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Cattle NK Cell Heterogeneity and the Influence of MHC Class I

Alasdair J. Allan, Nicholas D. Sanderson, Simon Gubbins, Shirley A. Ellis, and John A. Hammond

Primate and rodent NK cells form highly heterogeneous lymphocyte populations owing to the differential expression of germline-encoded receptors. Many of these receptors are polymorphic and recognize equally polymorphic determinants of MHC class I. This diversity can lead to individuals carrying NK cells with different specificities. Cattle have an unusually diverse repertoire of NK cell receptor genes predicted to encode receptors that recognize MHC class I. To begin to examine whether this genetic diversity leads to a diverse NK cell population, we isolated peripheral NK cells from cattle with different MHC homozygous genotypes. Cytokine stimulation differentially influenced the transcription of five receptors at the cell population level. Using dilution cultures, we found that a further seven receptors were differentially transcribed, including five predicted to recognize MHC class I. Moreover, there was a statistically significant reduction in killer cell lectin-like receptor mRNA expression between cultures with different CD2 phenotypes and from animals with different MHC class I haplotypes. This finding confirms that cattle NK cells are a heterogeneous population and reveals that the receptors creating this diversity are influenced by the MHC. The importance of this heterogeneity will become clear as we learn more about the role of NK cells in cattle disease resistance and vaccination. The Journal of Immunology, 2015, 195: 2199–2206.

Natural killer cells are a diverse lymphocyte population with fundamental roles in immunity, cancer, and reproduction (1). Extensive studies in humans and mice have shown that as part of the innate immune system, NK cells can recognize and kill transformed or infected cells, particularly those virally infected, and initiate subsequent immune responses through the release of cytokines. This cytokine release, in addition to direct interactions between NK cells and dendritic cells, also helps initiate the adaptive immune response mediated by B and T cells (2). In human reproduction, NK cells are also involved in forming the placenta through interaction with the extravillous trophoblast (1). These diverse NK cell functions are mediated by a plethora of activating and inhibitory cell surface receptors that recognize a diverse array of ligands. The balance of signals received from these receptors determines the activation status of an individual NK cell (3).

NK cells express a wide range of receptors and other cell surface markers, some of which are expressed on other lymphocytes, whereas others are restricted to NK cells and some T cell subsets. This is particularly true of the inhibitory receptors, which are often members of large polymorphic gene families, and a majority of their ligands are the polymorphic MHC class Ia molecules (4). The interaction between NK cell receptors and MHC class I is fundamental not only for the recognition and subsequent activation against target cells but also for functional education and receptor repertoire development (5–7). This highly polymorphic system creates differential specificity and avidity between receptor and ligand pairs within populations (8). Avidity is also influenced by the peptide presented by the MHC class I, which can significantly alter the extent of NK cell inhibition (9, 10). A further diversity-generating mechanism is the variegated expression of receptors between individual NK cells, creating a differentially responsive cell population (5, 11, 12). The overall outcome is immune diversity created by the variable expression of polymorphic germline-encoded receptors, which can contribute to differential susceptibility to viral diseases in humans and mice.

The extracellular domains of NK cell receptors that recognize MHC class I are either Ig-like or C-type lectin-like, and encoded within the leukocyte receptor complex (LRC) or NK complex (NKC), respectively. These gene complexes are located on different chromosomes in all mammals studied to date, and never on the same chromosome as the MHC. Simian primates, including humans, have evolved a diverse LRC-encoded killer cell Ig-like receptor (KIR) system. Rodents have convergently evolved an unrelated NKC-encoded Ly49 receptor system [encoded by the killer cell lectin-like receptor A gene (KLRA)] that recognizes MHC class I to control NK cell function (13). The lack of linkage between these codependent receptors and ligands can lead to individuals of a particular genotype containing receptors lacking strong ligands.

