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TITLE:

Profiling of the Human Natural Killer Cell Receptor-Ligand Repertoire

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SUMMARY:

Here we design two complementary mass cytometry (CyTOF) panels and optimize a CyTOF staining protocol with the aim of profiling the natural killer cell receptor and ligand repertoire in the setting of viral infections.

ABSTRACT:

Natural killer (NK) cells are among the first responders to viral infections. The ability of NK cells to rapidly recognize and kill virally infected cells is regulated by their expression of germline-encoded inhibitory and activating receptors. The engagement of these receptors by their cognate ligands on target cells determines whether the intercellular interaction will result in NK cell killing. This protocol details the design and optimization of two complementary mass cytometry (CyTOF) panels. One panel was designed to phenotype NK cells based on receptor expression. The other

panel was designed to interrogate expression of known ligands for NK cell receptors on several immune cell subsets. Together, these two panels allow for the profiling of the human NK cell receptor-ligand repertoire. Furthermore, this protocol also details the process by which we stain samples for CyTOF. This process has been optimized for improved reproducibility and standardization. An advantage of CyTOF is its ability to measure over 40 markers in each panel, with minimal signal overlap, allowing researchers to capture the breadth of the NK cell receptor-ligand repertoire. Palladium barcoding also reduces inter-sample variation, as well as consumption of reagents, making it easier to stain samples with each panel in parallel. Limitations of this protocol include the relatively low throughput of CyTOF and the inability to recover cells after analysis. These panels were designed for the analysis of clinical samples from patients suffering from acute and chronic viral infections, including dengue virus, human immunodeficiency virus (HIV), and influenza. However, they can be utilized in any setting to investigate the human NK cell receptor-ligand repertoire. Importantly, these methods can be applied broadly to the design and execution of future CyTOF panels.

INTRODUCTION:

Natural killer (NK) cells are innate immune cells whose primary role is to target and kill malignant, infected, or otherwise stressed cells. Through their secretion of cytokines such as IFNγ and TNFα, as well as their cytotoxic activity, NK cells can also shape the adaptive immune response to pathogens and malignancies. The NK response is mediated in part by the combinatorial signaling of germline-encoded inhibitory and activating receptors, which bind a myriad of ligands expressed on potential target cells. Several NK cell receptors have more than one ligand with new receptor-ligand pairs being identified regularly.

There is a particular interest in studying NK cells in the context of viral infections, where their ability to rapidly respond to stressed cells may limit viral spread or promote the development of NK cell evasion strategies. This interest in NK cell biology extends to the field of cancer immunotherapy where researchers are investigating the role of NK cells tumor immunosurveillance and in the tumor microenvironment[1]. However, the ability to profile NK cell-target cell interactions is complicated by the fact that human NK cells can express over 30 receptors which in turn can interact with over 30 known ligands[2]. The simultaneous detection of multiple NK cell receptors and their cognate ligands is, therefore, necessary to capture the complexity of the receptor-ligand interactions that control NK function. Consequently, we turned to mass cytometry (CyTOF), which allows for the simultaneous detection of over 40 markers at the single cell level. Our goal was to create two CyTOF panels to profile the NK cell receptor-ligand repertoire. We also wanted to design a protocol for effective processing and staining of clinical samples. Clinical human samples provide a wealth of information on how the body responds to viral infection. Therefore, we developed this protocol to investigate expression of NK cell receptors and their cognate ligands in parallel for better standardization, improved recovery, reduced reagent consumption, and limited batch effects.

Several flow cytometry panels designed to characterize the phenotype of human NK cells have been published previously[3–8]. Most of these panels are limited in their ability to capture the breadth of the receptor-ligand repertoire, only allowing for the detection of a limited selection

of markers. Moreover, these panels are limited by signal overlap between fluorochromes. CyTOF uses antibodies conjugated to metal isotopes, which are read out by time-of-flight mass spectrometry, thus dramatically reducing spillover between channels.

