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Two flow cytometric approaches of NKG2D ligand surface detection to distinguish stem cells from bulk subpopulations in acute myeloid leukemia

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TITLE:

Two Flow Cytometric Approaches of NKG2D Ligand Surface Detection to Distinguish Stem Cells from Bulk Subpopulations in Acute Myeloid Leukemia

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NKG2DL, CD34, AML, LSC, fusion protein, FACS

SUMMARY:

We present two different staining protocols for NKG2D ligand (NKG2DL) detection in human primary acute myeloid leukemia (AML) samples. The first approach is based on a fusion protein, able to recognize all known and potentially yet unknown ligands, while the second protocol relies on the addition of multiple anti-NKG2DL antibodies.

ABSTRACT:

Within the same patient, absence of NKG2D ligand (NKG2DL) surface expression was shown to distinguish leukemic subpopulations with stem cell properties (so called leukemic stem cells, LSCs) from more differentiated counterpart leukemic cells that lack disease initiation potential although they carry similar leukemia specific genetic mutations. NKG2DL are biochemically highly diverse MHC class I-like self-molecules. Healthy cells in homeostatic conditions generally do not express NKG2DL on the cell surface. Instead, expression of these ligands is induced upon exposure to cellular stress (e.g., oncogenic transformation or infectious stimuli) to trigger elimination of damaged cells via lysis through NKG2D-receptor-expressing immune cells such as

natural killer (NK) cells. Interestingly, NKG2DL surface expression is selectively suppressed in LSC subpopulations, allowing these cells to evade NKG2D-mediated immune surveillance. Here, we present a side-by-side analysis of two different flow cytometry methods that allow the investigation of NKG2DL surface expression on cancer cells i.e., a method involving pan-ligand recognition and a method involving staining with multiple antibodies against single ligands. These methods can be used to separate viable NKG2DL negative cellular subpopulations with putative cancer stem cell properties from NKG2DL positive bulk leukemic cells.

INTRODUCTION:

NK cells are important effectors of the innate immune system that can recognize and eliminate malignant cells or stressed healthy cells (e.g., by a viral infection) without prior antigen stimulation[1]. This process is tightly regulated via a complex repertoire of activating receptors—such as natural cytotoxicity receptors (NCRs), NKG2D and CD16—and inhibitory receptors that are largely represented by killer immunoglobulin-like receptors (KIRs)[2]. Binding of KIRs to human leukocyte antigen (HLA) class I molecules on somatic cells ensures self-recognition and conveys NK cell tolerance. On the other hand, absence of self-recognition and increased binding of activating receptors to their ligands on the target cells trigger the release of cytotoxic granules leading to NK cell-mediated cytotoxicity[1]. Finally, NK cells can exert antibody-dependent cellular cytotoxicity (ADCC) by binding of the activating receptor CD16 to targets expressing the Fc portion of Ig (FcR)[2]. Apart from direct cytotoxicity, NK cells can also trigger cytokine release bridging the innate with the adaptive immune system[3].

NKG2D is a major activating receptor expressed on NK, NKT, γδ T, and naïve CD8+ T cells[4] that enables such cytotoxic immune cells to recognize and lyse NKG2D ligand (NKG2DL) expressing target cells. Healthy cells commonly do not express NKG2DL. Instead, NKG2DL expression is upregulated on malignant or virus-infected cells to make these amenable to immune clearance[5].

The NKG2DL family comprises eight known molecules among which the two MHC I chain-related molecules A and B (MICA and MICB[6]) and the cytomegalovirus UL16-binding proteins 1–6 (ULBP1-6[7]). The expression of NKG2DL is regulated on the transcriptional, post-transcriptional as well as the post-translational levels[8]. As such, while NKG2DL expression is commonly not detectable on the surface of healthy cells, NKG2DL mRNA[9] and intracellular protein expression were reported in healthy tissues. The functional relevance of such expression and the mechanisms underlying such discrepant expression patterns remain to be defined[10].

The mechanistic regulation of NKG2DL expression in the cancer cells is a fascinating area of investigation. Pathways known to be involved in either cellular stress, e.g., the heat shock stress pathway[9], or DNA damage-associated pathways, such as the ataxia telangiectasia mutated (ATM) and Rad3 related (ATR) pathway[11], as well as viral or bacterial infections have been directly linked to the induction of NKG2DL expression[12]. However, even if surface expression of NKG2DL has been effectively induced, this expression can be lost again through proteolytic-mediated shedding, a mechanism associated with immune escape and poor clinical prognosis in some cancers[13].

