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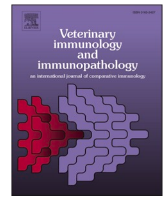

Cattle killer immunoglobulin-like receptor expression on leukocyte subsets suggests functional divergence compared to humans

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ABSTRACT

Cattle, sheep, and goats are the only species outside primates known to have an expanded and diversified family of killer immunoglobulin-like receptors (KIR). Primate KIR are expressed on the surface of NK and T cells and bind MHC-I to control activation. However, the surface expression, ligands and function of bovid KIR remain unknown. Cattle botaKIR2DL1 is the only functional KIR of the same DL-lineage as the expanded KIR in primates and we examined if leukocyte expression patterns were consistent with human. We raised a specific mouse anti-botaKIR2DL1 monoclonal antibody and assessed its utility in flow cytometry, ELISA, and western blot. Unlike primates, cattle DL-lineage KIR (botaKIR2DL1) is present on B cells and monocytes in addition to T cells and low-level expression on NK cells. Expression decreases after in vitro PBMC stimulation with IL-2. This suggests that botaKIR2DL1 has different functions, and potentially ligands, compared to primate KIR.

1. Introduction

Killer immunoglobulin-like receptors (KIR) are transmembrane proteins which are predominantly expressed on the surface of NK cells and subsets of CD4$^+$, CD8$^+$, and γδ T cells in primates (Anfossi et al., 2004; Hermes et al., 2013; Uhrberg et al., 2001; van Bergen et al., 2009; Vilches and Parham, 2002; Young et al., 2001). There are two lineages of *KIR* in extant species, DL and DX. Prosimian primates have significantly expanded and diversified the DL-lineage *KIR* while retaining a single DX-lineage *KIR*, which is pseudogenic in humans but putatively functional in other simian primates (Bruijnesteijn et al., 2020). The expanded DL-lineage KIR bind to major histocompatibility class I (MHC-I) molecules and are highly polymorphic with haplotype gene content also varying between individuals (Uhrberg et al., 1997). This interaction of KIR with MHC-I on NK cells is key to the early innate immune response to virally infected or cancerous cells (Jamil and Khakoo, 2011; Xu et al., 2020), with combinations of KIR/MHC-I shown to alter susceptibility to several diseases (Kulkarni et al., 2008; Rajagopalan and Long, 2005). In addition, different KIR/MHC-I combinations in humans effects trophoblast implantation and can impact the outcome of pregnancy (Colucci, 2017; Moffett-King, 2002).

Despite their critical biological functions in primates an expanded diverse repertoire of *KIR* is not universal. Cattle, sheep, and goats (all members of the family Bovidae) are currently the only known species outside of simian primates which have an expanded and diversified family of *KIR* (McQueen et al., 2002; Sanderson et al., 2014; Schwartz et al., 2019; Storset et al., 2003). In rodents (Yokoyama, 1995), swine (Schwartz and Hammond, 2018), and equids (Futas and Horin, 2013), *KIR* remain monogenic with as yet unknown function. In prosimians (Walter, 2011), felids (Jelinek et al., 2023) and pinnipeds (Hammond et al., 2009), *KIR* are pseudogenic, and in canids they are absent entirely (Jelinek et al., 2023). However, the restriction of NK cells by MHC-I is clearly a strong evolutionary pressure in mammals as in other species this function has been adopted by unrelated killer lectin-like receptor (KLR) families, a remarkable example of convergent evolution. In rodents, KLRA (Ly49) binds MHC-I (Yokoyama, 1995), and in prosimians KLRC/D perform the same function (Averdam et al., 2009; Walter, 2011).