Like us, other researchers have turned to CyTOF to study NK cells[9–14], though generally with fewer NK cell markers, which reduces the depth of phenotyping. While the general staining protocols used by these groups are similar to ours, there are some key differences. Other protocols do not involve isolating NK cells prior to staining even though the researchers are only interested in that subset[13,14]. Given that NK cells only make up 5-20% of peripheral blood mononuclear cells (PBMCs), staining whole PBMCs rather than isolated NK cells means that most of the collected events will not be NK cells. This reduces the amount of data generated on the subset of interest and results in inefficient use of machine time. Additionally, while many of these panels interrogate expression of NK cell receptors such as killer Ig-like receptors (KIRs), NKG2A/C/D, and the natural cytotoxicity receptors (NKp30, NKp44, and NKp46), expression of these markers is not put into a broader context due to the absence of data on expression of their respective ligands. Consequently, while these previously published methods for investigating NK cells via CyTOF are sufficient for broad NK cell phenotyping, used in isolation, they cannot provide a comprehensive picture of NK cell activity. This brings us to the major advantage of the methods described here, which is that up to this point there are no published flow cytometry or CyTOF panels focused on exploring the expression of ligands for NK cell receptors. Importantly, our ligand panel has several open channels to allow for the addition of markers to suit the unique needs of each experiment.

Considering that one of the main limitations of CyTOF is the inability to recover the sample after analysis, this method may not be appropriate for researchers who have limited samples with which they are interested in performing additional experiments. Additionally, the low throughput nature of CyTOF means that the data generated will be of poor quality if the starting number of cells is low. Barring these two limitations, this method will perform well in any setting to investigate receptor-ligand interactions between NK cells and target cells.

PROTOCOL:

Anonymized healthy adult PBMCs were obtained from leukoreduction system chambers purchased from the Stanford Blood Center. PBMCs from de-identified healthy pediatric donors and pediatric acute dengue patients were obtained from Gorgas Memorial Institute of Health Studies in Panama City, Panama and hospitals belonging to the Ministry of Health, the Social Security System in Panama City, and suburban areas. The dengue study protocol was approved by the IRB of Hospital del Niño (CBIHN-M-0634), then approved by the committees of ICGES, CSS, Santo Tomas Hospital, and Stanford University. PBMCs from HIV-infected patients on antiretroviral treatment were obtained from ACTG study A5321.

1. Antibody labeling, panel preparation, and storage

1.1. Antibody labeling with metal isotopes

NOTE: To increase inter-experimental standardization of staining, it is recommended to perform multiple conjugations for each antibody and then combining the products into a single master mix for long-term storage as described below.

1.1.1. Determine the concentration of each antibody by measuring the absorbance at 280 nm prior to conjugation. Antibodies used for this protocol are commercially available and were purchased from the vendors listed in the **Table of Materials**.

1.1.2. Label antibodies with metal isotopes using commercially available antibody labeling kits according to the manufacturer's instructions. Use 100 µg of antibody for each reaction.

1.1.3. Determine the final concentration of the recovered antibody by measuring the absorbance at 280 nm. Store antibodies for short-term at 4 °C.

1.2. Antibody titrations

NOTE: CyTOF technology is very sensitive to potential contaminating signals from environmental metals. Therefore, all buffers/reagents used should be prepared with ultrapure water and stored in plastic or glass containers that have never been washed with soap.

1.2.1. Prepare centrifuge tubes for each donor containing 9 mL of warm, complete RPMI (RPMI 1640, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin) and 20 µL of benzonase per vial of PBMCs to be thawed. Benzonase decreases viscosity and background from free DNA from lysed cells. Thaw the PBMCs in a water bath and add to tubes.

1.2.2. Centrifuge at 300 x *g* at room temperature for 5 min. Resuspend the PBMCs in 5 mL of complete RPMI media and count.

1.2.3. For each panel titration, plate 2-4 million PBMCs/well in 6 wells of a round bottom 96-well plate (one well for each titer and one for unstained). Centrifuge the plate at 600 x *g* at room temperature for 3 min. Flick the plate to remove the supernatant. Resuspend each well in 200 µL of CyPBS.

1.2.4. Perform cisplatin viability staining as described below.

NOTE: Cisplatin is used to discriminate live from dead cells in mass cytometry.

1.2.4.1. Resuspend cells in 100 µL of 25 µM cisplatin stock. Incubate at room temperature for 1 min.

1.2.4.2. Quench the cisplatin reaction by adding 100 µL of FBS to each well and mixing. Centrifuge and flick the plate.

NOTE: Perform all subsequent centrifuge steps at 4 °C.

1.2.4.3. Wash cells twice with 200 µL of CyFACS (1x PBS without heavy metal contaminants in ultrapure water with 0.1% BSA, 0.05% sodium azide). Centrifuge and flick the plate each time.