The absence of cell surface NKG2DL may also play important roles in patients with AML. Here, treatment with intensive chemotherapy often induces remission, but relapse often occurs from leukemic stem cells (LSC), which selectively survive chemotherapies and evade immune response. As we recently showed, LSCs, for example, escape NK cell lysis by suppressing NKG2DL surface expression[14].

Inversely, the absence of surface NKG2DL expression can be used as a method to identify and viably isolate putative stem-like subpopulations of cells from bulk counterpart leukemic subpopulations. Here, we present two flow cytometric approaches that can be used to detect NKG2DL surface expression and thereby identify NKG2DL negative stem cells in leukemia and perhaps also in other cancers: A method for pan-ligand surface recognition and a method involving staining with single or pooled antibodies recognizing individual known NKG2DL proteins.

PROTOCOL:

Patient samples were collected following approval from the Ethics Review Board of the University Hospitals of Basel and Tuebingen.

1. Biotinylation of the NKG2D fusion protein

NOTE: This step is performed with a biotinylation kit (see **Table of Materials**) according to the manufacturer's instruction. This step of the protocol must be performed at least 24 h prior to the staining. The biotinylated NKG2D fusion protein should be stored at -20 °C.

1.1. Thaw both the biotin, as well as the NKG2D fusion protein tubes at room temperature (RT).

NOTE: If the cells need to be sterile for further experimental use (injection in mice, colony forming unit assays, etc.), reconstitute the fusion protein under a laminar flow hood to avoid contamination. Otherwise, each step can be performed on a bench.

1.2. Quickly spin down the NKG2D-Fc powder. Add 500 µL of phosphate-buffered saline (PBS) to reconstitute the powder and mix thoroughly using a P1000 micropipette.

1.3. Add 100 µL of the NKG2DL fusion protein to a biotin tube to obtain a final concentration of 10 µg/mL.

1.4. Mix the solution thoroughly by continuous resuspending with a P100 micropipette.

1.5. Incubate the antibody/biotin solution at a controlled RT for 24 h. The fusion protein is now ready to use.

2. Thawing of primary AML cells

NOTE: Approximately 5,000,000 frozen leukemic cells per patient stored in liquid nitrogen were thawed and then used for the assays right away. Leukemic cells were obtained and frozen as previously described[15]. Briefly, peripheral blood samples collected from patients with AML and high blast cell percentages (>90% of blasts among mononuclear cells) were processed with a density gradient separation to obtain mononuclear cells and subsequently frozen in fetal calf serum (FCS) containing 10% dimethyl sulfoxide solution (DMSO). Cell numbers highly varied between patients in dependence on the leukocyte concentration in the patient (range: 1,000,000 to 30,000,000 leukemic cells per mL blood).

2.1. Pre-warm RPMI medium containing 10% of FCS at 37 °C using a water bath. For each vial of AML cells (up to 30,000,000 cells in 1 mL FCS + 10% DMSO), add 10 mL of pre-warmed medium to a 15 mL reaction tube.

2.2. Remove the cryovial containing primary AML from the liquid nitrogen storage and immediately thaw the cells using a 37 °C water bath. Gently move the tube back and forth in the water, allowing the contents of the vial to thaw until there is only a small ice crystal left.

2.3. Immediately transfer the thawed cells into the medium-containing tube and rinse the vial using 1 mL of medium.

2.4. Centrifuge the cells at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

2.5. Wash the cells with 5 mL of RPMI medium containing 10% of FCS and centrifuge at 300 *x g* for 10 min.

3. Cell counting

3.1. Resuspend the cell pellet in a known volume of RPMI medium containing 10% of FCS.

3.2. Transfer a small volume of the cell suspension to a 1.5 mL microcentrifuge tube and dilute at a known ratio with trypan blue or any other alternative dye that allows live cell/dead cell discrimination.

3.3. Use any device to count the cells.

3.4. Centrifuge the cells at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

4. Staining of primary AML cells using the biotinylated NKG2D fusion protein

4.1. Prepare the staining buffer by supplementing 500 mL PBS with bovine serum albumin (BSA) and Ethylenediaminetetraacetic acid (EDTA) to a final concentration of 0.07 mM and 2 mM, respectively. Adjust the pH (7.2–7.4) if necessary.

4.2. Resuspend the cell pellet with staining buffer to a final concentration of 0.5 x 10^7 cells/mL.

NOTE: Optionally, prior to staining, the cells can be incubated with an FcR-blocking agent.

4.3. Transfer 100 µL of the cell suspension to a cell culture 96-well U-bottom plate and centrifuge the plate at 300 *x g* for 10 min. Discard the supernatant without disturbing the pellet.

4.4. Prepare a master mix of biotinylated NKG2D fusion protein so that cells are resuspended in a final volume of 50 µL per well with a final concentration of 10 µg/mL per well.

