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Flow cytometry analysis of NK cells

Cross-reactivity of KIR antibodies

Cross-reactivity of killer cell immunoglobulin-like receptor (KIR) antibodies

Angelique Hölzemer, Maja Ziegler, and Sebastian Lunemann
Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

Introduction

Killer cell immunoglobulin–like receptors (KIRs) are type 1 transmembrane receptors expressed on natural killer cells (NK cells) and T cells. They consist of an extracellular domain, a transmembrane domain, and a cytoplasmic tail. The conventional classification is based on these structural features. The extracellular domains of KIRs consist of either two or three immunoglobulin (Ig)-like domains, hence the denotation 2D or 3D in the KIR nomenclature. The length of the cytoplasmic domain is reflected by an L for long or S for short. Depending on their structure, KIRs can either be inhibitory or activating. Inhibitory KIRs carry a long cytoplasmic tail, which contains one or two immunoreceptor tyrosine-based inhibitory motif(s) (ITIM). An exception to this is KIR2DL4, which contains an ITIM in its cytoplasmic tail, but also can associate with the activating Fc receptor gamma. Activating KIRs carry a charged amino acid in the transmembrane domain, which allows for association with the accessory molecule DAP-12, mediating activating signaling through its immunoreceptor tyrosine-based activating motif (ITAM). Expression of activating and inhibitory KIRs on NK cells is stochastic and can be tuned by the host's genetic environment. Differential expression of KIRs is associated with different outcomes in various diseases, such as viral infections or autoimmunity, or after transplantation.[1,2]

Flow cytometric assessment of KIR$^+$ NK cell populations is an important technique in NK cell research. However, because of the high sequence homology between KIRs, antibody cross-reactivity can pose a major obstacle for the unambiguous identification of KIR surface expression on NK cells. To shed light upon antibody cross-reactivity, we tested different KIR antibodies provided by Miltenyi Biotec for their capacity to stain Jurkat cell lines transduced with various types of KIR.

Methods

Generation of cell lines expressing a single type of KIR

Jurkat cells (clone E6.1; ATCC; b2m-deficient through previous CRISPR/Cas9-mediated gene knockout) were transduced using lentiviral vectors to achieve stable expression of single KIR types.[3] KIR chimeric constructs (KIRζ) were obtained from GeneArt® Gene Synthesis service (Thermo Fisher Scientific) based on the protein sequence of the KIR of interest and cloned into a transfer plasmid (pLVX-SFFV-IRES-Puro, kindly provided by Thomas Pertel, Harvard Medical School, Boston, MA, USA). Gene expression was under the control of an SFFV promoter, and puromycin resistance was IRES-driven. Lentiviral particles were harvested on day 3 following lipofection of 293T cells (ATCC) with the transfer plasmid, a lentiviral packaging plasmid (psPAX2, NIH AIDS Reagent Program), and a VSV-G envelope plasmid (pHEF-VSV-G, NIH AIDS Reagent Program). Transduced Jurkat cells were selected in 1 µg/mL of puromycin.

KIRζ were constructed by fusing the extracellular and transmembrane domain of inhibitory KIR (KIR3DL1, KIR2DL3, KIR2DL2, and KIR2DL1) to the cytoplasmic tail of the activating CD3ζ-chain. To ensure surface expression of activating KIR independent of the adaptor DAP12, the extracellular domains of KIR3DS1 and KIR2DS4 were fused to the transmembrane domain of KIR3DL1, whereas the extracellular domain of KIR2DS1 was fused to the transmembrane domain of KIR2DL1. All constructs contain the CD3ζ-chain as cytoplasmic tail (table 1).

Construct	Protein
KIR2DS4(ECD)-KIR3DL1(TMD)-ζ	KIR2DS4*001
KIR3DL2ζ	KIR3DL2*001
KIR2DL3ζ	KIR2DL3*001
KIR3DS1(ECD)-KIR3DL1(TMD)-ζ	KIR3DS1*013
KIR3DL1ζ	KIR3DL1*001
KIR2DL1ζ	KIR2DL1*001
KIR2DS1(ECD)-KIR2DL1(TMD)-ζ	KIR2DS1*002

Table 1: Constructs used for expression of KIRs in Jurkat cells. The sequence for the respective KIR is based on the indicated protein. All constructs were subjected to mammalian gene optimization. ECD: extracellular domain; TMD: transmembrane domain.