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In contrast to primates, Bovidae species have expanded the DX-lineage *KIR*, with only cattle retaining a single functional DL-lineage *KIR*, *botaKIR2DL1* (Schwartz et al., 2019). In addition, cattle, sheep, and goats also have expansions of the *KLRC* and *KLRD* genes (Schwartz et al., 2019, 2017) the products of which bind MHC-I in prosimians. Consequently, it is not clear if one, both, or neither of these unrelated gene families encode receptors for MHC-I and control NK cell function as current research has been limited to genetic analysis, partly due to a lack of suitable reagents (Entrican et al., 2020).

Here we describe the generation of a multipurpose monoclonal antibody (mAb) to the sole likely functional DL-lineage KIR present in all Bovidae, cattle KIR2DL1 (botaKIR2DL1), and determined its expression pattern on PBMC.

2. Materials and methods

2.1. Ethics statement

All animal work was approved by The Pirbright Institute animal welfare review board and carried out in accordance with the U.K. Animal (Scientific Procedures) Act 1986.

2.2. Immunisation and hybridoma generation

BotaKIR2DL1 antigen for immunisation was created as an Ig-fusion protein as previously described (Staines et al., 2013). Briefly, botaKIR2DL1 extracellular domains were amplified by PCR from existing plasmid constructs, purified, and ligated to the pMIg vector containing the mouse IgG2b Fc domain. 293T cells were transfected with botaKIR2DL1-Fc DNA construct using Lipofectamine 2000 (Invitrogen). BotaKIR2DL1-Fc fusion protein was purified from the supernatant by protein G affinity purification and buffer exchanged for PBS using the Amicon Ultra 10 kDa ultrafiltration system (Merck).

BALB/c mice were immunised intra-peritoneally with 50 μg botaKIR2DL1-Fc emulsified 1:1 with TiterMax Gold adjuvant (Sigma-Aldrich). Three subsequent boost immunisations were administered at day 14, 28, and 70. The final boost was adjuvant free. Mouse spleen was harvested 4 days post final boost and single cell suspensions prepared by flushing prior to fusion with the SP2/0-Ag14 mouse myeloma cell line. Individual hybridomas were obtained through limiting dilution culture and supernatants screened against botaKIR2DL1 or botaKIR3DXL1 transfected P815 cell lines. The clone with the highest specificity for botaKIR2DL1 was selected (clone AA7; https://www.immunologicaltoolbox.co.uk/antibody/ITB02145). Monoclonal antibody was purified from the supernatant by protein G affinity purification using the ÄKTA pure 25 M system (Cytiva).

2.3. Transfection of P815 cell line

Constructs for botaKIR2DL1-FLAG and botaKIR3DXL1-FLAG were generated by PCR amplification from bovine PBMC cDNA and cloned into the pcDNA3.1 + vector. The mouse mastocytoma P815 cell line was transfected with botaKIR2DL1-FLAG or botaKIR3DXL1-FLAG in pcDNA3.1 + by electroporation (270 V, 500 μF pulse) using a Gene Pulser (Bio-Rad), and resultant colonies clonally expanded to create stable KIR expressing cell lines. Surface expression of KIR was validated by flow cytometry staining using anti-FLAG antibody (M2).

P815 cell lines were maintained in high glucose DMEM (Sigma-Aldrich) with 10 % v/v FBS and 1 % v/v penicillin-streptomycin-glutamine (Gibco) at 37 °C and 5 % CO_2. Cell lysates were prepared by incubating cells with 1 % v/v IGEPAL CA-630 (Sigma-Aldrich) at a density of 1–2 × 10^8 cells/ml.

2.4. PBMC isolation

Heparinised peripheral blood samples were obtained by venipuncture from healthy Holstein Friesian cattle (Bos taurus) from a herd of MHC-homozygous cattle generated previously by backcrossing (Ellis et al., 1999). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation using Histopaque-1083 (Sigma-Aldrich) as previously described (Werling et al., 1999). PBMC were preserved in 90 % v/v fetal bovine serum (FBS; Gibco) with 10 % v/v dimethyl sulfoxide (DMSO; Sigma-Aldrich). Freshly isolated PBMC were maintained in RPMI-1640 (Sigma-Aldrich) with 10 % v/v FBS and 1 % v/v penicillin/streptomycin at 37 °C and 5 % CO_2. PBMC were stimulated using 100 IU/ml recombinant human IL2 (rhuIL-2; Invitrogen). Cell lysates were prepared by incubating cells with 1 % v/v IGEPAL CA-630 (Sigma-Aldrich) at a density of 1–2 × 10^8 cells/ml.

