self-MHC, thereby activating both CD8 and CD4 T cells.

MHC proteins are allogeneic both as intact peptide-MHC complexes and as processed antigens and therefore play a role in all mechanisms of allorecognition. There is a high frequency of alloantigen specific T cells compared to nominal antigens (11-14), which is inherent to the MHC restriction of T cell receptors (TCR). During T cell development there is first positive selection for T cells capable of interacting with MHC. Next, there is negative selection for T cells that respond strongly to MHC-self peptide complexes to eliminate self-reactive T cells. (Reviewed by Stritesky et al. (15) Although the mechanism of selecting T cells specific for foreign peptides restricted to self-MHC would appear to select for self-MHC specific TCR only; cross reactivity of TCR to self and allogeneic MHC-peptide complexes (16-18) also leads to high frequencies of allospecific T cells. There are two theories for the cross reactivity/degenerate specificity of TCR for allogeneic MHC-peptide complexes. The first, the "high determinant density model" (19), proposes that since MHC molecules are generally expressed at high levels on donor cells, the affinity of TCR specific for non-self MHC required to activate the T cell can be lower than TCR specific for foreign-peptide self MHC. The second theory, the "multiple binary complex model" (20), proposes that alternative self-peptide allogeneic-MHC complexes resemble foreign-peptide self-MHC complexes and are recognized by "cross reactive" T cells. It is likely that both mechanisms or a mix of both models determine alloreactivity. More recent studies have indicated that interaction with both MHC and peptide determine TCR recognition and that TCR's can be activated by a (relatively small) number of different peptide-MHC complexes (discussed by Nikolich-Zugich (21)).

Allogeneic MHC molecules are also recognized in the indirect pathway. As discussed previously, MHC genes are highly polymorphic and multiple proteins are codominantly expressed. In combination with the high expression level and the expression on almost all cell types, MHC class I is also highly immunogenic in the indirect pathway. Allogeneic MHC class I presented in self-MHC class II to self CD4 T cells is essential for activation and class switching of alloreactive B cells (10).

Regulation of materno-fetal alloimmunity during pregnancy

During bovine pregnancy the semi-allogeneic fetus is in intimate contact with the dam for the better part of 280 days without being rejected by the maternal immune system. The maternal immune system has to be tightly regulated to tolerate the fetus, while maintaining the ability to respond to infections. There are three basic mechanism assuring the acceptance of the fetus (22-25) i) anatomical separation of the fetus from the maternal immune system ii) downregulation of alloantigen expression by the fetus iii) regulation of the maternal immune response in the uterus. The mother is not completely tolerant to the fetus, nor is the maternal immune system completely shut down in the uterus. The first would leave the mother vulnerable to attack by the fetal immune system and the second would leave both mother and fetus vulnerable

to infectious organisms (22, 26). Indeed, pregnant rats and rabbits readily rejected fetal tissue transplanted to extra-uterine tissue (27) and bovine dams can mount an immune response to placental infection with *Neospora caninum* while the fetus survives (28). Species like horse, cattle and humans developed long gestation lengths after the evolutionary separation of these species. Therefore, mechanisms to regulate materno-fetal immunity have evolved separately and are likely to be species specific (22). Thus, care should be taken to extrapolate results from other species to cattle.

In the bovine placenta fetal trophoblasts and maternal endometrium form a continuous epithelial lining across the whole placenta (Fig 2.) (29). Specialized structures called placentomes form through interdigitation of maternal (caruncle) and fetal (cotyledon) epithelium, thereby increasing surface area for exchange of waste and nutrients (29). Bovine placental histology is in strong contrast to the human placenta, where fetal trophoblasts are directly in contact with maternal blood and extravilluous trophoblasts that invade the uterine tissue and reshape maternal blood vessels (30). The anatomy of the bovine placenta assures there is minimal contact between the maternal immune system and fetal cells.

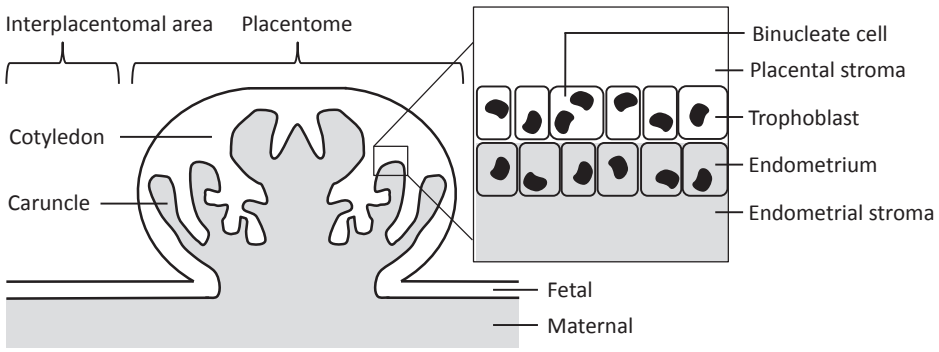


Figure 2. Bovine placentomes. Placentomes are formed through interdigitation of maternal (caruncle) and fetal (cotyledon) tissue. The apposition of fetal trophoblasts to the maternal endometrium forms a continuous epithelial lining across the placenta. Binucleate cells, specialized trophoblasts, can migrate to the maternal epithelium.

MHC class I is the quintessential alloantigen and in several species it has been shown that MHC class I is down regulated on fetal trophoblasts, e.g. humans (31), horse (32), pig (33). In cattle MHC class I expression on fetal trophoblasts is down regulated in early pregnancy, but towards mid gestation expression becomes apparent in interplacentomal regions and rises towards the end of gestation (34, 35). In the placentomes, at the area of most intimate contact, there is no MHC class I expression on the trophoblasts (34-36). Maternal endothelium expresses MHC class I throughout pregnancy in the interplacentomal area (34, 35). Findings regarding maternal MHC class I expression in the placentomes are conflicting, with studies reporting no expression (34), downregulation

(35) and normal expression (36). In an elegant study by Davies et al. (8) it was shown that interplacentomal trophoblasts transcribe very high levels of non-classical MHC class I, indicating part of the MHC class I proteins expressed by bovine trophoblasts are non-classical. Ellis et al. (37) detected transcription of MHC class I in late gestation placentome derived trophoblasts, but could not detect expression of MHC class I with ILA88, a monoclonal antibody that is pan specific for bovine MHC class I, and hypothesized this could reflect expression of NC-MHC class I. ILA88 has been shown to recognize some (9, 38), but not all non-classical MHC class I alleles (39). Since there are no NC-MHC class I specific antibodies, it is currently impossible to differentiate classical and non-classical MHC class I protein expression in the bovine placenta. In human pregnancies HLA-G, a NC-MHC class I, is highly expressed on trophoblasts both on the cell membrane and in soluble form and plays in important role in immune regulation, suppression and tolerance induction (25, 40). Davies et al. (8) found multiple splice variants of one non-classical allele, including a variant with a deletion of the transmembrane domain, indicating soluble bovine NC-MHC class I may be expressed. It is probable that NC-MHC class I expression on bovine trophoblasts has a similar role and that expression of both NC-MHC class I and restricted expression of classical MHC class I by the fetus contributes to the regulation of maternal immunity.

Binucleate cells (BNC), specialized cells formed from uni-nucleate trophoblasts and unique to ruminants, can migrate to the endometrium and fuse with maternal cells temporarily forming trinucleate cells (29, 41). BNC produce an array of secretory molecules, including placental lactogen, pregnancy associated glycoproteins and many hormones, and likely play a pivotal role in feto-maternal crosstalk (41-43). BNC have been found to express MHC class I in 'a term' collected placentomes (37, 44) and transcribed both classical and non-classical MHC class I (44). On the other hand, Davies et al. (34) and Chavatte-Palmer et al (36) could not detect MHC class I expression on BNC. However, these studies looked at BNC around 230 days of gestation and after dexamethasone induced parturition, respectively. In the study by Bainbridge and colleagues (44) is was found that not all BNC expressed MHC class I and at present it remains unknown if BNC express MHC class I at the moment of fusion with maternal cells. If this would be the case, this presents an interesting situation for allorecognition, as this enables the presentation of fetal antigens on both fetal and maternal MHC class I and the expression of maternal antigens on fetal MHC class I, thereby increasing the chance of allorecognition. Although the results regarding the MHC class I expression of BNC are not conclusive, invasive trophoblasts in horse also upregulate MHC class I (32) and Bainbridge (22) hypothesized that the upregulation of MHC class I on invasive trophoblasts possibly contributes to induction of tolerance to paternal MHC class I. Indeed, for the induction of antigen specific regulatory T cells the cognate antigen of the T cell has to be present (45). Expression of classical MHC class I on invasive trophoblast cells, in combination with the expression of NC-MHC class I and the

immunosuppressive and tolerogenic environment of the placenta, could lead to the induction of paternal MHC class I specific regulatory T cells in the dam.

MHC class I downregulation is a common immune evasion method of infectious organisms and NK cells can detect an kill cells with low or no MHC class I expression (46). The human NC-MHC class I gene HLA-G is known to inhibit NK cells and cytotoxic T cells and can induce regulatory T cells (25, 40). Although numbers were low, NK cells have been detected in bovine pregnancies (47) and expression of NC-MHC class I on bovine trophoblasts may inhibit NK cells and contribute to the induction of regulatory T cells. In humans reduced levels of regulatory T cells in the placenta and peripheral blood are associated with pre-eclampsia and preterm labor (48) and depletion of regulatory T cells in mice leads to gestation failure (49, 50), showing the importance of regulatory T cells for successful pregnancy. Foxp3+ (a marker for regulatory T cells) has been detected in the bovine placenta (47) and levels of CD4 CD25 T cells (another marker for regulatory T cells) rise in peripheral blood of pregnant cows (51). However, there is evidence that CD4 CD25 Foxp3 T cells do not have regulatory functions in cattle (52). Instead, γδ T cells were shown to act as regulatory cells (52, 53), of which low numbers have been detected in the bovine placenta (47).

Many soluble factors are released at the feto-maternal interface and systemically during pregnancy (e.g. uterine serpins, pregnancy hormones) and contribute further to the modulation of the maternal immune system. However, these are outside the scope of this thesis and are discussed in Oliveira et al (54) and Hansen et al. (55).

Transfer of passive immunity

Apart from direct contact with the fetus during pregnancy, there is indirect contact between the maternal immune system and the neonatal calf during transfer of passive immunity. In cattle there is no transfer of maternal antibodies across the placenta and calves are born agammaglobulinemic (56, 57). Therefore, calves rely solely on absorption of maternal alloantibodies from the colostrum for transfer of passive immunity. High concentrations of antibodies are present in colostrum (56, 58) and following ingestion, antibodies are absorbed in the intestines and antibody levels in the serum of calves quickly rise. Efficiency of the absorption of antibodies from the intestines declines after approximately 12 hours post-partum (57, 58). There is a strong association between failure of passive transfer, i.e. low serum antibodies levels, and mortality and morbidity in calves (59, 60).

Materno-fetal alloimmunity in bovine disorders

Maternal alloantibodies against paternal alloantigens are induced in up to 64% of multiparous cattle (61, 62). They can be detected as early as the second trimester of gestation (62) and are present at low levels in colostrum (61). The induction of maternal alloantibodies shows that materno-fetal alloimmunity is regulated and not fully suppressed. Moreover, a materno-fetal immune response is normally not harmful to the calf, both during pregnancy and during the transfer of passive maternal immunity. However, changes in the normal materno-fetal alloimmune response can have detrimental effects on pregnancy or the neonatal calf. Two disorders in which materno-fetal alloimmunity plays an important role are Retained Fetal Membranes and Bovine Neonatal Pancytopenia.

Retained fetal membranes

Normally the fetal membranes are expelled within 6 hours after the calf is born (63). Retention of the fetal membranes longer than normal is called retained fetal membranes (RFM) and is most commonly defined as retention longer than 24 hours postpartum (63-65). With estimated incidences ranging from 1.3% to 39.2% (65, 66), RFM is a common diseases of cattle and in Dutch dairy cattle the incidence is estimated to be around 5%. The occurrence of RFM is associated with a reduction in milk yield (63, 67), reduced fertility (68, 69) and most importantly an increased risk of (endo)metritis (63, 68). Important risk factors associated with the occurrence of RFM are short gestation length/abortion, caesarian section and induction of parturition (64, 70), but there is an extensive list of risk factors (64, 65, 71) and these can be summarized as anything that is suboptimal during pregnancy and parturition. Many treatments are practiced for RFM (reviewed by Peters and Laven (72), but all are symptomatic and have little or no effect. In the Netherlands common treatments for RFM are intra uterine application of antibiotics and manual removal of the fetal membranes, but both methods likely have no or even an adverse effect (67, 73-76).

Loss of adherence between the fetal and the maternal epithelium together with contractions of the uterus lead to the expulsion of the fetal membranes. The first indications for the involvement of the maternal immune system in the loss of fetal maternal adherence were provided by a series of elegant experiments performed by Gunnink (77-80). Gunnink investigated the chemotaxis of leukocytes towards cotyledon extracts and found a reduced chemotactic activity of cotyledons obtained from RFM cows. Also chemotaxis of leukocytes obtained from RFM cows towards cotyledons from healthy animals was hampered and this could already be observed a week before parturition. Similar results were found by Heuwieser and colleagues (81, 82). Kimura et al (83) found that the functioning of neutrophils from RFM cows was impaired and that this was also already apparent before parturition. Slama et al (84) found lower levels

of Leukotriene B4, a potent chemotactic factor, in caruncular tissue of RFM cows. However, the best indication for the direct involvement of the maternal immune system in placental separation, was given by a study by Joosten and coworkers (85) wherein it was found that the occurrence of RFM was associated with MHC class I compatibility between dam and calf. MHC class I is expressed by fetal trophoblasts at the end of gestation (34, 35) and the results by Joosten et al (85) indicated that expression of allogeneic MHC class I on fetal trophoblasts aids in the loss of fetal-maternal adherence around parturition. Conversely, the absence (or reduction) of allogeneic differences between dam and calf in MHC class I compatible pregnancies leads to the persistence of fetal-maternal adherence and consequently to RFM.

The loss of fetal maternal adherence not only depends on the maternal immune response, but is believed to involve several processes: i) Collapse of the fetal-placental circulation, leading to shrinking of the placentomal villi (65) ii) Placental-maturation, characterized by a decrease in the number and the height of maternal epithelial cells (65, 86) and a drop in BNC numbers (87, 88) iii) Breakdown of the extracellular matrix linking the fetal and maternal epithelium (71). Hormonal changes associated with the initiation of parturition lead to increased collagenase activity, e.g. relaxin (89) and decline in progesterone leading to increased activity of matrix metalloproteinases (MMP) (90). Although many studies found differences in concentrations of 'pregnancy' hormones between RFM cows and cows with normal placental separation, results are conflicting and inconsistent (65). Therefore differences in hormone patterns do not appear to be a major determinant in the development of RFM.

Bovine Neonatal Pancytopenia

Bovine Neonatal Pancytopenia (BNP) was first described in Germany in 2007 when an increase in the number of calves with a bleeding syndrome was observed (91). Soon similar cases were seen all over Europe (92-94) and in 2011 BNP calves were also reported in New-Zealand (95). The first signs of bleeding are typically seen in calves around 7-16 days of age and are most apparent from injection sites and after ear tagging (91, 94). Clinical signs are bleeding and petechiae in skin and mucosa, melena and signs associated with hemorrhagic diathesis (e.g. pale mucosal membranes, lethargy) (91, 92, 94, 96). The mortality of calves with BNP may be up to 90% and death usually occurs around 24-48 hours after onset of clinical signs (92, 94). Hematology revealed that affected calves had leukopenia, thrombocytopenia and anemia, i.e. pancytopenia (91, 92, 94, 96). Upon post mortem examination disseminated bleeding throughout all internal organs was evident and histology of bone marrow revealed a severe hypoplasia of all cell lineages (91, 92, 94). Kappe et al (96) found an association between a novel circovirus and the occurrence of BNP, but other studies could not detect circovirus or any other virus in BNP calves (92, 94, 97). Incidental cases of pancytopenia or hemorrhagic diathesis in cattle had been reported before, for example due to BVD

(98, 99) or dichlorovinylcysteine poisoning (100). However, all previously known causes were excluded for BNP calves (91, 92, 94, 96).

Feeding calves colostrum from dams that had previously given birth to a calf affected with BNP reproduced the disease (101-104). This finding led to the hypothesis that the colostrum of these dams contained alloantibodies that were able to induce BNP in the calf. Indeed, serum and colostrum of BNP dams, dams that had previously given birth to a BNP calf, contained alloantibodies able to bind leukocytes (102, 105-108), platelets (107, 108) and bone marrow cells (105-107). Injecting calves with IgG isolated from the serum of BNP dams showed antibodies alone were sufficient to induce BNP in the calf (107). This left the question what prompted the alloantibody response in the BNP dams. Results from a large multi country case-control study performed in Belgium, France, Germany and the Netherlands indicated that the use of Pregsure© BVD vaccine in the dam was strongly associated with the occurrence of BNP in the calf (93) and Kasonta et al (109) showed that the incidence of BNP was higher in herds that received multiple Pregsure© BVD vaccinations. Pregsure© BVD (Pfizer Animal Health) contained an inactivated BVD type 1 virus (110) that was grown on the bovine MDBK cell line (111). Bovine proteins were detected in the Pregsure© BVD vaccine (107, 112) and alloantibodies from BNP dams were shown to recognize MDBK cells (106, 107, 112). Experimental immunizations of calves with Pregsure© BVD confirmed Pregsure© BVD vaccination induced alloantibodies that bind MDBK cells and leukocytes (106, 109). Immunoprecipitation of target antigens on the surface of MDBK cells (112) and peripheral blood leukocytes (107) using sera from BNP dams and subsequent mass spectrometry analysis of precipitated protein, identified MHC class I as a target of BNP alloantibodies.

Together, these results indicated that alloantigens present in the Pregsure© BVD vaccine induced maternal alloantibodies that, upon ingestion of colostrum, caused BNP in calves. Following reports that associated the occurrence of BNP to the use of Pregsure© BVD, the vaccine was taken off the market in 2010.

Scope of the thesis

This thesis explores the role of materno-fetal alloimmunity in bovine immune mediated disorders, with an emphasis on the effect of MHC class I (in)compatibility between dam and calf on materno-fetal alloimmunity. To get a better understanding of adverse effects of materno-fetal alloimmunity on pregnancy and on the neonate, two disorders representing different aspects of improper regulation of materno-fetal alloimmunity were studied: Retained Fetal Membranes, associated with absence (or reduction) of materno-fetal alloimmunity and Bovine Neonatal Pancytopenia, caused by iatrogenic boosting of materno-fetal alloimmunity.

To explore in more detail the role of MHC class I compatibility between dam and calf on the occurrence of RFM, next-generation sequencing was used to type MHC class I haplotypes of calf–dam–granddam combinations and assess the effect of non-inherited maternal antigens and two-way compatibility between dam and calf on the development of RFM (**Chapter 2**).

The chance of MHC class I compatibility between dam and calf increases if dam and calf have common ancestors, i.e. have a higher coefficient of relationship. Therefore, the effect of the coefficient of relationship between dam and calf on the occurrence of RFM in the dam was examined (**Chapter 3**).

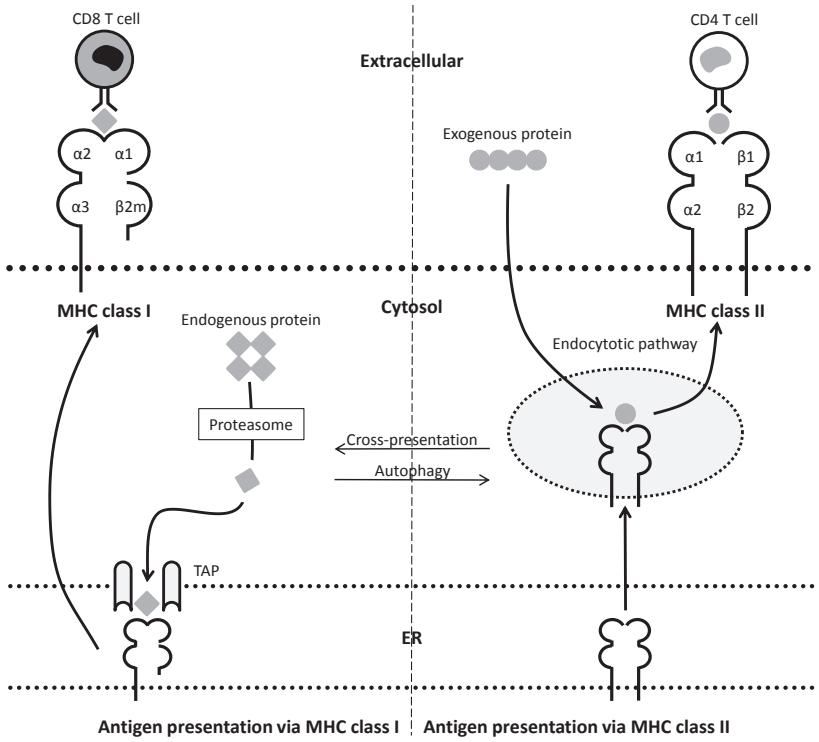
The high incidence of RFM after hormonal induction of parturition has led to the use of induction of parturition as a common model to study RFM (70, 76, 86, 113). We hypothesized that impaired materno-fetal alloimmunity plays an important role in the occurrence of RFM after induction of parturition. To test this hypothesis, we compared the chemotactic activity of cotyledons isolated from non-RFM animals following spontaneous parturition and non-RFM and RFM animals following induction of parturition with glucocorticoids (**Chapter 4**).

Despite the widespread use of the Pregsure© BVD vaccine, the incidence of BNP is low (109), indicating factors other than vaccination alone play a role in the etiology of BNP. We examined whether genetic differences between non-BNP and BNP dams and calves were associated with the occurrence of BNP (**Chapter 5**).

To elucidate the pathophysiology of BNP in the calf, the specificity of BNP alloantibodies was assessed and was linked to the pathology of BNP (**Chapter 6**)

Finally, the results reported in this thesis are summarized and discussed (**Chapter 7**).

Box 1. Antigen presentation by MHC class I and II

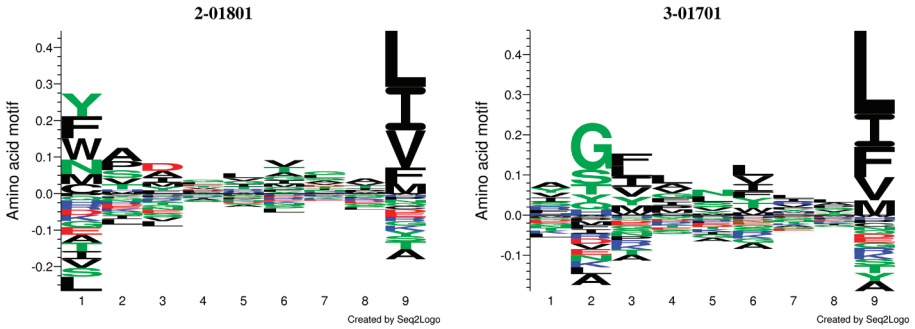


Central to adaptive immunity is the presentation of endogenous antigens to CD8 T cells and exogenous antigen to CD4 T cells by MHC class I and II molecules, respectively.

The MHC class I molecule consists of a variable alpha chain that non-covalently associates with a constant beta chain, the beta-2-microglobulin (B2M), and is expressed on all nucleated cells. The alpha-1 and alpha-2 domain form the peptide binding cleft and can present peptides that are eight to nine amino acids long. Peptides generated by degradation of intracellular proteins by the (immuno)proteasome are transported from the cytosol to the endoplasmic reticulum and are loaded onto the MHC class I by the MHC class I loading complex (containing amongst others the transporter associated with antigen processing (TAP)). The loaded MHC class I complex is then transported to the cell surface and in this way presents a sampling of the intracellular protein repertoire, allowing the screening of these cells by CD8 T cells for infections or aberrant protein expression. (114, 115)

MHC class II consist of a variable alpha and beta chain that are non-covalently associated and are both encoded by genes in the MHC. The open ended peptide binding cleft of MHC class II is formed by the alpha-1 and beta-1 domain of the two MHC class II chains and allows for the binding of peptides of varying lengths. MHC class II is mainly expressed on antigen presenting cells and presents peptides derived from extracellular proteins. Proteins that enter the endocytic or phagocytic pathway are degraded and peptides are loaded onto MHC class II in the mature/late endosome. The loaded MHC class II is subsequently expressed on the cell surface. (115) In exception to the general antigen routing above, both MHC class I and II can present intra- and extracellular antigens. MHC class II can present intracellular antigens via autophagy. Extracellular proteins can be presented via MHC class I trough 'communication' between phagosomes and the ER and the translocation of extracellular antigens to the cytosol. This process, called cross-presentation, is particularly effective in dendritic cells and is essential for the priming of CD8 T cells. (114, 115)

Box 2. MHC class I polymorphism and peptide binding



NETMHCpan 2.8 (116) was used to predict nine amino acid long peptides (derived from Ovalbumin, *Mycobacterium avium* subspecies *paratuberculosis* HSP70 and Bovine Viral Diarrhea virus) with strong binding to bovine MHC class I alleles 2*01801 and 3*01701. A plot of the peptide sequence motif of both alleles was constructed with Seq2Logo 2.0 (117). The horizontal axis shows the amino acid position in the peptide and the vertical axis depicts the amino acids that are predicted to have strong (positive) or weak (negative) binding at that position.

Binding efficiency of peptides to MHC molecules depends on the sequence motif of the peptide, most notably at so called anchor-positions of the peptide (118, 119) (e.g. position 9 in the figure). Clear differences in the sequence motif of peptides predicted to bind allele 2*01801 and 3*01701 can be seen. The amino acids that make up the peptide binding cleft of the MHC class I molecule determine the range of (pathogen) derived peptides that bind and can be presented by an MHC molecule (118, 119) and variation between MHC class I alleles is highest in sequences that encode the peptide binding cleft (5). It is believed that selective pressure by infectious organisms leads to variation in MHC and that it is advantageous both for individuals as for the population to be able to present a wide peptide repertoire and therefore to have a wide variety of MHC molecules (120). This can be achieved by multiple loci with a high degree of polymorphism, codominant expression of MHC alleles and variation in haplotype composition between individuals (5, 120).

Box 3. Human non-classical MHC class I

NC-MHC class I genes are mono or oligomorphic, have a restricted cellular expression pattern, and have very distinct functions from their classical counterparts (121). Three non-classical loci, HLA-E, -F, and –G, are present in humans. HLA-E and –G are known to interact with T cell receptors and NK cell receptors. HLA-E preferably presents peptides derived from signal peptides of MHC class I. This complex activates a suppressive receptor on NK cells, preventing lysis of cells with normal MHC class I expression. Downregulation of MHC class I, an immune evasion tactic of viruses, can be sensed through the down-regulation of HLA-E. HLA-E also presents pathogen derived peptides, since HLA-E restricted pathogen specific T cells have been detected. (121, 122) HLA-G, expressed on human fetal trophoblasts, can bind receptors on NK-cells, DC's and CD8 and CD4 T cells and causes immunosuppression and can lead to the induction of regulatory T cells. (25, 40)

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2

Two-Way Calf to Dam Major Histocompatibility Class I Compatibility Increases Risk for Retained Placenta in Cattle

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ABSTRACT

Problem

In cattle, retained placenta (RP) is suggested to arise from failure of immune-mediated rejection of the fetal membranes by the maternal immune system and is associated with major histocompatibility (MHC) class I compatibility between calf and dam.

Method of study

To study the association between RP and different MHC class I compatibilities between calf–dam–granddam combinations, massively parallel pyrosequencing was used to determine the MHC class I haplotypes of cows with and without RP.

Results

Two-way calf to dam MHC class I compatibility gave a high risk for RP. There was a tendency for a higher risk for RP with calf to dam MHC class I compatibility.

Conclusions

We concluded that in two-way compatible pregnancies, the maternal immune system fails to reject the fetal membranes, and the fetal immune system does not mount an immune response against maternal MHC class I antigens that could influence the immune-mediated rejection of the fetal membranes by the maternal immune system. The lack of immune-mediated rejection of the fetal membranes by the maternal immune system increases the risk of occurrence of RP.

Introduction

Retained placenta, failure of the timely expulsion of the fetal membranes, is a disease of the bovine reproductive tract leading to reduced fertility (1-3), increased veterinary costs and reduced milk yields (2, 3). Reported herd incidences of retained placenta range from 1.3%-39.2%, with a median of 8.6% (4). Normally, around parturition the fetal membranes detach from the uterus and are expelled within hours after the calf is born. Several studies have shown that immune-mediated rejection of the fetal membranes by the maternal immune system plays an important role in the breakdown of the fetal-maternal attachment. Failure of this immune-mediated rejection can lead to retained placenta. (5-7).

Joosten et al. (8, 9) found that even after normal pregnancy and parturition, excluding known risk factors of retained placenta, retained placenta still occurred in 4.1% of calvings. In a subsequent study they showed that retained placenta after normal pregnancy and parturition was associated with Major Histocompatibility Complex (MHC) class I compatibility of the calf to the dam (10). Pregnancies were defined as MHC class I compatible when all MHC class I products of the calf were also present in the dam. In the retained placenta group there was MHC class I compatibility in 60% of the calvings, whereas in the control group this was the case in only 20% of the calvings. MHC class I proteins are expressed on the fetal membranes at the end of gestation (11). Non-self MHC class I proteins can elicit a strong immune response (12) and Joosten and Hensen (13) hypothesized that a maternal immune response directed against paternally derived MHC class I proteins expressed on the fetal membranes is required for the detachment of the fetal membranes. A study by Davies et al. (14) using immunohistochemical staining to compare MHC class I compatible and incompatible pregnancies supports this hypothesis.

The maternal MHC class I genes not inherited by the fetus are called non-inherited maternal antigens (NIMA). It has been shown that during pregnancy and/or the suckling period immunological tolerance to NIMA can be induced (15-17). When a calf is MHC class I incompatible to the dam, but the incompatible MHC class I antigens are compatible to the NIMA of the granddam, the maternal immune system may be tolerant to these antigens leading to failure of immune-mediated rejection of the fetal membranes and to the occurrence of retained placenta. Joosten and colleagues (10) studied the effect of NIMA compatibility, i.e. the MHC class I antigens of the calf incompatible to the dam being compatible to the NIMA of the granddam, on retained placenta in a limited number of calvings. In retained placenta cases three out of five incompatible pregnancies were NIMA compatible whereas in the control group none out of nine incompatible pregnancies were NIMA compatible. This suggests that NIMA compatible pregnancy increases the risk of retained placenta.

The bovine fetal immune system, although immature, is fully functional at parturition and able to respond to a wide variety of diseases in utero (18). In mice, studies have shown that the fetus triggers the onset of parturition. When at the end of gestation the fetal lungs mature, surfactant protein A is secreted into the amniotic fluid where it activates fetal macrophages. These fetal macrophages migrate to the maternal side of the uterus and produce cytokines, triggering an inflammatory reaction and the onset of parturition (19). As accumulation of fetal macrophages in the placenta at the end of gestation is demonstrated in cattle(20), a similar inflammatory reaction may occur. Also, MHC class I compatibility is associated with the occurrence of retained placenta (10) and MHC class I compatibility influences the behavior of fetal macrophages around parturition (14). We hypothesize that around parturition the immune system of the calf responds to foreign MHC class I proteins expressed by maternal tissue and that cytokines released in this immune response influence the immune-mediated rejection of the fetal membranes. Because the fetal immune system is immature and the 'output' of the fetal immune system is much lower than that of the maternal immune system (18), we expect that the effect of the fetal immune response only influences the immune-mediated rejection when there is no/or a lowered maternal immune response. Therefore, we hypothesize that MHC class I compatibility of the dam to the calf, when the calf is either MHC class I compatible to the dam or NIMA MHC class I compatible to the dam, increases the risk of retained placenta in the dam.

In the present case-control study, massively parallel pyrosequencing was used to sequence parts of the MHC class I gene complex to determine the MHC class I haplotypes of individual calves, dams and maternal granddams. These data were subsequently used for analyses of MHC compatibility within calf-dam-granddam combinations in relation to the occurrence of retained placenta in the dams, to gain insight into the role of the maternal and fetal immune system in the occurrence of retained placenta.

Materials and Methods

Experimental animals

In the period between September 2008 and March 2009 sixty-five calf-dam-granddam combinations were selected from commercial dairy farms throughout the Netherlands. All animals were Holstein-Friesian or Holstein-Friesian cross breeds. Twin calvings were excluded to avoid MHC typing complexities due to chimaerism.

The study was designed as a case-control study, with a 1:2 ratio of cases to controls. Sample size was based upon the data from Joosten et al. (10). Controls (n=44) were defined as dams which expelled the fetal membranes within 6 hours post partum (21) following the birth of a live calf. Cases (n=21) were dams with retained placenta,

defined as a failure to expel the fetal membranes within 24 hours post partum. In the cases the calving was without difficulties (minor assistance was allowed), and the calf was born alive. Apart from retained placenta there were no periparturient diseases in the dam during the first 2 weeks post partum. Cases had to meet these criteria in order to exclude other major risk factors for retained placenta.

Cows were bled using heparin coated evacuated blood collection tubes (Vacutainer system with Lithium Heparin tube, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) as the source material for isolation of genomic DNA.

The described use of the animals in this study was approved by the Animal Ethical Committee of Utrecht University and conducted according to their regulations.

Sequencing

MHC genotyping was conducted using a novel protocol based on massively parallel pyrosequencing similar to other recently published protocols (22-25). Briefly, genomic DNA was isolated from peripheral leukocytes using the Promega Wizard Genomic DNA Purification Kit (Promega corporation, Madison, WI, USA). Exons 2 and 3 of the MHC class I genes were amplified from this genomic DNA by PCR and the resulting amplicons were sequenced on a Genome Sequencer FLX system (454 Life Sciences, Roche Diagnostics, Branford, CT, USA) according to the manufacturer's protocols. The resulting sequences were aligned and assembled into consensus reads using Amplicon Variant Analyzer software (454 Life Sciences, Roche Diagnostics, Branford, CT, USA). The consensus reads were identified by using BLAST to compare them to a custom database containing all known bovine MHC sequences. Once alleles were identified, MHC haplotypes were assigned using the Cytofile cluster analysis computer programs developed by C.J. Davies (26).

Statistical methods

MHC class I compatibility was determined using the haplotypes assigned to each animal. There is compatibility when all MHC class I haplotypes are compatible. Calf-dam-granddam combinations were grouped into the following MHC class I compatibility subclasses:

Maternal Compatibility (MC)

The MHC class I haplotypes of the calf are compatible to the dam. The calf has no MHC class I haplotypes that are not present in the dam. In this situation there is MHC class I compatibility between calf and dam from the point of view of the maternal immune system, because no foreign haplotypes are present on the fetal membranes.

Non-Inherited Maternal Antigen compatibility (NIMAC)

The MHC class I haplotypes of the calf that are not compatible to the dam are compatible to the non-inherited MHC class I haplotypes of the granddam. In this situation there is MHC class I compatibility from the point of view of the maternal immune system, because of tolerance of the dam to NIMA.

Calf Compatibility (CC)

The MHC class I haplotypes of the dam are compatible to the calf. The dam has no MHC class I haplotypes that are not present in the calf. In this situation there is MHC class I compatibility between calf and dam from the point of view of the immune system of the calf, because no foreign haplotypes are present on the endometrium.

Two-Way Compatibility (TWC)

The MHC class I haplotypes of the calf are compatible to the dam (MC) and the MHC class I haplotypes from the dam are compatible to the calf (CC). MHC class I compatibility from the point of view of the immune system of both the dam and the calf.

Two-Way Compatibility through Non-Inherited Maternal Antigens (NTWC)

The MHC class I haplotypes of the calf that are not compatible to the dam are compatible to the non-inherited MHC class I haplotypes of the granddam and the MHC class I haplotypes of the dam are compatible to the calf. MHC class I compatibility from the point of view of the immune system of both the dam and the calf.

No Compatibility (NC)

Immunological recognition can occur in both directions. The MHC class I haplotypes of the calf are not compatible to the dam, neither are they to the non-inherited MHC class I haplotypes of the granddam. In addition, the MHC class I haplotypes of the dam are not compatible to those from the calf.

Because of the binary nature of the outcome variable we chose a logistic regression model to analyze the data. The following model was used:

$$\ln\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta_1 * Comp$$

π = Fraction of the calvings leading to retained placenta; α = Intercept; $\beta 1$ * Comp = Effect of MHC class I compatibility on retained placenta (MC, NIMAC, CC, TWC, NTWC, NC)

After fitting the model we looked at which MHC class I compatibility subclass within the compatibility variable (Comp) had the highest p-value. This compatibility subclass was dropped and the calf-dam-granddam combinations within this subclass were added to the NC subclass. Subsequently the model was fitted with the new data. This procedure was repeated until there were no compatibility subclasses with a p-value ≥ 0.1.