Studies in other species reveal that not all mammals have an expanded NK cell receptor gene family (14, 15). All the known gene expansions have occurred independently within receptor families predicted to bind MHC class I. In contrast to simian
primates, the LRC of prosimians contains only one KIR gene, which is nonfunctional. However, alongside a nonfunctional KLRD1 gene, the NKC contains a large KLRD1 gene expansion (16). Horses are the only nonrodent species known to have expanded the KLRD1 genes (17). The most extensive expansion described to date is in cattle, the only species known to have significantly expanded two MHC class I receptor gene families, the KIR and KLRD1, each encoded within a different gene complex (18–22).

Cattle classical MHC class I haplotypes—unlike those in humans and, to a large extent, mice—vary in the number of genes they contain (23). In humans the HLA-A, B, and C genes are highly polymorphic but present on almost all haplotypes. MHC class I diversity in cattle is generated by six relatively polymorphic classical genes, with between one and three present on any one haplotype. However, there is no evidence of one gene being more or less important for Ag presentation, although some genes appear dominant over others at restricting T cell responses to certain pathogens. This diversity presents significant challenges for the coevolution of germline-encoded MHC receptors that segregate independently. In cattle, this receptor ligand system has the potential to generate an enormous diversity of differentially responsive NK cells (23).

The influence of this diversity on cattle NK cell function is yet to be understood. Cattle NK cells are defined by their expression of NCR1, a member of the natural cytotoxicity receptor family (24). Early work revealed that CD2 expression differentiated phenotypic NK cell subsets; 80% of peripheral blood NK cells are CD2bright in a resting state, less activated by IL-2 stimulation and a poorer producer of IFN-γ than CD2dim cells, although both subsets are equally cytotoxic (25, 26). Very little is currently known about the expression of other cattle NK cell receptors (18, 19, 24, 27), particularly the KIR and KLRC/D receptors that are predicted to bind MHC class I. To begin to address this, we examined the transcription of a panel of 25 NK cell receptors and other NK cell–associated molecules between and within individual animals with different MHC class I genotypes.

Materials and Methods

Animals

Heparinized peripheral blood samples were obtained by venipuncture from 14 Holstein–Friesian cattle (Bos taurus) with no overt signs of disease at the time of sampling. These cattle were part of an established MHC-defined homozygous herd that was generated by backcrossing (28). All animal experiments were approved by The Pirbright Institute Ethics Committee and carried out in accordance with the U.K. Animal (Scientific Procedures) Act 1986.

NKMC isolation

PBMCs were separated from whole blood by density gradient centrifugation (1,083 g/ml Histopaque; Sigma-Aldrich, Gillingham, U.K.). NK cells were positively selected from PBMCs by labeling with a mAb recognizing bovine NCR1 (CD335) (AbD Serotec, Kiddlington, U.K.) at 3 μg/ml and rat antimuscle IgG1 MicroBeads (Mitenyi-Biotec, Bergisch Gladbach, Germany) at 2.5 μl/106 cells, to purities >85%. These cells were then stimulated in RPMI 1640 plus 10 mM HEPES plus 50 μM 2-ME (Sigma-Aldrich) containing either recombinant bovine IL-2 (0.5 μg/ml), a mix of recombinant bovine IL-12 and recombinant human IL-18 (eBioscience, San Diego, CA) (both 0.1 μg/ml) or recombinant human IL-15 (0.1 μg/ml) (eBioscience) for up to 7 d.

NK cell dilution cultures

To generate single-cell NK cell cultures, NK cells positively selected from PBMCs were taken after 24-h cytokine stimulation (either IL-2 or IL-15) for limiting dilution assays. Cells were diluted in 96-well plates to 10 cells per 100 μl, or 0.33 cell per 100 μl before the addition of 5 × 107 allogeneic irradiated PBMC feeder cells and the same cytokine they were stimulated in overnight. Wells exhibiting growth after 14 d were expanded on 1 × 104 allogeneic feeder cells for an additional 14 d, before total cell counts were taken and a single NK cell origin and NCR1 expression were assessed by flow cytometry.