2.5. Production of botaKIR2DL1 proteins

The extracellular domains of botaKIR2DL1*001 and botaKIR3DXL1*001 were amplified by PCR from the botaKIR+ P815 cell lines (botaKIR2DL1-Fc_Fwd: ATATAT**GAATTC**GGGTGGTCATGACAAGCC, botaKIR2DL1-Fc_REV ATATAT**CCATGG**CGCTGTTGGAGTTTGGTTTT; botaKIR3DXL1-Fc_Fwd ATATAT**GAATTC**GGGTGAATATGAGAAGCTGTCTT, botaKIR3DXL1-Fc_REV ATATATA**GATCTT**TCTGTGGTGTGTGGATCCA; botaKIR2DL1-mon_FWD ATATAT**CCATGG**GTGGTCATGACAAGCCCTCCCTGTCAG, botaKIR2DL1-mon_REV TATATA**CCTAGG**CGACAACCTCAAACCAAAACACAC), and sequenced for fidelity. For KIR-Fc production, the amplified extracellular domains were ligated to the pFUSE-hIgG1-Fc2 plasmid backbone (InvivoGen). Constructs were transfected into the Expi293F cell line using PEI max 40k transfection agent (Polysciences) as previously described (Rosalind Franklin Institute online protocol) and maintained in Expi293 serum free expression media (Gibco) at 37 °C and 8 % CO_2 with orbital shaking at 120 rpm. KIR-Fc proteins were purified from the supernatant by protein G affinity purification using the ÄKTA pure 25 M system (Cytiva). Chicken DC-SIGN-Fc protein was kindly provided by Dr Zhiguang Wu (The Roslin Institute).

For monomer production, the extracellular domain of botaKIR2DL1 was cloned into the prokaryotic expression vector pET23d (Novagen) and recombinant botaKIR2DL1 proteins were generated as inclusion bodies in *Escherichia coli* Rosetta strain (Novagen). Inclusion bodies were thoroughly washed and fully denatured and reduced in 6 M guanidine before refolding. For in vitro refolding, the inclusion bodies were denatured using 20 mM dithiothreitol (DTT) and added to refolding buffer (8 M urea, 400 mM L-arginine, 150 mM NaCl, 20 mM tris pH 7.8, 5 mM reduced glutathione, 0.5 mM oxidised glutathione) in dialysis tubing. The resulting protein/buffer solution was dialysed against refolding buffer without urea or glutathione for 24 h. Correctly folded proteins were purified by repeated FPLC (Pharmacia) size-exclusion chromatography using preparatory grade SD75 26/60 and analytical grade SD75 GL 10/300 gel filtration columns (GE Healthcare).

2.6. Flow cytometry

Monoclonal antibodies were conjugated to their fluorophores using LYNX conjugation kits (Bio-Rad). Biotinylated antibodies were prepared using the EZ link biotinylation kit (Thermo Fisher). Wild type P815, botaKIR2DL1-FLAG-P815, botaKIR3DXL1-FLAG-P815 cell lines, and bovine PBMCs were stained with AA7-DL488 (0.5 μg/ml), BG11-PE (2.5 μg/ml), and live-dead near IR viability stain (1/2000). Expression of the botaKIR-FLAG protein was validated through staining with anti-FLAG (M2 40 μg/ml; Sigma-Aldrich). PBMCs were additionally stained for subset markers using MM1A-APC (anti-CD3; 2 μg/ml) and AKS1-biotin (anti-NKP46; 4 μg/ml), or CCG33-APC (anti-CD14; 1 μg/ml) and IL-