The fitted models were compared using both Akaike's information criterion (AIC) and the Likelihood ratio test (LRT). The model with the lowest AIC was considered to be the model best supported by the data. With the LRT we checked if excluding a compatibility subclass had a significant effect on the prediction of the dependant variable. Results were considered significant at $P<0.05$.

The program R (http://www.r-project.org) was used for all statistical computations.

Table 1. Two calf-dam-granddam combination with assigned haplotypes.

Compatibility*	Animal	MHC class I haplotypes
CC	Calf	AH011A, AH015A
	Dam	AH015A
	Granddam	AH014A, AH015A
MC	Calf	AH020C
	Dam	AH010A, AH020C
	Granddam	AH010A, AH020C

CC = Calf Compatibility, MC = Maternal Compatibility

Table 2. Compatibility between calf-dam-granddam combinations.

Compatibility*	RP+ (n=21)	Control (n=44)
MC	4 (19%)	3 (7%)
NIMAC	2 (10%)	6 (14%)
CC	2 (10%)	8 (18%)
TWC	5 (24%)	1 (2%)
NC	8 (38%)	26 (59%)

* MC = Maternal Compatibility, NIMAC = Non Inherited Maternal Antigen Compatibility, CC = Calf Compatibility, TWC = Two-Way Compatibility, NC = No Compatibility
+ RP = Retained Placenta

Results

In table 1 two examples of calf-dam-granddam combinations with assigned MHC class I haplotypes are shown. As the calf in the first combination had a MHC class I haplotype (AH011A) that was present in neither the dam, nor the granddam and the dam had no MHC class I haplotypes different from the calf; this calf-dam-granddam was grouped into the compatibility subclass CC. Following this approach, the second calf-dam-

granddam combination was assigned to the compatibility subclass MC. An overview of all the calf-dam-granddam combinations and the compatibility subclass to which they have been assigned can be seen in table 2. There was no two-way compatibility through NIMA between animals and the NTWC subclass was discarded.

The initial logistic regression model is summarized in table 3. In this model TWC gave a significant higher risk of retained placenta with an odds ratio (OR) of 16.25 and there was a tendency for a higher risk of retained placenta for MC with an OR of 4.33. The compatibility subclasses CC and NIMAC did not have a significant effect on the occurrence of retained placenta and had an OR of 0.81 and 1.08, respectively.

Table 3. Summary of the initial logistic regression model A (Comp = NC, CC, NIMAC, MC, TWC)[+] predicting the occurrence of Retained Placenta, with P-values indicating the significance of an estimate to be different from zero.

Exposure variable		B[a]	SE β[b]	OR[c]	95% CI[d]	P-value[e]
Intercept		-1.18	0.40			0.004**
Comp	NC	Referent				
	CC	-0.21	0.89	0.81	0.11 – 4.13	0.81
	NIMAC	0.08	0.91	1.08	0.14 – 5.88	0.93
	MC	1.47	0.86	4.33	0.80 – 26.25	0.090-
	TWC	2.79	1.17	16.25	2.21 – 336.79	0.017*

Null deviance: 81.79 on 64 degrees of freedom. Residual deviance: 71.07 on 60 degrees of freedom. AIC: 81.07. Cox & Snell $R^2 = 0.15$. Nagelkerke $R^2 = 0.21$.

[a] Parameter estimate

[b] Standard error of the parameter estimate

[c] Odds ratio

[d] 95% confidence interval of the odds ratio

[e] -p < 0.1, *p < .05, **p < .01

[+] NC = No Compatibility, CC = Calf Compatibility, NIMAC = Non Inherited Maternal Antigen Compatibility, MC = Maternal Compatibility, TWC = Two-Way Compatibility

Table 4. Akaike's information criterion (AIC) for the different logistic regression models

Model	**Subclasses within Comp***	**AIC**
A:	NC,CC,NIMAC,MC,TWC	81.07
B:	NC,CC,MC,TWC	79.08
C:	NC,MC,TWC	77.15[1]

[1] Model with the best fit using AIC

* NC = No Compatibility, CC = Calf Compatibility, NIMAC = Non Inherited Maternal Antigen Compatibility, MC = Maternal Compatibility, TWC = Two-Way Compatibility

Table 5. The Likelihood ratio test (LRT) comparing the logistic regression models and the log-likelihood for each model.

	Model[1]		
Model	A	B	C
A		0.93	
B			0.80
Log-likelihood	-35.54	-35.54	-35.57

[1] See Table 4 for the models

Subsequently different models were tested to find the best model. These models are shown in table 4. In model A, compatibility subclass NIMAC had the highest p-value (0.93) and was dropped. Compatibility subclass CC had the highest p-value (0.80) in model B and was therefore dropped in the next model. In the final model C, the compatibility variable contained the subclasses NC, MC and TWC. The final model had the lowest AIC (Table 4). Using the LRT to compare the different models showed that dropping the subclasses NIMAC and CC from the model did not have a significant effect on the prediction of the model on the occurrence of retained placenta (Table 5).

Table 6 summarizes the final model. The effect of TWC on retained placenta was significant (p=0.014) and gave a high risk for the occurrence of retained placenta with an OR of 16.67. Calf-dam-granddam combinations within the MC subclass tended (p=0.073) to have a higher risk for the occurrence of retained placenta than combinations within the NC subclass. The OR of MC was 4.44.

Table 6. Summary of the final logistic regression model C (Comp = NC, MC, TWC)[+] predicting the occurrence of Retained Placenta, with P-values indicating the significance of an estimate to be different from zero.

Exposure variable		β[1]	SE β[2]	OR[3]	95% CI[4]	P-value[5]
Intercept		-1.20	0.33			<0.001***
Comp	NC	Referent				
	MC	1.49	0.83	4.44	0.87 - 25.34	0.073-
	TWC	2.81	1.14	16.67	2.39 - 336.32	0.014*

Null deviance: 81.79 on 64 degrees of freedom. Residual deviance: 71.15 on 62 degrees of freedom. AIC: 77.15. Cox & Snell $R^2 = 0.15$. Nagelkerke $R^2 = 0.21$.

[1] Parameter estimate

[2] Standard error of the parameter estimate

[3] Odds ratio

[4] 95% confidence interval of the odds ratio

[5] -p < 0.1, *p < .05, **p < .01, ***p < .001

[+] NC = No Compatibility, MC = Maternal Compatibility, TWC = Two-Way Compatibility

Discussion

The data presented in this study indicate that there is a significant effect (p=0.014) of two-way MHC class I compatibility on the occurrence of retained placenta in cattle. Two-Way Compatibility (TWC) indicated that the MHC class I haplotypes of the calf were compatible to the dam and also that the MHC class I haplotypes of the dam were compatible to the calf. In this combination MHC class I compatibility existed from the point of view of the immune system of both the dam and the calf, taking into account calves are born with a fully functional immune system (18).

As calf compatibility by itself had no statistically significant effect on the occurrence of retained placenta, we conclude that the immune-mediated rejection of the fetal membranes is not determined by the fetal immune system alone. Calf compatibility only increases the risk for retained placenta when there is maternal MHC class I compatibility as well. This in turn raises the question whether maternal compatibility alone can also lead to the occurrence of retained placenta. Although there is a tendency for an effect of maternal compatibility on the occurrence of retained placenta in our study, this association is not statistically significant. A similar result was obtained in an earlier study (Davies C.J., unpublished data), in which serology was used to identify MHC class I haplotypes for evaluation of the effect of MHC class I compatibility between the calf and dam on the occurrence of retained placenta. Although there was no relation between maternal compatibility and the occurrence of retained placenta, there was a tendency ($p=0.060$, $n=80$) for the association of two-way compatibility with the occurrence of retained placenta.

Joosten et al. (10) found a significant association between maternal compatibility and the occurrence of retained placenta, but did not discriminate between maternal compatibility and two-way compatibility. By not discriminating between these two types of compatibility, Joosten et al. studied the combined effect of the two types of compatibility and may have overestimated the effect of maternal compatibility. More research is necessary to determine whether maternal compatibility alone leads to retained placenta. If maternal compatibility does lead to retained placenta, it is likely to give a lower probability of retained placenta than two-way compatibility, because an immune response of the fetal immune system against maternal MHC class I antigens could trigger the immune-mediated rejection of the fetal membranes by the maternal immune system. This can also be seen in our model for the prediction of the occurrence of retained placenta, where the OR's for maternal compatibility and two-way compatibility are 4.44 and 16.67, respectively.

Besides an immune-mediated rejection of the fetal membranes, breakdown of the fetal-maternal attachment is also influenced by hormonal changes and structural changes in the uterus and fetal membranes at the end of gestation (3). In one two-way compatible pregnancy the fetal membranes were expelled normally (Table 2) and in this case the fetal membranes may have detached even though there was no immune-mediated rejection of the fetal membranes. Another possibility is that, while fetus and dam were assigned to the same MHC class I haplotype, there were differences in MHC class I genes (i.e. the fetus and dam were actually of two closely-related haplotypes that were not differentiated). Lastly, it is possible that the dam responded to minor histocompatibility antigens triggering an immune-mediated rejection of the fetal membranes.

Immunological tolerance to non-inherited maternal antigens can be induced during fetal development and/or during the suckling period (15-17). Joosten et al. (10)

hypothesized that tolerance to NIMA could affect the immune-mediated rejection of the fetal membranes through an effect on MHC class I compatibility. Even though the number of animals in the study by Joosten et al. (10) was low, the results were suggestive of an effect of NIMA compatibility on retained placenta. Our results, however, did not show any effect of NIMA compatibility on the occurrence of retained placenta. The NIMAC subclass had a p-value of 0.930 and was the first subclass to be dropped. Therefore, it seems likely that tolerance to NIMA does not influence the occurrence of retained placenta in cattle.

Based on the results from this study we hypothesize that both the maternal and the fetal immune system play a role in the immune-mediated rejection of the fetal membranes around parturition. In mice it has been shown that cytokines produced by fetal macrophages trigger an inflammatory reaction leading to the onset of parturition (19). The bovine fetal immune system is fully functional at parturition (18) and there is an accumulation of fetal macrophages in the placenta at the end of gestation in cattle (20). Oliveira et al. (27) have shown that maternal macrophages in the second trimester of pregnancy are in a state that supports immune regulation and tissue remodelling. At the end of pregnancy the state of the maternal macrophages, under influence from signals either from the fetus or the dam, could change into a state supporting inflammation. MHC class I compatibility between dam and calf may influence the behavior of fetal macrophages and the release of inflammatory cytokines from both fetal and maternal macrophages in the uterus around parturition (14). The association of two-way compatibility with retained placenta, supports our theory that cytokines produced by the fetal immune system, in response to foreign maternal MHC class I antigens, influence the immune-mediated rejection of the fetal membranes by the maternal immune system. In the case of two-way compatibility, the maternal immune system does not reject the fetal membranes and the fetal immune system does not mount an immune response against maternal MHC class I antigens which could influence the immune-mediated rejection of the fetal membranes by the maternal immune system. Possible involvement of the fetal immune system in the breakdown of the fetal-maternal attachment gives new insight into bovine parturition and could be a promising lead for novel therapies to prevent the occurrence of retained placenta.

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3

Heritable and non-heritable genetic effects on retained placenta in Meuse-Rhine-Yssel cattle

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ABSTRACT

Failure of the timely expulsion of the fetal membranes, called retained placenta, leads to reduced fertility, increased veterinary costs and reduced milk yields. The objectives of this study were to concurrently look at the heritable and non-heritable genetic effects on retained placenta and test the hypothesis that a greater coefficient of relationship between dam and calf increases the risk of retained placenta in the dam.

The average incidence of retained placenta in 43,661 calvings of Meuse-Rhine-Yssel cattle was 4.5%, ranging from 0% to 29.6% among half-sib groups. The average pedigree based relationship between the sire and the maternal grandsire was 0.05 and ranged from 0 to 1.04. Using a sire-maternal grandsire model the heritability was estimated at 0.22 (SEM = 0.07) which is comparable with estimates for other dual purpose breeds. The coefficient of relationship between the sire and the maternal grandsire had an effect on retained placenta. The coefficient of relationship between the sire and the maternal grandsire was used as a proxy for the coefficient of relationship between dam and calf, which is correlated with the probability of major histocompatibility complex (MHC) class I compatibility between dam and calf. MHC class I compatibility is an important risk factor for retained placenta. Although the MHC class I haplotype is genetically determined, MHC class I compatibility is not heritable. This study shows that selection against retained placenta is possible and indicates that preventing the mating of related parents may play a role in the prevention of retained placenta.

Introduction

At parturition, the attachment between the fetal membranes and the uterus is normally broken down and the fetal membranes are expelled soon after the calf is born. Failure of the timely expulsion of the fetal membranes, called retained placenta (RP), leads to reduced fertility, increased veterinary costs and reduced milk yields (1-3). Reported incidences of RP in Meuse-Rhine-Yssel (MRY) and Holstein cattle are around 7% (4) and 5% (5, 6), respectively.

Important risk factors for RP, such as gestation length and calving difficulty (2), are genetically determined and have high heritability estimates (7, 8). Therefore, it is expected that RP is also heritable. Indeed, reported estimates of heritability of liability for RP range from 0.03 to 0.10 (5, 9-13). Joosten et al (14) and Benedictus et al (15) have shown that major histocompatibility complex (MHC) class I compatibility between dam and calf increases the risk for RP in MRY and Holstein cattle, respectively. Major

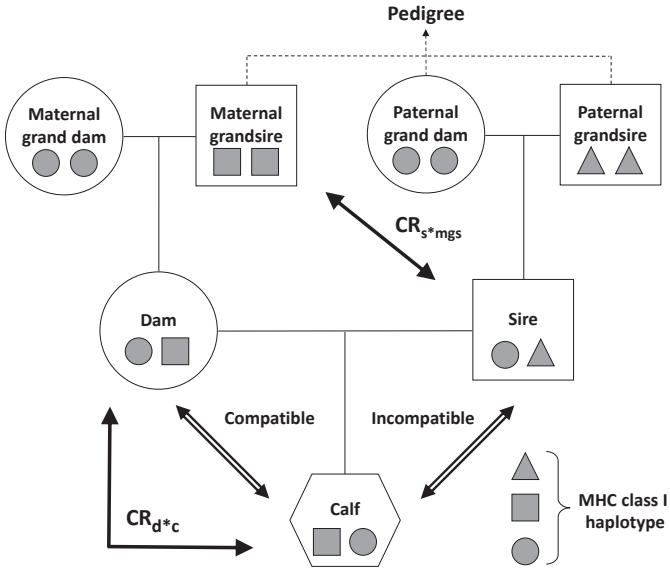

Fig. 1. MHC class I compatibility and coefficient of relationship. Major histocompatibility complex (MHC) class I compatibility is an important risk factor for retained placenta. Although the MHC class I haplotypes of an individual are genetically determined, MHC class I compatibility is not heritable as it depends on the combination of two animals. In this figure the MHC class I haplotype of the sire that is inherited by the calf is compatible to the dam (gray circle). Therefore the dam and calf are MHC class I compatible. Notice that the sire and the dam are incompatible, because the MHC class I haplotype of the dam that is inherited by the calf is incompatible to the sire (gray square). The coefficient of relationship between dam and calf (CRd*c) is correlated with the probability that dam and calf are MHC class I compatible. The coefficient of relationship is defined as the proportion of alleles held in common by two individuals as a result of a common ancestor. The coefficient of relationship between sire and maternal grandsire (CRs*mgs) was used as a proxy for the coefficient of relationship between dam and calf and was calculated with the pedigree data.

histocompatibility complex class I compatibility between dam and calf is genetically determined, but in contrast to other genetically determined risk factors for RP, MHC class I compatibility is not heritable (Figure 1). Major histocompatibility complex class I compatibility between calf and dam depends on whether the paternal MHC class I haplotype inherited by the calf is compatible to the MHC class I haplotypes of the dam. In this respect, MHC class I compatibility is akin to inbreeding. Inbreeding is genetically determined, but depends on the specific combination of parents and is therefore not heritable.

Given the results obtained by Benedictus et al. (15) for Holstein cattle, previously collected data in MRY cattle were reanalyzed (4). Tracing back the pedigree allowed us to analyze the effect of MHC compatibility on RP in a large dataset. The probability of MHC class I compatibility increases if dam and calf are related, i.e have common ancestors. The coefficient of relationship (CR) between dam and calf (CRd*c) is correlated with the probability that the MHC class I haplotypes of the calf are identical to the MHC class I haplotypes of the dam and thus the probability that dam and calf are MHC class I compatible by descent (figure 1). In the present research, it was hypothesized that a greater CRd*c increases the risk of RP in the dam. The objectives of this study were to concurrently determine heritable and non-heritable genetic effects on retained placenta and test the hypothesis that a greater coefficient of relationship between dam and calf increases the risk of RP in the dam.

Materials and methods

Data

Birth registrations for MRY cows were available from an artificial insemination centre from the south of the Netherlands over the period from November 1975 through May 1984, as described in detail by Joosten et al (4). Submission of birth registration records was obligatory at that time and farmers were instructed in the use of the registration records.

Only birth registration records on calvings with a gestation length of 260 to 300 days producing a single live calf were used. Sires (n = 22) and maternal grand sires (n = 6) were included such that all 132 half-sib groups had a minimum of 10 calvings. Retained placenta was defined as retention of the fetal membranes for more than 24 hours post-partum. The final data contained 43,661 records.

Pedigree file

The pedigree of the sires and maternal grandsires were traced back up to 18 generations and the first three generations were 95% complete. The resulting pedigree file included information on 634 individuals.

Coefficient of relationship

The coefficient of relationship is defined as the proportion of alleles held in common by two individuals as a result of a common ancestor (16). Using the pedigree file the CR between sire and maternal grandsire (CRs*mgs) were calculated with a computer algorithm written in Fortran 95 using the tabular method (17). The CRs*mgs was used as a proxy for CR between dam and calf (Figure 1), because no information regarding the dams was available.

Statistical methods

The data were analyzed with a threshold-liability sire-maternal grandsire model using the software package ASReml (18), a statistical package that fits generalized linear mixed models using Residual Maximum Likelihood. Retained placenta was fitted as a binomial variable using the logistic link function. In the first model CRs*mgs was fitted as a fixed class effect to check whether CRs*mgs had a linear effect on RP:

$$\lambda_{ijk} = \mu + CR_i + sire_j + mgs_k + e_{ijk}$$

where λijk is a vector of unobserved liabilities to RP; μ is the general mean; CRi is the fixed effect of the ith class of CRs*mgs, divided into 4 classes (CRs*mgs <0.05, 0.05 to <0.10, 0.10 to <0.15, ≥0.15); sirej is the random effect of the jth sire; mgsk is the random effect of the kth maternal grandsire and eijk is the vector of residuals.

For a subset of the data a second model was fitted with CRs*mgs as a continuous variable. In this second model CRi is the ith CRs*mgs and b is the regression coefficient of CRi. All other factors were the same as in Model 1.

The distributional assumptions of the random effects were:

$$\begin{bmatrix} sire \\ mgs \\ e \end{bmatrix} \sim N\left(\begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}, \begin{bmatrix} A\sigma_s^2 \\ A\frac{1}{4}\sigma_s^2 \\ I\sigma_e^2 \end{bmatrix} \right)$$

where sire is the vector of random sire effects, mgs is the vector of random maternal grandsire effects, e is the vector of residuals, A is the sire relationship matrix, I is the identity matrix, is the sire variance and is the residual variance.

Heritability was calculated using the variance components of the model, correcting for the underlying residual variance of the logistic scale of ϖ2/3, as follows:

$$h^2 = \frac{4\sigma_s^2}{\sigma_s^2 + \frac{1}{4}\sigma_s^2 + \frac{\pi^2}{3}}$$

Table 1. The coefficient of relationship between sire and maternal grandsire, the percentage of retained placenta and the number of calvings for 132 half-sib groups

	Maternal grand sire																		
	A			B			C			D			E			F			Total	
Sire	CR	RP	n	CR	RP	n	CR	RP	n	CR	RP	n	CR	RP	n	CR	RP	n	RP	n
a	.0088	4.4	409	.2711	3.8	234	1.0032	0.0	76	.0092	2.7	187	.0049	2.2	46	.0320	3.4	87	3.5	1039
b	.1372	2.0	49	.0171	2.3	44	.0182	1.3	79	.0025	3.8	26	.2522	0.0	50	.0079	2.8	360	2.3	608
c	.0054	2.6	469	.1432	4.7	427	.1443	2.1	435	.0673	2.2	366	.0056	1.3	80	.0548	0.8	119	2.7	1896
d	.1328	8.1	209	.0044	4.4	250	.0063	6.5	200	.0017	2.7	260	.0664	5.9	102	.0022	4.4	68	5.2	1089
e	.0088	6.4	1133	.1523	9.9	1144	.1537	4.6	1014	.0160	5.3	760	.0044	5.5	220	.1671	4.9	162	6.6	4433
f	.0044	6.1	1845	.0309	7.6	1530	.0320	5.5	1288	.0394	3.9	1520	.0022	8.2	441	1.0371	5.4	56	6.0	6680
g	.0000	5.8	329	.0000	3.8	1061	.0277	2.0	615	.0013	2.1	378	.0059	6.7	504	.0000	4.5	550	4.0	3437
h	.0088	3.6	222	.0254	4.7	277	.0270	3.5	257	.0016	2.7	183	.0161	7.1	56	.0114	1.5	67	3.8	1062
i	.0098	2.8	1236	.0338	2.4	1407	.0348	2.6	1268	.0648	2.3	899	.0127	3.6	302	.0507	3.1	324	2.6	5436
j	.0132	1.2	251	.1692	0.5	660	.1707	0.7	419	.0067	0.6	343	.0081	3.5	255	.0292	0.0	10	1.0	1938
k	.1372	10.3	78	.1001	6.8	132	.1026	4.0	100	.0097	4.2	95	.0687	6.7	45	.0271	4.2	260	5.5	710
l	.0005	4.8	166	.0091	6.2	129	.0095	8.2	159	.0426	4.5	88	.0188	2.0	51	.1540	6.6	301	6.0	894
m	.0674	5.5	548	.0190	5.4	827	.0200	5.4	596	.0806	1.9	426	.1362	6.3	284	.0754	4.3	561	4.8	3242
n	.0442	3.2	62	.0419	0.0	19	.0428	2.1	140	**.0033**	**14.3**	**14**	.0221	3.2	31	.0172	2.7	842	2.8	1108
o	.0092	5.0	159	.0739	6.3	208	.0746	3.4	203	.0245	2.7	146	.0140	5.4	129	.0216	3.5	603	4.1	1448
p	.0005	5.0	363	.0016	5.2	553	.0274	1.6	565	.0070	2.1	280	.0020	3.0	168	.0056	3.2	1211	3.4	3140
q	.0686	26.9	26	.0219	9.4	32	.0229	9.2	76	**.0230**	**29.6**	**27**	.1276	20.0	10	.1676	10.8	415	12.3	586
r	.0999	7.3	55	.0054	1.4	72	.0068	4.5	89	.0171	2.7	37	.1925	0.0	26	.0115	3.6	337	3.6	616
s	.0664	12.1	58	.0022	11.2	89	.0034	4.9	102	.0009	5.0	40	.1328	5.6	36	.0011	3.9	619	5.3	944
t	.0686	4.6	65	.0696	5.6	89	.0712	0.9	116	.0057	0.0	51	.1310	0.0	29	.0198	3.6	274	3.0	624
u	.0000	7.7	235	.0175	8.8	295	.0183	5.6	340	.0320	3.3	153	.0007	4.1	121	.1828	5.1	806	5.8	1950
v	.0664	3.0	66	.0054	8.7	149	.0157	3.2	124	.0067	4.1	49	.1331	1.4	71	.0688	9.0	322	6.5	781
Total		5.1	8033		5.4	9628		3.7	8261		3.2	6328		5.2	3057		4.3	8354	4.5	43661

CR, coefficient of relationship between sire and maternal grandsire; RP, percentage of cows with retained placenta; n, number of calvings
Outliers have been highlighted in bold

A Wald test was used to test whether CRs*mgs improved the fit of the model. The effect was considered significant at $P < 0.05$ and a tendency at $P < 0.1$. Values are means ± the standard error of the mean between brackets.

Results and Discussion

The average incidence of RP in 43661 calvings was 4.5%, ranging from 0% to 29.6% among half-sib groups (Table 1). This is comparable to previously reported incidences of RP in MRY and Holstein cattle (4-6). The average weighed CR_{s*mgs} was 0.05 and ranged from 0 to 1.04 (Table 1).

To check whether CR_{s*mgs} had a linear effect on the occurrence of RP, CR_{s*mgs} was fitted as a fixed class effect in Model 1, with half-sib groups divided into classes based on the CR_{s*mgs}. The estimates of the incidence of retained placenta for the different classes are shown in Figure 2. The estimated incidence of RP increases for higher CR_{s*mgs} classes, except for the class containing $CR_{s*mgs} > 0.15$, which showed a lower estimate for the occurrence of RP. The chance of MHC compatibility increases with a higher CR_{s*mgs} and it is expected that the estimated incidence of RP increases for higher CR_{s*mgs}. However, at the higher CR_{s*mgs} inbreeding depression may have influenced the outcome of pregnancy. Inbreeding depression leads to reduced fertility (19) and in cattle has been shown to increase mortality and fetal death (20, 21). For the present study, birth registration records of successful pregnancies with a gestation length of 260 to 300 days producing a single live calf were used. At the higher CR_{s*mgs} inbreeding depression may have led to increased first trimester pregnancy failure and still births and in combination with the selection criteria to a bias towards reduced incidence of RP in the data. In current breeding practice inbreeding is minimized (22). Because inbreeding depression may have had a confounding effect at the higher CR_{s*mgs} and the lower coefficients of relationship are more relevant to the current dairy industry, $CR_{s*mgs} >0.15$ were excluded in subsequent analyses.

The results from the second model, where CR_{s*mgs} was fitted as a continuous variable and all $CR_{s*mgs} >0.15$ were excluded, are shown in Table 2. The estimated heritability of RP in this model was 0.25 (±0.07) and the estimate for the regression coefficient of CR_{s*mgs} was 1.55 (±0.97), with a p-value of 0.11.

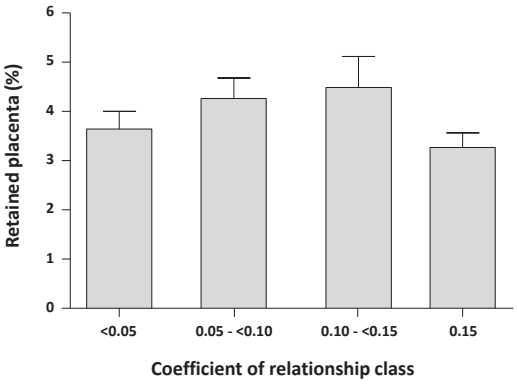

Fig. 2. Column chart depicting the estimated incidence of retained placenta for the different classes of coefficient of relationship between sire and maternal grandsire in Model 1. However, this relationship was not significant, even if the group with the highest value was excluded ($P = 0.08$).

Table 2 The sire variance and heritability estimate of retained placenta and the parameter estimates for the effect of coefficient of relationship between sire and maternal grandsire on retained placenta for different sire-maternal grandsire models

	CR class	σ^2_s	h^2 (SE)	β (SE)	
Model 1[a]	1: <0.05	0.28	0.31 (0.09)	Referent	
	2: 0.05-<0.10			0.16	(0.10)
	3: 0.10-<0.15			0.22	(0.14)
	4: ≥0.15			-0.11	(0.09)
Model 2[b]		0.22	0.25 (0.07)	1.55	(0.97)

σ^2_s, sire variance; h^2, heritability estimate; β, parameter estimate; CR, coefficient of relationship
[a] CR_{s*mgs} fitted as a fixed class effect with 4 classes
[b] CR_{s*mgs} fitted as a continuous variable

In both models the same two half-sib groups were identified as outliers. These half-sib groups had a residual more than five times the approximated standard deviation. The groups had an incidence of RP of 14.3% and 29.6%, which is much greater than the average incidence of retained placenta of 4.3% and contained a relatively low number of birth registration records of 14 and 26, respectively. Therefore, omitting these two half-sib groups leads to a better estimate of the relationship between CR_{s*mgs} and the incidence of RP. Running the final model, Model 2 excluding the outliers, resulted in an estimated heritability of RP of 0.22 (±0.07) and an estimate for the regression coefficient of CR_{s*mgs} of 1.69 (±0.97), with a p-value of 0.08.

Joosten et al (23) found an effect of sire and maternal grandsire on the incidence of RP in normal calvings of MRY cattle, suggesting that RP is a heritable trait in MRY cattle. Indeed, the estimated heritability of RP in the final model was 0.22. This is slightly greater than previous estimates of heritability of RP that range from 0.01 in first lactation Holstein cattle (24) to 0.14 in first lactation Simmental cattle (25). Meuse-Rhine-Yssel and Simmental cattle are both dual purpose breeds. Dual purpose breeds have more calving difficulties, which is a risk factor for RP (2) and a heritable trait (7). Therefore, heritability of RP may be greater in dual purpose breeds. Heritability estimates differ substantially between and even within breeds. Wassmuth et al (13) reported heritability estimates for Danish Jersey, Danish Holstein, and Danish Red of 0.03, 0.09 and 0.10 respectively. Heritability estimates for Norwegian Red cattle reported in the years 2005, 2009 and 2010 were 0.06, 0.09 and 0.08, respectively (9-11).

Immune-mediated rejection of the fetal membranes by the maternal immune system plays an important role in the breakdown of the fetal–maternal attachment and RP can be linked to failure of immune-mediated detachment of the fetal membranes (26-29). Joosten et al (14) and Benedictus et al (15) have shown that MHC class I compatibility between dam and calf is an important risk factor for RP. Non-self MHC class I protein can elicit a strong immune response and it has been postulated that the fetus is identified as 'foreign' through the recognition of paternal MHC class I

proteins expressed on the fetal membranes (26, 30). When dam and calf are MHC class I compatible, the calf has no MHC class I proteins different from the dam and the fetal membranes are not recognized as 'foreign' leading to retention of the fetal membranes. The CR_{s*mgs} was used as an estimate for the CR between dam and calf, which is correlated with the probability of MHC class I compatibility between dam and calf. In the final model, excluding the two outliers, there is a tendency for an effect of CR_{s*mgs} on RP.

Sevinga et al (31) found that in Friesian horses RP is associated with MHC class I compatibility and they showed a positive linear relationship between the CR between mare and foal and the incidence of RP in the mare (32). In Friesian horses the incidence of RP and the CR between mother and foal is much greater than in cattle (32). Nevertheless, the effect of the coefficient of relationship between mare and foal on the incidence of RP was small (32). The CR between sire and dam is half that of the CR between sire and maternal grandsire. From the definition of the CR follows that a 0.1 increase in CR between sire and maternal grandsire, the difference between the first and the third CR class in Model 1, would increase the chance of MHC class I compatibility between dam and calf by 5%. In previous studies about 70% of MHC class I compatible pregnancies led to RP (14, 15). This would mean that a 0.1 increase in CR between sire and maternal grandsire could in theory lead to a 3.5% increase in the incidence of RP. In the final Model 2 a 0.1 increase in CR between sire and maternal grandsire leads to a 0.65% increase in the incidence of RP. This is much lower than the theoretical calculation but more comparable to the results found in Friesian horses. A reason for this difference may be that part of the effect of MHC class I compatibility on RP is accounted for by the genetic effect in the model. Although the MHC class I gene complex is highly polymorphic, the haplotype diversity in a given cattle population is limited and some haplotypes are very common (33, 34). Therefore MHC class I compatibility between dam and calf can arise randomly as well as through common ancestry. By using the CR, only the chance of compatibility through common ancestry is measured. If a sire has a MHC class I haplotype which is present in a high frequency in the population, this would increase the chance of MHC class I compatibility and thus RP in pregnancies serviced by this sire. Because this haplotype is heritable, this effect is accounted for by the genetic effect in the model. However, when a sire has a common MHC class I haplotype and also a high CR with the dam, part of the effect of the CR on the chance of MHC class I compatibility is absorbed into the genetic effect of the model. Therefore the effect of CR on the incidence of RP is underestimated and this could explain the difference in the theoretical effect of CR on RP and the prediction from the model. Also, the CR between sire and maternal grandsire was used as a proxy for the CR between dam and calf and there was no information on other risk factors for RP. This could explain why we only saw a tendency for an effect of CR_{s*mgs} on RP in this study. Repeating this study with data containing information on CR between dam

and calf and on risk factors for RP would probably increase the power of showing an effect of the CR between dam and calf on RP.

In Holsteins, the predominant dairy breed, many important risk factors for RP, e.g. dystocia and nutrition (2), are less important than in dual purpose breeds. Therefore the relative importance of MHC compatibility for the occurrence of RP is probably larger in Holsteins than in dual purpose breeds such as MRY cattle.

Conclusions

In the present study, heritable and non-heritable genetic effects on retained placenta were assessed. The estimated heritability of retained placenta in MRY cattle is 0.22. This is the first time that a heritability estimate of retained placenta in MRY cattle is reported and the high heritability suggests that genetic selection against retained placenta in MRY cattle is possible.

Major histocompatibility complex class I compatibility is genetically determined, but is not heritable. The probability of major histocompatibility complex class I compatibility between dam and calf, an important risk factor for retained placenta, is correlated with the coefficient of relationship between sire and maternal grandsire. The present study showed a tendency for an effect of the coefficient of relationship between sire and maternal grandsire on retained placenta. This result indicates that preventing the mating of related parents may play a role in the prevention of retained placenta caused by major histocompatibility complex class I compatibility between dam and calf.

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4

Chemotactic activity of cotyledons for mononuclear leukocytes related to occurrence of Retained Placenta in Dexamethasone induced parturition in Cattle

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ABSTRACT

Induction of parturition with glucocorticosteroids in cattle is used for research purposes, in diseased or injured pregnant cows and as a management tool to time parturition. A negative side effect of induction of parturition with glucocorticosteroids is the high incidence of retained placenta that occurs after these calvings. Reaction of the maternal immune system against the 'foreign' foetal membranes contributes to the breakdown of the foetal-maternal attachment. Several studies indicate that failure of this immune assisted detachment increases the occurrence of retained placenta. We hypothesized that retained placenta occurring after induction of parturition with glucocorticosteroids is caused by failure of immune assisted detachment of the foetal membranes. The chemotactic activity of cotyledons for mononuclear leukocytes was used as a parameter to see whether immune assisted detachment of the foetal membranes had occurred. Cotyledons were collected from spontaneously calving non retained placenta cows and from dexamethasone induced non retained placenta and retained placenta cows. The study showed that the chemotactic activity of cotyledons for mononuclear leukocytes was lower ($P<0.001$) in cotyledons obtained from retained placenta cows in which parturition was induced with dexamethasone compared to the chemotactic activity of cotyledons obtained from spontaneously calving non retained placenta cows, whereas the chemotactic activity of cotyledons obtained from induced non retained placenta cows was not lower ($P=0.10$) than the chemotactic activity of cotyledons obtained from spontaneously calving non retained placenta cows. We concluded that induction of parturition with dexamethasone causes a failure of immune assisted detachment of the foetal membranes and the accompanying release of chemotactic factors. As a result, the chemotactic activity of cotyledons for mononuclear leukocytes is lower in induced retained placenta cows than in cotyledons from non retained placenta cows in which successful immune assisted detachment of the foetal membranes occurs.

Introduction

Induction of parturition with glucocorticosteroids in cattle is used for research purposes, in diseased or injured pregnant cows (1), and as a management tool to time parturition (2-4). A negative side effect of induction of parturition with glucocorticosteroids is the high incidence of retained placenta (RP) that occurs after these calvings (3, 5, 6). At parturition, the attachment between the foetal membranes and the uterus is normally broken down and the foetal membranes are expelled soon after the calf is born. It is believed that a maternal immune response against the 'foreign' foetal membranes contributes to the breakdown of the foetal-maternal attachment (7). Studies have shown that RP occurring under several conditions, can be linked to failure of immune assisted detachment of the foetal membranes (8-11). It is unknown how induction of parturition by glucocorticosteroids results in RP (12).