Flow cytometry

NK cell and NK cell dilution culture subsets were identified by labeling with mAbs CD335-PE (NCR1; AbD Serotec), CC42-AF647 (CD2; conjugated using AF647, Life Technologies, Carlsbad, CA), and CC63-APC-Cy5.5 (CD8; conjugated using APC Cy5.5, Innova Biosciences, Cambridge, U.K.). TRT1 (anti-turkey rhinotracheitis) (IgG1) and TRT6 (IgG2a) were used as isotype control mAbs. Cell populations were visualized using forward light scatter (FSC)–A and side light scatter–A to identify lymphocyte populations before removing cell doublets using FSC-A and FSC-H. Cell viability was confirmed using the nuclear stain DAPI. Quadrants and gates for NCR1+ and CD2 expression were determined by gating on Ig-matched isotype control Abs TRT1 (IgG1) and TRT6 (IgG2a), respectively (Supplemental Fig. 1). For NCR1 and CD2 profiling of ex vivo and in vitro stimulated MACS-isolated NK cells, ≥5,000,000 events within the live gate were counted. For NK cell limiting dilution cultures, ≥20,000 events were counted. Flow cytometry was performed using an LSRFortessa II (Beckton-Dickinson, Oxford, U.K.) and analyzed using FCS express v3 (De Novo software, Los Angeles, CA) or FlowJo v10.2 (TreeStar, Ashland, OR).

RNA isolation, quality assessment, and cDNA synthesis

A total of 3 × 10⁶ cells from each NK cell population were resuspended in 1 ml TRizol (Life Technologies), and RNA was extracted according to the manufacturer’s protocol. RNA quality and concentration were assessed by 260/280 nm absorbance, and integrity was confirmed with an RNA 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was reverse transcribed using random hexamers (ImProm-II RT kit; Promega; Madison, WI). cDNA synthesis reactions were performed in triplicate using 1 μl of total RNA per assay.

FIGURE 1. Ex vivo NK cells are predominantly CD2bright and CD8 expression increases upon stimulation with IL-2. PBMCs from 14 MHC class I–defined cattle were assessed for the expression of NCR1, CD2, and CD8 immediately ex vivo (unstimulated, filled bars) or after 7-d in vitro IL-2 stimulation (open bars). (A) The percentage of CD2bright and CD8+ cells within the NCR1+ population. Statistical significance was determined using a linear mixed model, with expression as the response variable and CD2/CD8 status and stimulated/unstimulated as fixed effects, plus an interaction between them and the animal as a random effect. (B) Overall change in the percentage of CD2bright and CD8+ within the NCR1+ population after cytokine stimulation. Statistical significance was determined using paired t tests. *p < 0.001.
using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized from 1 μg RNA using a SuperScript II Kit with oligo (dT12-18) primers (Life Technologies) according to the manufacturer’s guidelines.

**Primer generation and PCR**

PCR primers for 25 NK cell receptor genes or NK-associated genes were adopted from previous studies (18, 19) or designed based on sequences consistent with previously published data showing that NK cells from homozygous cattle. These animals represented three different haplotypes, four A18 containing only gene 6, four A14 containing a gene 1, 2 and 4 and six A31 containing a gene 1 and 2 but with different alleles to the A14. In all the animals, ~80% of NCR1+ cells were CD2high ex vivo and CD2 expression was reduced nonsignificantly (p = 0.27) after stimulation with IL-2 (Fig. 1). In contrast, CD8α expression increased in vitro under IL-2 when compared with ex vivo cells (p < 0.001) (Fig. 1), as has been shown with other cytokines (25). These NK phenotypes are consistent with previously published data showing that NK cells from determined the cell surface expression of CD2 and CD8α on ex vivo and in vitro stimulated NCR1+ PBMCs from 14 MHC homozygous cattle. These animals represented three different haplotypes, four A18 containing only gene 6, four A14 containing a gene 1, 2 and 4 and six A31 containing a gene 1 and 2 but with different alleles to the A14. In all the animals, ~80% of NCR1+ cells were CD2high ex vivo and CD2 expression was reduced nonsignificantly (p = 0.27) after stimulation with IL-2 (Fig. 1). In contrast, CD8α expression increased in vitro under IL-2 when compared with ex vivo cells (p < 0.001) (Fig. 1), as has been shown with other cytokines (25). These NK phenotypes are consistent with previously published data showing that NK cells from