4.5. Add the master mix prepared in step 4.4 using a 100 µL pipette and resuspend the cell pellets with a 300 µL multichannel pipette.

NOTE: Scale down the final volume according to the number of cells to reduce the amount of fusion protein necessary for the staining.

4.6. Incubate the cell suspension for 15 min at RT or 30 min at 4 °C.

4.7. Wash the cells by adding 200 µL of staining buffer per well with a 300 µL multichannel pipette.

4.8. Centrifuge the plate at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

4.9. Repeat steps 4.7 and 4.8.

NOTE: The staining described here is performed in a cell culture 96-well U-bottom plate. Therefore, washing steps are performed twice because of the small volume capacity of such plates. The staining can also be performed in other tubes and washing steps might then be performed only once using a larger volume.

4.10. Prepare a master mix using Streptavidin-PE (see **Table 1** for dilutions) so that cells are resuspended in a final volume of 50 µL.

4.11 Add the master mix prepared in step 4.10 using a 100 µL pipette and resuspend the cell pellets with a 300 µL multichannel pipette.

4.12. Incubate the cell suspensions for 15 min at RT or 30 min at 4 °C in the dark.

4.13. Wash the cells by adding 200 µL of staining buffer per well with a 300 µL multichannel pipette.

4.14. Centrifuge the plate at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

4.15. Repeat steps 4.13 and 4.14.

4.16. Prepare a solution of staining buffer including 7-AAD (7-Aminoactinomycin D, 1:1000) or any reagent to distinguish between live and dead cells.

4.17. Resuspend cell pellets in 200 µL staining buffer + 7-AAD prepared in step 4.16 in using a 300 µL multichannel micropipette.

4.18. Analyze the cells using a flow cytometry device.

5. Staining of single or pooled single anti-NKG2DL antibodies

5.1. Use the same cell suspension as in step 4.2.

5.2. Transfer 100 µL of the cell suspension to a cell culture 96-well U-bottom plate using a 300 µL multichannel pipette and centrifuge the plate at 300 *x g* for 10 min. Discard the supernatant without disturbing the pellet.

5.3. Prepare an antibody master mix for each primary antibody or pooled antibodies (see **Table 2** for dilutions) so that the cells are resuspended in a final volume of 50 µL.

5.4. Add the master mix prepared in step 5.3 using a 100 µL pipette and resuspend the cell pellets with a 300 µL multichannel pipette.

5.5. Incubate the cells for 25 min at RT.

5.6. Wash the cells by adding 200 µL of staining buffer per well using a 300 µL multichannel pipette.

5.7. Centrifuge the cell culture 96-well U-bottom plate at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

5.8. Repeat steps 5.6 and 5.7.

5.9. Prepare an antibody master mix by adding the secondary antibody (see **Table 2** for dilutions) so that the cells are resuspended in a final volume of 50 µL per well.

5.10. Add the master mix prepared in step 5.9 using a 100 µL pipette and resuspend the cell pellets with a 300 µL multichannel pipette.

5.11. Incubate for 15 min at RT or 30 min at 4 °C in the dark.

5.12. Wash by adding 200 µL of staining buffer per well using a 300 µL multichannel pipette.

5.13. Centrifuge the cell suspension at 300 *x g* for 10 min and discard the supernatant without disturbing the pellet.

5.14. Repeat steps 5.12 and 5.13.

5.15. Resuspend cell pellets in 200 µL staining buffer containing 7-AAD prepared in step 4.16.

5.16. Analyze the cells using a flow cytometry device as described in step 6.

6. Data acquisition

NOTE: Make sure that weekly quality controls of the flow cytometer are performed to ensure that lasers are functioning properly. For the protocol presented here, a weekly Cytometer and Tracking (CTS) process is performed with relative beads to check laser performances on all channels. After the CTS, 10,000 events are recorded using the 8-peak beads as an internal control. All peaks should be in the same position inside their gates and well separated from each other.

6.1. Create the matrix compensation to subtract spectral overlap between detectors with beads or with cells[16].

6.2. Record 10,000 events for the unstained cells and the single stained beads. For the fluorescence minus one (FMO) controls, record 50,000 events and for the full-stained samples record up to 100,000 events.

NOTE: Alternatively, single stained samples can be performed on the cells. If so, make sure that the cells have a positive signal for the desired marker.

6.3. Acquire a single stained cell or bead sample for each fluorophore used in the experiment to reveal the amount of spectral overlap. Use the compensation creator of the flow cytometry device to calculate spill-over values and apply the compensation matrix to all the measured samples.

NOTE: Secondary antibodies cannot be used for compensation creation using beads if the compensation is calculated with beads. Depending on the type of compensation beads, secondary antibodies cannot be used for the compensation with beads.