1.2.5. Titrate the surface antibody panel as described below.

NOTE: Separate master mixes should be made for the NK surface panel and the ligand panel.

1.2.5.1. Make a master mix of all the surface antibodies at a concentration of 10 µg/mL using CyFACS. Aim for a final volume of 150 µL. Make serial 1:2 dilutions using CyFACS, to obtain the following concentrations: 10, 5, 2.5, 1.25, and 0.625 µg/mL.

1.2.5.2. Filter antibody cocktails through a centrifugal filter unit (0.1 µm pore size) at 10,600 x *g* for 3 min prior to staining.

1.2.5.3. Resuspend the plated cells in 50 µL of the surface antibody cocktail at the respective titer. Resuspend the unstained well in CyFACS. Incubate at 4 °C for 30 min.

1.2.5.4. Wash cells with 150 µL of CyFACS. Centrifuge and flick the plate.

1.2.5.5. Wash cells with 200 µL of CyFACS. Centrifuge and flick the plate.

1.2.6. Perform fixation of cells by resuspending each well in 100 µL of 2% paraformaldehyde (PFA) in CyPBS. Incubate the plate at room temperature in the dark for 20 min. Wash cells with 100 µL of CyFACS. Centrifuge at 700 x *g* for 5 min.

CAUTION: PFA is suspected of causing genetic defects as well as cancer. Additionally, it is harmful if it gets in contact with the eyes, skin, or is inhaled. Handle appropriately by ensuring good ventilation, opening the receptacle carefully, and preventing the formation of aerosols.

NOTE: All subsequent centrifuge spins are performed at 700 x *g* for 5 min at 4 °C.

1.2.7. Permeabilize cells by resuspending in 200 µL of 1x Permeabilization Buffer (Perm buffer) diluted in ultrapure water. Centrifuge and flick the plate. Wash cells with 200 µL of Perm buffer. Centrifuge and flick the plate.

NOTE: Incubation in the Perm buffer is not necessary.

1.2.8. Intracellular antibody panel titration

1.2.8.1. Make a master mix of all the intracellular antibodies at a concentration of 10 µg/mL using Perm Buffer. Aim for a final volume of 150 µL. Make serial 1:2 dilutions using Perm Buffer, to obtain the following five different concentrations: 10, 5, 2.5, 1.25, and-0.625 µg/mL.

1.2.8.2. Filter antibody cocktails through a centrifugal filter unit (0.1 µm pore size) at 10,600 x *g* for 3 min prior to staining.

1.2.8.3. Resuspend the plated cells in 50 µL of the intracellular antibody cocktail at the respective titer. Resuspend the unstained well in Perm Buffer. Incubate at 4 °C for 45 min.

NOTE: If an intracellular panel is not going to be titrated, resuspend wells in 50 µL of the Perm buffer.

1.2.8.4. Wash cells with 150 µL of Perm buffer. Centrifuge and flick the plate.

1.2.8.5. Wash cells with 200 µL of Perm buffer. Centrifuge and flick the plate.

1.2.8.6. Wash cells twice with 200 µL of CyFACS. Centrifuge and flick the plate.

1.2.9. DNA intercalator staining

NOTE: Intercalator binds to cellular nucleic acid and is used to identify nucleated cells in mass cytometry.

1.2.9.1. Resuspend cells in 200 µL of intercalator diluted 1:10,000 in CyPBS and 2% PFA. Incubate the plate overnight at 4 °C.

1.2.9.2. Store plates, if needed, at 4 °C covered with paraffin film for up to a week.

NOTE: Perform all subsequent centrifuge steps at 700 x *g* for 5 min at 4 °C.

1.2.9.3. Before running the samples on CyTOF, remove the paraffin film from the plate and centrifuge at 700 x *g* for 5 min at 4 °C. Flick the plate. Wash cells once with 200 µL of CyFACS. Centrifuge and flick the plate.

1.2.9.4. Wash cells three times with 200 µL of ultrapure water. Centrifuge and flick the plate. Resuspend cells in 200 µL of ultrapure water. Immediately before running the sample, adjust the concentration to approximately 6 x 10^5 cells/mL in normalization beads diluted to a 1x concentration in ultrapure water.