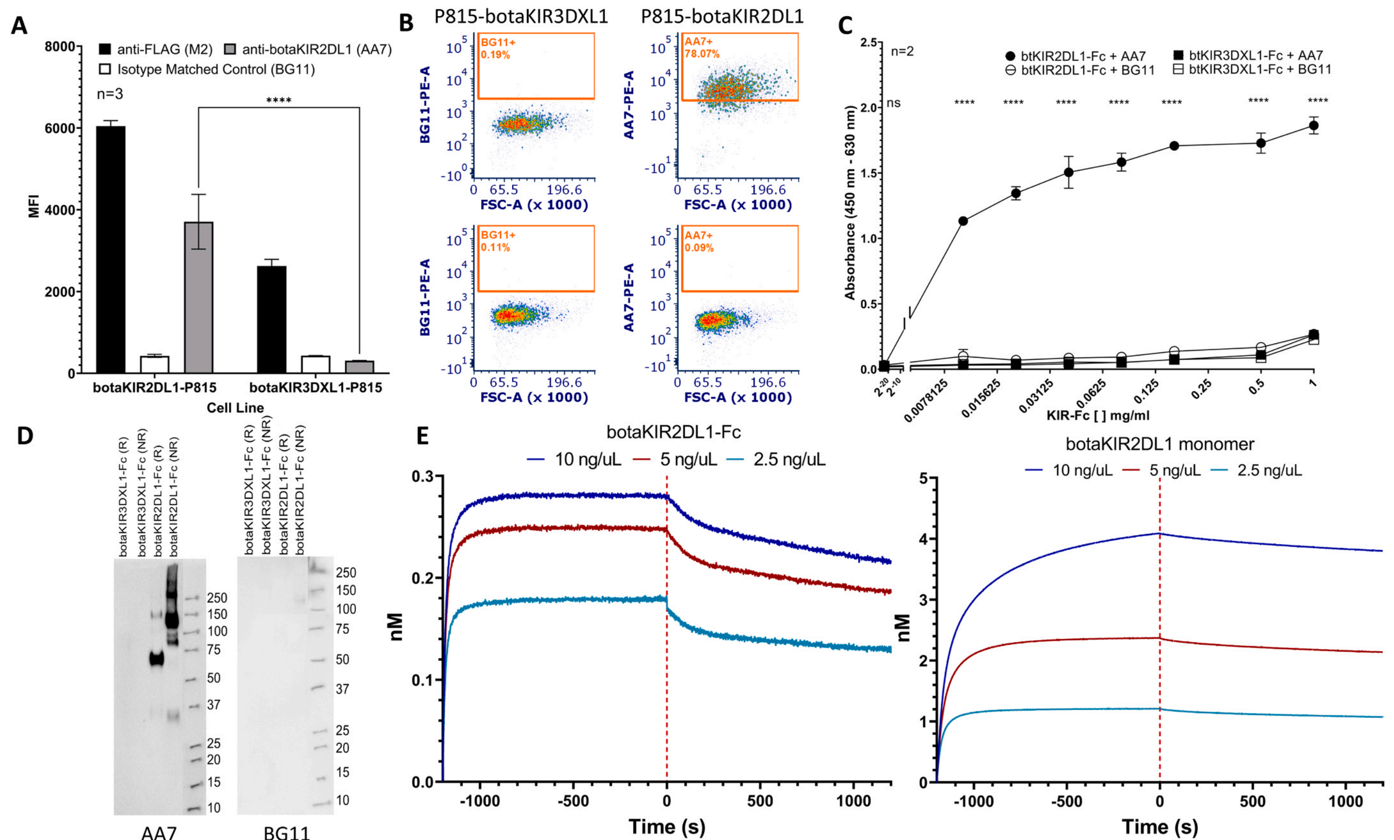

Fig. 1. AA7 is a multipurpose antibody specific to botaKIR2DL1 (A) Flow cytometry analysis of botaKIR2DL1 and botaKIR3DXL1 transfected P815 cell lines. The fluorophore MFI for AA7-PE (grey), BG11-PE (white), and M2-PE (black) are shown. Both cell lines were positive for the expression of the FLAG tagged protein. AA7 staining was significantly higher in botaKIR2DL1$^+$ P815 cells compared to botaKIR3DXL1$^+$ P815 cells. Statistical analysis performed using 2-way ANOVA with multiple comparisons in GraphPad Prism v9. * ** * = $p < 0.0001$. N = 3. (B) Representative flow cytometry plots of botaKIR2DL1 and botaKIR3DXL1 P815 cell lines stained with AA7-PE or BG11-PE. (C) ELISA of anti-botaKIR2DL1 (AA7; black shapes) and isotype matched irrelevant control (BG11; open shapes) antibodies detecting botaKIR2DL1-Fc (circle) or another cattle KIR (botaKIR3DXL1-Fc; square) protein. Absorbance at 450 nm, with background absorbance at 630 nm subtracted, is shown. Statistical analysis performed using two way ANOVA with multiple comparisons in GraphPad Prism v9 * ** * = $p < 0.0001$, n = 2. (D) Western blot of botaKIR2DL1-Fc and botaKIR3DXL1-Fc proteins using AA7 or BG11. Bands at approximately 65 kDa and 130 kDa for reducing (R) and non-reducing (NR) are visible when botaKIR2DL1-Fc is detected by AA7. No detection is observed of botaKIR3DXL1-Fc, and neither KIR-Fc protein was detected using the isotype matched irrelevant control antibody (BG11). (E and F) Biolayer interferometry association and dissociation data for AA7 binding