**Results**

**NK cells from MHC class I homozygous cattle have a conventional CD2 and CD8 expression profile**

As this study exploited a specially bred herd of MHC homozygous Holstein-Friesian cattle, it was important to establish that NK cells isolated from these animals were not substantively different from those used in previous studies (25, 26). Cattle NK cells are defined as NCR1+, with variable CD2 and CD8α expression (25, 26). We

<table>
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<th>Gene(s)</th>
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<th>Antisense Primer (5′–3′)</th>
<th>Reference No.</th>
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<td>GCTTCCCTCTGTGTTTTCCAGAAGCA</td>
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<td>19</td>
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</table>

**Table I. NK cell receptor and associated genes studied and the primers used for PCR amplification**

**FIGURE 2.** The variable transcription of NK cell receptor genes in ex vivo NK cells. The transcription of 25 NK cell receptor and associated genes was determined by PCR in NK cells from 14 MHC class I-defined animals (4 A18, 4 A14, and 6 A31). Genes that showed consistent variation are not shown. The dots represent transcription in immediately ex vivo NK cells (black), NK cells stimulated for 7 d in recombinant bovine IL-2 (orange), recombinant bovine IL-12, and recombinant human IL-18 (gray) or recombinant human IL-15 (blue).
these homozygous cattle have a broadly conventional phenotype and are responsive to cytokine stimulation.

Receptor transcription in NK cells with divergent MHC class I genotypes are indistinguishable

To determine the receptor mRNA expression profile of ex vivo NK cells, the transcription of 25 NK cell receptor genes and other NK cell markers (Table I) were assessed immediately ex vivo and after cytokine stimulation in 14 cattle. Analysis by FACS confirmed that these cells were expressing NCR1 on the cell surface. For the purposes of this study, we divided these genes into three groups: the conventional NK cell molecules (CKM), the KLR, and the KIR. Although the majority of genes were transcribed regardless of MHC class I genotype or cytokine stimulation, some exceptions were noted.

Five genes showed variable mRNA expression patterns that were independent of MHC class I background (Fig. 2). NCR1 expression on CD3+ cells currently defines cattle NK cells, but we observed that NCR1 transcription was not ubiquitous, and in cells from animal 982 we were not able to detect NCR1 transcription following isolation based on NCR1 expression. The activating receptor gene 2B4 was always transcribed by ex vivo NK cells but was partially or completely lost in cytokine-stimulated NK cells from eight animals. Conversely, TIM-3 was absent in the ex vivo populations from 10 of the 14 animals but was consistently transcribed by all animals after in vitro cytokine stimulation. CD8β was consistently transcribed by ex vivo and IL-2 and IL-15 in vitro stimulated NK cells, but in four animals, transcription was lost upon stimulation with IL-12/18. Only KIR3DL6 appeared to have variable transcription with no discernible pattern (Fig. 2). Although KLRA appears differentially transcribed, two distinct allele lineages are found in cattle. We have previously genotyped this group of animals for the presence of each KLRA lineage, and these NK population differences are due to genotype rather than differential regulation.

Cattle NK cells are a diverse population

As in other species, differential receptor transcription is likely to create a heterogeneous NK population in cattle. To examine NK cell receptor transcription patterns at the single-cell level, we maintained a total of 67 NCR1+ short-lived limiting dilution cultures from two A14 and four A18 homozygous cattle, the two most divergent haplotypes in this study based on gene content. Cloning efficiency varied between animals and cytokine regimen, from 3% up to 25%, which is consistent with similar studies in mice (29). To confirm that these cells were of an NK origin, and not from the recently reported population of CD3+ NCR1+ T cells, we assessed each clone for transcription of the TCRβ-chain of the TCR, using previously published methods (30). TCRβ transcription was found in a single culture, 375_2H3, which was subsequently removed from further analysis. Flow cytometry of the remaining 66 dilution cultures confirmed that both NCR1+ CD2high and NCR1+ CD2low populations were present at a ratio of ≈75:25 (data not shown). This finding is consistent with our bulk culture data and the previously published ratio for ex vivo NK cells, confirming that both these major populations were at least partially represented (26).