1.2.10. Run the samples on CyTOF.

1.2.11. Analyze data and choose appropriate titers for each antibody by selecting the lowest antibody titer which results in the highest signal intensity and the best separation between positive and negative populations based on visual assessment.

NOTE: Titrations for the NK and ligand panels are shown in **Figure 1** and **Figure 2** respectively. If a clear distinction between positive and negative populations is not identified, titers can be

assessed on multiple cell types or on cell lines, to allow for identification of both positive and negative cell populations.

1.2.12. Antibody panel storage

1.2.12.1. Combine titrated antibodies into a master mix and filter through a sterile 0.1 µm syringe filter unit. Separate master mixes should be made for the NK surface panel, the NK intracellular panel, and the ligand panel.

1.2.12.2. For the long-term storage of panels follow one of the two acceptable options:

1.2.12.3. Send master mix to a third-party company for lyophilization. This method is used for the NK panel. Antibodies not conjugated in-house cannot be lyophilized, due to the presence of antibody stabilizer, which interferes with the lyophilization process. These antibodies are added to the panel on the day of staining.

1.2.12.4. Use a repeater pipette to make 50 µL aliquots of each master mix and store them at -80 °C.

2. Staining protocol

2.1. Thaw peripheral blood mononuclear cells (PBMCs) as described in steps 1.2.1 and 1.2.2. Set aside at least 1 million PBMCs for ligand panel staining in a 15 mL centrifuge tube. Keep these PBMCs on ice during the NK cell isolation.

2.2. NK cell isolation

NOTE: The following NK cell isolation steps are a modified version of a specific vendor's protocol. However, any kit or protocol that performs magnetic-based negative selection of NK cells would be a suitable alternative. Additionally, this step is optional as this protocol is also suitable for the phenotyping of NK cells from whole PBMCs.

2.2.1. Spin the remaining PBMCs at 450 x *g* for 5 min. Resuspend cell pellet in 40 µL of MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) per 10^7 total cells.

2.2.2. Add 10 µL of NK cell Biotin-Antibody Cocktail per 10^7 total cells. Mix well and incubate for 5 min on ice.

2.2.3. Add 30 µL of MACS buffer per 10^7 total cells and 20 µL of NK cell MicroBead Cocktail per 10^7 total cells. Mix well and incubate for 10 min on ice.

2.2.4. Prepare elution columns by rinsing with 500 µL of MACS buffer. Add 2 mL of warm complete RPMI to 15 mL collection tubes.

2.2.5. Add MACS buffer to tubes containing cells to bring the volume up to 500 µL. Pipette the entire 500 µL volume onto the prepared elution column. Rinse out the tube with another 500 µL of MACS buffer and transfer to the column.

2.2.6. After flow has stopped, rinse the elution column with 500 µL MACS buffer twice. After flow has stopped, count NK cells.

2.3. Plate and wash cells.

2.3.1. Centrifuge isolated NK cells and PBMCs at 300 x *g* at room temperature for 10 min. Resuspend cells at a concentration of 5 million cells/mL in CyPBS (1x PBS without heavy metal contaminants in ultrapure water). Plate cells in a U-bottom, 96-well plate.

NOTE: Each aliquot of the NK cell panel can stain up to 3 million cells. If six individual NK cell samples are being barcoded and pooled prior to staining, the combined total number of NK cells should not exceed 3 million. The ligand panel can stain up to 6 million PBMCs per sample. If six individual samples are being barcoded and pooled prior to staining, the combined total number of PBMCs should not exceed 6 million.

2.3.2. Centrifuge plate at 600 x *g* at room temperature for 3 min. Flick the plate to remove supernatant.

NOTE: Perform all subsequent centrifuge spins at 600 *x g* for 3 min until step 2.7.

2.3.3. Resuspend cells in 200 µL of CyPBS. Centrifuge and flick the plate.

2.4. Perform cisplatin viability staining as described in step 1.2.4.

2.5. Barcoding staining

NOTE: These panels are used in conjunction with a modified two-of-four, Palladium-based barcoding method on live unfixed cells to minimize batch effects and maximize cell recovery[15]. However, this step is optional as barcoding is not necessary to obtain quality data.

2.5.1. Resuspend each well in 50 µL of respective premixed barcode and incubate at 4 °C for 30 min. Wash cells with 150 µL of CyFACS. Centrifuge and flick the plate.