6.4. For the FMO controls and the full stained samples with the fusion protein, firstly, select the cells based on their forward scatter (FSC) and side scatter (SSC). After the exclusion of doublets, gate the cells based on their negativity for 7-AAD (PercP-Cy5.5) signal to exclude the dead cells

(see step 5.15). The final plot shows the expression of CD34 (Y-axis) and NKG2DL (X-axis) on living singlets.

6.5. For the single anti-NKG2DL-antibodies, apply the same strategy to the samples but note that ligand positivity lies in Alexa Fluor 488 and not the PE channel.

6.6. Adjust gates according to the FMO controls for gating strategy from step 6.4 and 6.5 (see **Table 3**) for appropriate FMO controls to perform for this experiment: In the analysis software, draw a gate on the negative fraction. While recording the full-stained sample, the cells above the previously created gate are positive for the analyzed marker.

REPRESENTATIVE RESULTS:

Both the protocols presented here allow the enrichment of AML LSC by flow cytometric analyses using CD34, a known marker of LSC[17], in combination with NKG2DL surface expression by either utilizing pan-ligand recognition or staining with pooled antibodies against individual ligands. In **Figure 1**, we show that the analyzed AML samples are positive for CD34 and NKG2DL, but negative subpopulations also exist, which is shown by the presence of four different populations in total. Our data shows the typical gating strategy for such a staining, starting with the selection of the main population of cells via their FSC and SSC. Doublets and dead cells are excluded in the downstream analysis (**Figure 1A**). Gates are adjusted using FMO controls to ensure proper identification of positive cells (**Figure 1B**).

In **Figure 1C**, we highlight the fluorescence intensities of cells positive for CD34 (APC, Y-axis) vs NKG2DL (PE, X-axis). AML cells that are positive for CD34 show a lower surface expression of NKG2DL (**Figure 1C)**, which is in line with previous findings from our laboratory, showing that NKG2DL expression is associated with lack of stemness[14].

Figure 2 indicates the robustness of both the NKG2DL staining methods. In **Figure 2A**, we investigate three primary AML samples side-by-side showing 19.8, 49.8, and 89.4%, respectively of positive events for the fusion protein staining vs 20.4, 50.4, and 90.6% for the pooled anti-NKG2DL antibody staining (pooled antibodies against MICA, MICB, ULBP1, ULBP2/5/6, and ULBP3), respectively. On the other hand, single ligand (against either MICA, MICB, ULBP1, ULBP2/5/6, or ULBP3) staining shows a range of positive events up to 92% (**Figure 2B**). This percentage varies depending on the ligand itself and the patient, for example, ULBP3 (**Figure 2B**). Of note, a single cell can express multiple NKG2DL antibodies.

When multiple anti-NKG2DL antibodies are pooled and combined into one single tube, the percentage of positive cells is comparable to the percentage of the fusion protein (**Figure 2A**). As such, both protocols work well and may provide similar results regarding NKG2DL expression on primary AML cells. In some AML, the fusion protein method may allow higher sensitivity since it can allow the recognition of further NKG2DL proteins.

FIGURE AND TABLE LEGENDS:

Figure 1: **Typical gating strategy of primary AML samples**. (**A**) Cells are first gated based on their size (X-axis) and complexity (e.g., granularity, Y-axis). Doublets are then excluded by plotting the height or width against the area for forward scatter. Finally, living cells are selected based on their negativity for PercP-Cy5.5 (7AAD, X-axis) vs their size (Y-axis). (**B**) FMO controls are used to set up the gates helping to discriminate between positive and negative populations for indicated markers. The gating strategy remains identical as depicted in **Figure 1A**. (**C**) Flow cytometry plot showing positive and negative events for CD34 (APC, Y-axis) vs NKG2DL (PE, X-axis).

Figure 2: Staining protocols comparison. (**A**) Bar graphs showing the percentage of positive events for the fusion protein vs the pooled anti-NKGD2L antibodies (n = 3 AML samples) from gated single living cells. (**B**) Bar graphs displaying the percentage of positive events for single ligand staining for all known anti-NKG2DL antibodies and pooled single ligands (see **Table of Materials** for detailed antibody information). Gating was again performed on single living cells.

Table 1: Materials

Table 2. Tubes required to stain samples with the NKG2D fusion protein. This table shows the tubes required in order to set up the experiment.

Table 3. Tubes required to stain samples with the anti-NKG2DL antibodies. This table shows the tubes required in order to set up the experiment.

Table 4. Tubes required in order to set up the essential controls. This table shows the tubes required in order to set up proper FMO, primary, and secondary antibody controls. All the required controls (FMO) must be added by the experimenter to obtain valid results and interpretable data.