to sensors coated with E) 10, 5 or 2.5 ng/uL of botaKIR2DL1-Fc, or F) 10, 5, or 2.5 ng/uL of botaKIR2DL1-monomer. Both indicate a concentration dependent association and dissociation of botaKIR2DL1 with AA7. The background interference from uncoated sensors was subtracted prior to analysis.

A158-biotin (anti-CD40; 1 μg/ml). Biotinylated antibodies were detected using streptavidin-BV421 (0.1 μg/ml). Data was analysed in FCS express version 6 and graphs generated in GraphPad Prism version 9. Statistical analysis was performed using a t-test or two-way ANOVA as appropriate.

2.7. ELISA

MaxiSorp ELISA plates (ThermoFisher Scientific) were coated with botaKIR2DL1-Fc, botaKIR3DXL1-Fc, or chicken DC-SIGN-Fc, in PBS and blocked overnight with 3 % w/v milk. Detection was achieved using 40 μg/ml of AA7, BG11 or IL-A88 (anti-BoLA-I), and a goat-anti-mouse HRP secondary antibody (1/50,000 of stock), followed by TMB detection reagent and ELISA stop (Thermo Scientific). Absorbance at 450 nm was determined using the Tecan Infinite 200 PRO. Background absorbance at 630 nm was subtracted. Graphs were generated in GraphPad Prism version 9. Statistical analysis was performed using a t-test or two-way ANOVA as appropriate.

2.8. SDS-PAGE and western blot

Native samples were prepared for SDS-PAGE by adding non-reducing loading dye. Reduced samples were prepared by adding loading dye containing NuPAGE sample reducing agent (Invitrogen) and boiling. Samples were separated on a 4–12 % Bis-Tris gel (Invitrogen) at 180 V for 35 min and stained using Instant Blue (Abcam).