2.5.2. Wash cells twice with 200 µL of CyFACS. Centrifuge and flick the plate. Resuspend all wells in 30 µL of CyFACS. Combine up to six wells of cells stained with unique barcodes into one well and perform centrifugation and flicking of the plate.

2.6. Surface staining

2.6.1. Dissolve the surface NK panel lyosphere in 50 µL of CyFACS with additional surface

antibodies spiked in (anti-CD16, anti-HLA-DR, anti-LILRB1). Thaw ligand panel stored at -80 °C and spin down tube using a mini-centrifuge. Spike in additional ligand panel surface markers (anti-CD16, anti-CD19).

NOTE: Any antibody cocktail that has not been previously filtered (i.e., prior to lyophilization or freezing) should be filtered through a centrifugal filter unit (0.1 µm pore size) at 10,600 *x g* for 3 min prior to staining.

2.6.2. Resuspend each well in 50 µL of the respective panel. Incubate at 4 °C for 30 min. Wash cells with 150 µL of CyFACS. Centrifuge and flick the plate. Wash cells again with 200 µL of CyFACS. Centrifuge and flick the plate.

2.7. Fix cells as described in step 1.2.6.

NOTE: Perform all subsequent centrifuge spins at 700 *x g* for 5 min at 4 °C.

2.8. Permeabilize cells as described in step 1.2.7.

2.9. Intracellular staining

2.9.1. Dissolve the intracellular NK panel lyosphere in 50 µL of Perm buffer. Prepare an intracellular antibody cocktail for PBMC samples if so desired.

NOTE: Any antibody cocktail that has not been previously filtered (i.e., prior to lyophilization or freezing) should be filtered through a centrifugal filter unit (0.1 µm pore size) at 10,600 x *g* for 3 min prior to staining.

2.9.2. Resuspend wells in 50 µL of the respective intracellular panels. If an intracellular panel is not used in conjunction with the ligand surface panel, resuspend PBMC wells in 50 µL of the Perm buffer. Incubate at 4 °C for 45 min.

2.9.3. Wash cells with 150 µL of Perm buffer. Centrifuge and flick plate. Wash cells with 200 µL of Perm buffer. Centrifuge and flick the plate.

2.9.4. Wash cells twice with 200 µL of CyFACS. Centrifuge and flick the plate.

2.10. DNA intercalator staining. Perform DNA intercalator staining as described in step 1.2.9.1. Incubate plate overnight at 4 °C.

NOTE: Intercalator binds to cellular nucleic acid and is used to identify nucleated cells in mass cytometry. Plates can be stored covered with paraffin film for up to a week at 4 °C.

2.11. Before running the samples on CyTOF, wash cells as described in steps 1.2.9.3 and 1.2.9.4.

2.12. Run samples on CyTOF.

REPRESENTATIVE RESULTS:

Antibodies were conjugated to metal isotopes using commercially available labeling kits, according to the manufacturer's instructions. Antibody clones were validated by flow cytometry and mass cytometry prior to use in this panel. An initial list of clones was selected based on review of the literature and antibody availability. The expression levels of some ligands for NK cell receptors are low or undetectable on healthy PBMCs. Therefore, positive staining for some antibodies was validated by staining healthy PBMCs, chronic myeloid leukemia K562 cells, acute lymphoblastic leukemia NALM6 cells, or B cell acute lymphoblastic leukemia 697 cells (**Supplemental Figure 1**). Clones selected for the NK cell panel that did not produce an adequate stain or were too expensive were substituted for different ones, as detailed in **Supplemental Table 1** and shown in **Supplemental Figure 2**.

Metal-isotope pairing with antibodies for these panels was performed using the principles outlined by Takahashi et al.[16]. Lineage markers were of medium to high intensity. Consequently, they were mainly conjugated to low and medium sensitivity masses leaving high sensitivity masses available for conjugation to antibodies against more dimly expressed markers. A publicly available panel designer software was used to detect abundance sensitivity (M ± 1 bleed) or oxidation (M + 16 bleed) issues and antibody-metal pairs were re-assigned accordingly. Additionally, several markers were conjugated on different metals with minimal differences noted on signal intensities (**Supplemental Table 2** and **Supplemental Figure 3**). Antibody-metal pairings and clone information for the NK and ligand panels are listed in **Table 1** and **Table 2** respectively.