The experiments done by Gunnink (9, 13-15) were the first experiments that showed the involvement of the maternal immune system in the breakdown of the foetal-maternal attachment. Gunnink demonstrated that, in cows with the indication of an oversized living foetus that underwent a caesarean section, chemotactic activity of cotyledons was lower for RP cows than for non RP cows (13). Heuwieser et al. (10) compared the chemotactic activity of both cotyledons and caruncles from cows with the indication of an oversized living foetus, which underwent either a caesarean section or a forced extraction. They found that the chemotactic activity of both cotyledons and caruncles from RP cows were lower than that of cotyledons and caruncles from non RP cows. Kimura et al. (11) showed that plasma Interleukin-8 (IL-8) concentrations are lower in RP cows than in non RP cows from two weeks before until two weeks after parturition and that IL-8 is one of the chemotactic factors in cotyledons. In another study, Slama et al (16) suggested a role for Leukotriene B4, a product of inflammation which can stimulate B- and T-cells and is a potent chemotactic factor (16-18), in placental separation, as caruncular tissue from RP cows had significantly lower Leukotriene B4 concentrations compared to caruncular tissue from non RP cows. Together, these observations suggest that during immune assisted detachment of the foetal membranes chemotactic factors are released which determine the chemotactic activity of cotyledons. Apparently, these chemotactic factors are not released in RP cases and therefore the chemotactic activity of cotyledons will be lower in RP cows than in cows which expel the foetal membranes normally.

We hypothesized that RP after induction of parturition with glucocorticosteroids is caused by failure of immune assisted detachment of the foetal membranes. To test this hypothesis we compared the chemotactic activity of cotyledons from spontaneously calving cows and cows induced with dexamethasone. The chemotactic activity of the cotyledons was used as a parameter to see whether immune assisted detachment of the foetal membranes had occurred.

Material and Methods

Experimental animals

The described use of the animals in this study was approved by the Animal Ethical Committee of Utrecht University and conducted according to their regulation.

Between July 2007 and January 2009 all cows calving at the premises of the Faculty of Veterinary Medicine of the University of Utrecht were monitored and thirty five animals were included in this study. The cows, Holstein-Friesian or Holstein-Friesian cross bred, were used for educational purposes and either housed at the teaching dairy farm (n=12) or at the clinic for Farm Animal Health (n=23). In twenty animals from the clinic for Farm Animal Health, parturition was induced as part of the teaching program at day 275 of gestation using dexamethasone (Rapidexon 1mL/50kg im, Eurovet Animal Health, Bladel, The Netherlands). On these cows a caesarean section was performed thirty six hours after dexamethasone treatment or when the second phase of parturition, the expulsion of the foetus, started. The caesarean section was performed in the left flank using local infiltration anaesthesia (Alfacaine 2% + adrenaline 60 to 80 mL, Alfasan International BV, Woerden, The Netherlands). Before surgery ampicillin (Ampi-dry 5000, Dopharma, Raamsdonksveer, The Netherlands) was given intravenously and ketoprofen (Ketofen 3mL/100kg, Merial, Velserbroek, The Netherlands) was given intramuscularly. Non induced cows all calved without a caesarean section.

To look at the effect of induction of parturition on the chemotactic activity of cotyledons, cows were classified into three groups:

- Normal cows (NC, n = 15) expelled the foetal membranes within six hours and parturition occurred spontaneously.
- Induced cows (IC, n = 4) expelled the foetal membranes within six hours and parturition was induced using dexamethasone.
- Induced retained placenta cows (IRPC, n = 16) retained the foetal membranes for more than 24 hours and parturition was induced using dexamethasone.

Collection and preparation of samples

From the spontaneously calving cows, cotyledon samples were collected manually before the foetal membranes were expelled and within two hours post-partum. From the induced cows cotyledon samples were collected during the caesarean section. The stalk of a placentome was ligated, the placentome was cut off and cotyledon and caruncle were separated. All cotyledon samples were frozen at -20 °C within an hour and a half after collection.

To prepare the cotyledon samples for the chemotaxis assay, the samples were defrosted and kept on ice during the entire preparation process. First the cotyledons

were cut into small pieces using a scissor. Then, 1.0 g of sample was homogenized with 1 mL of Dulbecco's phosphate buffered saline (DPBS) (Lonza, Walkersville, MD, U.S.A.) using a tissue grinder (45 mL Potter-Elvehjem Tissue Grinder, Thomas Scientific, Swedesboro, NJ, U.S.A.). The resulting suspension was centrifuged for three minutes at 16.1 x 103 X g (Centrifuge 5415 R, Eppendorf, Hamburg, Germany) at 4 °C. Supernatants were collected and diluted to a standard protein concentration of 23±3 mg/mL and stored at -20 °C.

Leukocyte donor and mononuclear leukocyte preparation

A non-pregnant, clinically healthy lactating cow was used as a leukocyte donor. Twenty millilitre of blood was drawn from the jugular vein using heparin coated evacuated blood collection tubes (Vacutainer system with Lithium Heparin tube, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). As isolation of leukocytes following the protocol used by Gunnink (9), resulted in a leukocyte suspension dominated by mononuclear leukocytes (>95%, personal observation), we decided to only use mononuclear leukocytes in our experiments. Mononuclear leukocytes were isolated by density gradient separation. Fifteen millilitres of DPBS were pipetted into a 50 mL polypropylene tube (Corning Incorporated, Corning, NY, U.S.A.), 10 mL blood was added and 14 mL of Histopaque with a density of 1.077 g/mL (Histopaque-1077, Sigma-Aldrich, Steinheim, Germany) was pipetted below the diluted blood on the bottom of the 50 mL polypropylene tube. The 50 mL polypropylene tube was centrifuged for 20 minutes at 1500 X g (Allegra X-12R Centrifuge, Beckman Coulter, Fullerton, CA, U.S.A). At the Histopaque-plasma interface 14 mL fluid was removed and pipetted into another 50 mL polypropylene tube. This tube was centrifuged for 10 minutes at 1500 r.p.m. (= 524 X g) and the supernatant was discarded. To lyse any remaining red blood cells 500 µL of Milli-Q was added and after 15 seconds 15 mL of culture medium (GIBCO-42401, Invitrogen, Carlsbad, CA, U.S.A. with: 10% Fetal Calf Serum, Bodinco, Alkmaar, The Netherlands; 50 IU/mL penicillin, 50 µg/mL streptomycin, 2mM/L glutamine, Gibco BRL, Paisley, UK) was added. The tube was again centrifuged for 10 minutes at 1500 r.p.m., the supernatant was discarded and 3 mL of culture medium was added. The concentration of mononuclear leukocytes was determined using a Bürker counting chamber (Brand, Wertheim, Germany). Finally, cells were diluted with culture medium to a final concentration of 2 x 106 mononuclear leukocytes/mL. Time between drawing of the blood and use of the mononuclear leukocyte cell suspension in the chemotaxis assay was between three and four hours. The cells were kept at room temperature until used.

Serum standard

A pooled serum sample of five non pregnant, clinically healthy cows in the ninth week of lactation was used as a standard for chemotactic activity, to enable comparisons between assays. Fifty millilitres of blood were drawn from the jugular vein using

evacuated blood collection tubes (Vacutainer system with SST II Advance tube, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The blood samples were left to coagulate for 30 minutes and afterwards were centrifuged for 10 minutes at 2800g (Mistral 2000R, Measuring and Scientific Equipment, London, UK). Serum was collected, pooled and centrifuged again for 10 minutes at 2800 r.p.m. (Allegra X-12R Centrifuge). The supernatant was divided into aliquots and stored at -20 0C.

Each chemotaxis assay was loaded with three pooled serum samples and the mean of these samples was used as the chemotactic standard activity for that assay. The chemotactic activity of the samples was evaluated as the chemotactic activity expressed in percentage of the chemotactic standard activity, which was calculated as follows: Chemotactic activity in % of standard = chemotactic activity of sample / chemotactic standard activity x 100 % (19).

Chemotaxis assay

The chemotactic activity of the cotyledon samples was assessed using the Neuroprobe ChemoTx disposable chemotaxis system. The 96 well plates with 30 μL well capacity and 3.2 mm diameter sites were used in combination with polycarbonate track-etch membranes with 3 μm pore size. Twenty-nine microlitres of cotyledon sample or serum standard was loaded into the wells and the filter membrane was put in place. On top of each filter site 22 μL of cell suspension was added. The chemotaxis chamber was incubated for 60 minutes at 37 oC in a humidified incubator with 5% CO2. After incubation the cell suspension was rinsed off the filter using DPBS. To dislodge any cells attached to the bottom of the filter, the chemotaxis chamber was centrifuged for 10 minutes at 500 X g (Allegra X-12R Centrifuge). Chemotactic activity was assessed by measuring the concentration of cells in the lower wells using a flow cytometer (FACScan using CellQuest software, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). The flow cytometer was set to measure for a fixed amount of time and mononuclear leukocytes were recognized based on forward and side scatter. Cells were resuspended before counting using a pipette. All chemotaxis assays were performed in triplicate. The intra-assay CV was 57.2% and the inter-assay CV was 69.0%

Preparations of all the samples and loading of the samples onto the chemotaxis assay were performed in a laminar flow cabinet (Biological Safety Cabinet Class II type EF, Clean Air Techniek B.V., Woerden, The Netherlands), to prevent contamination with chemotaxis influencing substances.

Statistical methods

A linear model was fitted to analyze the effect of induction of parturition on the chemotactic activity of cotyledons. The initially fitted model was defined as:

$$y = \alpha + \beta 1 * CP + e$$

where:
y = Logarithm of the chemotactic activity
α = Intercept
CP = Effect of the course of parturition (NC, IC, IRPC)
e = Error term

Subsequently the effect of the course of parturition was excluded and this model and the initial model were compared using both Akaike's information criterion (AIC) and the F-test. The model with the lowest AIC was considered to be the model best supported by the data. With the F-test we checked if excluding the effect of the course of parturition had a significant effect on the prediction of the dependant variable.

The animals housed at different locations within the NC group were compared using a two sided Two-Sample t-Test. Normality of the data was tested using the Wilk-Shapiro test (WST) and equality of variances was tested using Levene's test (LT).

Because the data was skewed to the right a log transformation was done to achieve normality of the data and all statistical analyses were performed on the log transformed data. Results were considered significant at $P<0.05$. The program R (http://www.r-project.org) was used for all statistical computations.

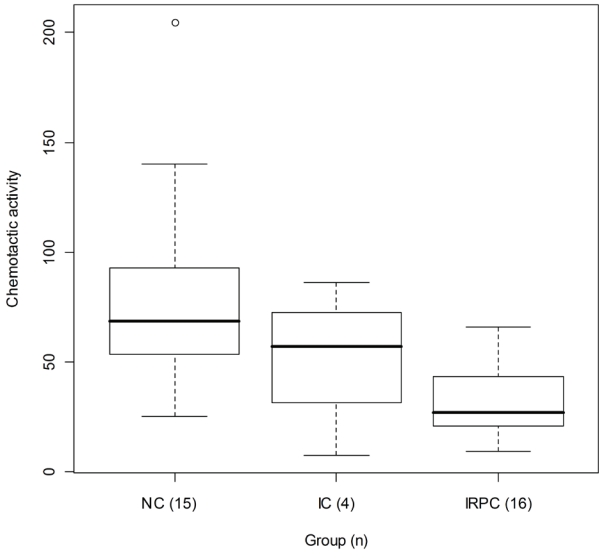

Fig. 1. Box-and-whisker plot of chemotactic activity, expressed in percentage of the standard activity, of cotyledon samples grouped according to the course of parturition. The box represents the 25th and 75th percentiles and the line within the box the median. The whiskers extend to the smallest and largest observation within one and a half interquartile range from the end of the box. Open points indicate values that are more than one and a half interquartile range from the end of the box.
NC = Normal cows, IC = Induced cows, IRPC = Induced retained placenta cows.

Results

The chemotactic activity of the cotyledons within the three groups (NC, IC, IRPC) is provided in Fig. 1. We found a significant effect of CP on the chemotactic activity of the cotyledon samples, illustrated by AIC and the F-test (Table 1). The final model, summarized in Table 2, shows that the chemotactic activity of the cotyledons from IC is not lower than that from NC (P=0.10) and that the chemotactic activity of the cotyledons from IRPC is lower than that from NC (P<0.001).

Results of a Two sided Two-Sample t-Test showed that there was no effect of housing and management on the chemotactic activity of cotyledons for animals within the NC group (P=0.68).

Table 1 Akaike's information criterion (AIC) for the linear models and the F-test comparing the two linear models.

Model	AIC	F-test
$\alpha + \beta_1 * CP + e^{a,b}$	-31.53	
$\alpha + e$	-21.38	P=0.002

[a] Model with the best fit using AIC
[b] Model with the best fit using the F-test

Table 2 Summary of the linear model ($\alpha + \beta_1 * CP + e$). P-values indicate the significance of an estimate to be different from zero.

Exposure variable		β^a	95% CI[b]	P-value
Intercept		4.22		<0.001
CP	NC	Referent		
	IC	-0.58	-1.28 – 0.13	0.10
	IRPC	-0.87	-1.32 – -0.43	<0.001

Null deviance: 17.94 on 34 degrees of freedom. Residual deviance: 11.98 on 32 degrees of freedom. CP deviance: 5.97 on 2 degrees of freedom. AIC: -31.53. Multiple R-squared: 0.33.
[a] Parameter estimate
[b] 95% confidence interval of the estimate ratio
CP= Course of parturition, NC = Normal cows, IC = Induced cows, IRPC = Induced retained placenta cows, AIC = Akaike's information criterion

Discussion

We tested the hypothesis that RP after induction of parturition with glucocorticosteroids is caused by failure of immune assisted detachment of the foetal membranes using the chemotactic activity of cotyledons as a parameter to see whether immune assisted detachment of the foetal membranes had occurred. The results from our study show that the chemotactic activity of cotyledons obtained from dexamethasone induced retained placenta cows is significantly lower than that from cotyledons obtained from spontaneously calving non retained placenta cows. The chemotactic activity of cotyledons obtained from dexamethasone induced non retained placenta cows is not significantly different from cotyledons obtained from spontaneously calving non retained placenta cows.

The observed lowered chemotactic activity of cotyledons obtained from retained placenta cows in which parturition was induced using dexamethasone is in accordance with previous observations of lowered chemotactic activity of cotyledons obtained from retained placenta cows with the indication of an oversized living foetus (9, 10, 19). A number of other studies have also shown a link between failure of immune assisted detachment of the foetal membranes and retained placenta (8, 11, 16, 20).

We hypothesise that dexamethasone interferes with the maturation of the placenta (12, 21, 22) and that this leads to failure of immune assisted detachment of the foetal membranes and thereby to failure of the accompanying release of chemotactic factors, causing a lowered chemotactic activity of the cotyledons. During the maturation of the placenta there has to be a transition of the immune system from acceptance of the foetal membranes to prevent premature detachment of the foetal membranes (23-25) towards immune assisted detachment of the foetal membranes during parturition, finally resulting in the loosening of the foetal-maternal attachment. Following this hypothesis, the placenta is not yet fully matured when parturition is induced, leading to failure of immune assisted detachment of the foetal membranes. This likely implies that induction close to the expected moment of parturition results in a more mature placenta, a more successful immune assisted detachment of the foetal membranes and a lower incidence of retained placenta. This is in accordance with other observations in the literature showing that the incidence of RP after induction of parturition with glucocorticosteroids is lower when parturition is induced at a more advanced stage of pregnancy (6). Also, the incidence of RP increases in spontaneous parturition with shorter gestation length (26, 27) and decreases after application of long acting glucocorticosteroids used to stimulate placental maturation before induction of parturition (4).

Dexamethasone influences lymphocyte functioning, suppresses cytokine production and has anti-inflammatory properties (28, 29). Although dexamethasone influences the immune system in many different ways, there is no evidence in the

scientific literature that dexamethasone causes failure of immune assisted detachment of the foetal membranes through a direct effect on the maternal immune system. Dexamethasone considerably changes the protein expression profile of neutrophils and while there are differences in the change in expression profiles of neutrophils after parturition and after dexamethasone treatment, there is also an overlap (30) indicating that there is a similarity in the changes in protein expression in neutrophils around parturition and after treatment with dexamethasone. Dexamethasone treatment changes the expression of genes in neutrophils that, amongst others, delay apoptosis, enhance bactericidal activity and promote tissue remodelling (31, 32). In a review by Burton et al (32) on the effect of endo- and exogenous glucocorticosteroids on the gene expression signatures of neutrophils in cattle, it is argued that the altered functioning of neutrophils exposed to glucocorticosteroids is beneficial, rather than detrimental, for a role during parturition and the release of the foetal membranes. IL-8 has been shown to play a role in the occurrence of RP (11) and infusion of the uterus of cyclic cows with human IL-8 led to the migration of a higher number of neutrophils into the uterus in dexamethasone treated cows than in control cows (33). Evidence from the scientific literature indicates a beneficial role for dexamethasone in the release of the foetal membranes and this argues against a direct effect of dexamethasone on the maternal immune system as a likely cause of RP.

Induction of parturition with progesterone receptor blockers (34, 35) and prostaglandin F2α analogues (36-38) lead to high incidences of RP, even though progesterone receptor blockers and prostaglandin F2α analogues have no known or minor stimulatory (39, 40) effects on the immune system, respectively. This supports our hypothesis that induction of parturition interferes with the maturation of the placenta and that this leads to failure of immune assisted detachment of the foetal membranes rather than that the substance used to induce parturition has a direct effect on the immune system leading to failure of the immune assisted detachment.

Given the relatively low coefficient of determination of the linear model (multiple R-squared: 0.33), our results show that a large part of the variation in chemotactic activity of the cotyledons is caused by other factors than the course of parturition. Other important factors are the natural biological variation between and within animals (41) and the variation introduced by the assay. The influence of the assay is quite substantial as can be seen from the high CV's, which are 57.2% and 69.0% for the intra and inter-assay CV respectively.

Cows within the experimental groups were unevenly distributed between the two housing locations and different housing and management conditions could have had an influence on the chemotactic activity of the cotyledons. However, we found no significant difference in chemotactic activity of the cotyledons between the two housing locations.

The cotyledons of all the cows in which parturition was induced were collected during a caesarean section. Gunnink showed that in cows on which a caesarean section was performed the chemotactic activity of cotyledons was lowered only in RP cows (13). In that study the chemotactic response of leukocytes against cotyledons was the same for normally calving cows and for cows on which a caesarean section was performed. Only when RP occurred after the caesarean section was this response lower before and after parturion (13, 14). From this, it can be concluded that a caesarean section does not influence the chemotactic activity of cotyledons and therefore the caesarean section was not considered to affect the chemotactic activity of cotyledons in our analyses.

All animals that underwent a caesarean section were treated with ampicillin and ketoprofen. Ampicillin has a slightly negative effect or no effect at all on chemotaxis (42, 43). Ketoprofen can negatively influence chemotaxis through the inhibition of prostaglandin synthesis and through an inhibition of IL-8 induced chemotaxis (44). As there is no significant difference in chemotactic activity between cotyledons from NC and IC the effect of ampicillin and ketoprofen on the chemotactic activity of the cotyledons seems limited.

Cotyledon samples were collected at parturition or 36 hours after induction in induced cows and within 2 hours in the NC cows. Most of the cotyledon samples in the NC cows were collected at parturition. In a study by Heuwieser, Grunert and Ehlert (10) testing cotyledon samples collected at parturition and 24 hours later, no differences were seen in chemotactic activity between the two time points. Another study done by Heuwieser and Grunert (19), wherein cotyledon samples collected at parturition and 3 hours later were tested, showed a slight increase in chemotactic activity in normally calving cows and no change in chemotactic activity in retained placenta cows. We therefore assume that the small time differences between the collection of the cotyledon samples did not influence the results.

This study shows that the chemotactic activity of cotyledons for mononuclear leukocytes was lower in cotyledons obtained from retained placenta cows in which parturition was induced with dexamethasone compared to the chemotactic activity of cotyledons obtained from spontaneously calving non retained placenta cows. It is likely that induction of parturition with dexamethasone causes failure of immune assisted detachment of the foetal membranes and the accompanying release of chemotactic factors. As a result, the chemotactic activity of cotyledons for mononuclear leukocytes is lower in induced RP cows than in cotyledons from non retained placenta cows in which successful immune assisted detachment of the foetal membranes occurs.

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5

Bovine Neonatal Pancytopenia is a heritable trait of the dam rather than the calf and correlates with the magnitude of vaccine induced maternal alloantibodies not the MHC haplotype

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ABSTRACT

Bovine Neonatal Pancytopenia (BNP), a bleeding syndrome of neonatal calves, is caused by alloantibodies absorbed from the colostrum of particular cows. A commercial BVD vaccine is the likely source of alloantigens eliciting BNP associated alloantibodies. We hypothesized that the rare occurrence of BNP in calves born to vaccinated dams could be associated with genetic differences within dams and calves. We found that the development of BNP within calves was a heritable trait for dams, not for calves and had a high heritability of 19%. To elucidate which genes play a role in the development of BNP we sequenced candidate genes and characterized BNP alloantibodies. Alloantigens present in the vaccine have to be presented to the dam's immune system via MHC class II, however sequencing of DRB3 showed no differences in MHC class II haplotype between BNP and non-BNP dams. MHC class I, a highly polymorphic alloantigen, is an important target of BNP alloantibodies. Using a novel sequence based MHC class I typing method, we found no association of BNP with MHC class I haplotype distribution in dams or calves. Alloantibodies were detected in both vaccinated BNP and non-BNP dams and we found no differences in alloantibody characteristics between these groups, but alloantibody levels were significantly higher in BNP dams. We concluded that the development of BNP in calves is a heritable trait of the dam rather than the calf and genetic differences between BNP and non-BNP dams are likely due to genes controlling the quantitative alloantibody response following vaccination.

Introduction

Since 2007 an increase in newborn calves with the bleeding syndrome Bovine Neonatal Pancytopenia (BNP) was observed all over Europe (1-3). Epidemiological studies showed a strong association between the occurrence of BNP in calves and vaccination of their dams with the PregSure© BVD vaccine (Pfizer Animal Health) (2). Symptoms of BNP are severe internal and external bleeding, first seen around 10-20 days of age. Hematological signs are severe leukopenia and thrombocytopenia. In addition, trilineage hypoplasia of the bone marrow can be observed upon post-mortem examination (3-5).

Colostrum of dams that had previously given birth to a calf which developed BNP contained alloantibodies recognizing bovine leukocytes (6-9). Feeding this colostrum to healthy neonatal calves induced the symptoms of BNP (4,8,10). Proteins from the bovine kidney cell line MDBK (11), used to grow the BVD type 1 virus present in PregSure© BVD, are the likely source of alloantigens that induce alloantibody production in vaccinated dams. The alloantibodies bind MDBK cells and it was shown that an important target of these antibodies were MHC class I proteins (7,9,12). Moreover, MDBK derived MHC class I proteins were detected in the PregSure© BVD vaccine (9,12) and immunization of calves with PregSure© BVD induced alloantibodies recognizing MDBK cells (7,13).

Since the incidence of BNP calves born to PregSure© BVD vaccinated dams was estimated to be lower than 0.3% (7,9,13), it was hypothesized that factors other than vaccination per sé play a role in the etiology of BNP. The prevailing hypothesis is that the pathogenesis of BNP resembles a histocompatibility (mis)match between dam and calf and is based on immunization of the dam with MDBK derived MHC class I (9,12). First, in the dam MDBK cell derived proteins, present in the Pregsure© BVD vaccine, are presented in the context of MHC class II. The resulting T cell help to B cells recognizing allogeneic differences between MDBK cells and the dam will result in the generation of alloantibodies which are also present in the colostrum. Due to tolerance to self-antigens, dams do not exhibit adverse effects after vaccination, i.e. the vaccine induced alloantibodies do not recognize alloantigens expressed in the dam. The maternal alloantibodies transferred to the calf via the colostrum will recognize alloantigens in case of a partial alloantigen match between MDBK cells and the calf. We hypothesized that the rare occurrence of BNP after Pregsure© BVD vaccination may depend both on the capability of the dam's immune system to present the MDBK alloantigens via MHC class II, as well as the degree of alloantigen (mis)match between the dam and the MDBK cell line (and the calf and the MDBK cell line, respectively) and the ensuing immune response of the dam. Since alloantigens (including MHC I and MHC class I associated B2M) and MHC class II are genetically determined and therefore heritable, we studied whether differences in these genes between dams and/or calves may explain why BNP only occurs in part of the calves born to PregSure© BVD

vaccinated dams. First we studied the heritability of the development of BNP in the calf as a potential dam or calf trait. Next, to elucidate if these genes genes play a role in the development of BNP we sequenced and compared the MHC and B2M candidate genes and characterized BNP associated alloantibodies.

Materials and methods

Heritability study

The data used for the heritability study were a subset of data from a large multi country epidemiological study on BNP (2) and concerned Dutch farms that participated in this study. Data on herd matched BNP and non-BNP calves were collected by on farm questionnaires. We looked at the heritability of the development of BNP within the calf as a trait of Pregsure© BVD vaccinated dams as well as of calves born to these dams. The definitions for BNP and non-BNP calves used, were according to Jones et al. (2). A BNP calf was defined as a calf that showed one or more BNP clinical signs on or before 28 days of age; bone marrow depletion as assessed by histopathology and/or thrombocytopenia ($< 150 \times 10^9$/litre) and leucopenia ($< 5 \times 10^9$/litre). A non-BNP calf was defined as a calf on the same farm as a case, aged 10–28 days at the time of case reporting, no clinical signs of BNP up to 28 days of age, and normal blood parameters (thrombocytes $\geq 300 \times 10^9$/litre, leucocytes $\geq 5 \times 10^9$/litre). To ensure that the correct phenotype, BNP or non-BNP, was assigned to the dam, only calves that were fed colostrum from their own dam were included. Furthermore, dam-calf combinations without pedigree information were excluded. Pedigrees of calves and dams were provided by the Dutch Cattle Improvement Organization (CRV, Arnhem, the Netherlands). The pedigree of dam-calf combinations meeting the inclusion criteria were traced back up to 21 generations and the final pedigree included 12 586 records. The first generation of the pedigree was a 100% complete for calves and 95% complete for dams. The data were analyzed using the software package ASReml (14), a statistical package that fits generalized linear mixed models using Residual Maximum Likelihood. The heritability of the development of BNP within the calf as a dam and calf trait was estimated from the dam and sire variance components of a sire-dam model. Only alloantigens inherited from the sire can be recognized by maternal alloantibodies and therefore the heritability of the development of BNP as a calf trait was estimated by calculating BNP as a sire trait. Variables included in the data set were:

- Vaccination history of the dam (Yes or No) with other BVD vaccines, Blue Tongue Virus, Rota/Corona virus, Infectious Bovine Rhinotracheitis virus or other.
- The number of Pregsure© BVD vaccinations (1, 2, 3, ≥ 4).
- Time since the last Pregsure© BVD vaccination of the dam (divided in classes of three months).

– Lactation number of the dam (1, 2, 3, 4, ≥ 5).

BNP was fitted as a binomial variable using the logistic link function to relate binomial outcome of BNP to the linear predictor used for the generalized linear mixed model. The following general model was used:

$$\text{Logit(BNP)} = \mu + (X_\nu)_n + \text{sire}_j + \text{dam}_k + e_{(i)n\ jk},$$

where BNP is the outcome of BNP, μ is the general mean, $(X_\nu)_n$ is one or more of the aforementioned variables, sire_j is the random effect of the jth sire; dam_k is the random effect of the kth dam and $e_{(i)n\ jk}$ is the vector of residuals. Heritability was calculated using the variance components of the model, as follows: BNP as dam trait $h^2 = \sigma^2_{dam} / \sigma^2_p$; BNP as a sire trait $h^2 = \sigma^2_{sire} / \sigma^2_p$; $\sigma^2_p = \sigma^2_{dam} + \sigma^2_{sire} + (\pi^2)/3$, where σ^2_p is the phenotypic variance, σ^2_{dam} is the dam variance, σ^2_{sire} is the sire variance and the residual variance was fixed at $(\pi^2)/3$.

First we looked at the effect of each individual variable on BNP in a sire-dam model. Next all variables with a *P*-value < 0.2 were included in the final sire-dam model. Immune responses normally decline with time and to test if the incidence of BNP after the last Pregsure© BVD vaccination also declines with time, the variable *Time since last Pregsure© BVD vaccination* was forced into the final model despite having a *P*-value higher than 0.2 in the univariate model. Because the estimates for *Time since last Pregsure© BVD vaccination* appear to have a linear effect on BNP, the variable was added as a linear covariable in the final model. There were only eight dams with one Pregsure© BVD vaccination and because the vaccination scheme consists of an initial prime and subsequent boost vaccination which may have been interpreted as one vaccination by the farmer, in the final model animals with one or with two Pregsure© BVD vaccinations were grouped.

Animals

Blood of calves was drawn as part of the multi country epidemiological study on BNP (2). Farms with more than one living BNP dam were revisited in 2013 to collect blood- and colostrum-samples from dams.

Throughout our study we used the following definitions for dams and calves:

– non-BNP dam – Dam that had been vaccinated with Pregsure© BVD and had not given birth to a calf that developed BNP following colostrum feeding.
– BNP dam – Dam that had been vaccinated with Pregsure© BVD and had given birth to a calf which developed BNP following colostrum feeding.
– Non-BNP calf – Calf born to a Pregsure© BVD vaccinated dam, that upon receiving colostrum from its dam did not show signs of BNP, confirmed via hematology and/or pathology.

– BNP calf – Calf born to a Pregsure© BVD vaccinated dam, that upon receiving colostrum from its dam showed clear signs of BNP, confirmed via hematology and/or pathology.

This study was approved by the Animal Ethical Committee of Utrecht University and conducted according to their regulations.

Sequence based typing of MHC class I, B2M and DRB3

Madin Darby Bovine Kidney cells (MDBK; ATCC-CCL22) were cultured in DMEM (Gibco, Life Technologies, Logan, USA), supplemented with Glutamax™, 50 IU/mL Penicillin, 50 ug/mL Streptomycin and 10% FCS. DNA was isolated from whole blood of animals and MDBK cells using the MagNA Pure Compact Instrument (Roche Diagnostics, Indianapolis, USA) according to manufacturer instructions.

Sequence based typing of MHC class I was done using gene specific primers aligning with intron 1 and intron 3 of MHC class I genes 1,2,3 and 6 (15) (Additional file 1). These primers amplify exon 2 and 3, which encode the most polymorphic regions of the MHC class I gene. For genes 1,2 and 3 PCR was carried out in 25 μL containing 1.4U Expand High fidelity Taq (Roche Diagnostics, Indianapolis, USA), 2.5mM $MgCl_2$, 0.5mM each dNTP and 0.4μM, or 0.2uM in the case of primers with ambiguous nucleotide, of each primer. The thermal cycling profile was 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 63 °C for 20 s, 72 °C for 60 s followed by 72 °C for 5 min. For gene 6 PCR conditions were similar, except 1.25U of AmpliTaq® 360 (Applied Biosystems, Life Technologies) was added, the $MgCl_2$ concentration was 1mM and the annealing temperature was 56 °C. Sequencing of PCR's resulting in a product were performed on the 3730 DNA Analyzer (Applied Biosystems) using the same primers used for the PCR and the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequence products were analyzed using SeqScape© (v2.5, Applied Biosystems). Forward and reverse sequences were aligned to a reference sequence to produce a consensus sequence. Using the IPD MHC database (16) a library of the exon 2 and 3 sequences of known MHC class I alleles was constructed using Seqscape©. Seqscape© is able to cope with ambiguous nucleotides and, in the case of heterozygous PCR products, matches the consensus read to the best combinations of alleles from the library. Consensus read basecalling of the amplified genomic DNA and library matches to known full length MHC class I cDNA sequences were checked. Using the assigned MHC class I alleles, MHC class I haplotypes were determined using haplotypes defined in Codner et al. (17) and this study (Additional file 2).

MHC class I haplotypes define a set of MHC class I alleles that are inherited together and haplotype differences between animals do not give information on differences in MHC class I as an alloantigen. To better estimate allogeneic differences between MDBK cells and dams/calves we looked at MHC class I protein differences between MDBK cells and dams/calves. Alloantibodies recognize the extracellular

part of expressed proteins and we therefore looked at protein differences within the extracellular part of MHC class I (Exon 2-4). DNA sequences of exon 2-4 were translated into protein sequences and the difference in protein sequence between two MHC class I alleles was calculated, expressed as percentage of the protein sequence that was different. Dams can recognize MDBK alleles (listed in Additional file 2) as non-self if there are differences between the dam and MDBK MHC class I and for dams we calculated the difference between the MDBK allele that was most different to the dam MHC class I alleles. For alloantibodies to recognize MHC class I in the calf, there has to be a (partial) match between the MDBK and paternally inherited calf MHC class I and for calves we calculated the difference between the most similar MDBK and paternally inherited calf MHC class I allele.

Beta-2-microglobulin (B2M) primers (Additional file 1) flanking exon 2 were designed using the bovine whole genome assembly UMD3.1. PCR was carried out in 50 μL containing 2.5U PfuTurbo Cx Hotstart DNA Polymeras (Agilent, Santa Clara, USA), 2mM $MgCl_2$, 0.2mM each dNTP and 0.5 μM each primer. The thermal cycling profile was 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 63 °C for 30 s, 72 °C for 60 s followed by 72 °C for 10 min. Sequencing was performed as described for MHC class I. Forward and reverse sequences were aligned to the UMD3.1 reference sequence using SeqScape©.

DRB3 sequence based typing was based on the method described by Miltiadou et al. (18). Primers aligning with intron 1 and 3 of the DRB3 locus (Additional file 1) amplify exon 2, the most polymorphic region of the DRB3 gene. PCR was carried out in 25 μL containing 0.6U AmpliTaq Gold (Applied Biosystems), 1.5mM $MgCl_2$, 0.4mM each dNTP and 0.4 μM each primer. The thermal cycling profile was 95 °C for 10 min, 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s followed by 72 °C for 5 min. Sequencing was performed as described for MHC class I. Sequence reads were analysed using SeqScape© as described for MHC class I.

Flow cytometry

Total alloantibody levels were assessed as serum antibody levels specific for MDBK cells. The latter were suspended in serum diluted 1:20 in PBS supplemented with 2% FCS and 0.1% sodium azide. Bovine IgG binding was detected using polyclonal biotinylated sheep anti-bovine IgG antibodies (Abd Serotec, Bio-Rad Laboratories Inc, Hercules, USA) and Streptavidin-Phycoerythrin (BD biosciences, Franklin Lakes, USA). Isotype specific alloantibodies were measured in a similar way. MDBK cells were suspended in serum or colostrum diluted 1:10 and alloantibody binding was detected by bovine isotype specific mouse monoclonal antibodies (19) and FITC conjugated polyclonal goat anti-mouse antibodies (BD Biosciences). Total leukocytes were isolated from blood collected from ten healthy randomly selected dams at the slaughterhouse by hypotonic lysis of erythrocytes. Whole blood was suspended in 9 parts of distilled water, after lysis

of erythrocytes isotonicity was restored using 1 volume of 10x PBS. Total leukocytes, used to detect alloantibody binding to Peripheral Blood Mononuclear Cells (PBMC), were suspended in serum or colostrum diluted 1:10. Alloantibody binding was detected by anti-bovine IgG1 mouse monoclonal antibodies and FITC conjugated polyclonal goat anti-mouse antibodies.

In all alloantibody binding experiments serum from non Pregsure© BVD vaccinated dams were used as (isotype) controls. Flow cytometry (BD FACSCanto™, BD biosciences) was used to measure alloantibody binding and data was analyzed using Flowjo software (Tree Star Inc., Ashland, USA). PBMC were selected based on Forward and Sideward scatter. Data are depicted as Geometric Mean Fluorescent Intensity (GMFI). In the case of alloantibody binding to PBMC depicted GMFI values are GMFI values subtracted by the GMFI of the isotype controls. In order to be able to compare alloantibody binding of PBMC irrespective of total alloantibody levels in serum or colostrum, relative alloantibody binding was calculated by dividing the GMFI of each sample by the GMFI of alloantibody staining of MDBK cells, representing total alloantibody binding. A positive PBMC sample was defined as a sample that had a higher geometric mean fluorescent intensity (GMFI) than the average of all measured samples or in the case of alloantibody level compensated values defined as having a higher relative signal than the average of the relative signal of all samples.

Statistics

The Wald test was used to test whether a variable improved the fit of the sire-dam model. Haplotype/allele frequencies were analyzed using Fisher's Exact test. Alloantibody binding levels were compared by two tailed simple T-tests for unequal variance. To adjust for multiple comparisons the false discovery rate (FDR) was controlled using the method by Benjamini and Hochberg (20). This method controls the chance of falsely declaring the result of a statistical test as significant. The largest *P*-value lower than its FDR-derived significance threshold and all *P*-values smaller were considered to be significant. The number of significant *P*-values that are false positive was controlled at 5%. Correlation was tested with Pearsons correlation. Normality was tested with D'Agostino and Pearsons omnibus normality test.