Although a majority of the dilution cultures appeared to be from a single-cell origin, with a single phenotype determined by expression...
of NCR1 and CD2 (Fig. 3A–C), one had a mixed CD2 phenotype (Fig. 3D) and two had mixed NCR1 phenotypes (Fig. 3E, 3F). This finding could indicate a multiple-cell origin or the transient expression of NCR1 and CD2. As further limiting dilution was not possible, all 66 populations are referred to as dilution cultures rather than clones for the remainder of this article.

Transcription of the same 25 NK cell markers and receptors previously examined in ex vivo NK cells was determined (Fig. 4). A majority of the CKM genes were differentially transcribed; CD2, CD8α, CD8β, CD25, and NCR1 were all variably present between dilution cultures from several individual animals. The variable pattern of TIM-3 and 2B4 transcription was generally consistent between the dilution cultures and ex vivo NK cells from the same animals. Within the KLR group, mRNA for KLRC1*2-7, KLRC2*2, KLRD*1, and KLRD*2 was ubiquitously present at the population level but variably transcribed between the dilution cultures. Transcription of the KLRA alleles was consistent with the NK cell population data, with animals heterozygous for both allele lineages showing variable transcription patterns. As predicted from ex vivo NK cell populations, KIR3DXL6 was variably transcribed between dilution cultures, as was KIR3DXL1 and, to a more limited extent, the KIR group containing KIR3DXL3/5/7.

**FIGURE 5.** The frequency of dilution cultures transcribing NK cell receptors correlates with MHC class I genotype (A) and CD2 expression (B). Gene transcription is displayed as a normalized percentage of the total number of cultures within each population. Samples in which expression was mixed and genes were transcribed by all the dilution cultures were omitted. Statistical significance was determined using Fisher’s exact tests. *p < 0.05.

**NK cell receptor transcription is associated with CD2 expression and MHC class I genotype**

Having established NK cell receptor transcriptional variability within the dilution cultures, we examined whether there was any association between receptor transcription and NK cell phenotype or genotype. The expression of CD2 is an important differentiator of functional NK cell subsets in cattle (26). Therefore, we grouped the data from each dilution culture by CD2 expression and MHC class I genotype. Besides the significant association of CD2 transcription with expression, KLRC1*2-7 and KIR3DXL1 were significantly more likely to be present on the CD2high NK cell subset regardless of MHC class I genotype (p < 0.05) (Fig. 5). The transcription of more inhibitory receptors correlates with the stronger inhibition of CD2high NK cells compared with CD2low (26).

The transcription of five receptor genes was also significantly correlated with MHC class I genotype (Fig. 5), using Fisher’s exact test. This correlation was independent of any animal-specific variation (Supplemental Fig. 2). The receptor KIR3DXL1 and the KLRA*02 allele lineages were more likely to be transcribed in dilution cultures from A14 MHC haplotype animals. This observation is consistent with the lack of KLRA*01 in the A14 animals and very few CD2low dilution cultures. However, KLRA*01,
KLRD, and the activating receptors KLRC2*2 and 2B4 were all significantly more likely to be transcribed in the dilution cultures from the A18 animals. KLRD forms heterodimers with members of the KLRC family, and an increase in transcription of both KLRC2*2 and KLRD points to a potential functional interaction. Although it was not possible to measure a functional consequence of these differences using this system, we have identified genes that are not exclusively, but are preferentially, transcribed in NK cells from one MHC class I background over another, and between functionally different NK cell subsets.