DISCUSSION:

Here we present two flow cytometric methods that can detect NKG2DL surface expression on human primary AML cells. We show that both detection methods can be used in conjunction with other antibody stainings (e.g., detecting CD34 expression). Similar stainings may also be performed on other primary cell types and cell lines.

We recently showed that the absence of NKG2DL on the surface of AML patient blasts can enrich LSC[14]. In AML, NKG2DL$^-$ but not NKG2DL$^+$ leukemic subpopulations possess clonogenic and in vivo leukemia-initiating capacities. Future research will show whether absence of NKG2DL surface expression can also be used as a tool to enrich stem cells in other cancer types.

CD34 has been classically shown to mark LSCs in AML, but AML is a highly heterogeneous disease and not all AML patients show CD34 expression (i.e., CD34 negative AML[18]). We have previously shown that absence of NKG2DL expression provides a novel tool to identify LSCs in this subgroup of CD34 negative AML. Here, in contrast, we focus on the subgroup of CD34-positive AMLs and show that different subpopulations can occur. Future research will show whether co-staining for

NKG2DL in conjunction with such other markers (e.g., CD34) can more effectively enrich LSC.

In flow cytometry, it is important to include adequate controls such as FMO to help the experimenter to correctly discriminate between positive and negative events. In addition, positive controls for every used antibody are necessary to ensure that the absence of staining is not due to a malfunctioning antibody. The protocol we present here uses only a limited number of surface markers, which can be extended according to the experimenter's need, for example, by addition of CD33 or other potential LSC markers[19]. Furthermore, the fluorophores used in this protocol can be adapted. Importantly, such changes should be accompanied by an antibody titration to determine the best dilution.

One critical step before the staining is the thawing of primary AML cells. Indeed, in addition to their fragility, such samples are conserved in dimethyl sulfoxide (DMSO), which is per se toxic for the cells. Therefore, the samples should be handled with care and should be rinsed thoroughly to avoid prolonged exposure to DMSO. Although our samples were processed and frozen according to a previously established protocol[15], it is important to carefully follow the steps described in the manuscript to ensure a high cell recovery. Working with fresh samples will ensure improved viability and is thus recommended. However, in most cases this is not practicable.

Another factor influencing the described method is sample-specific deviation that can result in different cell scattering profiles observed via flow cytometry. Due to disease heterogeneity, this is unavoidable but should be considered in the analysis. Additional variability can be introduced by freezing/thawing or other mechanical steps in sample processing. In general, rapidity is key to avoid cell death. Yet, dying cells and debris cannot be avoided and need to be taken into account and excluded in the analysis. Gating based on FSC/SSC should be done with thoroughness and a critical eye as it is the first step of sample analysis.

One major advantage of the fusion protein is its potency to detect all known and potentially unknown NKG2DLs, while specific anti-NKG2DL antibodies (e.g., MICA, MICB) introduce a selection bias. Both methods are equally feasible. For the analyzed limited number of samples, they also showed very comparable results. Using the NKG2D fusion protein has some major benefits: Less time-consuming, decreased reagent number, and number of staining samples as well as a lower probability of human mistakes (i.e., pipetting mistakes). Moreover, this method may also be more effective in capturing NKG2DL expression in some samples, since it should also capture expression of unknown proteins with the NKG2DL function, which may be expressed in some AML cases. While providing insight into the surface presence of NKG2DL, this method does not provide information about the intracellular levels of NKG2DL, their trafficking or regulation, and other assays should therefore be performed to gain further knowledge on such questions.

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DISCLOSURES:

The authors have nothing to disclose.