Effects were considered significant at $P < 0.05$. When applicable, values were given as mean ± the standard error of the mean, with the latter between brackets.

Results

Heritability of the development of BNP within the calf as a trait for Pregsure© BVD vaccinated dams and for calves

Based on the inclusion criteria 411 dam-calf combinations were selected for the heritability analysis. The 411 calves were born from 405 dams, fathered by 192 sires and comprised 102 BNP cases. The effect of each individual variable on BNP is summarized in Additional file 3.

Table 1 Summarizing results of the multivariable analysis of BNP using a sire-dam model (n = 411).

	Heritability estimate (SE)			
Sire	0.00 (0.00)			
Dam	0.19 (0.08)			
Variable	Category (n)	β (SE)	Odds Ratio	Wald test P-value
Lactation number	1 (67)	Referent	1	0.021
	2 (106)	0.49 (0.57)	1.63	
	3 (96)	1.10 (0.60)	3.00	
	4 (70)	0.77 (0.63)	2.17	
	5 ≥ (72)	-0.14 (0.68)	0.87	
Number of Pregsure© BVD vaccinations	≤ 2 (118)	Referent	1	0.014
	3 (134)	0.35 (0.42)	1.42	
	4 ≥ (159)	1.17 (0.45)	3.21	
Time since last Pregsure© BVD vaccination	Per month	0.03 (0.02)	1.03	0.214

The data included 102 BNP and 309 non-BNP dam-calf combinations.

The parameter estimates and odds ratios for the final model are shown in Table 1. For Pregsure© BVD vaccinated dams the heritability estimate for the development of BNP within the calf was 0.19 (0.08) and for sires it was 0.00 (0.00). The odds of BNP increased with an increased number of Pregsure© BVD vaccinations. The odds of BNP increased up to the third lactation and was lower for the fourth and fifth lactation. The effect of Time since last Pregsure© BVD vaccination on BNP was not significant.

Sequence based typing of MHC class I

MHC class I of Pregsure© BVD vaccinated dams

Sequence based typing was used to determine MHC class I haplotypes in vaccinated non-BNP and BNP dams (Table 2). The largest frequency differences between dams

were seen for variants of the A19 MHC class I haplotype, but with a P-value of 0.053, which was much higher than the FDR-threshold of 0.003, this was not significant. Assuming an incidence of BNP of 0.3% for Pregsure© BVD vaccinated dams (9), the positive predictive value of the A19 haplotypes was 0.007. Implying that BNP only occurred in 0.7% of calves born to Pregsure© BVD vaccinated dams with the A19 MHC class I haplotype.

The difference in protein sequence between the extracellular parts of the MDBK and dam MHC class I alleles was 13.6% (0.35%) for vaccinated non-BNP dams and 12.9% (0.40%) for BNP dams, with a P-value of 0.266 this was not significantly different between both groups (Additional file 4).

Table 2 Comparison of MHC class I haplotype frequencies in Pregsure© BVD vaccinated non-BNP and BNP dams.

MHC class I Haplotype[a]	Non-BNP Dams (n = 27)	BNP dams (n = 22)	P-value[b]	FDR-derived significance Thresholds[c]
A19 variants	5	11	0.053	0.003
H2	4	0	0.125	0.006
A13	2	6	0.135	0.009
UU6	0	2	0.199	0.012
UU5	0	1	0.449	0.015
A20v3 (UU)	5	2	0.454	0.018
UU1	2	0	0.500	0.021
A11	3	1	0.625	0.024
A10	4	2	0.688	0.026
H5v2 (UU)	4	2	0.688	0.029
A14	9	6	0.782	0.032
A15v1	9	8	1.000	0.035
A12vUU	3	2	1.000	0.038
UU3	1	0	1.000	0.041
A18v2	1	0	1.000	0.044
UU4	1	0	1.000	0.047
UU7	1	1	1.000	0.050

[a] Bovine MHC class I haplotypes are based on Codner et al. (17) and results from this study (detailed in Additional file 7).

[b] Ordered *P*-values from Fisher's exact test

[c] To adjust for multiple comparisons the False Discovery Rate (FDR) was controlled at 5% using the principle from Benjamini and Hochberg (19). The largest *P*-value lower than its FDR-derived significance threshold and all *P*-values smaller are significant.

MHC class I of calves born to Pregsure© BVD vaccinated dams

The paternal MHC class I haplotype frequencies of Non-BNP and BNP calves are shown in Table 3. Based on the Fisher's exact test the frequency of the A11 haplotype

was significantly higher in BNP calves, however with a P-value of 0.008 this value was higher than the FDR threshold of 0.004. Assuming an incidence of BNP of 0.3% for Pregsure© BVD vaccinated dams (9), the positive predictive value of the A11 haplotype was 0.014. Which implies that only 1.4% of calves with a paternally inherited A11 MHC class I haplotype born to Pregsure© BVD vaccinated dams get BNP.

In five non-BNP and three BNP calves fathered by the same sire, the MHC class I haplotypes were also typed (Additional file 5). Since all eight calves had the A11 MHC class I haplotype, it is likely that the sire was A11 homozygous. In that case both non-BNP and BNP calves inherited the A11 haplotype from their father and for these calves there was no association between the paternally inherited A11 haplotype and the development of BNP.

The protein difference between the extracellular part of the MDBK MHC class I alleles and paternally inherited calf MHC class I alleles was 9.44% (0.84%) for non-BNP calves and 9.37% (0.29%) for BNP calves, with a P-value of 0.938 this was not significantly different between both groups (Additional file 6).

Table 3 Paternal MHC class I haplotype frequencies of non-BNP and BNP calves born from Pregsure© BVD vaccinated dams.

Paternal MHC class I Haplotype[a]	Non-BNP calves (*n* = 21)	BNP Calves (*n* = 9)	*P*-value[b]	FDR-derived significance Thresholds[c]
A11	3	6	0.008	0.004
A14	0	1	0.300	0.007
UU3	0	1	0.300	0.011
UU9	3	0	0.535	0.014
UU8	2	1	1.000	0.018
A13	1	0	1.000	0.021
UU1	2	0	1.000	0.025
A18v2 (UU)	1	0	1.000	0.029
A12 (UU)	2	0	1.000	0.032
A15v1	1	0	1.000	0.036
A19variants	2	0	1.000	0.039
A20variant	1	0	1.000	0.043
H2	1	0	1.000	0.046
H5v2(UU)	2	0	1.000	0.050

[a,b,c] As in Table 2.

Sequence based typing of beta-2-microglobulin in Pregsure© BVD vaccinated dams and MDBK cells

Exon 2 of the beta-2-microglobulin (B2M) gene, encoding 97% of the mature protein, was sequenced in MDBK cells and in five vaccinated non-BNP dams and five BNP

dams that were farm matched. The B2M sequences of all vaccinated non-BNP dams, BNP dams and MDBK cells were identical.

Sequence based typing of DRB3 in Pregsure© BVD vaccinated dams

Results of the DRB3 typing of vaccinated non-BNP dams and BNP dams are shown in Table 4. The largest frequency differences were seen for the DRB3 alleles 1001 and 14011 both with a higher frequency in vaccinated non-BNP dams. However, the P-values were well above the FDR threshold and not significant.

Table 4 Comparison of DRB3 allele frequencies within Pregsure© BVD vaccinated dams and BNP dams.

DRB3 allele frequencies	Non-BNP Dams (*n* = 21)	BNP dams (*n* = 21)	*P*-value[a]	FDR-derived significance Thresholds[b]
1001	7	1	0.0574	0.004
14011	7	1	0.0574	0.008
2703	2	7	0.1555	0.013
0902	2	6	0.2646	0.017
1601	1	4	0.3597	0.021
0201	4	1	0.3597	0.025
0101	3	6	0.4827	0.029
0601	2	0	0.494	0.033
1101	9	11	0.7983	0.038
1201	3	4	1.000	0.042
0701	1	0	1.000	0.046
UU01[c]	1	1	1.000	0.050

[a] Ordered *P*-values from Fisher's exact test.

[b] To adjust for multiple comparisons the False Discovery Rate (FDR) was controlled at 5% using the principle from Benjamini and Hochberg (19). The largest *P*-value lower than its FDR-derived significance threshold and all *P*-values smaller are significant.

[c] Denotes a local name and is not included in the IPD Bovine MHC class II database.

Characterization of alloantibodies from Pregsure© BVD vaccinated dams

Total alloantibody levels in dams not vaccinated with Pregsure© BVD and in Pregsure© BVD vaccinated non-BNP and BNP dams were assessed as serum antibody levels specific for MDBK cells using flow cytometry (Figure 1). Alloantibody levels in BNP dams were significantly higher than in both non-BNP dams and dams not vaccinated with Pregsure© BVD, levels in vaccinated non-BNP dams are significantly higher than in dams not vaccinated with Pregsure© BVD.

Isotype specific alloantibody binding of MDBK cells is shown in Figure 2. IgG1 alloantibodies were most abundant and the levels were significantly higher in serum of non-BNP dams and in serum and colostrum of BNP dams compared to dams not vaccinated with Pregsure© BVD. IgG2 alloantibody levels were significantly

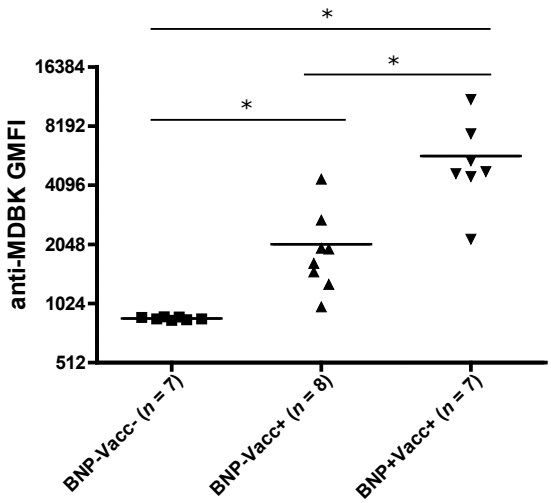

Figure 1 Serum of Pregsure© BVD vaccinated dams contain alloantibodies. Total IgG alloantibody binding of MDBK cells was measured in serum of i) dams not vaccinated with Pregsure© BVD (BNP-VAcc-) ii) Pregsure© BVD vaccinated non-BNP dams (BNP-Vacc+) and iii) Pregsure© BVD vaccinated BNP dams (BNP+Vacc+) using flow cytometry. The black bars denote the mean Geometric Mean Fluorescent Intensity (GMFI). Results were compared by two tailed simple T-tests for unequal variance. To adjust for multiple comparisons, the False Discovery Rate (FDR) was controlled at 5% using the principle from Benjamini and Hochberg (19). The largest *P*-value lower than its FDR-derived significance threshold and all *P*-values smaller are significant and are depicted by an asterisk (*).

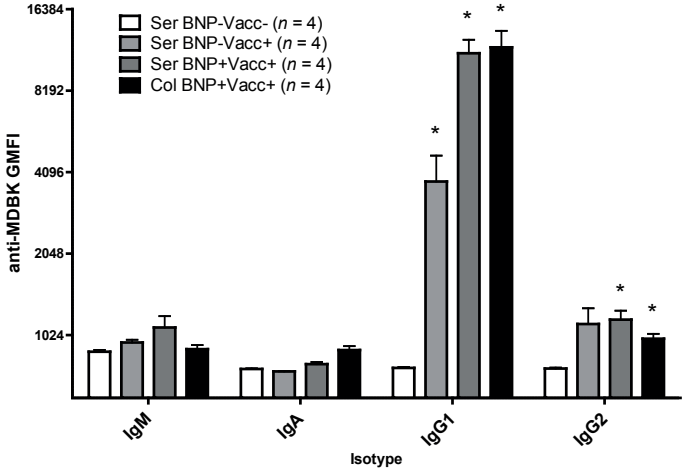

Figure 2 Isotype characterization of alloantibodies from Pregsure© BVD vaccinated dams. Flow cytometry was used to measure the isotype of alloantibodies binding to MDBK cells in serum (Ser) or colostrum (Col) from i) dams not vaccinated with Pregsure© BVD (BNP-VAcc-) ii) Pregsure© BVD vaccinated non-BNP dams (BNP-Vacc+) and iii) Pregsure© BVD vaccinated BNP dams (BNP+Vacc+). All results were compared by two tailed simple T-tests for unequal variance. Within each isotype, all groups are compared to the non Pregsure© BVD vaccinated dams (Ser BNP-Vacc-). To adjust for multiple comparison, the False Discovery Rate (FDR) was controlled at 5% using the principle from Benjamini and Hochberg (19). The largest *P*-value lower than its FDR-derived significance threshold and all *P*-values smaller are significant and are depicted by an asterisk (*).GMFI = Geometric Mean Fluorescent Intensity.

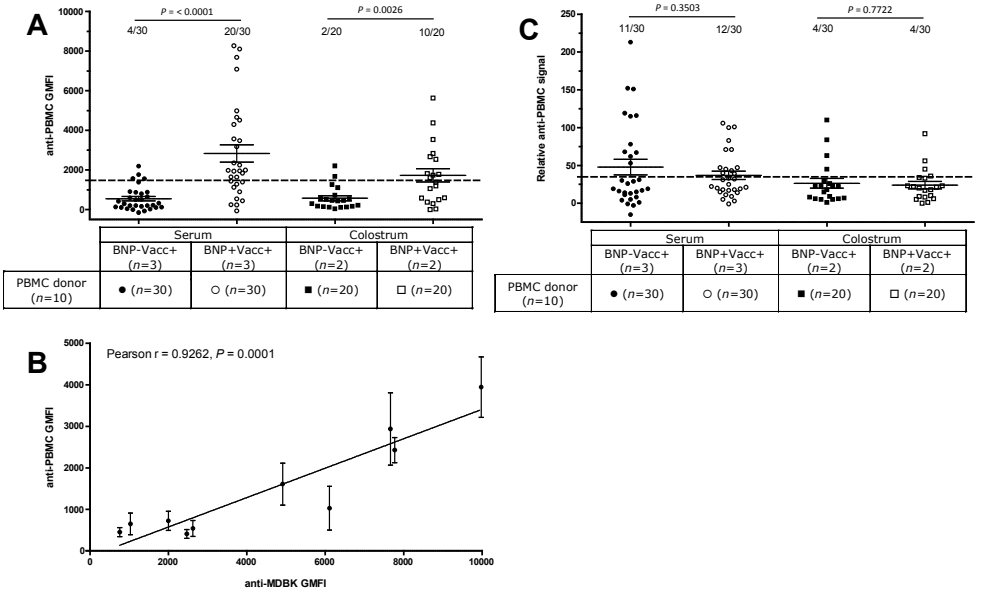

Figure 3 Binding of peripheral blood mononuclear cells by alloantibodies from Pregsure© BVD vaccinated dams. A: Peripheral Blood Mononuclear Cells (PBMC) from ten random dams were stained with serum (n = 3) and colostrum (n = 2) of different Pregsure© BVD vaccinated non-BNP dams (BNP-Vacc+, n = 5) and with serum (n = 3) and colostrum (n = 2) of Pregsure© BVD vaccinated BNP dams (BNP+Vacc+, n = 5). IgG1 alloantibody binding was measured by flow cytometry. GMFI subtracted by isotype control is plotted on the y-axis. The horizontal dotted line depicts the overall average geometric mean fluorescent intensity (GMFI) and the number above the plots describes the number of samples with a signal above the horizontal line. B: Correlation between the average IgG1 alloantibody binding of PBMC's from ten dams to IgG1 alloantibody binding of MDBK cells by serum or colostrum samples as in Figure 3A. C: The data from Figure 3A were divided by the GMFI signal of the alloantibody staining of MDBK cells by the respective serum or colostrum. The horizontal dotted line depicts the overall average relative signal and the number above the plots describes the number of samples with a signal above the horizontal line. Mean ± standard error of the mean is depicted in all graphs. Two tailed simple T-tests for unequal variance was used to compare serum or colostrum alloantibody binding of PBMC's between Pregsure© BVD vaccinated non-BNP and BNP dams. Correlation was tested with Pearsons correlation. Normality was tested with D'Agostino and Pearsons omnibus normality test.

higher in serum and colostrum of BNP dams compared to dams not vaccinated with Pregsure© BVD. IgG2 alloantibody levels tended to be higher in non-BNP dams as well, but due to higher variation among dams, did not differ significantly from that in dams not vaccinated with Pregsure© BVD. For IgM and IgA there were no significant differences between groups.

Antibodies present in serum and colostrum from non-BNP and BNP dams bind PBMC (Figure 3A). Alloantibody binding of PBMC was significantly higher for both serum and colostrum of BNP dams compared to non-BNP dams. The number of PBMC samples that were positive were also higher for both serum and colostrum of

BNP dams. However, average alloantibody binding of PBMC and alloantibody binding of MDBK cells had a high correlation (Figure 3B) and when alloantibody binding of PBMC was compensated for MDBK specific alloantibody levels to enable comparison of the binding of PBMC irrespective of total alloantibody levels, the relative signal was the same for BNP dams and non-BNP dams for serum as well as colostrum (Figure 3C). Also, the number of PBMC samples that were positive were similar in both groups for serum as well as colostrum. Results of individual serum and colostrum samples are shown in Additional file 7.

Discussion

We hypothesized that the rare occurrence of BNP after Pregsure© BVD vaccination depends both on the capability of the dam's immune system to present the MDBK alloantigens via MHC class II, as well as the degree of alloantigen (mis)match between the dam and the MDBK cell line (and the calf and the MDBK cell line, respectively) and the ensuing immune response of the dam. As a corollary we hypothesized that genetic differences in MHC class II in dams and alloantigens in dams and calves (e.g. MHC I and MHC class I associated B2M) would then explain why BNP only occurs in part of the calves born to PregSure© BVD vaccinated dams. The present study demonstrates that the development of BNP in calves is a heritable trait for Pregsure© BVD vaccinated dams with the high heritability estimate of 19%, which shows that genetic differences between dams explain in part why only the colostrum of some Pregsure© BVD vaccinated dams cause BNP in the calf. Genetic variation in the paternal haplotype of the calves is not related to the development of BNP in the calf, since the heritability of the development of BNP in calves born to Pregsure© BVD vaccinated dams is 0%. Demasius et al. (21) found that in an experimental German Holstein x Charolois crossbred herd with a limited number of sire lines, all BNP cases were restricted to a single maternal grandsire, also indicating the importance of the genetic background of the dam. In addition from a limited number of BNP dams was shown to induce BNP in randomly selected healthy calves (8-10) which supports the notion that the genetic background of the calf is not critical. The phenotype of the calf was based on very strict objective criteria, whereas the phenotype of the dam was based on the phenotype of the calf. BNP is caused by alloantibodies present in the colostrum and the phenotype of the calf therefore depends on the quality, quantity and source (own dam or other dam) of the ingested colostrum. This means that the phenotype of the calf, may not always be the proper phenotype of the dam. Much of this information was farmer reported and although we have tried to control for these aspects, the possibility exists that non-differential misclassification of the phenotype of

the dam occurred in this study and implies that the heritability for the development of BNP within calves of 19% for dams is potentially underestimated.

In our more in depth analyses of the genetic differences between Pregsure© BVD vaccinated non-BNP and BNP dams we sequenced a number of specific candidate genes. An important target of BNP alloantibodies is MHC class I (9,12), a highly polymorphic alloantigen (17). Hence MHC class I was genotyped to see if differences in MHC class I alloantigen repertoire of dams and/or calves were associated with the development of BNP in the calf. We did not find an association between the MHC class I of the Pregsure© BVD vaccinated dams and the occurrence of BNP. Although the number of BNP calves in the MHC class I haplotyping analysis was limited, it showed that BNP calves do not have a single paternal MHC class I haplotype and that most of the paternal haplotypes are shared between BNP and non-BNP calves (table 3, Additional file 5), together indicating that the paternally inherited MHC class I of calves is not associated with the occurrence of BNP. This result supports our finding that the heritability of the development of BNP in calves is zero and shows that BNP and non-BNP calves do not have a different allogeneic background. Ballingall et al. (22) found no differences in DRB3 allele frequencies between BNP and non-BNP calves. Since DRB3 and MHC class I are in linkage disequilibrium, this corroborates our MHC class I typing result in calves.

The binding of certain monoclonal antibodies to the B2M-MHC class I heavy chain heterodimer can depend on the associated B2M allele (23) or MHC class I allele (24). Although polymorphisms within the bovine B2M gene are known, none lead to changes in the amino acid sequence (25). Nevertheless we wanted to exclude the possibility that an unknown rare allelic variant of B2M influences the recognition and immune response to MDBK MHC class I proteins present in the vaccine. Since sequences of B2M were identical in the MDBK cell line and all typed Pregsure© BVD vaccinated non-BNP and BNP dams, it is highly unlikely that allelic variations of B2M play a role in the etiology of BNP.

Another aspect of immune recognition of MDBK alloantigens present in the Pregsure© BVD vaccine is their presentation to the dam's immune system via MHC class II. MHC class II haplotypes have been associated with disease resistance and susceptibility (26,27) and influence antibody responses after vaccination (28,29). We found no association between MHC class II haplotypes, as assessed by sequencing the highly polymorphic DRB3 locus, and the occurrence of BNP in Pregsure© BVD vaccinated dams.

Pregsure© BVD vaccinated BNP dams had significantly higher serum alloantibody levels compared to Pregsure© BVD vaccinated non-BNP dams. Nonetheless alloantibodies were produced both in Pregsure© BVD vaccinated non-BNP and BNP dams, confirming results from a previous study (7). Alloantibody production by all Pregsure© BVD vaccinated dams indicated there were allogeneic differences between

the bovine MDBK proteins and both Pregsure© BVD vaccinated non-BNP and BNP dams. This corroborated the sequencing results, where we did not find a difference between MHC class I or B2M between Pregsure© BVD vaccinated non-BNP and BNP dams. It also indicated that all dams were able to present alloantigens from the Pregsure© BVD vaccine in the context of MHC class II and fitted with the lack of an association between DRB3 and the occurrence of BNP within Pregsure© vaccinated dams.

The antibody isotype produced by B-cells depends on the cytokines that are produced during an (vaccine induced) immune response (30,31). The type of vaccine induced immune response may therefore influence the quality of the ensuing antibody response. As different antibody isotypes induce different biological effector functions, such as complement activation and neutralization, we studied the quality of the antibody response in Pregsure© BVD vaccinated non-BNP and BNP dams to determine if BNP dams only differ in alloantibody levels or also in the isotype and specificity of alloantibodies produced. BNP is caused by alloantibodies from colostrum and for BNP dams serum and colostrum alloantibodies were compared to see if results for serum alloantibodies can be extrapolated to colostrum derived alloantibodies. Alloantibody isotypes were similar in serum of Pregsure© BVD vaccinated non-BNP dams and serum and colostrum of BNP dams, indicating a similar response to vaccination in both groups. Likewise, studying cattle responding with high or low antibody levels after vaccination with hen-egg white lysozyme or Candida albicans extract Heriazon et al. (32) also did not find any differences in IgG1 and IgG2 levels between animals, whereas antibody levels following vaccination varied significantly.

When stained with serum or colostrum from Pregsure© BVD vaccinated BNP dams higher numbers of (random) PBMC samples were positive for alloantibody binding and on average staining intensity was higher than when stained with serum or colostrum from Pregsure© BVD vaccinated non-BNP dams. However, when compensated for alloantibody binding of MDBK cells to enable comparison of binding to PBMC irrespective of total alloantibody levels, numbers of positive PBMC samples and the relative staining intensity with alloantibodies were comparable between Pregsure© BVD vaccinated BNP and non-BNP dams, indicating that the specificity for allogeneic cells was also comparable. Based on the similar antibody isotypes and relative staining of PBMC we argue that alloantibodies from Pregsure© BVD vaccinated non-BNP and BNP dams are qualitatively similar and that only the level of alloantibodies is higher in Pregsure© vaccinated BNP dams. The high correlation between binding of alloantibodies to MDBK cells and PBMC corroborates the notion that the most important alloantigens in the Pregsure© BVD vaccine are derived from the producer cell line. Bastian et al. (7) found that BNP dams also had higher BVD neutralizing antibody levels than Pregsure© BVD vaccinated non-BNP dams, showing that BNP dams generally respond with higher antibody levels to components in the Pregsure©

BVD vaccine. In combination with the high heritability estimate for the development of BNP in calves for Pregsure© BVD vaccinated dams, it is likely that genetic differences between vaccinated non-BNP and BNP dams are due to genes that determine the level of antibody production after Pregsure© BVD vaccination. In cattle high heritability estimates have been found for antibody production after vaccination, these ranged from 13% to 88% (33,34). High antibody production after BRSV vaccination was associated with single nucleotide variants of TLR4 and TLR 8 (28). Likewise, differences in responsiveness of the innate immune system of BNP dams to the adjuvant of the Pregsure© BVD vaccine may have led to higher antibody production to antigens in the vaccine. The occurrence of BNP shows that in an outbred population some individuals may respond very differently to vaccination than the general population. This emphasizes the importance of monitoring adverse effects of both existing and new vaccines, but may on the other hand also provide opportunities for selective breeding for an increased humoral immune response.

Bovine MHC class I has an unusual organization, with six putative genes of which a variable number of genes are functionally present per haplotype (17), making MHC class I typing in cattle difficult. Several techniques with different (dis)advantages have been used to type MHC class I in cattle, including serology (35), cloning and sequencing of full length cDNA (36) and next generation sequencing of polymorphic regions (37). In this study we use gene specific primers for four of the six MHC class I genes to amplify exon 2 and 3 (15), encoding the most polymorphic region of the MHC class I. Alleles are distinguished based on exon 2 and 3 sequence and full length sequences are imputed from the IPD bovine MHC class I database (16), a method commonly used for HLA typing (e.g. (38)). Advantages of this typing method are that the amplified gene specific sequence normally only contains two alleles, allowing the use of traditional sanger sequencing, and that a relatively large number of samples can be typed, as was necessary for the present study. However, there are also some limitations to this method. The gene specific primers are only validated for Holsteins, which was not a problem in this study as all dams were of Holstein origin, and alleles from MHC class I gene 4 and 5 are not directly typed. However, genes 4 and 5 are the least polymorphic of the bovine MHC class I genes with only seven documented alleles of which only two have been reported in Holsteins (16). One of these alleles (4*02401) can be imputed based on haplotype and the other (5*03901) is amplified by gene 3 specific primers. MHC class I haplotypes define a set of MHC class I alleles that are inherited together and because different haplotypes can define very similar MHC class I alleles, haplotype differences between animals do not accurately represent allogeneic or immunological differences between animals. It has been hypothesized that the occurrence of BNP depends on allogeneic (mis)matches between the dam, the MDBK cell line and the calf (9,12). The likelihood of an alloimmune response is directly related to the number of epitope mismatches between the foreign alloantigen and the host (39) and in order to

better estimate allogeneic (mis)matches between animals and MDBK cells, we analyzed protein differences in the extracellular domain of the MHC class I protein between MDBK cells and dams/calves. Although this method is potentially a better estimate of allogeneic differences between animals than only relying on MHC class I haplotypes, accurate prediction of antibody epitopes is much more complicated and depends on many other factors, such as conformation, non-linear epitopes and flanking residues (40). Since genes 4 and 5 are not directly typed in the MHC class I method used in this paper, in some animals the presence of certain alleles was imputed from the defined haplotype for that animal and for newly defined haplotypes the presence of additional alleles cannot be excluded, giving an extra level of uncertainty to the analysis of the MHC class I protein differences. However, previously published haplotypes comprise the majority of the haplotypes typed in this study and the results from the MHC class I halpotyping corroborates the results from other experiments in this study. Together, the results indicate that the occurrence of BNP is not associate with a specific allogeneic background of BNP dams or calves. The only difference we found between Pregsure© BVD vaccinated non-BNP and BNP dams were in the alloantibody levels and this would imply that the development of BNP in the calf primarily depends on the alloantibody dose the calf absorbs. The finding by Jones et al. (2) that the odds of BNP increases with increased colostrum intake, and thus alloantibody intake, strengthens this hypothesis. The risk of BNP increases with increased number of Pregsure© BVD vaccinations and this can be explained by boosting of antibody production, increasing the alloantibody levels in the colostrum and thus increasing the alloantibody dose of the calf after colostrum ingestion. Furthermore, our findings that the heritability for the development of BNP in the calf was 0% for calves, whereas as a dam trait the heritability was 19% and the observation that BNP can be induced in unrelated healthy calves by alloantibodies/colostrum from BNP dams (8-10) show that the dam and not the calf plays a pivotal role in determining whether a calf gets BNP or not. We conclude that the development of BNP in calves is a heritable trait of the dam rather than the calf and that genetic differences between BNP and non-BNP dams are likely due to genes controlling the quantitative alloantibody response following vaccination.

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Additional files

Additional file 1

Primers used for the amplification of MHC class I genes, Bèta-2-Microglobulin and DRB3.

Gene	Forward Sequence (5′–3′)	Location	Reverse Sequence (5′–3′)	Location
MHC class I, Gene 1 (15)	TGCGAGGGGACCGCCCGA	Intron 1	AGGTGAGAACAGGCCTTGAGAA	Intron 3
MHC class I, Gene 2 (15)	GAACRAGCGACCCCGACT	Intron 1	CAAGTGGGGCAACTGGTC	Intron 3
MHC class I, Gene 3 (15)	TCGACCGCTTCCATCTCG	Intron 1	GAACAGGCCTTGAGAGAC	Intron 3
MHC class I, Gene 6 (15)	TCATTGACCCTCCGCCCA	Intron 1	GGCGCTGTTYCCACAGGC	Intron 3
B2M	GACCAAGGTAGCCCCAAGTG	Intron 1	TATATGCCGCAGCTGTGCTC	Intron 2
DRB3 (17)	TCCCGCATTGGTGGGTGT	Intron 1	CTCCACACTGGCCGTCCAC	Intron 2

Additional file 2

MHC class I haplotypes

List of MHC class I haplotype definitions used in this article. The newly defined haplotypes, containing an UU prefix or suffix, are provisional haplotypes. These haplotypes have not been confirmed using different MHC class I typing methods and because the gene specific primers used in this study do not amplify gene 4 and 5 and have not been validated for all known MHC class I alleles, the presence of additional alleles cannot be excluded. In some cases previously defined haplotypes (17) have been renamed to accommodate for additional haplotype variants within a group. Allele nomenclature refers to the IPD Bovine MHC class I database (16).

Haplotype	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5	Gene 6
A12(UU)		2*00801				
A15v1[a]	1*00901	2*02501		4*02401		
A15v2[a]	1*00902	2*02501		4*02401		
A18v1[a]						6*01301
A18v2[a]						6*01302
A19v1[a]		2*01601				6*01401
A19v2(UU)		2*01602				6*01402
A19v3(UU)		2*01602				6*01401
A20v1[a]		2*02601	3*02701			
A20v2[a]		2*02602	3*02702			
A20v3(UU)		2*02603	3*02702			
H5v2(UU)			3*03601			
UU1	UU1[c]					
UU3		2*0180x[b]				
UU4			3*05901			
UU5	UU5[c]	2*01602				
UU6		2*02501				
UU7		2*02603			5*03901	
UU8		2*05401			5*03901	
UU9			3*05002			

[a] Name of previously defined haplotype (17) was changed to accommodate additional haplotype variants within group.

[b] 2*01801 or 2*01802. These alleles are only different in the signaling peptide, not the expressed MHC class I, and cannot be differentiated by the method used in this study.

[c] Denotes a local name and is not included in the IPD Bovine MHC class I database. GenBank accession numbers: KM397369 (UU1), KM39730 (UU5).

MDBK MHC class I typing

The following MHC class I alleles were typed in the MDBK cell line:

2*04801

2*MDBK[a]

3*01101

3*05001

[a] Denotes a local name and is not included in the IPD Bovine MHC class I database. Genbank accession number KM397368.

Additional file 3

Summarizing results of the univariable analysis of the effect of independent variables on BNP, including sire and dam heritability estimates (n = 411).

	Heritability estimate (SE)			
Sire	0.00 (0.00)			
Dam	0.15 (0.08)			
Variable	**Category (*n*)**	**β (SE)**	**Odds Ratio**	**Wald test *P*-value**
Blue Tongue Virus vaccination	No (75)	Referent	1	0.741
	Yes (312)	0.19 (0.58)	1.21	
Other BVD vaccinations	No (229)	Referent	1	0.918
	Yes (182)	-0.02 (0.23)	0.98	
Rota/Corona vaccination	No (311)	Referent	1	0.671
	Yes (62)	-0.23 (0.51)	0.79	
Infectious Bovine Rhinotracheitis vaccination	No (261)	Referent	1	0.544
	Yes (119)	0.21 (0.50)	1.23	
Other vaccinations	No (310)	Referent	1	0.889
	Yes (64)	-0.04 (0.50)	0.10	
Lactation number	1 (67)	Referent	1	<.001
	2 (106)	0.92 (0.49)	2.51	
	3 (96)	1.90 (0.48)	6.66	
	4 (70)	1.67 (0.50)	5.31	
	5≥ (72)	0.81 (0.53)	2.24	
Number of Pregsure© BVD vaccinations	1 (8)	Referent	1	<.001
	2 (110)	-0.75 (0.86)	0.47	
	3 (134)	-0.23 (0.84)	0.79	
	4≥ (159)	0.52 (0.83)	1.68	
Time since last Pregsure© vaccination	≤3 months (7)	Referent	1	0.430
	>3 - 6 months (10)	0.46 (1.40)	1.50	
	>6 - 9 months (23)	0.97 (1.17)	2.63	
	>9 - 12 months (36)	0.18 (1.17)	1.20	
	>12 - 15 months (68)	0.53 (1.20)	1.70	
	>15 - 18 months (112)	0.60 (1.10)	1.81	
	>18 - 21 months (70)	0.73 (1.11)	2.08	
	>21 - 24 months (56)	1.48 (1.15)	4.40	
	>24 - 27 months (16)	0.69 (1.23)	2.00	
	>27 - 30 months (22)	0.57 (1.19)	1.77	
	>30 - 33 months (12)	1.80 (1.23)	6.00	
	>33 - 36 months (9)	-0.29 (1.51)	0.75	

The data included 102 BNP and 309 non-BNP dam-calf combinations.

Additional file 4

Comparison of the difference in protein sequence of the extracellular part of the MHC class I protein (Exon 2-4) between the the MDBK MHC class I allele that is most different to the MHC class I alleles of Pregsure© BVD vaccinated non-BNP and BNP dams.

	Protein difference	SD	P-valuea
Non-BNP dams (n =27)	13.6%	0.35%	0.266
BNP dams (n = 22)	12.9%	0.40%	

[a] Unpaired t-test with unequal variance.

Additional file 5

MHC class I haplotypes of calves (n = 8) fathered by the same sire.

Calf	MHC class I haplotypesa	
Non-BNP	A11	UU1
Non-BNP	A11	A15v1
Non-BNP	A11	A11
Non-BNP	A11	A19v
Non-BNP	A11	A20v
BNP	A11	A14
BNP	A11	A12 (UU)
BNP	A11	A13

a Bovine MHC class I haplotypes are based on Codner et al (16) and results from this study.

Additional file 6

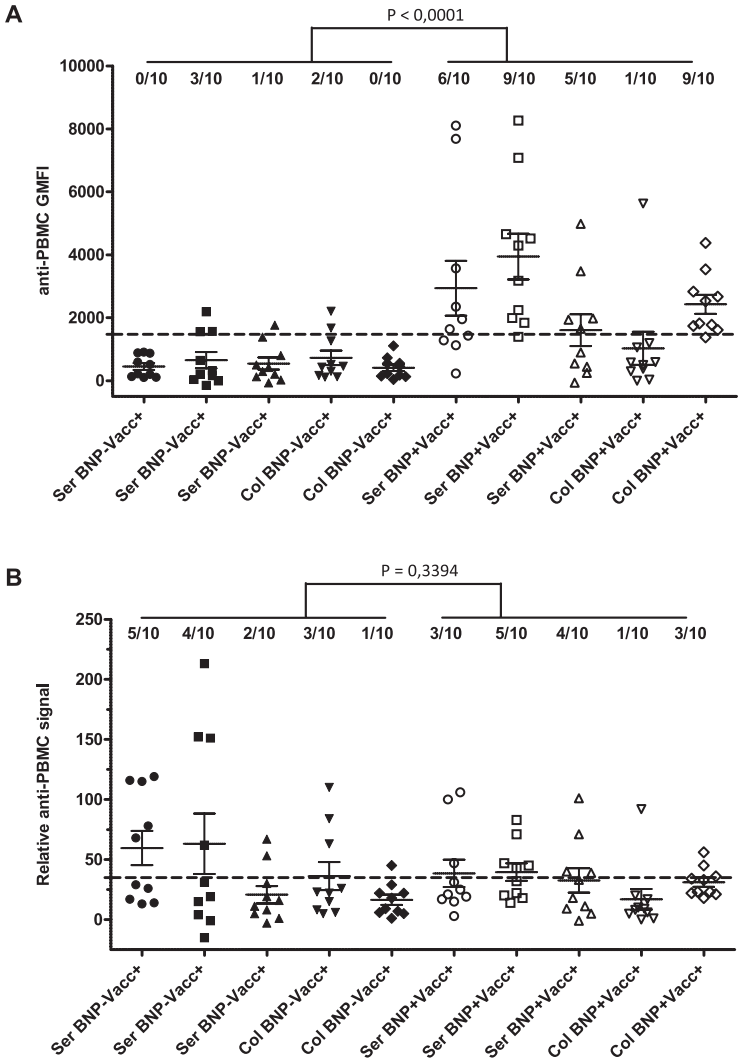
Comparison of the difference in protein sequence of the extracellular part of the MHC class I protein (Exon 2-4) between the most similar MDBK and paternally inherited MHC class I allele from non BNP and BNP calves.