Western blotting was performed using the iBlot2 (Life Technologies) with nitrocellulose membrane stacks (Invitrogen). Transfer was achieved using 20 V for 1 min, 23 V for 6 min, and 25 V for 2 min. Blots were blocked using 5 % w/v milk in phosphate buffered saline (PBS) containing 0.02 % v/v Tween 20 (Sigma-Aldrich). AA7, BG11, or anti-FLAG (M2) primary antibodies were incubated on blot overnight at a concentration of 1 μg/ml. Primary antibodies were detected using anti-mouse IgG-HRP (Bio-Rad) at 0.04 μg/ml, and ECL Prime detection reagent (Amersham).

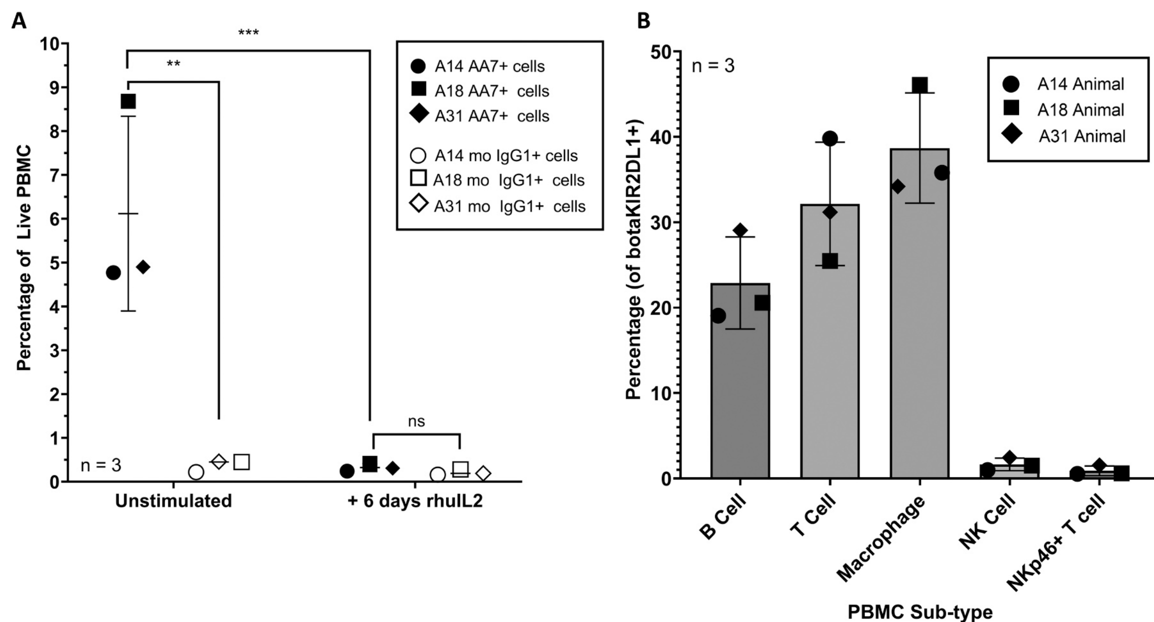

Fig. 2. Cell surface expression of botaKIR2DL1 in bovine PBMC (A) Flow cytometry analysis of PBMC stained with anti-botaKIR2DL1 (AA7) or a mouse IgG1 isotype control. AA7 staining was observed at significantly higher levels than that of the isotype control indicating specific staining of cattle PBMC by AA7. Mean percentage of live cells which are AA7$^+$ is shown ± standard deviation. Individual data points for each animal are also shown. Statistical analysis performed using two-way ANOVA with multiple comparisons in Graphpad Prism v9 * * = $p < 0.01$, * ** = $p < 0.001$. (B) Mean percentage of AA7$^+$ cells which are B cells, T cells, macrophages, and NK cells ± standard deviation. Individual data points for each animal are shown. Only data for the unstimulated AA7$^+$ cells is shown as all other stains/conditions were negative.