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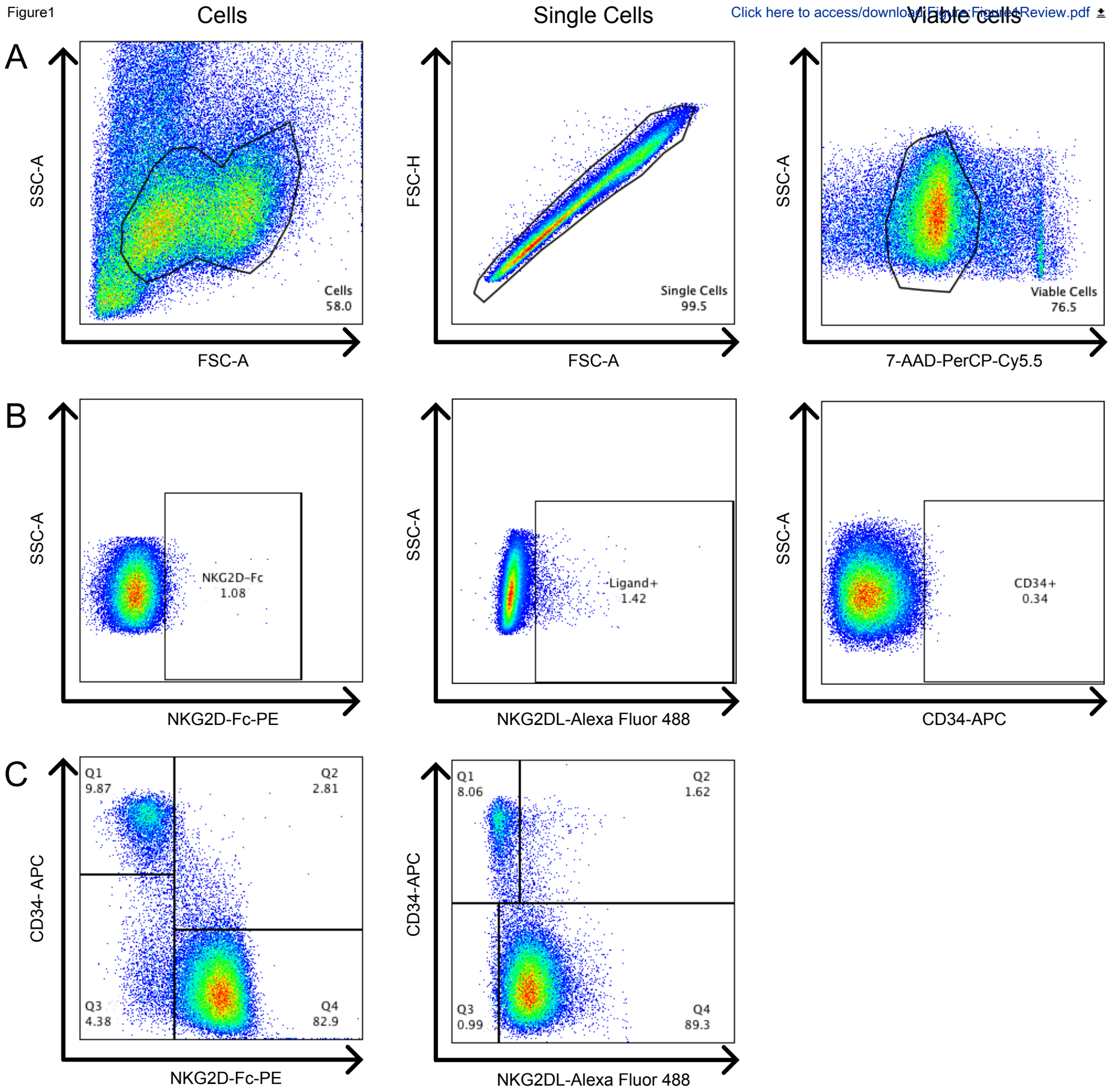

