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
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).

Flow cytometry analysis of NK cells

Cross-reactivity of KIR antibodies

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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 motifs (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.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Protein</th>
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<td>KIR2DL3ζ</td>
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<tr>
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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.

<table>
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<tr>
<th>Cell line</th>
<th>Anti-KIR2D</th>
<th>CD158a (KIR2DL1)</th>
<th>CD158a/h (KIR2DL1/DS1)</th>
<th>CD158b2 (KIR2DL3)</th>
<th>CD158b (KIR2DL2/DL3)</th>
<th>CD158d (KIR2DL4)</th>
<th>CD158f (KIR2DL5)</th>
<th>CD158i (KIR2DS4)</th>
<th>CD158e (KIR3DL1)</th>
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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 REA100), KIR2DL2/DL3 (clone REA106), KIR2DL3 (clone REA147), KIR2DL4 (clone REA168), KIR2DL5 (clone REA955), KIR2DS4 (clone REA860), KIR3DL1 (clone REA1005), KIR3DL1/DL2 (clone REA970), and KIR3DL1/DS1 (clone REA168).
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.

MACS® Antibodies used in this study

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<th>Antibody</th>
<th>Clone</th>
<th>Fluorochrome</th>
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<td>Anti-KIR2D</td>
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These antibodies are available conjugated to a wide range of other fluorochromes. For more information on MACS Antibodies visit [www.miltenyibiotec.com/antibodies](http://www.miltenyibiotec.com/antibodies).

References


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.