In-house conjugated antibodies were titrated on PBMCs at five different titers: 0.625, 1.25, 2.5, 5, and 10 µg/mL. The lowest antibody titer which resulted in the highest signal intensity and the best separation between positive and negative populations was selected based on visual assessment. Titrations for the NK and ligand panels are shown in **Figure 1** and **Figure 2** respectively. For certain markers, a clear distinction between positive and negative populations was not identified, due to the marker being dimly expressed or universally positive. To determine the most accurate working dilution for these antibodies, titers were assessed on multiple cell types (PBMCs, T cells, B cells, or NK cells), or on cell lines, to allow for identification of both positive and negative cell populations (**Supplemental Figure 4**). The staining index (SI) for each marker was not calculated as this metric is not applicable to CyTOF data[17,18].

The panels described here were designed to be compatible with sample barcoding. There are several barcoding methods available for CyTOF. The most commonly used are a commercially available Palladium-based kit, which requires fixation prior to barcoding, and the CD45-based barcoding method described by Mei et al.[15], which allows the barcoding of live cells. To assess which barcoding method best fit our needs, we tested the stability of NK cell marker staining after fixation in an early version of the NK cell panel (**Supplemental Figure 5**). We found that the expression of a majority of NK cell markers was affected by fixation. Consequently, we decided to use a modified two-of-four, CD45-based barcoding method on live cells[15]. This barcoding

method uses ^{102}Pd, ^{104}Pd, ^{106}Pd, and ^{108}Pd, and differs from the three-of-six method originally described by Mei et al., which used ^{104}Pd, ^{106}Pd, ^{108}Pd, ^{110}Pd, ^{113}In, and ^{115}In. Indium channels were not included in our barcoding scheme, as they interfered with the signal from ^{115}In-CD3. ^{110}Pd was not included as it interfered with the signal from the HLA-DR Qdot and the CD19 Qdot in the NK cell and ligand panels, respectively.

Although we recommend NK cell purification prior to staining, the NK cell panel is designed to allow for the phenotyping of NK cells from whole PBMCs. An example of our NK cell gating strategy is shown in **Figure 3A** using PBMCs from a healthy donor. Staining and gates for each of the NK markers are shown on healthy, isolated NK cells in **Figure 3B**. The ligand panel is designed to detect the expression of NK cell ligands on whole PBMCs. **Figure 4A** illustrates the gating strategy used to identify $CD4^+$ T cells, $CD8^+$ T cells, NK cells, monocytes, and $CD19^+$ B cells in PBMCs from a healthy donor. Representative staining examples for each ligand are shown in **Figure 4B** using PBMCs from acute dengue patients and HIV-infected individuals who were virologically suppressed.

To ensure panel stability over time, our protocol includes two possible options: lyophilization through a third-party company into single use beads or freezing of pre-made aliquots at -80 °C. For this protocol, the NK panel was lyophilized, and the ligand panel was frozen. Both methods were validated prior to using each panel on clinical samples.

We produced over 700 reactions of the NK panel from one master mix by performing multiple conjugations of each antibody in the panel. After validation and titration of each conjugated antibody, the antibodies were combined into a master mix, filtered through a sterile 0.1 µm syringe filter unit, and sent to a third party company for lyophilization. Two sets of single-stain lyospheres were made, one for surface staining, and one for intracellular staining. Antibodies not conjugated in-house (HLA-DR and CD16) could not be added to the lyosphere, due to the presence of antibody stabilizer, which interferes with the lyophilization process. These antibodies are added to the panel on the day of staining. A comparison between stains obtained pre- and post-lyophilization is shown in **Supplemental Figure 6**. The clone of LILRB1 antibody initially used in the lyospheres did not produce a sufficiently strong stain (**Supplemental Table 1** and **Supplemental Figure 2**). A better clone was subsequently identified, conjugated and added to the panel on the day of staining (**Table 1**). The KIR2DS2 polyclonal antibody used in the lyopsheres was noted to produce a non-specific stain after lyophilization and we do not recommend its use for subsequent analyses (**Supplemental Table 1** and **Supplemental Figure 2**). Most intracellular stains slightly increased in intensity following lyophilization (**Supplemental Figure 6**).