2.9. Bio-layer interference

Bio-layer interference assays were performed on the Octet RED96e (Sartorius). Streptavidin sensors were coated with biotinylated botaKIR2DL1 monomers, botaKIR2DL1-Fc, or an irrelevant Fc-isotype matched chicken-DC-SIGN-Fc control. Coated sensors were incubated in AA7 or BG11 to measure association kinetics, then in HEPES-buffered saline (Lonza) to measure dissociation kinetics. Background subtraction of non-coated streptavidin sensors was performed prior to calculating reaction kinetics.

3. Results and discussion

3.1. The monoclonal antibody AA7 strongly and specifically recognises botaKIR2DL1

Antibodies remain invaluable reagents to study complex immune responses but their availability for non-model species such as cattle continues to present a challenge in veterinary research (Entrican et al., 2020). Moreover, generating antibodies specific for individual receptors encoded within duplicated and diversified gene families such as *KIR*, presents an even greater challenge due to the presence of shared epitopes between highly similar genes that are species specific (i.e. no true orthologue even in closely related species).

To identify candidate antibodies for further validation, supernatants from hybridoma clones were screened by flow cytometry against wild-type and botaKIR2DL1 expressing P815 cells (data not shown). AA7 and BG11 were selected as positive and negative clones respectively and taken forward for further validation. To confirm the specificity of AA7 for botaKIR2DL1, purified antibody was labelled and used to stain botaKIR2DL1-FLAG and botaKIR3DXL1-FLAG (a cattle KIR from the expanded DX-lineage) expressing P815 cells. Antibody AA7 stained the botaKIR2DL1-FLAG$^+$ but not the botaKIR3DXL1-FLAG$^+$ cell line, BG11 did not stain either cell line (Fig. 1A&B). After confirming specificity, the utility of AA7 for ELISA and western blot was assessed using purified botaKIR2DL1-Fc protein. In an ELISA, AA7 recognised botaKIR2DL1-Fc in a concentration dependent manner (Fig. 1C). In a western blot, AA7 detected a protein of approximately 65 kDa under reducing conditions and 130 kDa under non-reducing conditions indicating the KIR-Fc is dimeric in its native form (Fig. 1D). The expected size of the reduced botaKIR2DL1-Fc monomer without post-translational modification was calculated as 51 kDa, therefore this result is consistent with predicted glycosylation based on demonstrated N-linked glycosylation mass of human KIR (VandenBussche et al., 2009). Finally, biolayer interferometry was used to establish the avidity of AA7 to botaKIR2DL1. Sensors were coated with botaKIR2DL1-Fc, botaKIR2DL1-monomer, or an irrelevant and Fc-isotype matched chicken-DC-SIGN-Fc. AA7 specifically and strongly bound both monomeric and dimeric botaKIR2DL1, with K_{dis} values of1.327E-05 and 2.437e-04 respectively, while the chicken-DC-SIGN-Fc response was below biological significance (K_{dis} 4.924E-01) (Fig. 1E&F). The dimeric nature of the botaKIR2DL1-Fc, although a useful reagent, likely creates some steric hindrance to antibody binding resulting in the faster dissociation compared to the receptors native monomeric conformation. In combination, the results demonstrate that AA7 is a multipurpose antibody that strongly and specifically recognises botaKIR2DL1. Importantly, it has also been demonstrated that AA7 is unlikely to bind closely related Ig-like receptors, such as 3DX lineage KIR, which are known to be transcribed by bovine NK cells (Allan et al., 2015) As such, AA7 represents a valuable resource for the further study of botaKIR2DL1 in the cattle immune response.