Staining of KIR-expressing cell lines

One million cells were washed once in PBS/2% FBS. The cells were then stained with 5 µL of KIR antibody (Miltenyi Biotec) in a total volume of 50 µL and incubated in the dark at 4 °C for 20 min. Afterwards the cells were washed twice with PBS/2% FBS, fixed with PBS/4% paraformaldehyde for 10 min at 4 °C, and analyzed by flow cytometry.

Results

Cell lines transduced with single KIR constructs were stained with different KIR antibodies. As shown in table 2, the cross-reactivity of each antibody varies. All constructs used the same promoter and an inhibitory transmembrane domain to ensure high KIR surface expression. However, when analyzing differences in antibody staining of the distinct KIRs, it should be noted that differences in KIR receptor surface levels between the cell lines may affect the degree of staining. Therefore, cross-reactivity for each antibody can be assessed, but median fluorescence intensity of KIR staining does not fully translate to differences in antibody affinity.

Whereas certain KIR antibodies display a low degree of cross-reactivity (e.g. CD158e1/e2 (KIR3DL1/DS1), CD158b2 (KIR2DL3), CD158b (KIR2DL2/DL3), CD158e (KIR3DL1), and CD158a (KIR2DL1) against the available cell lines, others, in particular CD158e/k (KIR3DL1/DL2; clone REA970) and CD158i (KIR2DS4; clone REA860) display cross-reactivity with other activating KIRs (table 2). This has been partly described before.[4] Of note, KIR3DS1 staining was detected after fixation due to high levels of KIR3DS1 surface expression on Jurkat cells. When using primary samples, fixation can impact the ability of this antibody to detect KIR3DS1 on the cell surface. Unfortunately, KIR2DL2–, KIR2DL5–, KIR2DL4–, and KIR2DS2-expressing cell lines were not available for testing, which is why cross-reactivities with these KIRs could not be assessed here. Examples of plots for the CD158a (KIR2DL1) antibody are presented in figure 1.

Cell line	Anti-KIR2D	CD158a (KIR2DL1)	CD158a/h (KIR2DL1/DS1)	CD158b2 (KIR2DL3)	CD158b (KIR2DL2/DL3)	CD158d (KIR2DL4)	CD158f (KIR2DL5)	CD158i (KIR2DS4)	CD158e (KIR3DL1)	CD158e/k (KIR3DL1/DL2)	CD158e1/e2 (KIR3DL1/DS1)
Jurkat (non-transduced)											
Jurkat KIR2DS1	+++	+	+++					++		++	
Jurkat KIR2DL1	++	++	++								
Jurkat KIR2DL3	+++			+++	+++						
Jurkat KIR2DS4	+++							+++		++	
Jurkat KIR3DS1										+	+
Jurkat KIR3DL1									++	+	++
Jurkat KIR3DL2										+	

Table 2: Jurkat cell lines transduced with different KIR constructs were stained with different KIR antibodies. The plus signs correspond to the following increases in median fluorescence intensity (MFI): + 2–10-fold, ++ 10–100-fold, and +++ >100-fold increase. The increase in MFI was calculated using the non-transduced Jurkat parental cell line as reference. Antibody clones used in this experiment were as follows: KIR2D (clone REA1042), KIR2DL1 (clone REA284), KIR2DL1/DS1 (clone REA1010), KIR2DL2/DL3 (clone REA1006), KIR2DL3 (clone REA147), KIR2DL4 (clone REA768), KIR2DL5 (clone REA955), KIR2DS4 (clone REA860), KIR3DL1 (clone REA1005), KIR3DL1/DL2 (clone REA970), and KIR3DL1/DS1 (clone REA168).