	Protein difference	SE	P-valuea
Non-BNP calves (n = 21)	9.44%	0.84%	0.938
BNP Calves (n = 9)	9.37%	0.29%	

a Unpaired t-test with unequal variance.

Additional file 7

Binding of peripheral blood mononuclear cells by alloantibodies from Pregsure© BVD vaccinated dams. A: Peripheral Blood Mononuclear Cells (PBMC) from ten random dams were stained with serum (Ser, n = 3) and colostrum (Col, n = 2) of different Pregsure© BVD vaccinated non-BNP dams (BNP-Vacc+, n = 5) and with serum (n = 3) and colostrum (n = 2) of Pregsure© BVD vaccinated BNP dams (BNP+Vacc+, n = 5). IgG1 alloantibody binding was measured by flow cytometry. GMFI subtracted by isotype control is plotted on the y-axis. The horizontal dotted line depicts the overall average geometric mean fluorescent intensity (GMFI) and the number above the plots describes the number of samples with a signal above the horizontal line. B: The data from Additional file 7A were divided by the GMFI signal of the alloantibody staining of MDBK cells by the respective serum or colostrum. The horizontal dotted line depicts the overall average relative signal and the number above the plots describes the number of samples with a signal above the horizontal line. Mean ± standard error of the mean is depicted in all graphs. Two tailed simple T-tests for unequal variance was used to compare alloantibody binding of PBMC's between Pregsure© BVD vaccinated non-BNP and BNP dams.



6

The major targets of Bovine Neonatal Pancytopenia-associated vaccine-induced alloantibodies are MHC class I and VLA-3; however, pathogenicity correlates with MHC class I expression

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Manuscript submitted

ABSTRACT

Bovine Neonatal Pancytopenia (BNP) is a fatal bleeding syndrome of neonatal calves first observed in Europe in 2006. The disease, caused by maternal alloantibodies absorbed from colostrum, is characterized by lymphocytopenia, thrombocytopenia and bone marrow hypoplasia. An inactivated viral vaccine is the likely source of alloantigens inducing BNP-associated alloantibodies in the dam. In this study the specificity of BNP alloantibodies was assessed and was linked to the pathology of BNP. We demonstrated that MHC class I was the major target of BNP alloantibodies and showed that, depending on the dam, these accounted for 40-90% of all induced alloantibodies. Very Late Antigen-3, an integrin α3/β1 heterodimer, was identified as an additional major target of BNP alloantibodies. However, BNP alloantibody binding to various bovine cell types correlated with MHC class I expression, rather than integrin β1 or α3 expression. Likewise, BNP alloantibody-dependent complement-mediated cell lysis correlated strongly with MHC class I expression. In addition examination of several tissues of third trimester bovine fetuses revealed that cells affected in BNP are characterized by high BNP alloantibody binding and high MHC class I expression. We conclude that in spite of the heterogeneous specificity of BNP-associated maternal alloantibodies, MHC class I-specific, rather than VLA-3 specific antibodies, mediate the pathogenicity of BNP in the calf and that cells with high MHC class I expression were preferentially affected in BNP.

Introduction

A fatal bleeding syndrome in neonatal calves named Bovine Neonatal Pancytopenia (BNP) is characterized by leukopenia, thrombocytopenia, bone marrow depletion and severe internal and external bleeding (1-3). BNP was first seen in 2006 and quickly emerged all over Europe (3-5). Epidemiological studies showed a strong association between the occurrence of BNP and vaccination of the mothers of affected calves with Pregsure© BVD (Pfizer Animal Health) (5). BNP has been reproduced in calves by feeding colostrum from dams that had previously given birth to calves that succumbed to BNP (1, 6) and several studies have shown the presence of alloantibodies recognizing calf leukocytes in the colostrum of these cows (7-9). Experimental immunization of calves with PregSure© BVD induced alloantibodies recognizing the MDBK cell line used for the production of the vaccine (8, 10). Bovine MHC class I (MHC I) was shown to be present in the PregSure© BVD vaccine and is a target of BNP-associated alloantibodies (9, 11). Therefore, vaccination of dams with Pregsure© BVD is the likely source of Ags that induce maternal alloantibodies which, upon ingestion of colostrum, elicit BNP in calves.

In a recent study, we did not find an association between the occurrence of BNP and MHC I haplotypes of dams or calves (12). MHC I is expressed on almost all nucleated cells, whereas BNP pathology is characterized by a loss of specific cell types (i.e. leukocytes, thrombocytes and bone marrow cells) (1-3). Some authors have therefore argued that alloantibodies with a different specificity than MHC I might better explain the pathogenesis of BNP (7, 13, 14). The goals of this study were to i) assess the relative importance of anti-MHC I Abs, ii) elucidate if BNP-associated alloantibodies recognize other targets and iii) link the alloantibody specificity to the pathology of Bovine Neonatal Pancytopenia.

Materials and Methods

Animals and sample collection

Peripheral blood, serum and colostrum were collected from BNP, non-BNP and control dams. The following definitions were used to categorize dams:

- BNP dam, vaccinated with Pregsure© BVD and given birth to a calf which developed BNP following ingestion of the dam's colostrum. BNP was diagnosed based on clinical signs and hematology and/or pathology.
- Non-BNP dam, vaccinated with Pregsure© BVD and given birth to healthy calves that showed no clinical signs of BNP upon ingestion of the dam's colostrum.
- Control dam, no Pregsure© BVD vaccination history and given birth to healthy calves.

Peripheral blood leukocytes (PBLk) were isolated by hypotonic lysis of erythrocytes. Whole blood was suspended in 9 parts of distilled water; after lysis of erythrocytes isotonicity was restored using 1 volume of 10x PBS. IgG was purified from serum and colostrum by liquid affinity chromatography using HiTrap™ Protein G columns (GE Healthcare) according to manufacturer's instructions. Colostrum was delipidated before IgG isolation using calcium chloride and dextran sulfate. IgG was biotinylated with biotin-7-NHS (Roche) in a 1:20 molar ratio. Samples were dialyzed using Vivaspin ultrafiltration spin columns (Sartorius) with a 50 kDa cut off to remove excess biotin.

Non-bovine cell lines (COS-7, CHO-K1, SP-20, THP-1, HEK-293) used to detect BNP alloantibody binding across species were maintained as appropriate. Horse PBLk were isolated by hypotonic lysis of erythrocytes.

To assess BNP alloantibody binding and MHC I expression of cells that were (un)affected in BNP, late gestation bovine fetuses (n=3) were collected at a commercial slaughterhouse and used to isolate bone marrow and endothelium. Gestation length, determined by measuring crown-rump length as described by Rexroad et al. (15), was 7, 8, and 9 months(/a term) of gestation, respectively. Endothelial cells were isolated from the aorta by gently scraping the endothelial lining with a surgical blade, a method described by Ryan and Maxwell (16). Both femurs were carefully opened to isolate bone marrow cells by rinsing the femur cortex with DMEM (Gibco) supplemented with 5U/ml heparin. Repeated centrifugation, resuspension and removal of debris was performed to obtain a single cell suspension. In addition, bone marrow cells of a neonatal calf that died during caesarean section were isolated from both femurs and cryopreserved in 10% DMSO at -80 ^{o}C before analysis.

This study was approved by the Animal Ethical Committee of Utrecht University and conducted according to their regulations.

Sequence based MHC class I haplotyping

Sequence based MHC I haplotyping of non-BNP and BNP dams was performed as described by Benedictus et al. (12). In short, gene-specific primers aligning with intron 1 and intron 3 of putative MHC I genes 1,2,3 and 6 (17) are used to amplify exons 2 and 3 encoding the most polymorphic region of the MHC I. PCR products were sequenced in forward and reverse on a 3730 DNA Analyzer (Applied Biosystems) using the same primers used for the PCR and the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). SeqScape© (v2.5, Applied Biosystems) was used to match consensus sequences to a library of exon 2 and 3 sequences of all known MHC I alleles documented on the IPD MHC database (http://www.ebi.ac.uk/ipd/mhc/bola/). In the case of heterozygous PCR products, Seqscape© matches the consensus sequence to a combination of alleles. Consensus read base-calling of the amplified genomic DNA and library matches to known full-length MHC I cDNA sequences were checked

manually. MHC I haplotypes were determined using haplotypes defined in Codner et al. (18) and Benedictus et al. (12).

MDBK and MDBK-derived cell lines

Madin Darby Bovine Kidney cell line (MDBK; ATCC-CCL22), the origin of the producer cell line used for Pregsure© BVD, was cultured in DMEM (Gibco), supplemented with Glutamax™, 50 IU/ml Penicillin, 50 ug/ml Streptomycin and 10% FCS. A MDBK-derived cell line expressing the bovine herpes virus 1 (BHV-1) derived TAP inhibitor UL49.5 was used as a MDBK cell line with constitutively reduced MHC I expression. This cell line has previously been described by us (19). In short, a retroviral vector was used to transduce the UL49.5 gene of BHV-1, a potent TAP inhibitor, in anti-sense (MDBK control cells) or sense orientation.

We employed the CRISPR/Cas9 system (20, 21) to generate β2-microglobulin (B2M) knockout MDBK cells. For this, we constructed a selectable lentiviral CRISPR/Cas vector which will be described elsewhere (manuscript in preparation). Briefly, we altered the lentiviral pSicoR vector (Addgene plasmid 11579, Tyler Jacks Lab, MIT) to express a human codon-optimized nuclear-localized Cas9 gene that was N-terminally fused to PuroR via a T2A ribosome-skipping sequence. This cassette was expressed from the human EF1A promoter. Additionally, we replaced the mouse U6 promoter with a human U6 promoter which drives expression of a guideRNA (gRNA) consisting of a bovine B2M-specific CRISPR RNA (crRNA; target sequence GAAATTGATTTGCTGAAGAA) fused to the trans-activating crRNA (tracrRNA) and a terminator sequence. MDBK cells were transiently transfected with this anti-B2M CRISPR/Cas9 vector using the Neon® transfection system (Invitrogen). After transfection, cells were stained for MHC I surface expression levels using PE-conjugated anti-MHC I mAb (W6/32, AbD Serotec) and sorted on a FACSAriaII (BD Biosciences). Cells were cloned by limited dilution and a clone showing no MHC I surface expression was selected for this study.

Cloning and transfection of MHC class I

In order to transfect HEK-293 with MDBK-derived MHC I, total RNA was isolated from MDBK cells using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad). Full-length MHC I was amplified from cDNA using a mixture of forward primers Bov 21a/g and Bov 21-BSF together with reverse primers Bov 3 and Bov 3-BSF (17), to account for known polymorphisms at the primer target sites, and were ligated into the pcDNA™ 3.1(+) vector (Invitrogen). 29 clones were selected and sequenced as described for the Sequence based MHC I haplotyping. Vector primers and internal primers Bov 9 and Bov 11 (17) were used for sequencing. Seqscape© was used to analyze the sequence files.

HEK-293 cells were transfected with Lipofectamine® 2000 (Invitrogen) according to manufacturer's instructions. Cells, transfected with representative clones of all different MDBK-derived MHC I alleles and a mock pcDNA™ 3.1(+) construct as a negative control, were analyzed 36 hours after transfection.

Flow cytometry and confocal microscopy

Mouse mAbs used in this study include anti-MHC I (ILA88 unlabeled and FITC conjugated), anti-CD31-PE (CO.3E1D4), anti-CD41 (ILA164 unlabeled and PE conjugated) acquired from AbD serotec; anti-MHC I (PT85a), anti-integrin β1 (FW4-101) acquired from WSU Monoclonal Antibody Center; anti-B2M (B1.1G6) described by Liabeuf et al. (22).

Cells were incubated with Abs diluted in PBS supplemented with 2% FCS and 0.01% Azide at 4 ^{0}C for 30 min. Serum and colostrum was diluted to a final concentration of 1:20 and Ab binding was detected using polyclonal biotinylated sheep anti-bovine IgG Abs (AbD Serotec) and Streptavidin-PE (Molecular Probes©). Binding of biotinylated IgG was directly detected using PE or A647 conjugated Streptavidin. Granulocytes and PBMC were gated on forward and sideward scatter. To compare MHC I expression and BNP Ab binding of endothelial cells, granulocytes and PBMC under uniform staining condition, PBLk isolated from a healthy donor were mixed with endothelial cells isolated from late gestation fetuses. In all experiments appropriate isotype-matched control Abs were included. Flow cytometry experiments were performed on a FACSCanto™ (BD Biosciences) and data were analyzed using Flowjo software (TreeStar Inc.)

For confocal imaging of bone marrow cells, cells were stained as for flow cytometry. Next, cells were fixed in 4% formaldehyde for 15min and nuclei were counterstained with DAPI (Sigma Aldrich). Cells were spotted on microscope slides using a Cytospin centrifuge (Shandon) and slides were mounted with coverslips using Fluorescent Mounting Medium (Dako). Images were acquired on a SPE-II confocal microscope (Leica).

Immunoprecipitation and visualization/identification of precipitated protein

Cell surface proteins of MDBK cells or PBLk were labeled with EZ-Link™Sulfo-NHS-SS-Biotin (Thermo Scientific) according to manufacturer's instructions. After biotinylation, cells were stained in serum diluted 1:20 and incubated at 4 ^{0}C for 45min on a head-over-head roller. To assure binding of extracellular proteins only, cells were washed four times to wash away unbound and non-specifically bound Abs. Cells were lysed with ice-cold lysis buffer (1.0% Triton X-100, 20 mM MES, 100 mM NaCl, 30 mM Tris, pH 7.5) supplemented with a protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktails, Roche Life Sciences) at 4 ^{0}C for 30 min on a head-over-head roller.

Supernatant, obtained after centrifugation (18,000 x g; 4 ^{0}C; 20 min), was incubated with Protein G coupled Dynabeads (Life Technologies) at 20 ^{0}C for 20 min on a head-over-head roller. After washing four times in lysis buffer, samples were boiled (95 ^{0}C; 5 min) in non-reducing lithium dodecyl sulfate sample buffer (Thermo Scientific). Beads were removed and samples were subjected to PAGE on Amersham ECL Gel 4-20% (GE Healthcare). Proteins were transferred to a nitrocellulose membrane (Protran, Whatman) using a semi-dry blotting system (Trans Blot Semi Dry, BioRad) according to manufacturer's instructions. The membrane was blocked with blocking reagent for ELISA (Roche). Biotinylated cell surface proteins were detected with alkaline phosphatase (AP) conjugated streptavidin (Sigma). MHC I was detected with anti-MHC I (ILA88) and AP conjugated goat anti-mouse IgG (Southern Biotech). Signals were developed with NBT/BCIP (Roche life sciences).

Non-biotinylated immunoprecipitated protein samples were processed in parallel and visualized using GelCode Blue Stain Reagent (Thermo Scientific) after PAGE. Bands corresponding to the bands found in the Western blots were excised as indicated in figure 4C and were sent for mass spectrometry analysis (Alphalyse Inc.). In short, protein samples were reduced and alkylated with iodoacetamide and trypsin digested. Digested peptides were subjected to nano-liquid chromatography on an Ultimate3000 system (Dionex) and subsequent MS/MS analysis on an Impact QTOF instrument (Bruker Maxis). The MS/MS spectra were analyzed using Mascot (Matrix Science) and the UniProt and NCBI protein database were searched to identify protein matches.

Antibody-dependent complement-mediated cell lysis

To assess antibody-dependent complement-mediated cell lysis, cells were incubated with Ab (monoclonal Abs anti-MHC I ILA88 & PT85a diluted to 0.5µg/ml, serum of BNP or control dams diluted 1:100 – 1:400 for incubation with cell lines and 1:10 for incubation with PBLk) for 30 min at RT. Next, baby rabbit serum (Abd Serotec) was added to a final dilution of 1:10 and cells were incubated at 37^{0}C and 5% CO_2 for 60 min. After washing and staining with DAPI to discriminate dead and live cells, flow cytometry was used to assess cell lysis. Fluorescent microspheres (Luminex) were added to the PBLk as a control to measure changes in the number of cells per bead, reflecting killing of cells.

Statistical analyses

Protein sequence homology was analyzed using Mega6 to construct a phylogenetic tree using the Neighbor-Joining method and tree distances were calculated using the number of amino acid differences. Protein alignments were performed in Bioedit 7.2.5. The use of specific statistical tests, calculated with GraphPad Prism 5.03 (GraphPad Software), is mentioned in the figures legends. P values < 0.05 were considered significant.

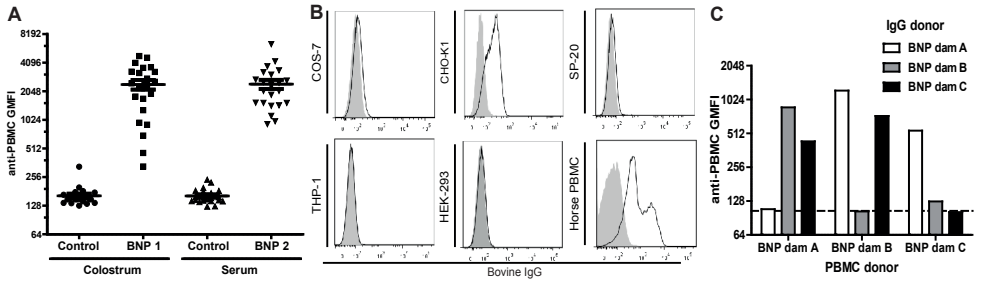


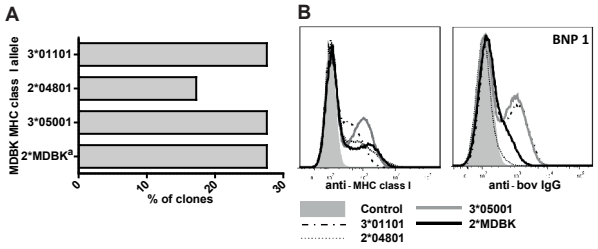
FIGURE 1. BNP alloantibodies recognize cells from a diverse MHC class I background. (A) Bovine PBMC isolated from MHC class I haplotyped animals (n=22) were stained with IgG isolated from the colostrum or serum of two dams. The MHC class I haplotypes of the PBMC donors and of the IgG donors are listed in supplementary table I. (B) Cells from different species were stained with IgG isolated from BNP dams. Data are representative for three different BNP Ab donors. COS-7 = African green monkey. CHO-K1 = Chinese hamster. SP-20 = Mouse. THP-1 = Human. HEK-293 = Human. Live cells were gated on forward and sideward scatter. (C) PBMC isolated from three dams were cross-stained with IgG isolated from the same animals to test whether alloantibodies recognize autoantigens. The horizontal dotted line depicts the average GMFI of PBMC staining by autologous IgG. In all experiments Ab staining was measured using flow cytometry. PBMC were selected based on forward and sideward scatter. GMFI = Geometric Mean Fluorescent Intensity

Results

BNP-associated alloantibodies recognize MHC class I and bind cells with a diverse MHC class I background

To test the specificity of BNP-associated alloantibodies and assess how frequently alloepitope (mis)matches between bovine cell donors, vaccine and BNP dams occur, binding of alloantibodies from colostrum or serum of BNP dams (n=4) to PBMC from non-BNP (n=12) and BNP (n=10) dams was assessed (Fig 1A). PBMC donors were selected to obtain a group of animals with a diverse MHC I background and results of the sequence based MHC I haplotyping are summarized in supplemental table I. Figure 1A shows the broad recognition and almost equal staining of PBMC by IgG isolated from serum or colostrum of BNP dams, despite the diverse MHC I background of cell donors. There was no difference in staining of PBMC isolated from BNP or non-BNP dams. To further test the specificity of BNP alloantibodies, cell lines from diverse animal origins were stained with IgG isolated from BNP dams (Fig. 1B). BNP alloantibodies stained Cho-K1 cells (Chinese Hamster) and Horse PBMC, showing BNP alloantibodies recognized targets across species. Because of tolerance to autoantigens we expected that there would be a "gap" in the repertoire of BNP alloantibodies. Isolating IgG and PBMC from three BNP dams and cross-staining Abs with PBMC from these three animals (Fig. 1C) showed alloantibodies did not bind self PBMC, confirming autoantigens were not recognized.

Since BNP alloantibodies recognized cells with very diverse MHC I backgrounds, we further examined the MHC I specificity of BNP Abs. For that purpose full-length MHC I alleles from the MDBK cell line, used for vaccine production, were cloned and expressed in HEK-293 cells. Bovine cells co-dominantly express between 2-6 classical MHC I alleles (18) and sequencing of 29 MHC I clones identified four classical MHC I alleles that appeared to be transcribed at equal levels in the MDBK cells (Fig. 2A). Transfection of the respective MHC I clones in HEK-293 cells and staining with Abs isolated from four different BNP dams revealed that all Ab pools recognized MHC I, but the recognition of specific MDBK-derived MHC I alleles was different between Abs isolated from different dams (Fig. 2B, 2C). We investigated if a common sequence or (linear) protein motif in the MHC I could be detected explaining the broad MHC I cross-reactivity of the alloantibodies. To identify regions within MHC I that may be involved in Ab binding, protein sequences of the MHC I extracellular domain (exon 2-4) of the responder dam were compared to protein sequences of the alloimmune response-inducing alleles (recognized MHC I alleles of MDBK cells) and alleles differentially recognized by alloantibodies isolated from the dam. However, phylogenetic trees and protein alignments revealed no apparent relation between Ab binding and differences in protein sequence between responder, alloimmune response-inducing and differentially recognized MHC class I alleles. An example of this analysis can be seen in supplementary figure 1A and B (phylogenetic tree and protein sequence alignments of the extracellular part of MHC I alleles, respectively).



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Antibody	Control	3*01101	2*04801	3*05001	2*MDBK
a-MHC class I	-	++	++	++	++
BNP 1	-	++	-	++	+
BNP 2	-	++	+	++	+
BNP 3	-	++	++	++	++
BNP 4	-	+	-	+	-

FIGURE 2. BNP alloantibodies recognize MHC class I alleles carried by the cell line used for vaccine production. (A) 29 full length MHC class I clones derived from MDBK cells, the cell line used for the production of Pregsure© BVD, were sequenced and the relative number of clones per identified allele are depicted. (B, C) Full length MDBK-derived MHC class I clones or a mock construct were transfected into HEK293 cells, stained with anti-bovine MHC class I mAb ILA88 as a positive control and IgG isolated from the serum of one (B) or four (C) BNP dam(s) and Ab binding was revealed using flow cytometry. BNP 1 in C is the same dam as in B. Results are based on two separate transfection experiments. [a]Denotes a local name (Genbank accession number KM397368).

BNP-associated alloantibodies recognize Integrin beta-1, alpha-3 and MHC I, but the majority of Abs have MHC I specificity

To assess the proportion of BNP alloantibodies that recognize MHC I, we used MDBK cells expressing the Bovine Herpes Virus-1-derived TAP inhibitor UL49.5 (19). TAP inhibition leads to MHC I down-regulation and MHC I expression was reduced with

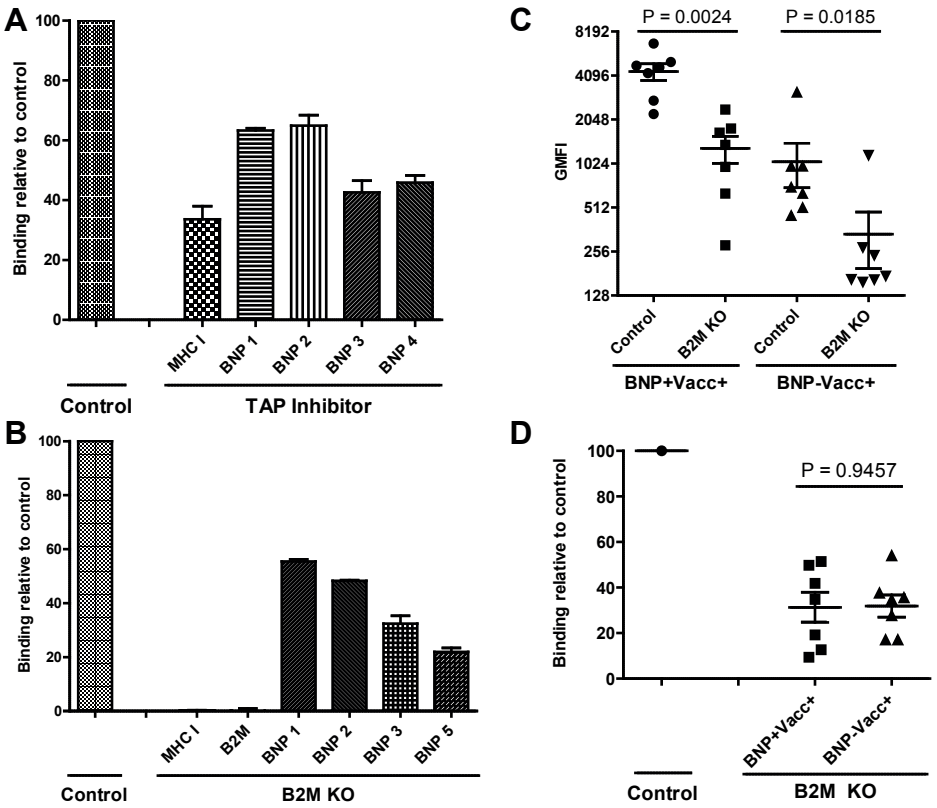

FIGURE 3. MHC class I is a major, but not the only, target of BNP-associated alloantibodies. (A) Comparison of MHC class I expression and binding of IgG isolated from BNP dams between MDBK cells transduced with a viral TAP inhibitor and MDBK cells that were transduced with a control vector. Data were expressed relative to binding of the control cell line and are average of three separate experiments. MHC class I expression was measured using mAb ILA88. (B) Comparison of MHC class I and B2M expression and binding of IgG isolated from BNP dams between wild-type MDBK cells (Control) and B2M KO MDBK cells. Data expressed as in A. B2M expression was measured using mAb B1.1G6. (C) Wild-type MDBK cells (Control) and B2M KO MDBK cells were stained with sera from Pregsure© BVD-vaccinated BNP (BNP+Vacc+, n=7) and non-BNP (BNP-Vacc+, n=7) dams. Alloantibody binding on the wild-type MDBK and the B2M KO MDBK was compared using a paired t-test. (D) Data from C were transformed to express Ab binding to B2M KO MDBK relative to wild-type MDBK. The relative alloantibody binding between Pregsure© BVD-vaccinated BNP and non-BNP dams was compared using an unpaired t-test for equal variance. In all experiments Ab binding was measured using flow cytometry and respective isotype controls were subtracted. GMFI = Geometric mean fluorescent Intensity.

66% on MDBK cells transduced with the viral TAP inhibitor (Fig. 3A). BNP Ab binding was reduced with 35-57% (Fig. 3A), again confirming that BNP alloantibodies recognized MHC I. However, the reduction in BNP Ab binding was lower than the MHC I down regulation, indicating that BNP alloantibodies also recognized non-MHC I targets.

Knocking-out B2M leads to retention of MHC I in the ER and almost completely abolishes MHC I expression at the cell surface (23). To confirm that BNP alloantibodies recognize non-MHC I targets we constructed a B2M knockout MDBK cell line (B2M KO) using the CRISPR/Cas9 gene editing technique. Using flow cytometry we confirmed that there was no residual B2M and MHC I expression on the B2M KO cell line (Fig. 3B). There was a marked reduction in binding of IgG isolated from serum of BNP dams to B2M KO cells compared to wild-type MDBK cells, with the residual signal on the B2M KO cells accounting for 22-55% of the signal on the wild-type MDBK cells (Fig. 3B). Staining cells with serum from Pregsure© BVD-vaccinated BNP (n=7) and non-BNP (n=7) dams showed a significant reduction in Ab binding on the B2M KO cells for both groups (Fig. 3C). Comparing the relative Ab binding on B2M KO to the wild-type MDBK cells showed that the residual signal on B2M KO cells was similar between Pregsure© BVD-vaccinated BNP and non-BNP dams and ranged between 9-54% (Fig. 3D). These results demonstrated that MHC I is the major target for BNP-associated alloantibodies and depending on the BNP dam accounts for 46-91% of the Ab specificity. Conversely, 9-54% of the Abs were specific for non-MHC I targets, depending on the BNP dam.

To identify the non-MHC I targets of BNP alloantibodies we immunoprecipitated target Ags on wild-type and B2M KO MDBK cells using sera from BNP dams. In B2M KO cells, MHC I is still expressed intracellularly (data not shown) and therefore care was taken to precipitate extracellular proteins only. Antigens precipitated from wild-type MDBK cells using sera from BNP dams were analyzed by Western blot and showed three prominent bands around 40, 130 and 140 kDa. The band around 40 kDa disappeared when using B2M KO cells, indicating these precipitated proteins were MHC I. This was confirmed with an MHC I mAb, resulting in a positive band around 40 kDa for wild-type MDBK cells, which was absent in B2M KO cells (Fig. 4B). Results were similar when using serum from a Pregsure© BVD-vaccinated non-BNP dam, although the bands of precipitated proteins were less intense (data not shown). To see if similar Ags are precipitated by sera from different dams, we repeated the above experiment with sera from several BNP dams (Fig. 4C) using B2M KO cells to focus on non-MHC I targets. Sera were ranked according to alloantibody staining intensity of B2M KO MDBK cells. As expected, the amount of precipitated extracellular protein roughly correlated with alloantibody binding of B2M KO cells. Two prominent bands around 130 and 140kDa were observed in the lanes of four of the seven BNP sera. To identify these non-MHC I targets of BNP alloantibodies, the region containing these

precipitated Ags were cut out of the PAGE gels and used for nano-LC-MS/MS. Two proteins were identified, Integrin alpha-3 and Integrin beta-1, that together form the receptor Very Late Antigen-3 (VLA-3) (Table I).

Table I. Identification of proteins precipitated with sera from BNP dams.

Protein[a]	Calculated MW	Sequence coverage(%)	Number of peptides
Integrin alpha-3	117 kDa	19% (Sample 1)	82
		20% (Sample 2)	86
Integrin beta-1	91 kDa	21% (Sample 1)	86
		21% (Sample 2)	75

[a]Precipitated target Ags of BNP sera on B2M KO MDBK cells were identified using nano-liquid chromatography and subsequent mass spectrometry.

Binding of BNP Abs to various cell types and BNP antibody-dependent complement-mediated cell lysis correlates with MHC I expression, not Integrin beta-1 expression.

We compared expression of MHC I and integrin beta-1 to BNP Ab binding of wild-type and B2M KO MDBK cells and PBLk (Fig. 5). MHC I expression was high on wild-type MDBK cells and on PBMC, but was very low on granulocytes and absent on B2M KO MDBK cells. BNP Ab binding of PBLk followed a pattern similar to MHC I expression, i.e. high binding of PBMC and low binding of granulocytes. Integrin beta-1 expression showed a different pattern, with comparable expression levels on PBMC and granulocytes and very high expression on both B2M KO and wild-type MDBK cells. Since BNP Ab binding on PBLk correlated with MHC I expression, rather than integrin beta-1 expression, we examined target Ags of BNP Abs on PBLk by immunoprecipitation to see if integrin beta-1 was recognized on PBLk. As shown in figure 4D, MHC I was immunoprecipitated on PBLk, whereas integrin beta-1 and alpha-3 were not, indicating BNP abs only bind MHC I on PBLk.

Almost all nucleated cell express MHC I, but as shown in figure 5 the expression levels of MHC I can differ greatly between cell types. We therefore investigated whether MHC I expression levels had implications for the pathogenic effects of BNP-associated alloantibodies. A recent study showed that BNP alloantibodies could induce complement-mediated lysis of MDBK cells (24). MHC I mAb dependent complement-mediated cell lysis of TAP-inhibited and control MDBK cells was compared. Cell lysis was lower in TAP-inhibited than in control MDBK cells (Fig. 6A), correlating with MHC I expression as found in figure 3A. Next, the same assay was performed using sera from several BNP dams (Fig. 6B). Complement-mediated cell lysis was significantly lower for TAP-inhibited MDBK cells compared to the control cell line ($p=0.0019$),

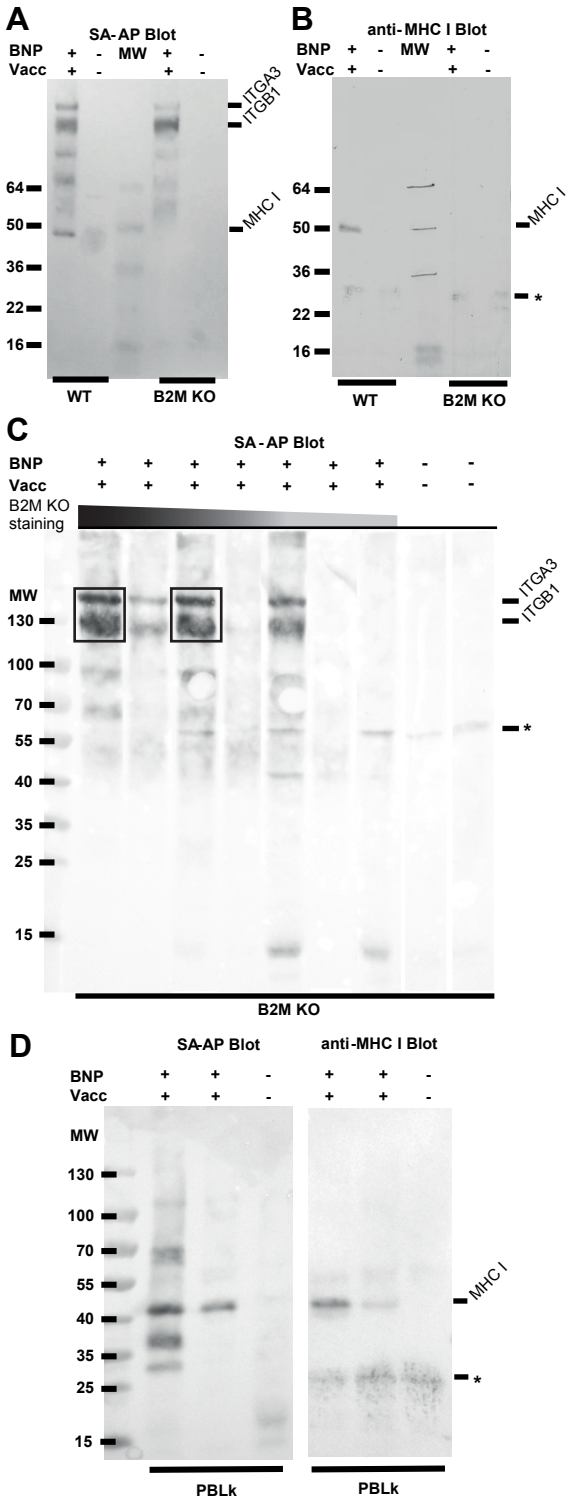

FIGURE 4. Sera from BNP dams recognize MHC class I and non-MHC class I targets. Cell surface proteins of wild-type MDBK (WT), B2M KO MDBK (B2M KO) or PBLk were biotinylated and subsequently stained with sera of Pregsure© BVD-vaccinated BNP dams (BNP+Vacc+) or control dams not vaccinated with Pregsure© BVD (BNP-Vacc-). After washing away unbound Abs, cells were lysed. Abs and bound Ag were precipitated using Protein G coupled dynabeads, separated by non-reducing gel electrophoresis and blotted on nitrocellulose membrane. (A) WT and KO MDBK cells were stained with serum from a BNP+Vacc+ dam or a BNP-Vacc- dam. Cell surface proteins were visualized using SA-AP. (B) As in A, but immunoprecipitated MHC class I was visualized using anti-bovine MHC class I mAb ILA88. (C) B2M KO MDBK cells were stained with sera from BNP+Vacc+ dams (n=7) and BNP-Vacc- dams (n=2). Cell surface proteins were visualized using SA-AP. The sera from BNP+Vacc+ dams were ranked according to alloantibody staining intensity of B2M KO MDBK cells as in figure 3C. Representative for two separate experiments. (D) PBLk from a healthy donor were stained with sera from BNP+Vacc+ dams (n=2) or a BNP-Vacc- dam. Cell surface proteins and MHC I were visualized using SA-AP and mAb ILA88, respectively. Representative for two separate experiments with different PBLk donors. MW = Molecular weight markers (kDa). ITGB1 = integrin β1. ITGA3 = integrin α3. * = nonspecific signal. Boxes in C indicate regions that were subjected to mass spectrometry analysis

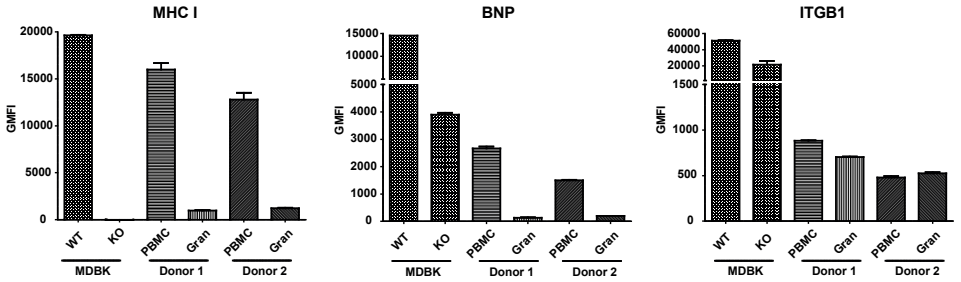

FIGURE 5. Expression of MHC class I, Integrin beta-1 and BNP Ab binding on wild-type (WT) and B2M knockout (KO) MDBK cells and PBLk. WT and B2M KO MDBK cells and PBLk (n=2, donor 1 & 2) were stained with mAbs against MHC class I (MHC I), Integrin beta-1 (ITGB1) or with BNP Abs. Expression/Ab binding was measured using flow cytometry. PBMC and granulocytes (Gran) were gated on forward and sideward scatter. GMFI = Geometric Mean Fluorescent Intensity

indicating that BNP Ab dependent complement-mediated cell lysis correlated with MHC I expression. PBMC have a much higher MHC I expression than granulocytes and comparing complement-mediated lysis of peripheral blood leukocytes using a mAb against MHC I showed a steep increase of the granulocyte/PBMC ratio (Fig. 6C, 6D), reflecting the killing of the high MHC I expressing PBMC rather than granulocytes. Similarly, incubating peripheral blood leukocytes with serum or colostrum from BNP dams significantly increased the granulocyte/PBMC ratio (Fig. 6D). Beads were added to the complement lysis assay in order to track the number of cells per bead and PBLk were incubated with a broad range of sera and colostrum samples from control and BNP dams. The number of PBMC per bead decreased, reflected by an increased granulocyte/PBMC ratio, whereas the number of granulocytes were unaffected (Fig. 6E). Demonstrating granulocytes are not lysed after BNP Ab mediated complement dependent cell lysis of PBLk, despite low BNP alloantibody binding and low MHC I expression. Together these data show that BNP antibody-mediated complement dependent lysis strongly correlates with MHC I expression and that cells with high MHC I expression are killed predominantly. In line with this, BNP antibody-mediated complement dependent cell lysis of B2M KO MDBK cells was lower than that of wild type-MDBK cells ($p < 0.001$, Fig. 5F). Despite the lack of MHC I expression, complement lysis of B2M KO MDBK cells appeared to be higher for sera from BNP dams than for control sera, although this effect was not statistically significant ($p = 0.0571$, Fig. 6F), indicating that non-MHC I alloantibodies may also activate complement and could in theory lead to cell lysis *in vivo*.