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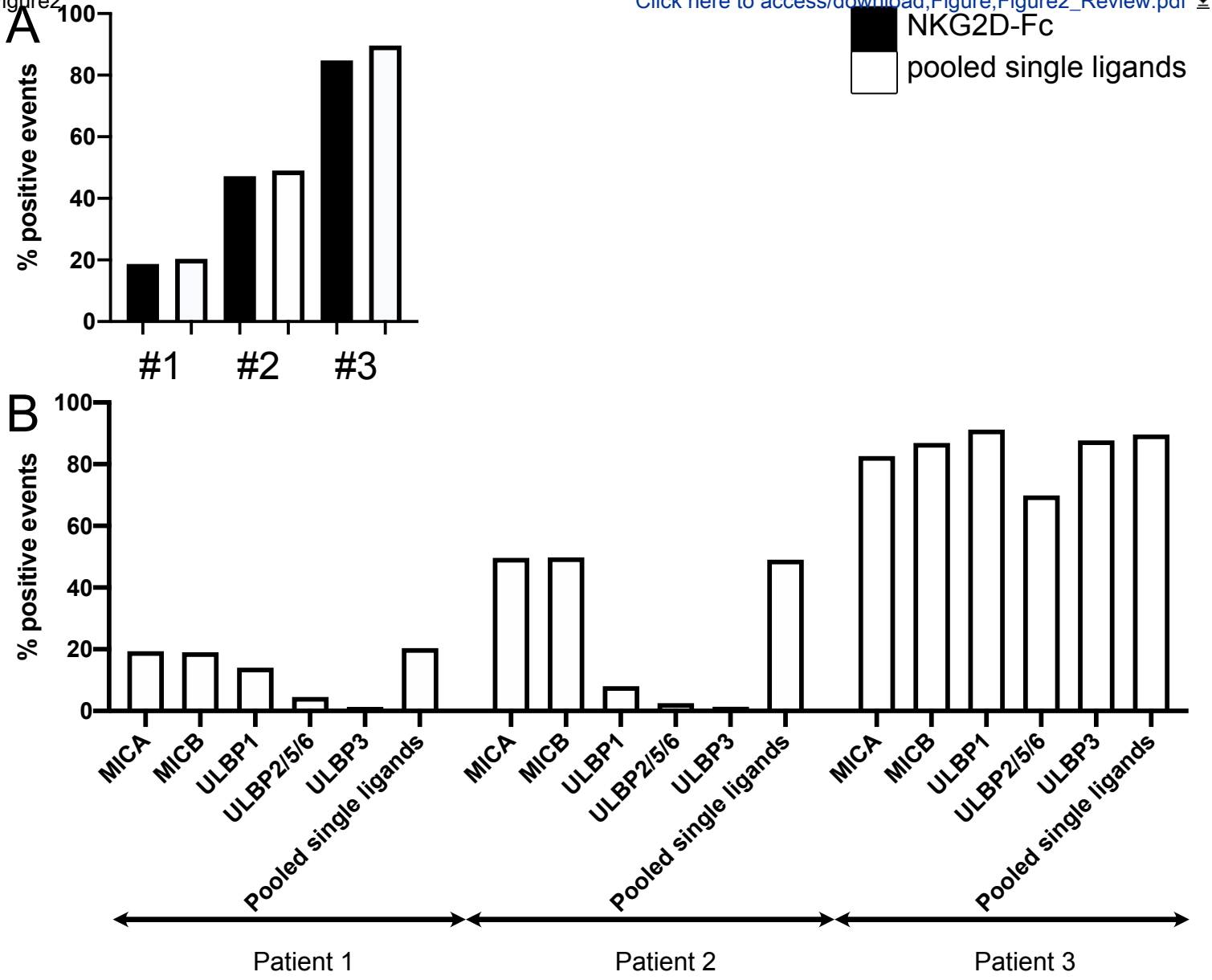

Table 1

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Tube name	Antigen	Fluorophore	Dilution/concentration
Unstained cells	-	-	-
Single stain PE	NKG2D-Fc	PE	10 µg/mL for primary step 1:100 for secondary step
Single stain PercPCy5.5	Live/Dead	PercpCy5.5	1:1000
Single stain APC	CD34	APC	1:25
Full stained cells	CD34 NKG2D-Fc Live/Dead	APC PE PercpCy5.5	1:25 1:10 for primary step 1:100 for secondary step 1:1000

Table 2

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Tube name	Antigen	Fluorophore	Dilution/concentration
Unstained cells	-	-	-
Single stain AF488	ULBP-1 ULBP-2/5/6 ULPB-3 MICA MICB	Alexa Fluor 488	10 µg/mL for primary step 4 µg/mL for secondary step
Single stain PercPCy5.5	Live/Dead	PercpCy5.5	1:1000
Single stain APC	CD34	APC	1:25
Full stained cells	CD34 NKG2DL Live/Dead	APC PE PercPCy5.5	1:25 10 µg/mL for primary step 1:100 for secondary step 1:1000

Table 3

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Tube name	Antigen	Fluorophore	Dilution/concentration
Unstained cells	-	-	-
FMO_APC	ULBP-1 ULBP-2/5/6 ULPB-3 MICA MICB NKG2D-Fc Live/Dead	Alexa Fluor 488 PE PercPCy5.5	10 µg/mL for primary step 4 µg/mL for secondary step 10 µg/mL for primary step 1:100 for secondary step 1:1000
FMO_PE	CD34 ULBP-1 ULBP-2/5/6 ULPB-3 MICA MICB Live/Dead	APC Alexa Fluor 488 PercPCy5.5	1:25 10 µg/mL for primary step 4 µg/mL for secondary step 1:1000
FMO_FITC	CD34 NKG2D-Fc Live/Dead	APC PE PercPCy5.5	1:25 10 µg/mL for primary step 1:100 for secondary step 1:1000