As the transcription of individual receptors was influenced by MHC class I genotype, we compared the entire transcription profiles by MHC class I background. Of the 66 dilution cultures described in this study, we observed 52 different transcription phenotypes using our panel of 25 genes, with no single phenotype seen more than twice (data not shown). When separated by MHC class I background, the A14 dilution cultures transcribed fewer genes than the A18, which was due to significantly less KLR gene transcription \((p < 0.05)\) and less transcription of the CKM genes, which approached significance \((p = 0.12)\) (Fig. 6).

The KLR group includes genes that can encode inhibitory (KLRC1) or activating (KLRC2) receptors, whereas the majority of the CKM are activating. Therefore, we separated the transcription profiles based on both signaling potential and MHC class I background (genes that did not fit into either group were excluded from this analysis; Supplemental Table I). Although no statistical differences were found between the MHC groups, when analyzed together there was a clear dominance of two activating receptor transcription phenotypes: all the genes analyzed or all the genes except CD25 (Supplemental Fig. 3). This finding suggests that cattle NK cell functional diversity is primarily driven by variable expression of inhibitory receptors.

**Discussion**

NK cell receptor genes predicted to recognize MHC class I ligands are highly diversified in cattle (23). We hypothesized that some of these receptors would be differentially transcribed to create a diverse NK cell population. Therefore, we determined the transcription of 25 NK cell receptors and other associated molecules from cattle with different MHC class I genotypes. A small number of genes were differentially transcribed after cytokine stimulation of entire ex vivo NK cell populations. Using limiting dilution cultures, we further identified mRNA from activating and inhibitory receptor genes, including members of the KIR and KLRC/D families, differentially expressed within and between animals. Comparing receptor transcription patterns revealed that the KLRC/D genes were the most variably present and, importantly, were preferentially transcribed by A18 MHC class I genotype NK cells compared with the A14. Overall, we have shown that cattle NK cells differentially transcribe several NK cell receptor genes and that MHC genotype can influence the frequency of receptor mRNA expression in the total NK population.

Haplotypic diversity in combination with allelic polymorphism creates extensive MHC class I diversity in cattle herds and breeds. Different combinations of haplotypes and almost all cattle will be heterozygous at the MHC and will inevitably vary in the genes that are expressed and therefore in the repertoire of antigenic peptides that can be presented. Although it is difficult to disentangle the effects of intensive breeding programs, evidence exists that certain haplotypes are more beneficial based on their penetrance in the population and the hierarchy of gene presence (31, 32). Our data suggest that this diversity will influence the receptor diversity of the circulating NK cell population. The transcription patterns of the paired receptors KIR3DXL1 and KIR3DXS1 effectively highlight the influence of MHC class I. KIR3DXS1 is the only functional activating KIR receptor gene in cattle (18, 33) and was

**FIGURE 6.** MHC class I haplotype influences the number of KLR receptors transcribed on individual dilution cultures. Dilution cultures were separated by MHC class I haplotype (A14, filled bars, and A18, open bars) and assessed for the total number of transcribed genes (top left) and also transcription of each NK receptor gene group: KIR, KLR, and CKM. The frequency has been calculated based on a normalized percentage of the dilution cultures with statistical significance between MHC class I haplotypes determined by a Fisher exact test. *\(p < 0.05\).
ubiquitously transcribed in all the dilution cultures, whereas KIR3DL1 was variably present in A18 NK cells. The significant presence of KLR receptor mRNA in more cultures from A18 animals, as well as suggesting these are functionally important receptors for MHC class I, indicates that MHC class I is involved in the development and function of cattle NK cells. Between individuals within a population there is likely to be a range of NK cell specificities and activation thresholds that will create subtly different responses to individual pathogens. This observation also adds to the body of genetic and comparative evidence all pointing to determinants of MHC class I being the ligands for the KIR, KLRA, and KLR/C receptors in cattle, as they are in other species (4).

The interactions between NK cell receptors and MHC class I have been extensively described in humans and mice. Recently, this work has advanced the missing-self hypothesis from which the concepts of NK cell education and licensing have arisen (7). The activation threshold for an individual NK cell is determined by the strength of inhibition, which in turn is determined by the number and avidity of expressed self-inhibitory receptors (34–36). If cattle NK cells are educated by MHC class I in an analogous mechanism, our findings may point to an important effect of MHC class I on the development of NK cell receptor repertoires, which in turn creates diversity in the immune response.