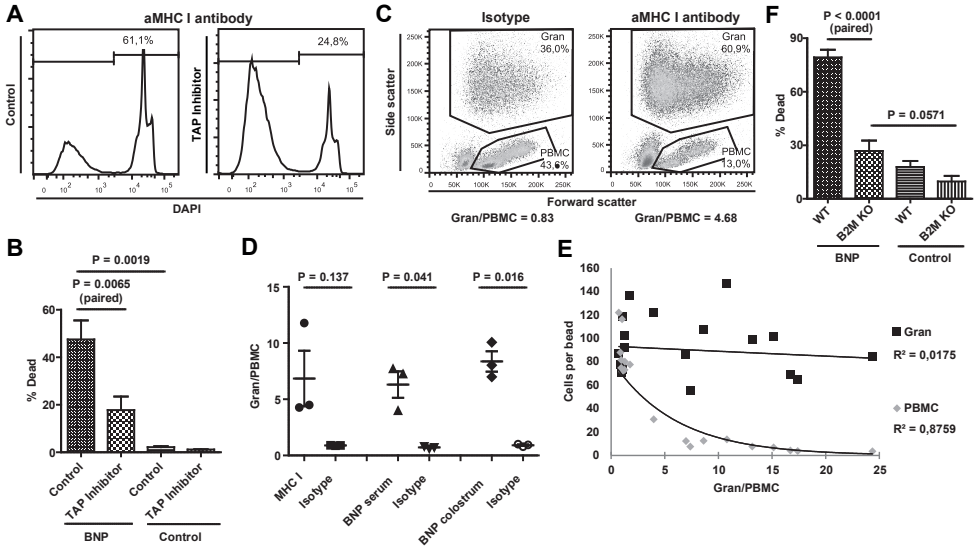

FIGURE 6. BNP antibody-dependent complement-mediated lysis of cells correlates with MHC class I expression. Cells were stained with antibodies and subsequently incubated with rabbit complement for 30 minutes. Antibody-dependent complement-mediated killing was measured as the percentage DAPI positive cells using flow cytometry. (A) MDBK cells transduced with the viral TAP inhibitor UL49.5 (TAP inhibitor) and MDBK cells that were transduced with a control vector (Control) were stained with an anti-MHC class I mAb (PT85a). (B) TAP inhibitor and control cells were stained with sera from Pregsure© BVD-vaccinated BNP dams (BNP, n=6) and with unvaccinated control sera (control, n=4). The difference in complement-mediated killing of Control and TAP inhibitor MDBK cells by sera from BNP dams was compared using a paired t-test and killing of control MDBK cells between BNP and control sera was compared using an unpaired t-test for unequal variance. (C) Peripheral blood leukocytes (PBLk) were stained with anti-MHC class I mAb (ILA88) or isotype control. Granulocytes (Gran) and PBMC were gated on forward and sideward scatter. The proportion of Gran to PBMC is given. (D) PBLk from calves (<6mnd, n=3) were stained with Abs from different sources. MHC I, Anti-MHC class I mAb and isotype control. BNP serum, serum from a Pregsure© BVD-vaccinated BNP dam and serum from an unvaccinated dam as isotype control. BNP colostrum, colostrum from a Pregsure© BVD-vaccinated BNP dam and colostrum from an unvaccinated dam as isotype control. The proportion of Gran to PBMC between Ab and isotype control is compared using a paired t-test. Data are representative for several different BNP sera and BNP colostra. (E) PBLk from a calf were incubated with fluorescent microspheres during the complement lysis assay as an internal control. The number of cells per bead (Gran or PBMC) are plotted against the proportion of gran to PBMC for several serum and colostrum samples from Pregsure© BVD-vaccinated BNP and non-BNP dams and unvaccinated dams. The number of cells per bead is expressed relative to the number of cells per bead for the medium control. An exponential trend line between cells per bead and the proportion Gran to PBMC is plotted and the R2 for the trend line is shown. Data are representative for three different animals. (F) Wild-type (WT) and B2M knockout (B2M KO) MDBK cells were stained with sera from Pregsure© BVD-vaccinated BNP dams (BNP, n=6) and with unvaccinated control sera (control, n=4). The difference in complement-mediated killing of WT and B2M KO MDBK cells by sera from BNP dams and of B2M KO MDBK cells between BNP sera and control sera was compared using a paired t-test and an unpaired t-test for equal variance, respectively.

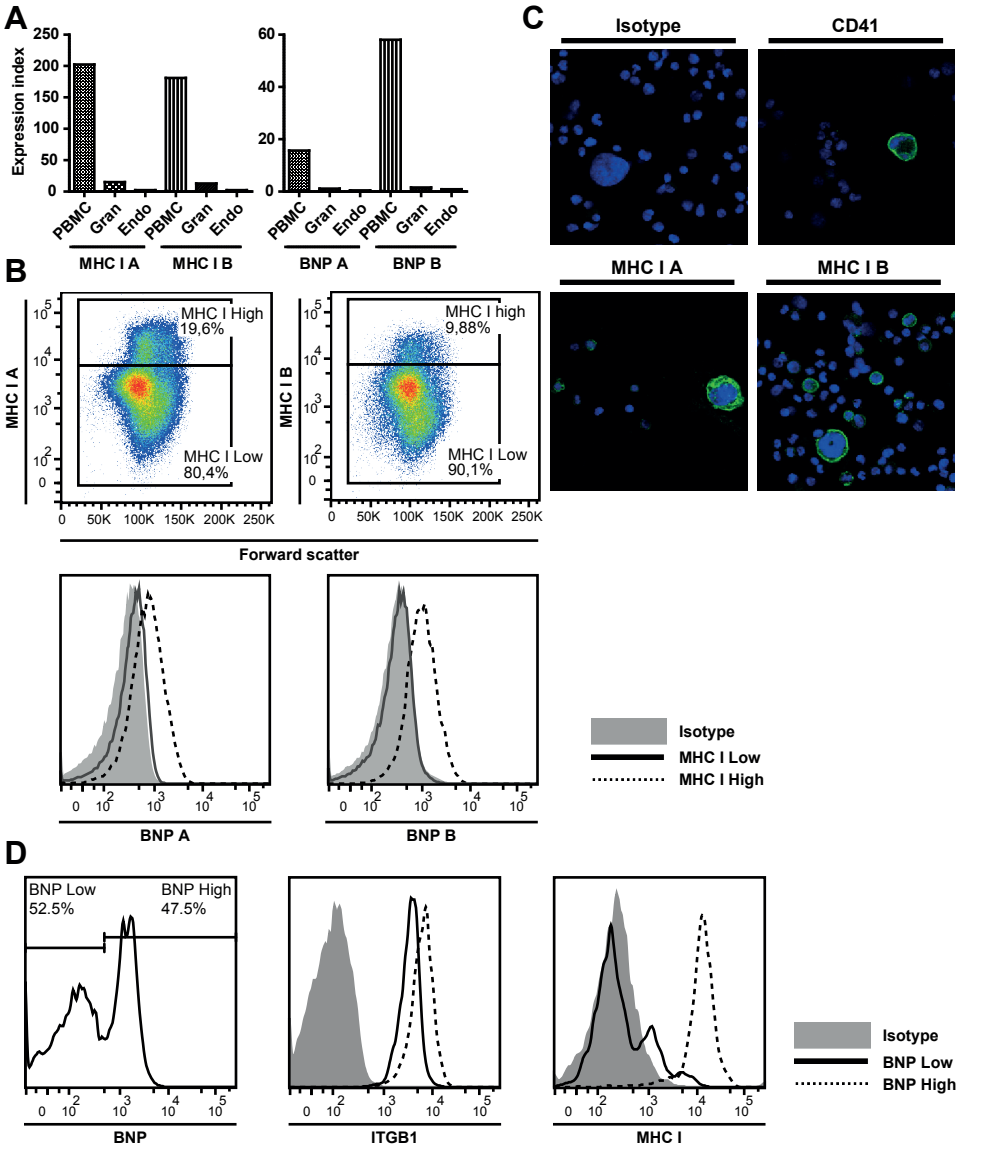

FIGURE 7. Cell types affected in Bovine Neonatal Pancytopenia are characterized by high MHC class I expression. (A-C) Bone marrow and endothelial cells were isolated from late gestation bovine fetuses (n=3). MHC class I expression and BNP Ab binding was assessed using flow cytometry and confocal imaging. Data are representative for three different animals. (A) Endothelial cells were mixed with peripheral blood leukocytes from a healthy donor to compare MHC class I expression and BNP Ab binding between tissues as measured by flow cytometry. Endothelial cells and PBMC/Granulocytes were selected on forward and sideward scatter and on $CD31^{high}$ and $CD31^{low}$ expression, respectively. Expression index = Δ Ab / Δ Isotype. (B) MHC class I expression and BNP Ab binding of bone marrow cells was measured by flow cytometry. Bone marrow cells divided into MHC class I^{high} and MHC class I^{low} expression (upper panel) were compered for BNP alloantibody binding (lower panel). Cells were gated on live cells based on forward and sideward scatter (confirmed with DAPI) and on autofluorescent-negative cells. (C) Confocal images of bone marrow cells stained with isotype, anti-MHC class I or anti-CD41 (Green). DAPI (blue) was used as

a nuclear stain. Megakaryocytes are cells with large nuclei and anti-CD41 was used as a positive control for megakaryocytes. (D) Cryopreserved bone marrow cells isolated from a neonatal calf were measured using flow cytometry to evaluate MHC class I and Integrin beta-1 expression of cells with low and high BNP Ab binding. Intact cells were gated based on forward and sideward scatter and dead cells were excluded using DAPI staining. Data are representative for Abs isolated from two different BNP dams.

Cells affected in Bovine Neonatal Pancytopenia are characterized by high MHC class I expression

After colostrum intake, Abs are absorbed via intestinal cells and are transported throughout the body via the circulation. Indeed, alloantibodies were detected in the blood of BNP calves (data not shown). However, only certain cell types are affected in BNP (i.e. lymphocytes, platelets and bone marrow cells), whereas endothelial cells which are in close contact with the absorbed alloantibodies are unaffected (1, 6). We investigated whether differential MHC I expression levels and BNP alloantibody binding of different cell types could explain these observations. Endothelial and bone marrow cells were isolated from third trimester fetal calves (7-9 months of gestation) and stained with MHC I mAbs and BNP alloantibodies (Fig. 7A-C). As shown in figure 7A, MHC I expression and BNP Ab binding was much lower on endothelial cells than on PBMC. Bone marrow cells were characterized by populations of high or low MHC I expression (Fig. 7B, upper panels) and these populations were also characterized by high or low BNP alloantibody binding, respectively (Fig. 7B, lower panels). Confocal images of bone marrow cells showed that megakaryocytes have high MHC I expression (Fig. 7C). MHC I bright and dim uni-nucleated cells, as seen with flow cytometry, were also observed on these images. Cryopreserved bone marrow cells isolated from a neonatal calf were divided into cells with high and low BNP Ab binding and we examined integrin beta-1 and MHC I expression in both populations (Fig. 7D). Although integrin beta-1 expression was somewhat lower in bone marrow cells with low BNP Ab binding, MHC I expression of bone marrow cells correlated much better to BNP Ab binding. Together these data showed that the cell types affected in BNP were characterized by high MHC I expression and high BNP alloantibody binding.

Discussion

BNP is a fatal bleeding syndrome in neonatal calves caused by Pregsure© BVD vaccine-induced maternal alloantibodies absorbed from the colostrum. In this paper, we investigated the specificity of BNP-associated alloantibodies and linked the specificity of these Abs to the pathogenesis of BNP.

To investigate the importance of MHC I specific Abs in the pathogenesis of BNP, we first assessed the relative quantity of MHC I specific BNP Abs. Knocking out B2M from MDBK cells using the CRISPR/Cas9 gene editing technique abolished MHC I expression and led to a significant drop in BNP alloantibody binding (Fig. 3). This showed that MHC I is a major target of BNP abs and depending on the BNP dam accounts for roughly 40-90% of all alloantibodies. The expression of MHC I-like molecules MR1 and CD1 also depends on B2M, but intra- and extracellular staining of MDBK cells with MR1 and CD1 specific Abs showed no expression of these proteins on MDBK cells (data not shown). Based on sequencing of the B2M gene, we previously concluded that it is highly unlikely that B2M itself is a target of BNP Abs (12). Conversely, BNP Abs also recognized non-MHC I targets and mass spectrometry analysis of Ags immunoprecipitated using BNP alloantibodies revealed VLA-3 is an additional target of BNP Abs. VLA-3 is a heterodimer consisting of integrins β1 and α3 and for both these integrins many allelic variants are documented (25).

To further investigate the role of the heterogeneous Ab specificity in the pathogenesis of BNP, we determined the expression of target Ags of BNP Abs in tissues that are differentially affected in BNP. BNP is characterized by the loss of specific cell types (i.e. lymphocytes, thrombocytes and bone marrow cells), whereas other cells exposed to BNP Abs are not affected (e.g. endothelium). Bell et al. (1) reproduced BNP in calves by feeding colostrum from BNP dams and found there was only a transient drop in neutrophil levels in blood, with levels restoring to normal within 12 hours after colostrum ingestion. Furthermore, they showed that in bone marrow, mature cells of the neutrophil, eosinophil and erythroid lineages were less affected, which was confirmed by other studies (9, 26). BNP Ab binding matched the pathology of BNP, with high Ab binding of cells that are affected in BNP such as PBMC and a subset of bone marrow cells, whereas endothelial cells and granulocytes showed very low BNP Ab binding (Fig. 5, 7). Although MHC I is expressed on all nucleated cells, the level of MHC I expression differed greatly between cell types and corresponded to BNP Ab binding (Fig. 5, 7). Interestingly, megakaryocytes, which are almost completely depleted in most BNP cases (1, 3), were characterized by very high MHC I expression (Fig. 7). VLA-3 is typically expressed on endo- and epithelium (27, 28) and also on many cancer cells and immortalized cell lines (29, 30). However, BNP Abs marginally bound endothelial cells (Fig. 7) and epithelial and endothelial linings are not damaged in the course of BNP. Integrin β1 can associate with several alpha integrins and is expressed on virtually all

cell types (31). We confirmed the high expression of integrin β1 on MDBK cells (Fig. 5) and hence it is likely to be present in the Pregsure© BVD vaccine. Expression of integrin β1 on PBLk and on bone marrow cells did not correlate with BNP Ab binding. Moreover, although both MHC I and integrin β1 are expressed on PBLk, MHC I, but not integrin β1, was immunoprecipitated from PBLk using BNP sera (Fig. 4). Summarizing these findings, the expression patterns of VLA-3 and integrin β1 did not match with the pathology of BNP and the binding of BNP-associated alloantibodies, whereas MHC I expression did. Furthermore, only four of seven sera from BNP dams clearly precipitated VLA-3 (Fig. 4C), indicating VLA-3 specificity is not a prerequisite for the development of BNP. The clinical and post mortem presentation of BNP is very consistent between cases (1-3) and does not indicate a difference in the clinically relevant alloantibody specificities between BNP dams. Therefore, we hypothesized MHC I specific alloantibodies drive the pathogenesis of BNP and susceptibility of cells to BNP-associated alloantibodies depends on MHC I expression levels.

Already 2 hours after ingestion of colostrum from BNP dams a steep drop in lymphocyte and thrombocytes can be seen (1, 6). This remarkably quick effect of BNP Abs suggests a pivotal role for antibody-dependent complement-mediated cell lysis of cells, rather than a cellular immune response (32), in the pathogenesis of BNP. We showed that BNP Abs can active complement and that subsequent cell lysis correlates with MHC I expression *in vitro* (Fig. 6). This corroborates our hypothesis that only cells with high MHC I expression are affected in BNP and also supports the notion that antibody-dependent complement-mediated cell lysis is likely to be an important effector mechanism of BNP alloantibodies *in vivo*.

Fetal/neonatal allo-immune thrombocytopenia (FNAIT) in humans is a syndrome caused by maternal anti-platelet alloantibodies and is characterized by a severe thrombocytopenia that can lead to life threatening hemorrhages (33). FNAIT has been compared to BNP, but whereas in FNAIT only thrombocytopenia is seen, BNP is additionally characterized by leukopenia and depletion of bone marrow cells. Alloantibodies in FNAIT are directed against platelet Ags, of which integrin β3 is the most important. Paternal MHC I specific Abs can be found in 10-30% of pregnant women and have been associated with incidental cases of FNAIT (33, 34). Nevertheless, it is generally accepted that MHC I specific Abs do not play a (important) role in FNAIT (33, 35). In this respect FNAIT resembles our view of BNP; in both diseases alloantibodies with heterogeneous specificity may be present, but a dominant specificity (platelets and MHC I in FNAIT and BNP, respectively) drives pathogenesis.

MHC I is expressed on the fetal membranes at the end of gestation (36) and naturally occurring alloantibodies against paternal alloantigens can be detected in up to 64% of multiparous cattle (37, 38). The risk of the occurrence of BNP increases with parity (5, 12) and exposure to fetally expressed paternal MHC I could contribute to the Pregsure© BVD induced alloimmune response. The recognition of several transfected

MHC I alleles was different between BNP dams (Fig. 2), showing that each animals has a specific alloantibody repertoire. This is expected as the alloantibody repertoire depends on the allogeneic background of the dam. However, binding of PBMC from animals with a diverse MHC I background (Fig. 1) showed that BNP alloantibodies have a very broad specificity. The MHC I specificity of pregnancy-induced alloantibodies broadens with multiple gestations (37, 39) and this effect has also been seen after repeated vaccinations with allogeneic lymphocytes (37). Through (multiple) Pregsure© BVD vaccination(s) and pregnancy, BNP dams are repeatedly exposed to alloantigens, which could explain the broad specificity of BNP alloantibodies we observed. We previously hypothesized that the quality of the alloantibody response is equal in Pregsure© BVD-vaccinated non-BNP and BNP dams (12). The finding that non-BNP dams have MHC I specific alloantibodies corroborates this hypothesis. The MHC I specificity of non-BNP dams has also been shown in a recent study by Kasonta et al. (24). However, BNP dams have considerable higher alloantibody levels (8, 12). In humans, complement fixation correlates with alloantibody levels (40) and high alloantibody levels are associated with increased risk of antibody-mediated rejection of allogeneic transplants (40, 41). Therefore, we hypothesized that the development of BNP in the calf primarily depends on the alloantibody dose the calf absorbs (12). The odds of BNP increases with increased colostrum intake (5) and as a corollary increased alloantibody intake, also indicating that the occurrence of BNP is alloantibody dose dependent. Incidental cases of calves with BNP symptoms without a history of Pregsure© BVD vaccination have been reported (1, 42, 43). Although speculative, these cases could be related to pregnancy induced MHC I alloantibodies. Another explanation could be the use of other vaccines that contained bovine alloantigens. Two studies could not detect alloantibodies in dams vaccinated with other inactivated BVD vaccines (8, 24). However, Pregsure© BVD induced BVD antibody titers significantly higher than alternative BVD vaccines (8, 44) and this may have favored the induction of high alloantibody levels. Following vaccination with different inactivated BVD vaccines, Bastian et al (8) detected low levels of antibodies that bound bovine leukocytes in the sera of guinea pigs and Deutskens et al (11) showed that the sera from some dams precipitated antigens from MDBK cells, thus indicating bovine proteins may also be present in other vaccines. In cows, maternal Abs are not transported across the placental barrier and are only absorbed from the colostrum in the first hours after birth. This sudden uptake of a large quantity of alloantibodies likely contributes to the sudden and severe presentation of BNP. In contrast, in humans, Abs are transported across the placental barrier during pregnancy. Consequently, pathologic alloantibodies can already affect the fetus during pregnancy and will likely lead to more chronic and less easily observed pathologies. In humans anti-paternal MHC I alloantibodies have been associated with pathology during pregnancy (e.g. chronic chorioamnionitis (45) and recurrent miscarriage (46)), but results between studies are heterogeneous (47). Influenza vaccination in humans has been shown to

induce alloimmune responses (48, 49), but to our knowledge alloimmune related adverse effects of human vaccines produced on human cell lines have not been reported. Nevertheless, as adverse effects may be difficult to monitor, it remains prudent to be vigilant of possible alloimmune-related adverse effects of vaccines grown on same-species cell lines.

In this study we show MHC I and VLA-3 are major targets of BNP-associated vaccine-induced alloantibodies. Whereas alloantibodies from all BNP dams recognize MHC I, not all BNP dams recognize VLA-3. Since the pathology of BNP is very consistent, it is likely that clinically relevant alloantibody specificities between BNP dams are also consistent. Considering that BNP Ab binding of peripheral blood leukocytes, endothelial cells and bone marrow cells conforms to MHC I expression, whereas VLA-3 and integrin β-1 have very different expression patterns, MHC I-specific alloantibodies appear to be most important in the pathogenesis of BNP. Antibody-dependent complement lysis assays showed that *in vitro* killing of cells correlates with MHC I expression and BNP Ab binding. We conclude that MHC I-specific BNP alloantibodies mediate the pathogenicity of BNP in the calf and that cells with high MHC I expression were preferentially affected in BNP.

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Supplementary files

Supplementary Table I

BNP alloantibodies stain bovine PBMC isolated from animals with a diverse MHC class I background.

MHC class I haplotype of PBMC donor[a]		BNP 1 (colostrum IgG)	BNP 2 (serum IgG)
A19v3	H5v2(UU)	459	988
A12 (UU)	A20v3UU	2478	1128
A14	A15v1	3663	2654
A20v3UU		4796	2505
A11		3611	3061
A14		331	3750
A15v1	UU1	2573	2066
A13	A10	4614	4220
A11		3291	3243
A10	H2	3189	2562
UU1	A18v2	2784	3393
A14		956	2071
A15v1		1926	923
A11	A20v3UU	4065	2408
A14		700	6407
A19v3		1708	1545
A15v1	A19v3	3275	1439
A19v3	UU7	2402	1542
A13	A12 (UU)	1800	1551
A15v1		2214	1455
A14	A19v3	910	1882
A13	A15v1	1296	2717

[a]PBMC isolated from MHC class I haplotyped animals (n=22) were stained by IgG isolated from the colostrum or serum of two dams and Ab binding was measured using flow cytometry. MHC class I haplotype of the IgG donors are A14 for BNP dam 1 and A12(UU) for BNP dam 2. GMFI = Geometric Mean Fluorescent Intensity. Bovine MHC class I haplotypes are based on Codner et al. (1) and Benedictus et al. (2). Haplotypes with a UU pre- or suffix are provisional haplotypes.

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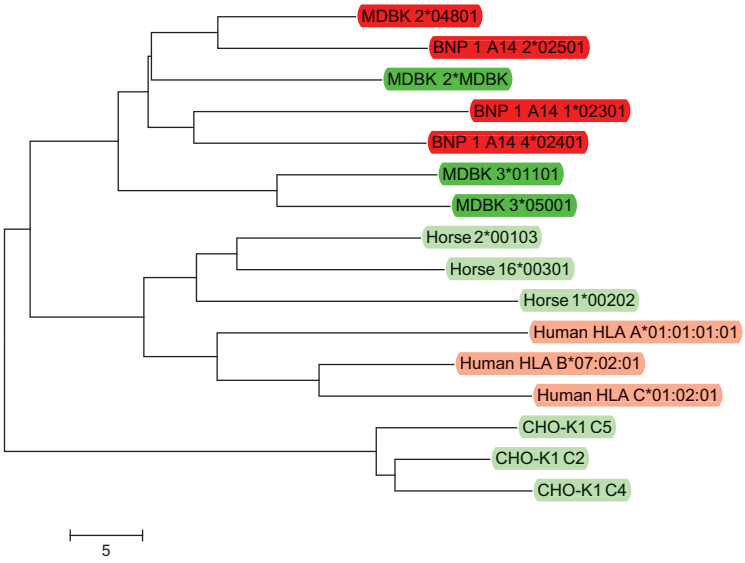
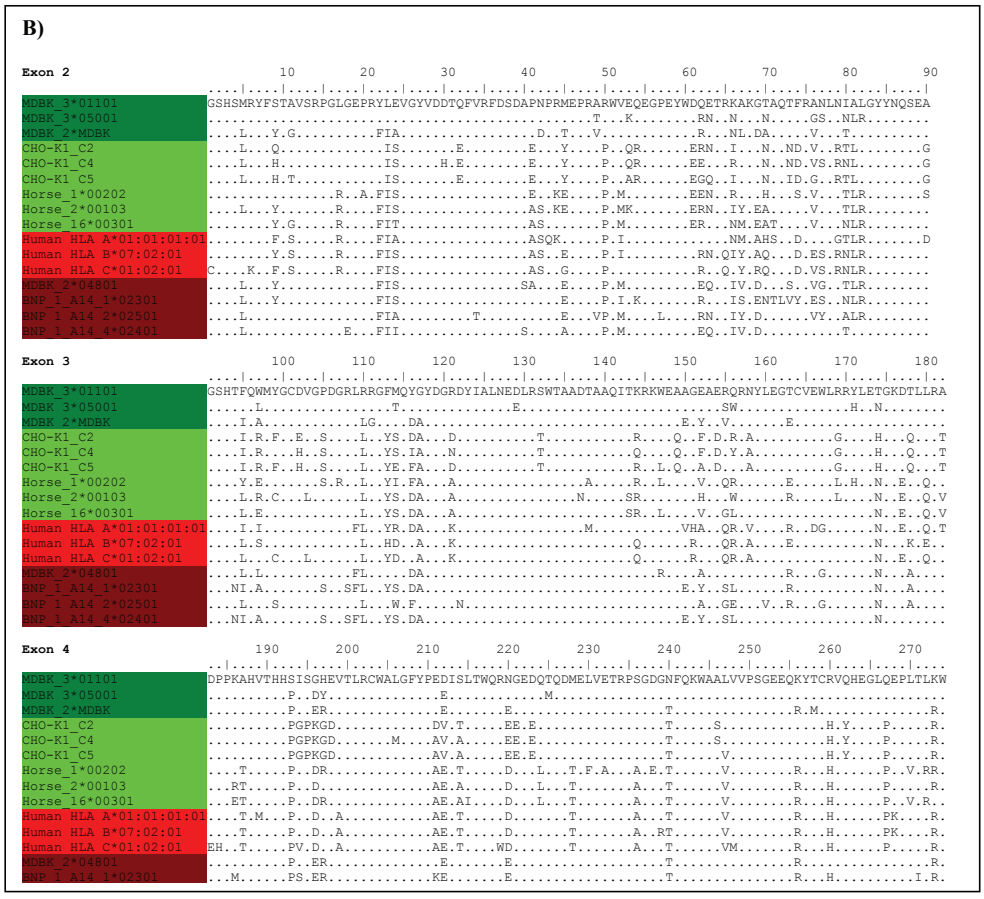
Supplementary figure 1

Phylogenetic tree (A) or alignment of the protein sequence (B) of the extracellular part of MHC class I alleles (exon 2-4) from several species. To identify regions within MHC I that may be involved in Ab binding, protein sequences of the extracellular domain (exon 2-4) of MHC I of the responder (the dam) were compared to protein sequences of the alloimmune response inducing alleles (recognized MHC I alleles of MDBK cells) and alleles differentially recognized by alloantibodies isolated from the dam. Bovine MHC class I alleles that were recognized are in green and alleles that were not recognized (including self alleles) are in red. MHC class I alleles from species that were or were not recognized are in light green and in light red, respectively. MHC class I alleles grouped according to species rather than on recognition by serum alloantibodies of BNP dam 1 and protein alignments revealed no apparent relation between Ab binding and differences in protein sequence between responder, alloimmune response inducing and differentially recognized MHC class I alleles. (A) The phylogenetic tree was constructed using the Neighbor-Joining method and tree distances were calculated using the number of amino acid differences.

(A)



(B) All sequences were compared to the first allele and dots represent identities. MHC class I alleles that were differentially recognized by alloantibodies from BNP dam 1 were based on the information from figure 1 and 2. For human, horse and CHO-K1 sequences it was not known which specific alleles were recognized. Horse alleles were selected from the most common classical MHC class I genes 1, 2 and 16 (1). For humans representative alleles of the classical HLA-A, HLA-B and HLA-C gene were selected from the IMGT/HLA Database (http://www.ebi.ac.uk/ipd/imgt/hla/). For CHO-K1 the expressed classical MHC class I alleles were added (2).



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7

Summarizing discussion

Part of this chapter is submitted as:

The role of placental MHC class I expression in immune assisted separation of the fetal membranes in cattle

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The capacity to distinguish self from non-self is central to the adaptive immune system. The fetus inherits and expresses paternal antigens and is therefore semiallogeneic to the maternal immune system. Acceptance of the 'foreign' fetus is imperative for a successful pregnancy and depends on regulation, rather than suppression of the materno-fetal alloimmune response. Improper regulation of the materno-fetal alloimmune response can have adverse effects on pregnancy and on the health of the neonatal calf. Two disorders representing different aspects of improper regulation of materno-fetal alloimmunity were studied: Retained Fetal Membranes, associated with absence (or reduction) of materno-fetal alloimmunity and Bovine Neonatal Pancytopenia, caused by iatrogenic boosting of materno-fetal alloimmunity. This chapter summarizes the research described in this thesis and adverse effects of materno-fetal alloimmunity in cattle are discussed.

Materno-fetal alloimmune assisted separation of the fetal membranes

Retained fetal membranes (RFM), persistence of the adherence between the fetal membranes and the maternal placenta after parturition, is a frequently occurring postpartum disorder in cattle (1, 2). Our research shows that MHC class I compatibility between fetus and dam gives a high risk of RFM in the dam (**chapter 2**, **chapter 3**). Comparison of cytokine levels and leukocyte subsets in placental tissue between MHC class I compatible and incompatible pregnancies approximately 24 hours before parturition showed that MHC class I compatibility had a direct influence on the maternal immune response at the placenta (3). These results indicate that around parturition allogeneic MHC class I expressed on fetal trophoblasts elicits a materno-fetal alloimmune response that aids in the "loss" of fetal-maternal adherence. Conversely, the absence (or reduction) of materno-fetal alloimmunity in MHC class I compatible pregnancies leads to RFM. In the following section we discuss how allorecognition of fetal MHC class I and the ensuing maternal immune response affects fetal-maternal adherence. Next, we hypothesize what prompts the alloimmune assisted separation of the fetal membranes at the end of gestation and finally we discuss methods to reduce MHC class I compatibility between dam and calf.

Immune assisted loss of fetal maternal adherence

First, we questioned which mechanism of allorecognition is (most) important for the fetal MHC class I driven materno-fetal alloimmune response? The maternal and fetal epithelia are largely intact following separation of the fetal membranes (2, 4, 5), indicating that the loss of fetal adherence is not a destructive process. Therefore, direct allorecognition of fetal MHC class I on trophoblasts by cytotoxic CD8 T cells

and subsequent killing is not a likely route of materno-fetal alloimmune assisted separation of the fetal membranes. CD8 positive T cells are present in the placenta during pregnancy (3, 6), but not in great numbers. Davies et al. (3) detected a drop in CD8 T cells around parturition in MHC class I incompatible pregnancies, but not in compatible pregnancies. The mechanism causing the drop in CD8 T cell numbers is not known, but appears to be related to the recognition of fetal MHC class I and could potentially be caused by non-classical MHC class I induced FAS receptor mediated apoptosis of activated CD8 T cells in incompatible pregnancies (7). Expression of MHC class I on trophoblasts of first trimester somatic-cell nuclear transfer (SCNT) cloned bovine fetuses can lead to immune mediated abortion (8). Characterization of lymphocyte populations in the placenta of SCNT pregnancies revealed that CD4 T cells were the dominant population (3), indicating indirect presentation of alloantigens via self MHC class II and activation of CD4 T cells is the most likely route of immune mediated abortion in SCNT pregnancies. Similarly, recognition of fetal MHC class I around parturition most likely involves the indirect pathway of allorecognition. Maternal macrophages residing in the placentomal endometrium are MHC class II positive (9) and MHC class I expression is upregulated on placentomal (binucleate) trophoblasts towards parturition (10, 11). Apoptosis of trophoblasts and subsequent phagocytosis by macrophages allows the presentation of fetal MHC class I via maternal MHC class II. The production of fetal alloantigen specific IgG antibodies, which can be detected during pregnancy (12), depends on self MHC II restricted CD4 T cell help and shows that the indirect pathway of allorecognition indeed occurs during pregnancy.