3.2. Expression of botaKIR2DL1 is not restricted to NK cells and T cells

Simian KIR expression is restricted to NK cells and subpopulations of CD4$^+$, CD8$^+$, and γδ T cells (Anfossi et al., 2004; Hermes et al., 2013; Uhrberg et al., 2001; van Bergen et al., 2009; Vilches and Parham, 2002; Young et al., 2001). However, it is not yet known whether cattle KIR perform the same immunological function as their human homologues. Similar botaKIR2DL1 expression on leukocyte subsets would indicate potential functional homology. AA7 staining of cattle PBMC subsets confirmed botaKIR2DL1 expression on NK cells (NKP46$^+$) and T cells

(CD3$^+$) including a sub-population of NKp46$^+$ T cells (Connelley et al., 2014). The low level of botaKIR2DL1protein expression observed on bovine NK cells is not unexpected, as human and primate studies have shown that KIR are typically low abundance at the protein and transcript level. However, macrophages (CD14$^+$) and B cells (CD40$^+$) also, unexpectedly, represented a large proportion of botaKIR2DL1$^+$ PBMC (Fig. 2B, Supplementary Fig. 1). As such, cattle botaKIR2DL1 has an overlapping expression profile with primate KIR but is clearly less restricted to NK and T cells. This observation was largely consistent across multiple replicates of the three animals with different MHC class I haplotypes (A18, A31 and A14) which adds confidence to the flow cytometry staining performed. A wider study incorporating larger numbers of animals and species would be beneficial in future to determine the consistency of this observation in larger numbers and in different cattle breeds.

Preliminary results as part of other studies using gene expression data from bovine PBMC subsets partially corroborate this observation, with transcripts for botaKIR2DL1 observed in NK cells and T cells with a low level transcription of some botaKIR3DX receptors observed on B cells and monocytes. However, this gene expression data did not originate from the same animals or conditions and is therefore not directly comparable to the present study. Considering the high levels of identity between cattle KIR transcript sequences, low level of transcription and a known lack of correlation between transcription and cell surface expression, a larger and more targeted study is required to confirm exquisite specificity. Therefore, it must still be considered possible that antibody AA7 cross reacts with another KIR or very closely related receptor, despite not binding to BotaKIR3DXL1.

Stimulating the PBMC with rhuIL-2 for six days (known to drive a type-I IFN response in cattle NK cell and T cells (Allan et al., 2015; Connelley et al., 2014)) resulted in the absence of significant AA7 staining on all PBMC subsets (Fig. 2A, Supplementary Fig. 1). This is in contrast to human KIR, where culture of NK cells with IL-2 increased expression of some inhibitory DL-lineage KIR and is hypothesised to perform an immunoregulatory function as part of the type-I IFN response to infection (Chrul et al., 2006; de Rham et al., 2007). The observation of downregulation in cattle suggests that botaKIR2DL1 does not play a direct role in regulating this arm of the cattle immune system.

The overlapping but contrasting expression patterns between botaKIR2DL1 and primate KIR may indicate different functions, and perhaps ligands other than MHC-I, as is observed with other receptors also encoded within the leukocyte receptor complex. For example, the leukocyte immunoglobulin-like receptors (LILR) are an expanded family of LRC receptors which are expressed on NK cells, T cells, B cells, and macrophages (Katz, 2006) and bind to a diverse range of endogenous and exogenous ligands (Burshtyn and Morcos, 2016). As such, botaKIR2DL1 expression on multiple leukocyte subsets may indicate that bovine KIR have evolved to perform a function more analogous to that of LILR than that of simian KIR. If DL-lineage KIR have diverged functionally and do not bind MHC-I in cattle, the role of DX-lineage KIR and the expanded KLR and KLRD families need to be better understood.

4. Conclusion

AA7 is a versatile monoclonal antibody that strongly and specifically recognises the cattle botaKIR2DL1 receptor, and not related botaKIR3DXL1. Application of this antibody to the study of botaKIR2DL1 expression by flow cytometry of bovine PBMC indicates that cell surface expression is not restricted to NK and T cells and its expression decreases upon stimulation with rhuIL-2. These results contrast with primate KIR expression patterns and indicate that botaKIR2DL1 likely has an alternative, and perhaps less specific, function within the bovine immune system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2023.110646.

Details and availability of AA7 can be found on the Immunological Toolbox website https://www.immunologicaltoolbox.co.uk/antibody/ITB02145

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