The method used to generate NK cell dilution cultures in this study relied on cytokine stimulation, and it must be assumed that this method has altered the resting phenotype of these cells. The dominance of the activation receptor phenotype including all, or almost all, the genes is likely a result of this. However, it is of note that the proportion of CD2 high cells was consistent with the ratio seen immediately ex vivo. Furthermore, although we could study only what is likely to be a fraction of the total number of NK cell phenotypes, it can be assumed that a majority of the ways we isolate are among the most common, at least of those that proliferate in response to cytokine stimulation, and functionally relevant.

Owing to the very high sequence similarity between receptor cDNA sequences, it was not possible to discriminate between certain groups of genes by PCR. In addition, the PCR methods used were not quantitative and simply measured transcript presence or absence. As a consequence, the diversity described in this article is likely to be an underestimate in terms of qualitative and quantitative gene transcription. However, it is likely that receptor transcription and expression for most genes are correlated. A study assessing the genetic control of human KIR expression found strong correlations between expression of KIR3DL1, KIR2DL3, and KIR2DS4 and transcriptional activity (37). This may not be the case for all the genes or individual animals though, as expression levels can even vary between alleles, regardless of transcript abundance (38). This finding adds to the potential phenotypic diversity of the cattle NK cell population.

This study provides evidence that cattle NK cells constitute a heterogeneous population that differentially expresses several receptors, including those for MHC class I. As the frequency of NK cells transcribing individual receptors significantly correlated with the MHC class I genotype of the animal, individual animals are likely to differ in the frequency of NK cell phenotypes. As we learn more about the functional consequences of this diversity in cattle, this information should be considered when studying responses to pathogens and vaccination.

Acknowledgments
We thank the animal services team at The Pirbright Institute for skilled assistance and Dr. Elfrain Guzman for help with flow cytometry.

Disclosures
The authors have no financial conflicts of interest.
expressed by bovine T-cell clones following a single semi-nested PCR reaction.


Supplemental Data:

Supplemental figure 1: Flow cytometry gating strategies for NCR1 and CD2 expression on lymphocyte subsets. Cell populations were visualised using FSC-A and SSC-A to identify lymphocyte populations (A) before removing cell doublets using FSC-A and FSC-H (B). Cell viability was confirmed using the nuclear stain DAPI (C). Quadrants and gates for NCR1+ and CD2 expression (D) were determined by gating on Ig-matched isotype control antibodies TRT1 (E) and TRT6 (F) respectively. For NCR1 and CD2 profiling of ex vivo and in vitro stimulated MACS isolated NK cells (Figure 1) a minimum of 50,000 events within the live gate were counted. For NK cell limiting dilution cultures (Figure 3) a minimum of 20,000 events were counted. Representative plots from one of 14 animals are shown.
Supplemental figure 2: Receptor transcription patterns for each animal are broadly similar and not skewed by a small number of individuals. Each dilution culture has been colour coded by the animal of origin. The total number of clones from each animal is provided in the key.
Supplemental figure 3: The variable transcription of activating and inhibitory NK receptor genes in dilution cultures. Transcription of 25 NK receptor and associated genes were separated into three groups depending on the predicted signalling potential of the gene (supplemental table I). Individual phenotypes were then ascribed an arbitrary number and the phenotype with the highest number of genes transcribed listed as phenotype 1 and subsequent phenotypes are described in descending order of genes transcribed. Genes with inhibitory (A) and activating potential (B) are displayed. Gene transcription is displayed as a percentage of the total number of dilution cultures with MHC class I haplotype defined (A14 filled bars and A18 open bars). Individual NK receptor phenotypes and the total number of transcribed genes are listed in the tables on the right hand side of the figure.
### NK receptor gene signal classification

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<th>CD16</th>
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**Supplemental table I:** The signalling classification of the 25 NK cell receptors used in this study.