The next question we addressed was how does the maternal alloimmune response facilitate the loss of fetal-maternal adherence? Breakdown of the extracellular matrix (ECM) linking the fetal and maternal epithelium is thought to be very important in the separation of the fetal membranes (13). Indeed many genes associated with the degradation of the ECM are upregulated around parturition (14) and disruption of the ECM by the infusion of collagenase into the placenta led to a marked reduction in retention time of fetal membranes in experimentally induced RFM (15, 16). Macrophages are potent producers of many cytokines and play an important role in breakdown and remodeling of the ECM (17, 18). Miyoshi et al. (19) found that a reduced function of uterine macrophages was associated with the occurrence of RFM. Oliveira and Hansen showed that during pregnancy large numbers of maternal macrophages accumulate in the uterus (9, 20) and that at least part of these macrophages have a phenotype that supports immune regulation and tissue homeostasis (21). However, under influence of the materno-fetal alloimmune response uterine macrophages may assume a more inflammatory phenotype towards parturition (17, 21) that aids in breakdown of the ECM. In human placental macrophages a shift from an immune regulatory towards an inflammatory phenotype at parturition is believed to aid in degradation of the ECM (22). Comparing MHC class I incompatible and compatible pregnancies, Davies et al.

(3) found higher numbers of maternal macrophages in incompatible than in compatible pregnancies. Furthermore, in incompatible pregnancies higher amounts of IL-2 were detected and macrophages stained less intense for TNF-α, which likely reflects the release of TNF-α (3). These results imply that maternal recognition of fetal MHC class I activates macrophages and induces cytokine production, which, through direct and indirect effects of macrophages, can lead to breakdown of the ECM and to loss of fetal maternal adherence.

Placental maturation is characterized by increased apoptosis of trophoblasts and maternal endothelium (23) and is one of the processes believed to be involved in the loss of fetal maternal adherence (2, 4, 23). Uterine macrophages produce TNF-α (3), which can induce apoptosis in cells (24) and as such may influence placental maturation. Matrix metalloproteinases (MMP) are enzymes capable of breaking down the ECM. MMP-2, MMP-9 and MMP-14 have been detected in the bovine placenta (25-27) and are upregulated before parturition (14). MMP's can be activated by many (inflammatory) cytokines (28, 29), including TNF-α. Maj and Kankoffer (26) found lower MMP-2 and MMP-9 enzyme activity in animals with spontaneous RFM, but after induced parturition Walter and Boos (27) and Dilly and colleagues (25) found no differences in MMP-2, MMP-9 and MMP-14 between non-RFM and RFM cows. However, the activity of MMP's is inhibited by tissue inhibitors of MMP's (TIMP) and both studies found the presence of TIMP-2 in the bovine placenta is restricted to binucleate trophoblast cells (BNC) (25, 27). In normal pregnancies there is a steep drop in BNC before parturition, while in RFM BNC numbers remain high (5, 30). The drop in BNC numbers before parturition may increase the activity of MMP's in the placenta trough the withdrawal of TIMP-2. Interestingly, around parturition BNC numbers were lower in MHC class I incompatible than in compatible pregnancies (3), indicating allorecognition of fetal MHC class I is directly related to the drop in BNC normally seen before parturition. Neutrophils also have the ability to remodel or break down the ECM (18, 31). In humans, IL-8 stimulates the release of MMP-9 from neutrophils. IL-8 is an important chemotactic factor for neutrophils in a term cotyledons (32) and normally, the expression of IL-8 in placentomes is upregulated around parturition (14). IL-8 serum levels around parturition were lower in RFM than in non-RFM dams. Furthermore, the chemotaxis towards cotyledons of neutrophils obtained from dams that develop RFM is lower than from dams that release the fetal membranes normally (32). Although the number of neutrophils in the placenta during parturition does not appear to be high (19), these data imply that neutrophils do play a role in the loss of fetal maternal adherence.

Together these data indicate that innate immune effector mechanisms are most important for the loss of fetal maternal adherence. Indeed, in cattle numerous genes associated with innate immunity are upregulated around parturition (14) and in humans inflammation and innate immune cells are believed to play a pivotal role in parturition as

well (33). In spite of this, the high risk of RFM in MHC class I compatible pregnancies (**chapter 2**, **chapter 3**, (34)) and the direct effect of MHC class I compatibility on the maternal immune response in the uterus (3) shows that an adaptive immune response to fetal MHC class I is critical for separation of the fetal membranes. Whereas T cell numbers in a term placenta's are low (19), macrophage numbers are high (19, 20). Therefore, we hypothesize that around parturition CD4 T cells, activated through indirect allorecognition of fetal MHC class I, stimulate uterine macrophages to produce cytokines and create an inflammatory milieu which subsequently activates even more macrophages. As detailed above, cytokines and activated macrophages (and possibly neutrophils) aid in the loss of fetal maternal adherence. We conclude that the alloimmune response against fetal MHC class I serves as a trigger that activates innate immune effector mechanisms leading to the breakdown of fetal maternal adherence (Fig. 1).

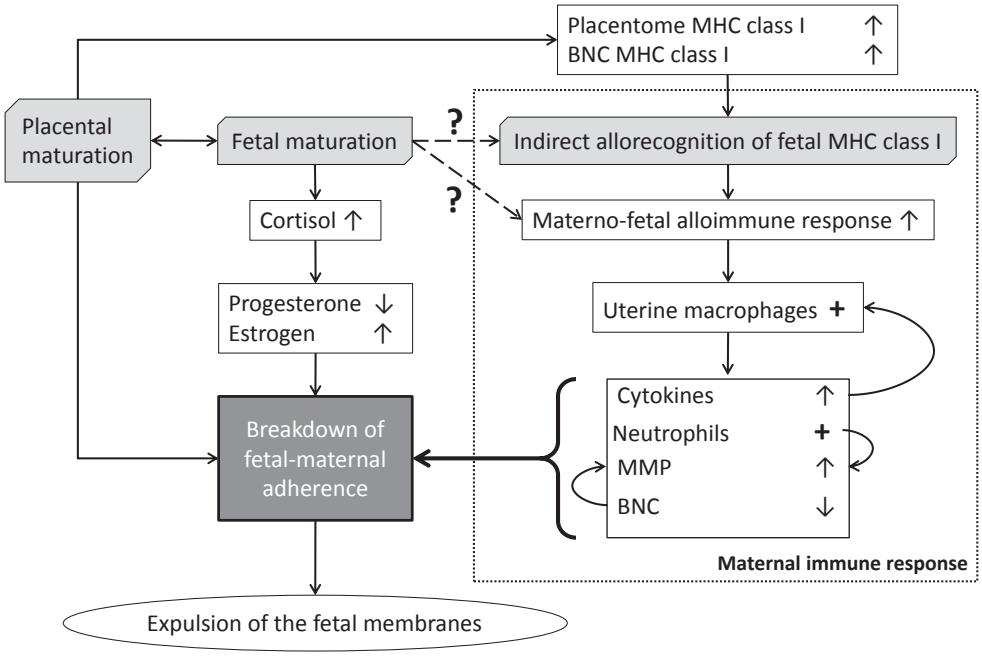

Figure 1. Processes leading to breakdown of fetal maternal adherence. ↑ Upregulation. ↓ Downregulation. + Activation.

What prompts the alloimmune assisted separation of the fetal membranes?

Fetal MHC class I is expressed on interplacentomal trophoblasts throughout the third trimester of pregnancy, while fetal maternal adherence is unaffected. Currently, it is not

known what prompts the shift from regulation of materno-fetal alloimmunity during pregnancy to a maternal immune response that aids in separation of the fetal membranes at parturition. Fetal BNC are the cells in most intimate contact with maternal tissue, since they migrate to the endometrium and fuse with maternal endometrial cells (35, 36). MHC class I expression on BNC is detected only around parturition (10, 37) and BNC numbers are affected by MHC class I compatibility between dam and calf (3). We hypothesize that allorecognition of MHC class I expressed on BNC at the end of pregnancy could be the trigger of the maternal alloimmune assisted separation of the fetal membranes. Of course many other changes occur at the end of pregnancy that could also influence the regulation of materno-fetal alloimmunity, including placental maturation (2, 4, 23), remodeling of the maternal and fetal epithelium, and the hormonal changes that occur around parturition (38, 39). In most RFM cases the calf is born normally and, therefore, separation of the fetal membranes and birth of the calf appear to be governed by (partially) separate mechanism. In cattle the mature fetus produces cortisol which triggers a cascade of hormonal changes that eventually initiates parturition (38, 39). Emulating these hormonal changes with corticosteroids (**chapter 3**), prostaglandins (40, 41) or progesterone receptor blockers (42) to induce parturition leads to successful birth of the calf, but is associated with a high rate of RFM. This shows that the hormonal changes seen around parturition alone are not sufficient for successful separation of the fetal membranes. Shenavai and colleagues (43) found that placental maturation, i.e. changes in maternal endometrium and a drop in the number of BNC, did not occur after induction of parturition. Above, we reasoned that the materno-fetal alloimmune response likely contributes to placental maturation and in **chapter 4** we showed that following induction of parturition with corticosteroids the occurrence of RFM is associated with reduced chemotactic activity of the fetal

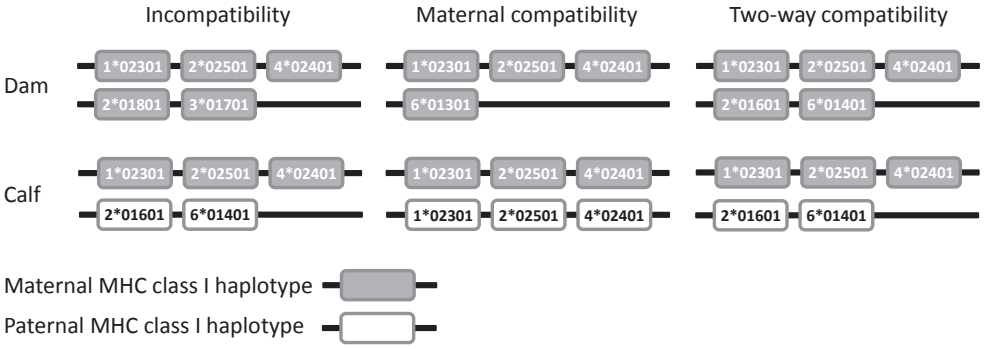

Figure 2. MHC class I compatibility. In MHC class I incompatible pregnancies the paternally inherited haplotype of the calf is not compatible to the dam, nor is the non-inherited maternal haplotype compatible to the calf. When the paternally inherited haplotype is compatible to the dam, there is maternal compatibility when the non-inherited maternal haplotype is not compatible to the calf and two-way compatibility when the non-inherited maternal haplotype is compatible.

cotyledons and, therefore, with impaired alloimmune assisted separation of the fetal membranes. We postulate that fetal maturation and the hormonal changes occurring around parturition are pivotal for the birth of the calf, whereas placental maturation and the materno-fetal alloimmune response are most important for separation of the fetal membranes (Fig. 1).

In **chapter 2** we found that the odds of RFM was much higher in two-way compatible than in maternal compatible pregnancies (fig. 2). Compatibility of the dam to the calf increased the odds of RFM and suggests that the fetal immune system also plays a role in the separation of the fetal membranes. The immune system of the calf is fully functional at the end of gestation (44) and the number of fetal macrophages in the fetal membranes rises towards the end of pregnancy (35). Spontaneous parturition in humans is associated with fetal monocyte activation (45, 46) and in mice surfactant protein-A production in the lungs of the fetus at the end of pregnancy is believed to activate macrophages, which migrate towards the maternal side of the placenta where they produce inflammatory cytokines that initiate parturition (47, 48). Therefore, we hypothesize that cytokines produced as a result of a fetal immune response against maternal alloantigens may contribute to the activation of the maternal innate immune response that leads to the breakdown of fetal maternal adherence.

Reducing MHC class I compatibility as a preventive measure for RFM

Currently, there is no effective treatment for RFM (13) and many of the identified risk factors for the occurrence of RFM are difficult to prevent. However, reducing MHC class I compatibility between dam and calf through controlled breeding may be a feasible measure to prevent RFM. The chance of MHC class I compatibility between dam and calf increases if dam and calf have a higher coefficient of relationship (CR). Indeed, results from **chapter 4** indicate that there might be a positive association between the CR between dam and calf and the occurrence of RFM. A similar association has been found in Frisian horses (49). However, the effects of CR on the occurrence of RFM are small and current breeding practice already minimizes the CR between dam and calf. Hence, reducing the CR between dam and calf will only have a minimal effect on the incidence of RFM. Although MHC class I haplotypes in a population are diverse, there are usually a handful of common haplotypes occurring at a high frequency (50, 51). Therefore, MHC class I compatibility occurring through chance is relatively high and higher than compatibility occurring through common ancestry. Selective breeding of MHC class I typed dams and sires would avert the occurrence of MHC class I compatible pregnancies. Since following normal parturition approximately half of the RFM cases are associated with MHC class I compatibility (**Chapter 2,** (34)), such an approach would be expected to substantially reduce the incidence of RFM. In this thesis we describe two new methods to type bovine MHC class I (**Chapter 2, Chapter 5**), but both methods are currently too expensive and labor intense for use in general

breeding practice. However, considering that sequencing costs are dropping rapidly, a sequencing approach may be cost effective in the near future. Moreover, since materno-fetal alloimmunity is hypothesized to have beneficial effects on the implantation of the embryo and on placentation (52, 53), increased pregnancy rates may be an additional advantage of reduction of MHC class I compatibility between dam and calf. In macaques it has recently been shown that pregnancy rates are higher in MHC incompatible than in compatible pregnancies (54). Results from Aguilar and colleagues (55) indicated that, following embryo transfer in cattle, pregnancy rates may be higher in fully MHC incompatible than in fully compatible pregnancies, but further research is necessary to establish whether MHC compatibility has an effect on pregnancy rates in natural pregnancies. In summary, MHC class I incompatibility is important for normal separation of the fetal membranes and may be associated with increased pregnancy rates. Hence, reducing MHC class I compatibility between dam and calf, through selective breeding of MHC class I typed animals, may reduce the incidence of RFM and increase pregnancy rates in cattle.

Bovine neonatal pancytopenia: Iatrogenic boosting of materno-fetal alloimmunity

Development of BNP in the calf depends on the alloantibody dose absorbed from colostrum

Bovine Neonatal Pancytopenia (BNP), a bleeding syndrome of neonatal calves, is caused by alloantibodies absorbed from the colostrum of particular cows. Pregsure© BVD is the likely source of alloantigens inducing BNP-associated alloantibodies in the dam. Despite the widespread use of Pregsure© BVD the number of BNP cases has been relatively low, with the incidence of BNP within calves born from Pregsure© BVD vaccinated dams estimated to be lower than 0.3% (56-58). Following the discovery that BNP associated alloantibodies bind MHC class I (**Chapter 6**, (57, 59)), it was hypothesized that differences in MHC class I between dam, calf and vaccine determined whether BNP would occur in the calf (57, 59). We explored whether genetic differences, including those in MHC class I, between BNP and non-BNP calves and Pregsure© BVD vaccinated BNP and non-BNP dams were associated with the occurrence of BNP (**Chapter 5**). The heritability estimate for the occurrence of BNP in a calf born from a Pregsure© BVD vaccinated dam as a calf trait was found to be zero, indicating that genetic variation in the paternally inherited genes is not associated with the occurrence of BNP in the calf. In accordance with this result, our MHC class I typing results revealed that BNP calves did not have a single paternal MHC class I haplotype in common and showed overlap in paternal MHC class I haplotypes between BNP and non-BNP calves. In addition, induction of BNP in randomly selected calves

by feeding colostrum from BNP dams (60-62) also indicates that the development of BNP does not depend on the genetic background of the calf. On the other hand, the heritability estimate of BNP as a dam trait was 19%, indicating that genetic differences between Pregsure© BVD vaccinated dams explain in part the development of BNP in the calf. This finding was supported by the study of Demasius (63) who showed that in an experimental herd with a limited number of maternal sire lines all BNP cases were restricted to a single maternal grandsire. Nevertheless, MHC class I typing of dams revealed no association between the MHC class I haplotype of the dam and the occurrence of BNP. A prerequisite for the induction of a humoral immune response against alloantigens present in the Pregsure© BVD vaccine is presentation of these antigens in the context of MHC class II and we hypothesized that the MHC class II haplotype of the dam may determine whether a dam can mount an alloantibody response. However, typing of MHC class II did not reveal differences between BNP and non-BNP dams. Moreover, we found that both non-BNP and BNP dams produced alloantibodies, which shows that non-BNP dams, as well as BNP dams, are allogeneically different from the MDBK cell line and present MDBK derived alloantigens in the context of MHC class II, thus corroborating both the MHC class I and the MHC class II typing results. We compared the alloimmune responses in both groups in more detail and found no differences in alloantibody isotype, nor in the binding to leukocytes isolated from randomly selected animals. In addition, both BNP and non-BNP dams target MHC class I and the proportion of MHC class I specific alloantibodies were similar (**Chapter 6**). Hence, qualitatively the alloantibody responses in BNP and non-BNP dams appear to be similar. The only striking difference we observed was that BNP dams had significantly higher alloantibody levels than non-BNP (**Chapter 5**, (56)). In addition, higher BVD specific neutralizing antibody levels in Pregsure© BVD vaccinated BNP dams (56) indicated that BNP dams responded with higher antibody levels following vaccination in general. Antibody levels following vaccination have been shown to be a heritable trait (64, 65) and, therefore, we hypothesized that the genetic differences between BNP and non-BNP dams are in genes controlling the (allo)antibody response following Pregsure© BVD vaccination. Since there are many genes associated with the regulation of the antibody response and there are no obvious candidate genes based on current literature, a generalized approach, e.g. whole genome sequencing, would be the most feasible to identify genes in the dam that are associated with the occurrence of BNP in the calf.

The alloantibody responses in BNP and non-BNP dams appear to be qualitatively similar, but BNP dams have significantly higher alloantibody levels. Hence, we hypothesized that the development of BNP in the calf depends on the alloantibody dose the calf absorbs (Fig. 3). In support of this hypothesis are the observations that the odds of BNP rose with increased colostrum intake (66) (i.e increased alloantibody intake) and with increased numbers of Pregsure© BVD vaccinations (58, 66) (i.e.

increased numbers of alloantibody boosts). If the development of BNP depends on alloantibody dose, one would expect that there is not a strict division between BNP affected and unaffected calves. Indeed, calves with subclinical signs of BNP have been observed both in Pregsure© BVD vaccinated herds (67-69) and following experimental reproduction of the disease (61, 62). These calves showed no signs of bleeding, but had one or more of the other symptoms typically seen in BNP (e.g. leukopenia, thrombocytopenia or bone marrow hypoplasia).

Our hypothesis that the development of BNP in the calf depends on the alloantibody dose absorbed by the calf raises the question whether the MHC class I/alloantigen background of dam and calf is irrelevant for the pathophysiology of BNP? We previously concluded that the allogeneic background of dam and calf does not play an important role in the development of BNP. Nevertheless, BNP alloantibodies do not bind autologous cells (**Chapter 6**, (56)), showing that self-tolerance is not broken and that there is a "hole" in the MHC class I/alloantigen specificity of BNP associated alloantibodies. Furthermore, results from **chapter 5** and **chapter 6** indicate that alloantibody binding of leukocytes is not equally strong to all leukocytes. Hence, it cannot be excluded that in some cases calves did not develop BNP despite absorbing a high alloantibody dose, due to alloantibodies that were not reactive with alloantigens of the calf. Notwithstanding, such cases are probably rare and we believe alloantibody dose is the most important determinant of the occurrence of BNP in the calf. Feeding calves

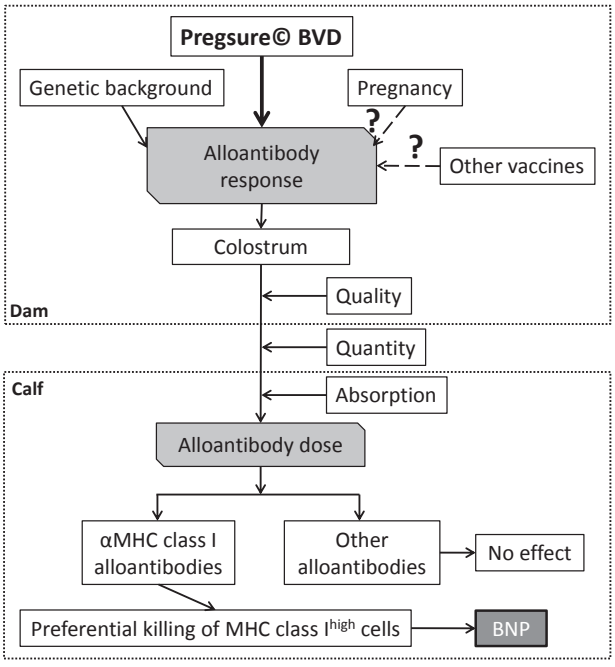

Figure 3. Schematic representation of the pathogenesis of BNP in dam and calf.

colostrum from BNP dams did not always induce BNP and it has been hypothesized that this was due to alloantibodies not recognizing the calf's alloantigens (61, 70). However, in these studies it was not tested whether the colostrum contained alloantibodies, the calf absorbed sufficient amounts of alloantibodies and the alloantibodies did not bind cells from the calf in vitro. Therefore, BNP may also not have developed due to an insufficient alloantibody dose, rather than due to the alloantigen background of the calf.

Despite the heterogeneous specificity of BNP alloantibodies, pathogenicity correlates with MHC class I expression

The MHC class I specificity of BNP alloantibodies has clearly been established (57, 59, 71). However, since MHC class I is expressed on all nucleated cells and BNP is characterized by the loss of specific cell types (i.e. leukocytes, thrombocytes, bone marrow) (68, 72-74), the questions arose how MHC class I specific alloantibodies cause the pathology seen in BNP and whether potential additional targets may better explain the pathology of BNP? We constructed a beta-2-microglobulin knockout MDBK cell line, which abolished the expression of MHC class I, and used this cell line to show that MHC class I was the dominant target of BNP alloantibodies and, depending on the dam, accounted for 40-90% of the alloantibody specificity (**Chapter 6**). This also revealed that not all BNP antibodies were MHC class I specific. Immunoprecipitation of antigens from the beta-2-microglobulin knockout MDBK cells using BNP alloantibodies showed that alloantibodies from roughly half of the BNP dams additionally bound Very Late Antigen-3 (VLA-3). VLA-3 is a heterodimer consisting of integrin chains β1 and α3 that has been shown to be expressed on endo- and epithelium (75, 76). Nevertheless, BNP alloantibodies only marginally bound endothelial cells (**Chapter 6**) and endo- and epithelial cells are not affected in BNP calves (68, 72-74). In contrast to the integrin α3 chain, which is only expressed as part of VLA-3, the integrin β1 chain may dimerise with many other integrin chains and is expressed on a plethora of cell types (77). Comparison of BNP alloantibody binding to the expression of MHC class I and integrin β1 on several different cell types showed that BNP alloantibody binding correlated with MHC class I expression, whereas it did not correlate with integrin β1 expression (**Chapter 6**). Moreover, although both MHC class I and integrin β1 are expressed on leukocytes, using BNP alloantibodies we could only precipitate MHC class I from leukocytes. Together these data indicate BNP alloantibodies do not bind VLA-3 or integrin β1 on bovine cells other than MDBK cells. Furthermore, the alloantibodies from only half of the tested BNP dams bound VLA-3, whereas the pathology of BNP is similar in all affected calves (68, 72, 73). Therefore, we conclude that it is unlikely that integrin α3/β1 specific alloantibodies play a role in the pathophysiology of BNP. Previous studies examining the specificity of BNP alloantibodies using immunoprecipitation did not identify VLA-3 as a target antigen (57, 59), which can be explained by the different methods applied in these studies. Deutskens et al. (59) examined precipitated proteins

of molecular weight lower than integrins β1 and α3 and Foucras et al. (57) precipitated antigens from leukocytes, from which we also did not precipitate integrin β1/VLA-3 . The alloantibody response following Pregsure© BVD vaccination is polyclonal and it is not known whether the epitopes recognized by BNP alloantibodies reside on integrin β1, α3 or on the VLA-3 heterodimer. Knowing the exact specificity of these alloantibodies will not change our conclusion, since the expression of none of these proteins correlates with BNP alloantibody binding or with the pathology of BNP in the calf.

BNP alloantibody binding correlated with MHC class I expression (**Chapter 6**) and a recent study demonstrated that binding of BNP alloantibodies can induce complement mediated lysis of MDBK cells (71). Utilizing several MDBK cell lines with different levels of MHC class I expression, we showed that BNP alloantibody dependent complement mediated cell lysis correlated with BNP alloantibody binding and MHC class I expression (**Chapter 6**). Complement mediated lysis of peripheral leukocytes revealed that PBMC, which have high MHC class I expression and as a consequence bind high numbers of BNP antibodies, were killed, whereas granulocytes, which have low MHC class I expression and bind low numbers of BNP antibodies, were not affected. We hypothesized that the pathogenic effects of BNP alloantibodies correlate with alloantibody binding and that only cells with high MHC class I expression are affected in BNP. To further explore this hypothesis we characterized BNP alloantibody binding and MHC class I expression on several cell types that are differentially affected in BNP (**Chapter 6**). Endothelial cells come into close contact with BNP alloantibodies, but are not affected in BNP calves. In line with these observations we found BNP alloantibody binding and MHC class I expression on endothelial cells was low. Although the bone marrow of BNP calves is often characterized as aplastic (60, 68, 74), many authors reported that not all lineages were affected equally (57, 61, 68, 70). Cellular depletion was strongest in megakaryocytes and precursor cells of all lineages (61, 68, 70), whereas Bell and colleagues (70) reported that granulocytes were still present. In line with the differential depletion of disparate bone marrow cells, we found differential BNP alloantibody binding and MHC class I expression, with low MHC class I expression and BNP alloantibody binding of granular cells and high MHC class I expression on megakaryocytes. Together these findings corroborate our hypothesis that cells with high MHC class I expression are preferentially affected in BNP. Despite the low binding of BNP alloantibodies to granulocytes, Foucras and colleagues (57) reported a marked drop in granulocyte numbers following injection of calves with BNP alloantibodies. However, Bell et all (70) showed granulocyte numbers drop both in calves fed colostrum from BNP dams as well as calves fed control colostrum and found no significant differences in granulocyte numbers between both groups from 12 hours onward. Moreover, they reported that following the ingestion of colostrum from BNP dams cellular depletion of the bone marrow progressed with time, indicating that the bone

marrow hypoplasia seen in BNP calves may be an end stage caused by a combination of depletion and exhaustion that is preceded by gradual depletion of specific (high MHC class I expressing) cell lines. Therefore, the granulocytopenia typically seen in BNP calves (68, 74) may be a consequence of the bone marrow hypoplasia and the short life span of granulocytes, rather than due to direct effects of BNP alloantibodies on granulocytes.

In conclusion, although BNP alloantibodies have heterogeneous specificities, pathogenicity appears to be mediated by MHC class I specific BNP alloantibodies alone. Furthermore, our results indicate that the pathogenic effects of BNP alloantibodies correlate with alloantibody binding and that cells with high MHC class I expression were preferentially affected in BNP. (Fig. 3)

The risk of using allogeneic cell lines in vaccine production

Bovine MHC class I proteins were detected in the Pregsure© BVD vaccine (57, 59) and experimental immunization of calves and guinea pigs confirmed that vaccination with Pregsure© BVD induces alloantibodies that recognize bovine leukocytes and MDBK cells, the cell line used for vaccine production (56, 58). From these findings follows the prevailing hypothesis that alloantigens from the production cell line present in the Pregsure© BVD vaccine induced the alloantibodies associated with the occurrence of BNP. The occurrence of BNP brings the use of allogeneic (same species) cell lines for vaccine production into discussion. More specifically the question is raised whether the induction of alloantibodies was a complication specific for the Pregsure© BVD vaccine or whether it is a general risk when using allogeneic cell lines in vaccine production? Viruses often have a restricted host tropism and only infect and replicate in cells from the host species and the use of allogeneic cell lines in vaccine production is, therefore, quite common. BNP symptoms have been reported in calves without a Pregsure© BVD history (70, 78-80). In these cases the dam of the calf was not vaccinated with Pregsure© BVD, nor did the calf receive colostrum from a Pregsure© BVD vaccinated dam. Jones et al. (66) even reported that in 5% of the BNP cases there was no link with the Pregsure© BVD vaccine. These cases could be related to alloantibodies naturally occurring as a result of pregnancy or to the use of other viral vaccines grown on bovine cell lines (Fig. 3). Fetal MHC class I is expressed on the trophoblast in the third trimester of pregnancy (11, 81) and in up to 64% of multiparous dams alloantibodies against fetal alloantigens can be detected in serum (12, 82). However, in colostrum the levels of these naturally occurring alloantibodies are very low (82) and it is questionable if these levels are high enough to induce pathology in the calf. Another explanation for BNP cases that cannot be linked to the use of Pregsure© BVD could be the use of other vaccines grown on allogeneic cell lines. Alloimmune mediated hemolytic anemia of newborn calves has been reported following ingestion of colostrum from dams that were vaccinated with an anaplasmosis vaccine contaminated with erythrocytes (83,

84). However, to our knowledge there are no reports of alloimmune related adverse effects of any other veterinary or human vaccines currently in use that are produced using allogeneic cell lines and clear alloantibody levels could not be detected in dams vaccinated with other BVD vaccines (56, 58, 59). Nevertheless, following vaccination of Guinea Pigs with an alternative BVD vaccine Bastian et al. (56) detected low levels of antibodies that bound bovine leukocytes and in some cases Deutskens et al. (59) precipitated antigens from MDBK cells using sera from dams vaccinated with a different BVD vaccine, albeit in much lower quantities than when using serum from Pregsure© BVD vaccinated dams; indicating that bovine proteins were present in these vaccines and, therefore, that these vaccines could potentially induce alloantibodies. The difference between Pregsure© BVD and other BVD vaccines may be the potent adjuvant that induced BVD antibody titers significantly higher than alternative vaccines (56, 85) and may also have favored the induction of high alloantibody levels.

In conclusion, BNP appears to be a problem associated with the use of Pregsure© BVD rather than the use of vaccines produced on allogeneic cell lines in general; bovine cell debris present in Pregsure© BVD in combination with a very potent adjuvant induced high alloantibody levels in a sub group of genetically predisposed high responder cows. Since all vaccines produced on allogeneic cell lines may potentially induce alloantibodies, we propose that all such vaccines should be carefully monitored for the induction of alloimmune related adverse effects. The adverse effects of Pregsure© BVD vaccination manifested itself in the calf and the incidence of BNP was low, showing that it may be difficult to expose alloimmune related adverse effects during vaccine trials and with current pharmacovigilance. However, we detected alloantibodies in almost all Pregsure© BVD vaccinated animals and monitoring animals for the induction of alloantibodies against the cell line used for vaccine production may be a feasible approach to monitor for vaccine induced alloimmunity. Alternatively, the induction of alloimmunity could be minimized through removal of alloantigens from the vaccine or the use of xenogeneic cell lines.

In conclusion

This thesis explored the role of materno-fetal alloimmunity in bovine immune mediated disorders, with an emphasis on the effect of MHC class I (in)compatibility between dam and calf. The maternal immune response against fetal antigens is regulated, rather than suppressed, and is normally beneficial, not detrimental to pregnancy or to the successful transfer of passive immunity to the calf. However, reduced or increased materno-fetal alloimmunity can have adverse effects.

Our research showed that two-way MHC class I compatibility between dam and calf gave a high risk of retention of the fetal membranes in the dam. Allorecognition

of fetal MHC class I and the ensuing immune response aids in the separation of the fetal membranes and this is impaired in MHC class I compatible pregnancies. Ensuring MHC class I incompatibility between dam and calf through controlled breeding can prevent RFM caused by MHC class I compatibility.

The data presented in this thesis show that the development of BNP in the calf depends on the alloantibody dose absorbed from colostrum and, although BNP alloantibodies have heterogeneous specificity, that MHC class I specific alloantibodies mediate pathogenicity. The occurrence of BNP in the calf is specifically linked to the use of Pregsure© BVD in the dam. Nevertheless, BNP cases without a Pregsure© BVD history have been described and there is some evidence that other vaccines contain alloantigens as well. We therefore propose to monitor vaccines produced on allogeneic cell lines for the induction of alloantibodies.

The fetus is semiallogeneic to the maternal immune system and regulation of the materno-fetal alloimmune response is essential for successful pregnancy and to avoid the transfer of pathogenic maternal alloantibodies.

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Samenvatting

Materno-foetaal alloimmuun gemedieerde aandoeningen in het rund

MHC klasse I (in)compatibiliteit in Retentio Secundinarum en Boviene Neonatale Pancytopenie

De foetus erft alloantigenen van zijn vader en brengt deze tot expressie, waardoor de foetus lichaamsvreemd is voor het immuunsysteem van de moeder. Het voorkomen van immunologisch afstoting van de foetus door de moeder is essentieel voor een succesvolle zwangerschap. Na de geboorte staat het maternale immuunsysteem in contact met de foetus door de overdracht van maternale antilichamen naar het pasgeboren dier via de biest. Regulering van de materno-foetale alloimmuniteit is cruciaal voor een succesvolle dracht en om de overdracht van pathogene maternale antilichamen te voorkomen. In dit proefschrift wordt er gekeken naar schadelijke effecten van materno-foetale alloimmuniteit in koeien op dracht en op het pasgeboren kalf, met een nadruk op de rol van MHC klasse I (in)compatibiliteit tussen koe en kalf op materno-foetale alloimmuniteit. Twee aandoeningen, Retentio Secundinarum en Boviene Neonatale Pancytopenie, respectievelijk geassocieerd met hypo- en hyper-materno-foetale alloimmuniteit, zijn bestudeerd.

Gedurende de dracht is de semi-allogene foetus in intiem contact met de moeder zonder dat het afgestoten wordt door het maternale immuunsysteem (**Hoofdstuk 1**). Het maternale immuunsysteem moet strak gereguleerd worden om de foetus te tolereren, terwijl het ook moet kunnen reageren tegen (intra-uteriene) infecties tijdens de dracht. Er zijn drie basis mechanismen die de acceptatie van de foetus verzekeren: i) anatomische scheiding tussen moeder en foetus, ii) downregulatie van alloantigen expressie door de foetus en iii) regulatie van de maternale immuunrespons. Bij het rund vormen de foetale trofoblast en het maternale endometrium een continue dubbele epitheliale belijning over de gehele placenta, wat er voor zorgt dat er minimaal contact is tussen het maternale immuunsysteem en foetaal weefsel. Major Histocompatibility Complex (MHC) klasse I eiwitten zijn alloantigenen bij uitstek. Ze spelen een centrale rol in de adaptieve immuunrespons door het presenteren van intracellulaire peptiden aan T-cellen. Er is een grote variatie in MHC klasse I eiwitten; een koe brengt 2-6 verschillende MHC klasse I allelen tot expressie en tussen dieren is er een grote verscheidenheid aan MHC klasse I allelen. Daarnaast komt MHC klasse I tot expressie op bijna alle cellen en is er door de MHC restrictie van T cellen een hoge frequentie van MHC specifieke T cellen. Dit alles maakt MHC klasse I eiwitten tot zeer potente alloantigenen. In het begin van de dracht komen er geen MHC klasse I eiwitten tot expressie op de trofoblasten, wat bijdraagt aan de immunologische acceptatie van de foetus. Naarmate de dracht vordert stijgt de MHC klasse I expressie op trofoblasten en op het einde van de dracht komt MHC klasse I ook tot expressie op binucleaire trofoblasten (BNC). Voor runderen is het onbekend hoe het maternale immuunsysteem in de placenta exact gereguleerd wordt. Niet-klassiek MHC klasse I komt tot expressie op trofoblasten en kan bijdragen aan onderdrukking van het immuunsysteem en de inductie van regulatoire T cellen. Verschillende T cel populaties met een regulatoir fenotype zijn aangetoond in de placenta van het rund. Daarnaast worden er systemisch en op de maternale-foetale interface verscheidene factoren

uitgescheiden die bijdragen aan de modulatie van het maternale immuunsysteem (o.a. uteriene serpines, progesteron). Na de dracht is er indirect contact tussen het maternale immuunsysteem en het kalf via de overdracht van passieve immuniteit. Een kalf wordt geboren zonder noemenswaardige hoeveelheden antilichamen en is afhankelijk van de absorptie van maternale antilichamen uit biest voor immunologische bescherming in de eerste levensdagen.

Antilichamen tegen paternale alloantigenen worden in de meeste drachtige koeien geïnduceerd en dit laat zien dat het maternale immuunsysteem inderdaad gereguleerd wordt en niet onderdrukt. Daarnaast blijkt hieruit dat materno-foetale alloimmuniteit normaliter niet schadelijk is voor het kalf, zowel tijdens de dracht als gedurende de overdracht van de passieve immuniteit. Echter, verstoringen in de normale materno-foetale alloimmuun respons kunnen schadelijke effecten hebben op de dracht en het neonatale kalf. Twee aandoeningen waarbij materno-foetale alloimmuniteit een rol speelt zijn Retentio Secundinarum en Boviene Neonatale Pancytopenie.