Table of Materials

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Name of Material/ Equipment/Software	Company	Catalog Number
7-amminoactinomycin D (7-AAD)	Invitrogen	A1310
96 well plate U bottom	Sarstedt	833925500
APC Mouse Anti-Human cd34	BD	555824
Bovine Serum Albumin	PanReac AppliChem	A1391,0050
Ethylenediaminetetraacetic acid	Roth	8043.1
Fetal Calf Serum (FCS)	BioConcept	2-01F10-I
FlowJo 10.2	BD	/
Goat- anti-Rabbit IgG (H+L) Alexa Fluor 488	Thermo Scientific	A21222
Human NKG2D Fc Chimera Protein, CF	R&D	1299-NK-050
Human ULBP-1 Antibody	R&D	AF1380
Human ULBP-2/5/6 Antibody	R&D	AF1298
Human ULBP-3 Antibody	R&D	AF1517
MICA Polyclonal Antibody	Thermo Scientific	PA5-35346
MICB Polyclonal Antibody	Thermo Scientific	PA5-66698
One-step Antibody Biotinylation Kit 1 strip, for 8 reactions	Miltenyibiotec	130-093-385
Phosphate Buffered Saline	Sigma Aldrich	D8537-500ML
Rabbit anti-Goat IgG (H+L) Alexa Fluor 488	Thermo Scientific	A11034
Rainbow Calibration Particles (8-peaks) 3.0 um	Spherotech Inc.	RCP-30-20A
RPMI medium	Sigma Aldrich	R8758-500ML
RayBright Universal Compensation Beads	Raybiotech	137-00013-100
Streptavidin, R-Phycoerythrin Conjugate (SAPE) - 1 mg/mL	Invitrogen	S866

Comments/Description	RRID					
Viability dye	/					
96 Well plate for our Flow cytometer	/					
Antibody detecting CD34	AB_398614					
Component of the staining buffer	/					
Component of the staining buffer	/					
Component of the supplemented RPMI medium	/					
Software enabling data analysis for flow cytometry experiment	/					
econdary antibody detecting the primary antibodies for MICA and MIC	AB_10373853					
Fusion Protein detecting all NKG2DLs	/					
Antibody detecting ULBP1	**AB_354765**					
Antibody detecting ULBP2/5/6	AB_354725					
Antibody detecting ULBP3	AB_354835					
Antibody detecting MICA	AB_2552656					
Antibody detecting MICB	AB_2663413					
Biotinylation kit for the NKG2DL fusion protein	/					
Component of the staining buffer	/					
Secondary antibody detecting the primary antibodies for the ULBPs	AB_2576217					
Beads used for flow cytometry device maintainance						
Cell culture medium	/					
Beads used to create the compensation matrix	/					
Seondary step for the biotinylated NKG2DL fusion protein detection	/					

Rebuttal Letter

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Rebuttal:

We thank the editors for all the work and comments on the manuscript. Please note that we have made changes according to the Reviewers' and Editors' suggestions and all changes performed in this revision have been marked blue.

Please include how each step is performed.

We have decided to leave the acquisition of the flow cytometry out of the video and have therefore not included a step by step protocol on how to use the device used for flow cytometry. If the editors think that it is necessary for readers to understand the protocol, we can add this information.

Figure 2B: What are MICA, MICB, ULB1, ULB2/5/6, ULB3, pooled ligand?

We added more details in the description of figure 2B and it should now be clear, what MICA, MICB, ULBP1, ULBP2/5/6 and ULBP3, as well as the pooled ligands are, and why we do this staining. This is also further elaborated in the discussion.

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UNIVERSITÄT BASEL

DEPARTEMENT BIOMEDIZIN

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Senior Science Editor
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September 1st, 2020

Dear Mr. Werth

Herewith, we would like to submit our revised manuscript „ **Two flow cytometric approaches of NKG2D ligand surface detection to distinguish stem cells from bulk subpopulations in acute myeloid leukemia"** for publication as a video article in JoVe.

Please note that we have made changes according to the Reviewers' and Editors' suggestions and all changes performed in this revision have been marked blue.

We have decided to leave the acquisition of the flow cytometry out of the video and have therefore not included a step by step protocol on how to use the device used for flow cytometry. If the editors think that it is necessary for readers to understand the protocol, we can add this information.

We added more details in the description of figure 2B and it should now be clear, what MICA, MICB, ULBP1, ULBP2/5/6 and ULBP3, as well as the pooled ligands are, and why we do this staining. This is also further elaborated in the discussion.

Thank you very much for your consideration.

Sincerely,

Claudia Lengerke

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UKBB Universitäts-Kinderspital beider Basel

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June 30th, 2020

Dear Mr. Werth

Herewith, we would like to submit our revised manuscript „ **ABSENCE OF IMMUNE ACTIVATING NKG2D LIGANDS AS A TOOL TO IDENTIFY LEUKEMIC STEM CELLS"** for publication as a video article in JoVe.

Please note that we have made significant changes according to the Reviewers' and Editors' suggestions. Furthermore, we have added two more co-authors that were involved during the revision phase. However, we were not able to include them in the system, but they should for sure be included. Please, also note the equal contribution of 2 co-authors for this new manuscript.

Thank you very much for your consideration.

Sincerely,

Claudia Lengerke

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UKBB Universitäts-Kinderspital beider Basel