Prior to storage of the ligand panel master mix at -80 °C, we tested two different storage conditions. We prepared a smaller master mix of this panel and stored aliquots at -80 °C and in liquid nitrogen for approximately two months. After two months we stained whole PBMCs with the frozen aliquots. We compared the staining to that of PBMCs from the same donor stained with the freshly prepared panel (**Supplemental Figure 7**). We found storage at -80 °C and in liquid nitrogen does not change the signal intensity for most markers. In fact, the signal intensity of anti-pan HLA class I, anti-CD7, anti-CD4, anti-HLA-Bw4, anti-CD14, anti-CD11b, and anti-LFA-3 is

higher upon freezing, particularly in the case of samples stored at -80 °C. We could not determine whether the signal intensities of anti-LLT-1, anti-Nectin-1, anti-MICA/B, anti-DR4/5, anti-ULBP-1,2,5,6, anti-Nectin-2, anti-CD155, and anti-B7-H6 were affected by freezing, due to the fact that healthy PBMCs do not express high levels of these markers. However, validation of these markers on cell lines (**Supplemental Figure 1**) was performed using conjugated antibodies stored at -80 °C. Consequently, we were confident that freezing did not result in a significant loss of signal. Signal intensity did decrease upon freezing for five markers: anti-CD8, anti-ICAM-1, anti-CCR2, anti-CD33, and anti-CD56. However, in all of these cases the clear separation between the positive and negative populations remained. Given that metal-conjugated antibodies are not stable at 4 °C for long periods of time, freezing was necessary to preserve panel stability long-term, and despite a decrease in staining intensity in a subset of markers, we were able to retain sufficient staining separation. Importantly, the loss of signal intensity of anti-CD8, anti-CCR2, and anti-CD56 was greater in the samples stored in liquid nitrogen compared to those stored at -80 °C. Based on this data, we decided to store the panel at -80 °C.

FIGURE AND TABLE LEGENDS:

Figure 1: Titration of in-house conjugated antibody-metal conjugates for NK panel. Titrations of in-house conjugated antibodies were performed on PBMCs from a healthy donor using five different concentrations: 0.625, 1.25, 2.5, 5, and 10 μg/mL. Titers for anti-CD3, anti-CD14, anti-CD33, anti-CD19, anti-PD-1 and anti-CD56 were determined by gating on live cells. Titers for anti-CD4 and anti-CD8 were determined by gating on T cells. Titers for the remaining antibodies were determined by gating on NK cells. Since NKp44 is not expressed on resting NK cells, titers were determined on PBMCs stimulated with IL-2 and shown on NK cells. The red arrows indicate the titer selected for each antibody.

Figure 2: Titration of in-house conjugated ligand panel antibodies. Titrations of in-house conjugated antibodies were performed on PBMCs from a healthy donor using five different concentrations: 0.625, 1.25, 2.5, 5, and 10 μg/mL. Titers for anti-HLA-DR, anti-ICAM-1, anti-CCR2, anti-CD14, anti-CD11b, and anti-LFA-3 were determined by gating on $CD3^{-}CD7^{-}$ cells. Titers for anti-CD3, anti-pan HLA class I, anti-CD7, anti-CD48, anti-LLT-1, anti-HLA-C,E, anti-HLA-E, anti-FasR, anti-Nectin-1, anti-MICA/MICB, anti-DR4/DR5, anti-ULBP-1,2,5,6, anti-Nectin-2, anti-CD155, anti-HLA-Bw4, anti-HLA-Bw6, anti-CD33, anti-CD56, and anti-B7-H6 were determined by gating on live cells. Titers for anti-CD4 and anti-CD8 were determined by gating on $CD3^{+}$ cells. The red arrows indicate the titer selected for each antibody.

Figure 3: NK panel gating strategy and performance. (**A**) Serial negative gating from whole PBMCs to NK cells is shown in a healthy donor. Intact, bead and event-length gates ensure successful gating to single cells. Cisplatin staining was performed as a Live/Dead stain. T cells and B cells were excluded using CD3 and CD19. Monocytes were excluded by negative gating on CD4 and CD14/CD33 and by further negative gating of CD56-/HLA-DR^{bright} cells. CD56 and CD16 were used to identify different subsets of NK cells ($CD56^{bright}$, $CD56^{dim}$ and $CD56^{-}$). (**B**) Examples of expression of NK cell receptors on NK cells from one healthy donor purified by magnetic-bead isolation.