Retentio Secundinarum

Normaal worden de foetale membranen (de nageboorte) binnen 6 uur na de geboorte van het kalf uitgescheiden. Retentio Secundinarum (RetSec, aan de nageboorte blijven staan) is het vertraagd afkomen van de foetale membranen. De incidentie van RetSec bij Nederlands melkkoeien ligt rond de 5%. RetSec is geassocieerd met een verminderde melkgift, verminderde fertiliteit en een verhoogd risico op (endo)metritis. Een maternale alloimmuunrespons gericht tegen alloantigenen die tot expressie komen op de foetale membranen helpt waarschijnlijk in het afbreken van de verbinding tussen de foetale membranen en de uterus, wat essentieel is voor het uitscheiden van de foetale membranen. Het ontstaan van RetSec is geassocieerd met een verminderde (allo) immuunrespons van de moeder.

In **hoofdstuk 2** wordt het effect van MHC klasse I compatibiliteit tussen kalf, moeder en grootmoeder op het ontstaan van RetSec bestudeerd. MHC klasse I dat tot expressie komt op de foetale membranen induceert hoogstwaarschijnlijk de maternale immuunrespons die bijdraagt aan de expulsie van de foetale membranen. Als het MHC klasse I van het kalf compatibel (gelijk) is aan dat van de moeder wordt het kalf niet als lichaamsvreemd herkend en wordt er geen (/een verminderde) maternale alloimmuunrespons geïnduceerd, wat vervolgens kan leiden tot het ontstaan van RetSec. Tijdens de dracht kan er in de foetus tolerantie geïnduceerd worden tegen alloantigenen van de moeder. Om te kijken of dit effect een rol speelt in MHC klasse I compatibiliteit als oorzaak van RetSec zijn de grootmoederdieren ook meegenomen in dit onderzoek. Om de associatie tussen RetSec en verschillende vormen van MHC klasse I compatibiliteit te bestuderen, zijn de MHC klasse I haplotypes van kalf, koe en grootmoeder combinaties van moeders met en zonder RetSec getypeerd met behulp van nieuwe generatie sequence technieken. Het MHC klasse I haplotype van de grootmoeder

had geen effect op het optreden van RetSec. Er was een significant hoger risico op het optreden van RetSec als zowel het kalf MHC klasse I compatibel was aan de moeder en het MHC klasse I van de moeder compatibel aan het kalf, terwijl er een tendens voor een hoger risico op het optreden van RetSec was in het geval van alleen MHC klasse I compatibiliteit van het kalf tot de moeder. Er wordt geconcludeerd dat MHC klasse I compatibiliteit van het kalf tot de moeder en van de moeder tot het kalf er toe leidt dat het maternale immuunsysteem geen foetaal MHC klasse I herkent, waardoor er geen materno-foetale alloimmuunrespons geïnduceerd wordt, en dat daarnaast de foetus geen alloimmuun respons heeft tegen maternaal MHC klasse I, welke het maternale immuunsysteem zou kunnen activeren. Samen leidt dit tot het falen van de immuun geassisteerde afbraak van de verbinding tussen de foetale membranen en de uterus en zodoende tot RetSec.

De kans op MHC klasse I compatibiliteit tussen koe en kalf neemt toe als koe en kalf gemeenschappelijke voorouders hebben; i.e. als ze een hogere relatie coëfficiënt hebben. De relatie coëfficiënt wordt genetisch bepaald, maar is niet erfelijke, en daarom zijn erfelijke en niet erfelijk genetische effecten op RetSec bestudeerd (**Hoofstuk 3**). De gemiddelde incidentie van Ret. Sec in 43.661 kalvingen van Maas-Rijn-Ijssel koeien was 4.5%, en varieerde van 0% tot 29.6% tussen halfzus groepen. De gemiddelde relatie coëfficiënt tussen stier en de grootvader van de koe was 0,05 en varieerde van 0 tot 1,04. Met behulp van een stier-koe grootvader model werd de erfelijkheid van RetSec op 0,22 (SEM = 0.07) geschat. De relatie coëfficiënt tussen stier en de grootvader van de koe, gebruikt als benadering voor de relatie coëfficiënt tussen koe en kalf, had een effect op de incidentie van RetSec. Deze studie laat zien dat genetische selectie tegen RetSec mogelijk is en geeft aan dat het voorkomen van paren van verwante ouderdieren een rol kan spelen in het voorkomen van RetSec bij koeien.

Het inleiden van de partus in runderen met corticosteroïden wordt gebruikt voor onderzoek, bij zieke of verwonde dieren en als managementtool. Een groot nadeel van het inleiden van de partus is de hoge incidentie van RetSec. Wij stelden als hypothese dat RetSec na het inleiden van de partus ontstaat door verminderde materno-foetale alloimmuniteit. Om deze hypothese te testen hebben wij gekeken naar de chemotactische activiteit van cotyledonen voor mononucleaire leukocyten als maat voor het plaatvinden van immuun geassisteerd loslaten van de foetale membranen (**Hoofdstuk 4**). Cotyledonen werden verzameld van niet-RetSec koeien die spontaan gekalfd hadden en van dexamethason ingeleide koeien met en zonder RetSec. De studie liet zien dat de chemotactische activiteit van cotyledonen voor mononucleaire leukocyten significant lager was in cotyledonen verkregen van ingeleide koeien met RetSec in vergelijking met spontaan gekalfde niet-RetSec koeien ($P<0.001$), terwijl de chemotactische activiteit van cotyledonen verkregen van ingeleide koeien zonder RetSec niet lager was dan dat van spontaan gekalfde niet-RetSec koeien ($P=0.10$). We concludeerden dat het inleiden van de partus met dexamethason leidt tot het falen van

de materno-foetale alloimmuun respons en tot het falen van loslaten van chemotactische factoren, wat leidt tot verminderde chemotactische activiteit van de cotyledonen en tot het falen van het alloimmuun geassisteerd afbreken van de verbinding tussen de foetale membranen en de uterus.

Boviene Neonatale Pancytopenie

Vanaf 2007 werd er een toename van pasgeboren kalveren met hemorragische diathese gezien in heel Europa en deze aandoening werd Boviene Neonatale Pancytopenia (BNP) genoemd. Symptomen van BNP zijn ernstige interne en externe bloedingen, voor het eerst zichtbaar vanaf 10-20 dagen leeftijd. Het hematologisch beeld kenmerkt zich door een ernstige leukopenie en trombocytopenie. Daarnaast kan bij post-mortem onderzoek hypoplasie van alle type beenmergcellen geobserveerd worden. Serum en colostrum van moederdieren van kalveren die BNP ontwikkelden bevat alloantilichamen. Het voeren van gezonde kalveren met colostrum van deze BNP koeien reproduceert het BNP ziektebeeld in het kalf. Uit epidemiologisch onderzoek is gebleken dat er een zeer sterke associatie is tussen het optreden van BNP in kalveren en het gebruik van Pregsure© BVD in de moederdieren. Eiwitten van de runder niercellijn MDBK, gebruikt om het BVD virus op te groeien, waren aanwezig in het Pregsure© BVD vaccin en dit is de meest waarschijnlijke alloantigeen bron die de BNP geassocieerde alloantilichamen heeft geïnduceerd in de BNP moederdieren. Het is aangetoond dat BNP alloantilichamen runder MHC klasse I herkennen.

De incidentie van BNP in kalveren van Pregsure© BVD gevaccineerde koeien wordt lager geschat dan 0.3%, wat suggereert dat er andere factoren dan Pregsure© BVD vaccinatie alleen een rol spelen in de etiologie van BNP. In **hoofdstuk 5** werd de hypothese dat dat de ontwikkeling van BNP afhankelijk is van genetische verschillen binnen koeien en kalveren onderzocht. Er werd aangetoond dat de ontwikkeling van BNP in het kalf een erfelijke eigenschap is van de koe, in plaats van het kalf, met een erfelijkheid van 19%. Om op te helderen welke genen een rol spelen in de ontwikkeling van BNP zijn kandidaat genen gesequenced en zijn BNP alloantilichamen gekarakteriseerd. Alloantigenen die aanwezig zijn in het Pregsure© BVD vaccin moeten gepresenteerd worden aan het immuunsysteem via MHC klasse II, maar sequencen van DRB3 liet geen verschillen zien in MHC klasse II haplotypes tussen BNP en niet-BNP koeien. Een belangrijke specificiteit van BNP alloantilichamen is MHC klasse I, een sterk polymorf eiwit. Met behulp van een nieuwe sequence gebaseerde MHC klasse I typeringsmethode konden we geen relatie aantonen tussen BNP en de MHC klasse I haplotype verdeling binnen koeien dan wel kalveren. Alloantilichamen konden zowel aangetoond worden in Pregsure© BVD gevaccineerde BNP als niet-BNP koeien en we konden geen verschillen in alloantilichaam isotype of mate van binding aan leukocyten tussen beide groepen aantonen. Het enige verschil wat we aan konden tonen was dat de alloantilichaam niveaus significant hoger waren in BNP koeien dan in niet-BNP koeien.

We concludeerden dat de ontwikkeling van BNP in het kalf een erfelijke eigenschap van de moeder is, in plaats van het kalf, en dat de genetische verschillen tussen BNP en niet-BNP koeien waarschijnlijk geassocieerd zijn met genen die de kwantitatieve alloantilichaam respons na vaccinatie bepalen.

Het is eerder aangetoond dat BNP geassocieerde alloantilichamen MHC klasse I binden, maar het is onbekend hoe MHC klasse I specifieke antilichamen leiden tot BNP in het kalf. MHC klasse I komt tot expressie op bijna alle cellen, maar BNP kenmerkt zich door het verlies van zeer specifieke celtypes. In **hoofdstuk 6** werd de specificiteit van BNP alloantilichamen onderzocht en gekoppeld aan de pathologie van BNP. Er is aangetoond dat MHC klasse I de belangrijkste specificiteit is van BNP alloantilichamen en dat, afhankelijk van de BNP koe, 40-90% van alle alloantilichamen MHC klasse I herkenen. Very Late Antigen-3, een integrine α3/β1 heterodimeer, werd geïdentificeerd als een belangrijk aanvullende specificiteit van BNP alloantilichamen. Echter, het binden van BNP alloantilichamen aan diverse runder celtypen correleerde met MHC klasse I expressie, in plaats van integrine β1 of α3 expressie. Daarnaast correleerde BNP alloantilichaam-afhankelijke complement-gemedieerde cel lysis sterk met MHC klasse I expressie. Diverse weefsels van derde trimester runder foetussen zijn onderzocht en hieruit bleek dat celtypes die aangetast zijn in BNP gekenmerkt worden door hoge BNP alloantilichaam binding en hoge MHC klasse I expressie. Er wordt geconcludeerd dat ondanks de heterogene specificiteit van BNP geassocieerde maternale alloantilichamen, MHC klasse I specifieke antilichamen, in plaats van Very Late Antigen-3 specifieke antilichamen, de pathogeniciteit in het kalf mediëren en dat cellen met hoge MHC klasse I expressie preferentieel aangetast worden in BNP.

Conclusies

In de samenvattende discussie (**Hoofdstuk 7**) zijn de resultaten van dit proefschrift in perspectief geplaatst en bediscussieerd.

We concluderen dat upregulatie van MHC klasse I op foetale (binucleaire) trofoblasten op het einde van de dracht een materno-foetale alloimmuunrespons induceert welke cruciaal is voor het afbreken van de verbinding tussen de foetale membranen en de uterus. We hypothetiseren dat CD4 T cellen, geactiveerd door indirecte alloherkenning van foetaal MHC klasse I, uteriene macrofagen stimuleren en dat geactiveerde macrofagen een centrale rol spelen in de afbraak van de extracellulaire matrix die het foetale en maternale epitheel verbindt, zowel direct als indirect door het secreteren van cytokines en het activeren van downstream mechanismen. De afwezigheid (of reductie) van de maternale alloimmuunrespons kan leiden tot persistentie van de foetaal-maternale verbinding en zodoende tot RetSec. Er is momenteel geen effectieve behandeling voor RetSec en een beter begrip van de immuun geassisteerde afbraak van de verbinding tussen de foetale membranen en de uterus zou kunnen leiden tot aanknopingspunten voor nieuwe therapieën tegen RetSec.

De alloantilichaam respons van Pregsure© BVD gevaccineerde BNP en niet-BNP koeien lijkt kwalitatief gelijk te zijn, maar BNP koeien hebben significant hogere alloantilichaam niveaus. We concluderen dat de ontwikkeling van BNP in het kalf afhankelijk is van de alloantilichaam dosis die het kalf absorbeert uit colostrum en dat de alloantigen achtergrond van het kalf niet een belangrijke rol speelt. Cellen met hoge MHC klasse I expressie worden preferentieel aangetast in BNP en ondanks de heterogene specificiteit van BNP geassocieerde maternale alloantilichamen is het waarschijnlijk dat alleen MHC klasse I specifieke antilichamen BNP veroorzaken. Het ontstaan van BNP lijkt een aandoening die specifiek geassocieerd is met het gebruik van Pregsure© BVD, in plaats van het gebruikt van vaccins die geproduceerd zijn op allogene cellijnen in het algemeen; rundercel componenten aanwezig in Pregsure© BVD in combinatie met een zeer potent adjuvant hebben zeer hoge alloantilichaam niveaus geïnduceerd in een specifieke groep genetisch gepredisponeerde koeien.

De foetus is semiallogeen voor het maternale immuunsysteem en regulatie van de materno-foetale alloimmuunrespons is essentieel voor een succesvolle dracht en om de overdracht van pathogene maternale alloantilichamen te voorkomen.

Samenvatting voor leken

Ziektes bij koeien die geassocieerd zijn met een afweerreactie van de koe tegen het kalf

Het afweersysteem beschermt het lichaam tegen ziekteverwekkers. Ziekteverwekkers zijn anders dan de eigen cellen, ze zijn 'lichaamsvreemd'. Als het afweersysteem lichaamsvreemde objecten herkent ontstaat er een afweerreactie die er voor zorgt dat het lichaamsvreemde object onschadelijk wordt gemaakt en opgeruimd wordt. Een kind erft eigenschappen van zowel zijn vader als zijn moeder en is hierdoor een unieke combinatie van beide ouders. Een kind lijkt op zijn ouders, maar is toch anders. Dit betekent dat een kind lichaamsvreemd is voor zijn moeder. Als het afweersysteem van de moeder in contact komt met cellen van het kind, dan kan het deze herkennen als lichaamsvreemd en zal er een afweerreactie tegen de cellen van het kind ontstaan. Maar hoe zit dit dan tijdens de dracht? Hoe wordt een kalf (/baby) beschermd tegen het afweersysteem van de moeder? Er zijn een aantal manieren waarop het kalf tijdens de dracht beschermd wordt tegen het afweersysteem van de moeder: i) het kalf is in de baarmoeder grotendeels afgesloten (verscholen) van het afweersysteem van de moeder, ii) er worden in de baarmoeder stoffen uitgescheiden die het afweersysteem van de moeder onderdrukken en leren om het kalf niet aan te vallen en iii) cellen van het kalf die in de baarmoeder met het afweersysteem van de moeder in contact komen proberen zo weinig mogelijk eiwitten te maken die het afweersysteem van de moeder kunnen activeren.

Ondanks al deze maatregelen maakt een zwangere koe toch antistoffen aan tegen het kalf, wat betekent dat er wel een afweerreactie tegen het kalf plaatsvindt. Normaal heeft deze afweerreactie geen nadelige gevolgen voor het kalf of de koe. In dit proefschrift zijn twee aandoeningen onderzocht waarbij een verstoring in de normale afweerreactie van de koe tegen het kalf kan leiden tot ziekte, 'aan de nageboorte blijven staan' (Retentio Secundinarum) en 'bloederkalveren' (Boviene Neonatale Pancytopenie).

Aan de nageboorte blijven staan

Tijdens de dracht wordt het kalf omsloten door de nageboorte (de moederkoek of foetale membranen), een vlies dat als een soort ballon om het kalf zit. Via dit vlies zit het kalf verbonden met de baarmoeder van de koe. Normaal wordt nadat het kalf geboren is de nageboorte door de baarmoeder naar buiten geperst. Als de nageboorte niet binnen 24 uur af komt wordt dit 'aan de nageboorte blijven staan' genoemd. Aan de nageboorte blijven staan na het kalven komt bij ongeveer 5% van de Nederlands melkkoeien voor en kan leiden tot een verminderde melkgift, een verminderde vruchtbaarheid (de koe kan niet makkelijk weer zwanger worden) en tot baarmoederontsteking. Eerder onderzoek heeft aanwijzingen gegeven dat de afweerreactie van de moeder tegen het kalf helpt in het loslaten van de nageboorte. De afweerreactie van de moeder tegen het kalf helpt om de verbinding tussen de baarmoeder en de nageboorte (de foetale membranen) kapot te maken, waardoor de nageboorte los komt en door de baarmoeder naar buiten geperst kan worden.

In **hoofdstuk 2 tot 4** van dit proefschrift is verder onderzoek gedaan naar de rol van de afweerreactie van de moeder tegen het kalf in het loslaten van de nageboorte. MHC klasse I (Major Histocompatibility klasse I) eiwitten van het kalf wekken een hele sterke afweerreactie op in de koe. In het begin van de dracht zitten er geen MHC klasse I eiwitten op de nageboorte, zodat het afweersysteem van de moeder niet geactiveerd wordt. Op het einde van de dracht maken cellen van de nageboorte wel MHC klasse I eiwitten aan. De afweerreactie van de moeder tegen de MHC klasse I eiwitten op de nageboorte zorgt er voor dat de verbinding tussen de nageboorte en de baarmoeder afgebroken wordt en dat de nageboorte loslaat. Als de MHC klasse I eiwitten van het kalf gelijk zijn aan die van de koe is er een grote kans dat de koe aan de nageboorte blijft staan. Het afweersysteem van de moeder herkent de MHC klasse I eiwitten op de nageboorte niet als lichaamsvreemd en de verbinding tussen de nageboorte en de baarmoeder blijft intact, waardoor de koe aan de nageboorte blijft staan. Als de vader en de moeder van een kalf familie van elkaar zijn is er sprake van inteelt in het kalf. Inteelt verhoogd de kans dat de MHC klasse I eiwitten van het kalf gelijk zijn aan die van de moeder en leidt daardoor tot een verhoogde kans op aan de nageboorte blijven staan. Als de geboorte van een kalf ingeleid wordt, de geboorte wordt opgewekt door de koe een medicijn te geven, is er een grote kans dat de koe aan de nageboorte blijft staan. Wij hebben gevonden dat het inleiden van de geboorte met corticosteroïden leidt tot een verminderde afweerreactie van de koe tegen het kalf en dit is waarschijnlijk een belangrijke oorzaak van het aan de nageboorte blijven staan van koeien na het inleiden van de geboorte.

Bloederkalveren

Vanaf 2007 werd er in heel Europa een toename van pasgeboren kalveren met een bloederziekte gezien. De aandoening werd boviene neonatale pancytopenie of bloederkalveren genoemd. Typische symptomen van een bloederkalf worden voor het eerst gezien op een leeftijd van 10 tot 20 dagen en zijn bloedingen in de huid en slijmvliezen en bloed in de mest. Het kalf wordt steeds suffer en overlijdt na een aantal dagen. Bij bloedonderzoek wordt er een sterke vermindering van het aantal bloedplaatjes en witte bloedcellen gevonden en bij autopsie zijn bloedingen in alle organen en een verlies van het aantal cellen in het beenmerg te zien. In het bloed en in de biest van koeien die een bloederkalf hebben gekregen (bloederkalf-moeders) zitten antistoffen die koeiencellen aanvallen, anti-koe antistoffen. Als een gezond kalf biest drinkt van een bloederkalf-moeder, dan krijgt dat kalf door de anti-koe antistoffen in de biest ook de bloederziekte. Onderzoek naar de oorzaak van bloederkalveren liet zien dat er een hele sterke relatie was tussen het vaccineren van koeien met Pregsure© BVD en het ontstaan van bloederkalveren. Het BVD virus voor dit vaccin wordt gekweekt op koeiencellen en in het Pregsure© BVD vaccin zaten, naast het BVD virus, ook delen van koeiencellen. Vaccinatie met het Pregsure© BVD vaccin heeft, doordat er delen van

koeiencellen in het vaccin zaten, waarschijnlijk de anti-koe antistoffen opgewekt in de bloederkalf-moeders.

Van de kalveren van moeders die gevaccineerd waren met Pregsure© BVD kreeg minder dan 0,3% een bloederziekte. Dit geeft aan dat er waarschijnlijk nog andere factoren zijn dan alleen vaccinatie van de moeder met Pregsure© BVD die bepalen of een kalf een bloederkalf wordt. In **hoofdstuk 5** is er gekeken of genetische verschillen tussen koeien en kalveren kunnen verklaren waarom sommige koeien een bloederkalf krijgen. Er werd gevonden dat het ontstaan van een bloederkalf een erfelijke eigenschap is van de moeder en niet een erfelijke eigenschap van het kalf. Zowel Pregsure© BVD gevaccineerde moeders die een bloederkalf krijgen als gevaccineerde moeders die geen bloederkalf krijgen hebben anti-koe antistoffen in hun bloed. We konden geen verschillen vinden tussen het type en de eigenschappen van de anti-koe antistoffen van bloederkalf-moeders en moeders van gezonde kalveren. Het enige verschil was dat moeders van bloederkalveren veel meer anti-koe antistoffen hebben. Waarschijnlijk hebben bloederkalf-moeders een specifieke genetische achtergrond waardoor ze veel anti-koe antistoffen aanmaken als ze gevaccineerd worden met Pregsure© BVD. Kalveren van deze moeders krijgen de bloederziekte doordat ze veel anti-koe antistoffen via de biest binnen krijgen.

In **hoofdstuk 6** is onderzocht hoe de anti-koe antistoffen leiden tot het ontstaan van een bloederkalf. In eerder onderzoek was al ontdekt dat de anti-koe antistoffen MHC klasse I eiwitten herkennen, maar het was onbekend hoe de anti-koe antistoffen precies het ziektebeeld van een bloederkalf veroorzaken en of er ook nog andere eiwitten herkend worden. Wij hebben gevonden dat de anti-koe antistoffen naast MHC klasse I eiwitten ook andere eiwitten herkennen. Echter, het grootste deel van de anti-koe antistoffen, 40% tot 90%, herkent MHC klasse I eiwitten. MHC klasse I zit op alle cellen van een kalf. Hoe kan het dan dat alleen bloedplaatjes, witte bloedcellen en beenmergcellen aangetast zijn in bloederkalveren? Waarom zijn niet alle cellen van het kalf aangetast door de anti-koe antistoffen? Uit ons onderzoek blijkt dat die cellen die aangevallen worden door de anti-koe antistoffen heel veel MHC klasse I eiwitten hebben. We concluderen dat alleen de anti-koe antistoffen die MHC klasse I eiwitten herkennen leiden tot een bloederkalf en dat de anti-koe antistoffen alleen cellen aanvallen die veel MHC klasse I eiwitten hebben.

In het kort

Een kalf is verschillend van zijn ouders en als het afweersysteem van de koe in contact komt met cellen van het kalf, dan kan het deze herkennen als lichaamsvreemd en zal er een afweerreactie tegen de cellen van het kalf ontstaan. Het normale verloop van de afweerreactie van de koe tegen het kalf heeft geen negatieve gevolgen voor het (ongeboren) kalf of de koe, maar een verstoring van deze afweerreactie kan tot ziekte leiden bij de koe of het kalf. Afwezigheid van de afweerreactie van de koe tegen het kalf

kan leiden tot aan de nageboorte blijven staan en een versterking van de afweerreactie kan leiden tot bloederkalveren. Door het onderzoek beschreven in dit proefschrift begrijpen we beter hoe het komt dat koeien aan de nageboorte blijven staan en hoe bloederkalveren ontstaan. De resultaten van dit onderzoek dragen bij aan het voorkomen van deze ziektes in de toekomst.

Gearfetting foar leken

Sykten by kij dy't assosjearre binne mei in ôfwarreaksje fan de ko tsjin it keal

It ôfwarsysteem beskermet it lichem tsjin sykteferwekkers. Sykteferwekkers binne oars as de eigen sellen, hja binne lichemsfrjemd. At it ôfwarsysteem lichemsfrjemde objekten werkent, ûntstiet der in ôfwarreaksje dy't der foar soarget dat it lichemsfrjemde objekt ûnskealik makke wurdt en opromme wurdt. In bern ervet eigenskippen fan sawol de heit as de mem en is hjirtroch in unike kombinaasje fan beide âlders. In bern liket op syn âlders, mar is dochs oars. Dit betsjut dat in bern lichemsfrjemd is foar syn mem. At it ôfwarsysteem fan de mem yn kontakt komt mei sellen fan it bern, dan kin it dizze werkenne as lichemsfrjemd en sil der in ôfwarreaksje tsjin de sellen fan it bern ûntstean. Mar hoe sit dit dan yn de dracht. Hoe wurdt in keal (/poppe) beskerme tsjin it ôfwarsysteem fan de mem? Der binne in oantal wiizen wêrop't it keal yn de dracht beskerme wurdt tsjin it ôfwarsysteem fan de mem: i) it keal is yn it skûlliif (liifmoer) grutdiels ôfsluten (ferskûle) fan it ôfwarsysteem fan de mem, ii) der wurde yn it skûlliif stoffen útskaat dy't it ôfwarsysteem fan de mem ûnderdrukke en leare om it keal net oan te fallen en iii) sellen fan it keal dy't yn it skûlliif yn kontakt steane mei it ôfwarsysteem fan de mem besykje sa min mooglik aaiwiten te meitsjen dy't it ôfwarsysteem fan de mem aktivearje kinne.

Nettsjinsteande al dizze maatregels makket in drachtige ko dochs antystoffen oan tsjin it keal, wat betsjut dat der wol in ôfwarreaksje tsjin it keal ta stân komt. Gewoanwei hat dizze ôfwarreaksje gjin neidielige gefolgen foar it keal of de ko. Yn dit proefskrift binne twa oandwaningen ûndersocht wêrby't in fersteuring yn de normale ôfwarreaksje fan de ko tsjin it keal liede kin ta sykte, 'mei it fûlens stean bliuwe' (Retentio Secundinarum) en 'bliedkeallen' (Boviene Neonatale Pancytopenie).

Mei it fûlens stean bliuwe

Yn de dracht wurdt it keal omsluten troch it fûlens (de foetale membranen), in flues dat as in soarte ballon om it keal hinne sit. Fia dit flues sit it keal ferbûn mei it skûlliif fan de ko. Gewoanwei wurdt nei't it keal berne is it fûlens troch it skûlliif nei bûten parse. As it fûlens net binnen 24 oeren ôf komt wurdt dit 'mei it fûlens stean bliuwe' neamt (mei it ûngâns stean bliuwe). Mei it fûlens stean bliuwe komt by ûngefear 5% fan de Nederlânske molkkij foar en liedt ta in fermindere molke jefte, fermindere fruchtberens (de ko kin net maklik mear drachtich wurde) en ta skûlliifûntstekking. Earder ûndersyk hat oanwizingen jûn dat de ôfwarreaksje fan de mem tsjin it keal helpt yn it loslitten fan it fûlens. De ôfwarreaksje fan de mem tsjin it keal helpt om de ferbining tusken it skûlliif en it fûlens ôf te brekken, wêrtroch't it fûlens loslit en troch it skûlliif nei bûten dreaun wurdt.

Yn **haadstik 2 oant 4** fan dit proefskrift is fierder ûndersyk dien nei de rol fan de ôfwarreaksje fan de mem tsjin it keal yn it loslitten fan it fûlens. MHC klasse I (Major Histocompatibility klasse I) aaiwiten fan it keal wekke in hiele sterke ôfwarreaksje op yn de ko. Yn it begjin fan de dracht sitte der gjin MHC klasse I aaiwiten op it fûlens, sadat it ôfwarsysteem fan de mem net aktivearre wurdt. Op it ein fan de dracht meitsje

sellen fan it fûlens wol MHC klasse I aaiwiten oan. De ôfwarreaksje fan de mem tsjin de MHC klasse I aaiwiten op it fûlens soarget der foar dat de ferbining tusken it fûlens en it skûlliif ôfbrutsen wurdt en it fûlens loslit. As de MHC klasse I aaiwiten fan it keal gelyk binne oan dy fan de ko is der in grutte kâns dat de ko mei it fûlens stean bliuwt. It ôfwarsysteem fan de mem werkent de MHC klasse I aaiwiten op it fûlens net as lichemsfrjemd en de ferbining tusken it fûlens en it skûlliif bliuwt yntakt, wêrtroch't de ko mei it fûlens stean bliuwt. As de heit en de mem fan in keal famylje fan inoarren binne is der sprake fan yntylt yn it keal. Yntylt ferheget de kâns dat de MHC klasse I aaiwiten fan it keal gelyk binne oan dy fan de mem en liedt dêrtroch ta in ferhege kâns op mei it fûlens stean bliuwe. As de berte fan in keal ynlaat wurdt, de berte wurdt opwekke troch de ko in medisyn te jaan, is der in grutte kâns dat de ko mei it fûlens stean bliuwt. Wy hawwe fûn dat it ynlieden fan de berte mei kortikosteroïden liedt ta in fermindere ôfwarreaksje fan de ko tsjin it keal en benammen dit is nei alle gedachten in wichtige oarsaak fan it mei it fûlens stean bliuwen by dizze kij.

Bliedkeallen

Fan 2007 ôf naam yn hiel Europa it tal nijberne keallen mei in bloedsykte ta. De oandwaning waard boviene neonatale pancytopenie of bliedkeallen neamd. Typyske symptomen fan in bliedkeal wurde foar it earst sjoen op 10 oant 20 dagen leeftiid en binne bliedingen yn de hûd en slymfluezen en bloed yn de dong. It keal wurdt hieltyd sleauwer en stjert nei in pear dagen. By bloedûndersyk wurdt der in sterke fermindering fan it oantal bloedplaatsjes en wite bloedsellen fûn en by autopsje binne bliedingen yn alle organen en in ferlies fan sellen yn it moarchbonke te sjen. Yn it bloed en yn de bjist fan kij dy't in bliedkeal krigen hawwe (bliedkeal-memmen) sitte antystoffen dy't kowesellen oanfalle, anty-ko antystoffen. As in sûn keal bjist drinkt fan in bliedkeal-mem, dan kryt dat keal troch de anty-ko antystoffen yn de bjist ek de bloedsykte. Ûndersyk nei de oarsaak fan bliedkeallen lit sjen dat der in hiele sterke relaasje wie tusken it faksinearjen fan kij mei Pregsure© BVD en it ûntstean fan bliedkeallen. It BVD firus foar dit vaccin wurdt kweekt op kowesellen en yn it Pregsure© BVD vaccin sieten, neist it BVD firus, ek dielen fan kowesellen. Faksineare mei it Pregsure© BVD vaccin hat, trochdat der dielen fan kowesellen yn it faksin sieten, nei alle gedachten de anty-ko antystoffen opwekke yn de bliedkeal-memmen.

Fan de keallen fan memmen dy't faksinearre binne mei Pregsure© BVD krige minder as 0,3% in bloedsykte. Dit jout oan dat der nei alle gedachten noch oare faktoaren binne as faksinearen fan de mem mei Pregsure© BVD allinnich dy't bepale of in keal in bliedkeal wurdt. Yn **haadstik 5** is der sjoen oft genetyske ferskillen tusken kij en keallen ferklearje kinne wêrom't guon kij in bliedkeal krije. Der is fûn dat it ûntstean fan in bliedkeal in erflike eigenskip is fan de mem en net in erflike eigenskip fan it keal. Sawol Pregsure© BVD faksinearre memmen dy't in bliedkeal krije as faksinearre memmen dy't gjin bliedkeal krije hawwe anty-ko antystoffen yn harren bloed. Wy koene gjin ferskillen

fine tusken it type en de eigenskippen fan de anty-ko antystoffen fan bliedkeal-memmen en memmen fan sûne keallen. It iennige ferskil wie dat memmen fan bliedkeallen folle mear anty-ko antystoffen hawwe. Nei alle gedachten hawwe de bliedkeal-memmen in spesifike genetyske eftergrûn wêrtroch't hja in soad anty-ko antystoffen oanmakken at se faksineare waarden mei Pregsure© BVD. Keallen fan dizze memmen krije de bloedsykte trochdat hja in soad anty-ko antystoffen fia de bjist binnen krije.

Yn **haadstik 6** is ûndersocht hoe de anty-ko antystoffen liede ta it ûntstean fan in bliedkeal. Yn earder ûndersyk is ûntdekt dat de anty-ko antystoffen MHC klasse I aaiwiten werkenne, mar it wie ûnbekend hoe de anty-ko antystoffen presys it syktebyld fan in bliedkeal feroarsake en of't der ek noch oare aaiwiten werkend wurde. We hawwe fûn dat de anty-ko antystoffen neist MHC klasse I aaiwiten ek oare aaiwiten werkenne. Lykwols, it grutste diel fan de anty-ko antystoffen, 40% oant 90%, werkent MHC klasse I aaiwiten. MHC klasse I sit op alle sellen fan it keal. Hoe kin it dan dat allinnich bloedplaatsjes, wite bloedsellen en moarchbonke oantaaste binne yn bliedkeallen? Wêrom binne net alle sellen fan it keal oantaaste troch de anty-ko antystoffen? Út us ûndersyk docht bliken dat dy sellen dy't oanfallen wurde troch de anty-ko antystoffen in hiel soad MHC klasse I aaiwiten hawwe. Wy konkludeare dat allinnich de anty-ko antystoffen dy't MHC klasse I aaiwiten werkenne liede ta in bliedkeal en dat de anty-ko antystoffen allinnich sellen oanfalle dy't in soad MHC klasse I aaiwiten hawwe.

Yn it koart

In keal is ferskillend fan syn âlders en as it ôfwarsysteem fan de ko yn kontakt komt mei sellen fan it keal, dan kin dizze werkend wurde as lichemsfrjemd en sil der in ôfwarreaksje tsjin de sellen fan it keal ûntstean. It normale ferrin fan de ôfwarreaksje fan de ko tsjin it keal hat gjin negative gefolgen foar it (ûnberne) keal of de ko, mar in fersteuring fan dizze ôfwarreaksje kin ta sykte liede by de ko of it keal. Ôfwêzigens fan de ôfwarreaksje fan de ko tsjin it keal kin liede ta mei it fûlens stean bliuwe en in fersterking fan de ôfwarreaksje kin liede ta bliedkeallen. Troch it ûndersyk beskreaun yn dit proefskrift begripe wy better hoe't it komt dat kij mei it fûlens stean bliuwe en hoe bliedkeallen ûntsteane. De resultaten fan dit ûndersyk drage by oan it tefoaren komme fan dizze sykten yn de takomst.

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Curriculum Vitae

Lindert Benedictus was born on the 7th of January in 1987 in Drachten, the Netherlands. He graduated *cum laude* from high school (VWO) in 2004, Liudger college, Drachten. In the same year he started his study Veterinary Medicine at Utrecht University and graduated for his doctoral exam in 2008. Lindert was admitted to the research Honours Program of the Faculty of Veterinary Medicine (Excellent Tracé) and studied "*The Role of the Immune System in the Aetiology of Retained Placenta in Cattle"* under the supervision of Dr. A.P. Koets and Dr. R. Jorritsma. During the Honours Program he did an internship *"Sequence based MHC class I typing in cattle*" at Utah State University (Logan, USA) under supervision of Dr. C.J. Davies. In 2010 he started his residency program at the Faculty of Veterinary Medicine, Utrecht University and received his Doctorate in Veterinary Medicine, with a specialization in Farm Animal Health, in 2012. Immediately thereafter, Lindert started his PhD studies into "*Bovine materno-fetal alloimmune mediated disorders*" under supervision of Dr. A.P. Koets, Prof. Dr. V.P.M.G. Rutten and Prof. Dr. M. Nielen at the division of Immunology, Faculty of Veterinary Medicine, Utrecht University. Results of this research are described in this thesis and are published in peer-reviewed scientific journals. During his PhD studies Lindert did a short internship *"Bioinformatics of Next Generation Sequencing data"* at the Animal Genetics department of Zoetis (Kalamazoo, USA) under supervision of Dr. G. Rincon and Dr. D.L. Foss. In 2015, Lindert will continue his post-doctoral career at the division of Immunology and the department of Medical Microbiology (University Medical Centre, Utrecht), where he will participate in a project to develop innovative new vaccination strategies against *Staphylococcus aureus* mastitis in dairy cattle.

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