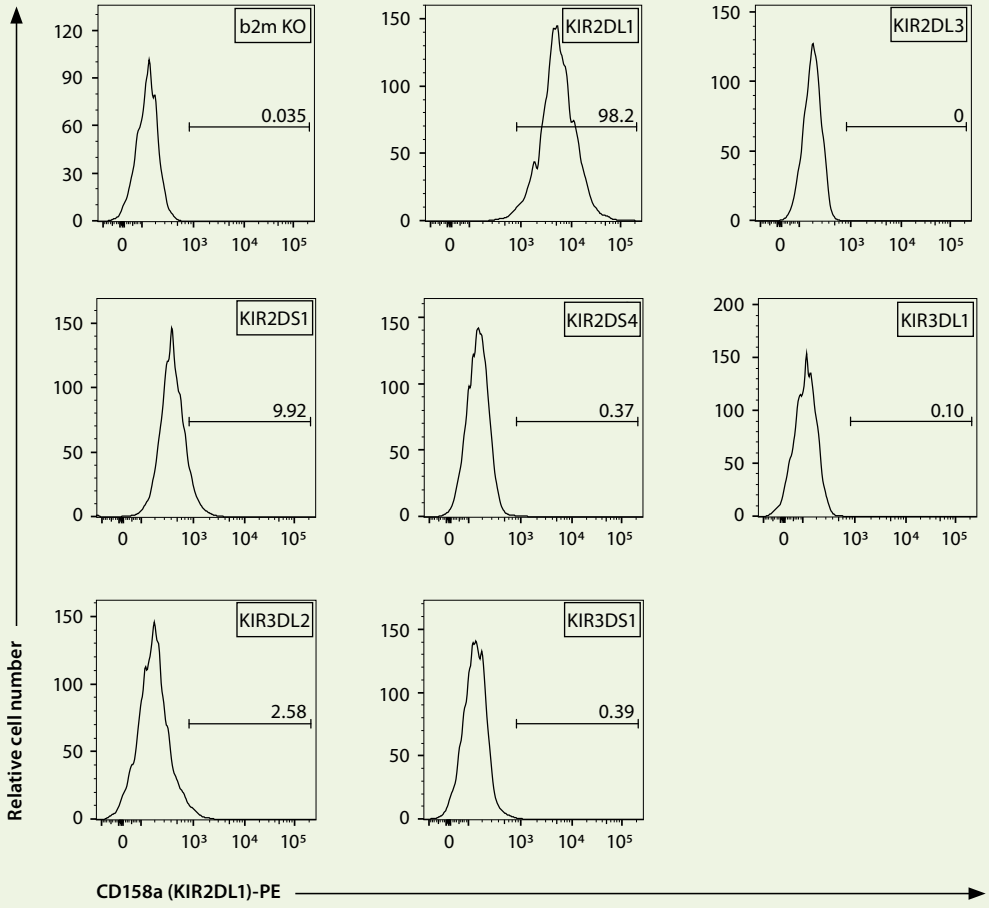

Figure 1: KIR2DL1 antibody staining of Jurkat cell lines transduced with different KIR constructs. The type of KIR expressed by the respective cell line is indicated in the box within the histogram. Cell lines were stained with CD158a (KIR2DL1)-PE and analyzed by flow cytometry. Numbers within the histograms specify percentages of cells detected by the antibody.

MACS® Antibodies used in this study

Antibody	Clone	Fluorochrome
Anti-KIR2D	REA1042	PE
CD158a (KIR2DL1)	REA284	PE
CD158a/h (KIR2DL1/DS1)	REA1010	PE
CD158b (KIR2DL2/DL3)	REA1006	PE
CD158b2 (KIR2DL3)	REA147	PE
CD158d (KIR2DL4)	REA768	PE
CD158f (KIR2DL5)	REA955	PE
CD158i (KIR2DS4)	REA860	PE
CD158e (KIR3DL1)	REA1005	PE
CD158e/k (KIR3DL1/DL2)	REA970	PE
CD158e1/e2 (KIR3DL1/DS1)	REA168	PE

These antibodies are available conjugated to a wide range of other fluorochromes. For more information on MACS Antibodies visit **www.miltenyibiotec.com/antibodies**.

Conclusions

- We have examined cross-reactivity of KIR antibodies against Jurkat cell lines engineered to express high levels of single KIR constructs.
- MACS® Antibodies tested in this study enable the reliable detection of KIRs on NK cells and vary in their degree of cross-reactive binding to the KIRs tested. The knowledge of cross-reactivities will be helpful to scientists to achieve a meaningful analysis and evaluation of KIR expression on NK cells.

References

1. Carrington, M. and Norman, P. (2003) The KIR gene cluster. National Center for Biotechnology Information (US), Bethesda, MD. Available at www.ncbi.nlm.nih.gov/books/NBK10135/ (last accessed October 09, 2018).
2. Jost, S. and Altfeld, M. (2013) Control of human viral infections by natural killer cells. Annu. Rev. Immunol. 31: 163–194.
3. Garcia-Beltran, W.F. *et al.* (2016) Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. Nat. Immunol. 17: 1067–1074.
4. Czaja, K. *et al.* (2014) A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs. Genes Immun. 15: 33–37.

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Miltenyi Biotec GmbH | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macs@miltenyibiotec.de | **www.miltenyibiotec.com**

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