Figure 4: Ligand panel gating and performance. (**A**) Gating of major immune cell subsets from PBMCs derived from a healthy donor following normalization, calibration bead removal, and debarcoding. (**B**) Expression of ligands for NK cell receptors as well as several myeloid markers on live PBMCs. Staining for all ligands except Nectin-1 and B7-H6 is shown on PBMCs from acute dengue patients. Staining for Nectin-1 and B7-H6 is shown on PBMCs from HIV-infected individuals who were virologically suppressed.

Supplemental Figure 1: Verification of antibody clones for ligand panel on cell lines. Antibodies against ligands for NK cell receptors that are expressed at low levels on healthy PBMCs were validated by staining cell lines. Chronic myeloid leukemia K562 cells were stained with anti-ICAM-1, anti-MICA/MICB, anti-DR4/DR5, anti-ULBP-1,2,5,6, anti-Nectin-2, anti-CD155, and anti-B7-H6. Acute lymphoblastic leukemia NALM6 cells were stained with anti-LLT-1 and B cell acute lymphoblastic leukemia 697 cells were stained with anti-Nectin-1. Dot plots and histograms showing staining on healthy PBMCs are in blue. Dot plots and histograms showing staining on the respective cell line are in red. The percentage of cells of the respective cell line that are positive for a given marker are provided.

Supplemental Figure 2: Validation of antibody clones. (**A**) Different antibody clones were tested in healthy donors to identify the clone with the best specificity. 2B4, CXCR6, KIR2DS4, NKG2A and TIGIT are shown on NK cells. CD56 and LILRB1 are shown on live cells. (**B**) The KIR2DS2 antibody clone showed a non-specific stain after lyophilization. An example of staining on the same donor is provided pre- and post-lyophilization.

Supplemental Figure 3: Optimization of antibody/metal pairs. Staining of PBMCs from healthy donors are shown. (**A**) Antibody/metal pairs tested for the panel. (**B**) Antibody/metal pairs used in the panel. LILRB1 and PD1 are shown on live cells. All the other markers are shown on NK cells.

Supplemental Figure 4: Titration of dimly expressed and mostly positive NK cell markers. Titers for antibodies against NK cell markers that did not show a clear positive and negative population were assessed both on NK cells (red) and on either B cells (blue) or PBMCs (grey) from healthy donors. The arrows indicate the titer selected for each antibody.

Supplemental Figure 5: Optimization of barcoding protocol. Epitope stability was tested before and after fixation with 2% paraformaldehyde on PBMCs from a healthy donor. (**A**) CD3, CD14 and CD56 staining was similar before (red) and after (blue) fixation, CD4 and CD16 staining was significantly affected by fixation. (**B**) Many NK cell markers were affected by fixation, including CD2, CD38, KIR3DL2, CD62L, KIR2DS4, NKp46, NKG2C, NKp30, NKG2D, KIR3DL1, TIGIT, KIR2DL1, KIR2DL3 and NTB-A.

Supplemental Figure 6: Confirmation of panel stability after lyophilization. The stability of in-house antibody conjugates was confirmed by staining PBMCs from the same blood bank donor pre-lyophilization (blue) and post-lyophilization (red). Anti-CD3, anti-CD14, anti-CD33, anti-CD19, anti-PD-1, anti-CD56 stains are shown on live cells. Anti-CD4 and anti-CD8 are shown on $CD3^{+}$

cells. Titers for the remaining antibodies are shown on NK cells, gated according to the gating scheme shown in Figure 1. Notably, anti-KIR2DS2 stained non-specifically after lyophilization and therefore has not been used for subsequent analyses.

Supplemental Figure 7: Confirmation of ligand panel stability at -80 °C. The stability of in-house antibody conjugates was confirmed by staining healthy PBMCs from the same donor with a freshly prepared master mix as well as the same mix after storage at -80°C or in liquid nitrogen. Anti-HLA-DR, anti-ICAM-1, and anti-LFA-3 staining is shown on $CD3^-CD7^-$ cells. Anti-CD3, anti-pan HLA class I, anti-CD7, anti-CD48, anti-LLT-1 anti-HLA-E, anti-FasR, anti-Nectin-1, anti-MICA/B, anti-DR4/5, anti-ULBP-1,2,5,6, anti-Nectin-2, anti-CD155, anti-HLA-Bw4, anti-HLA-Bw6, and anti-B7-H6 staining is shown on live cells. Anti-CD8 and anti-CD4 staining is shown on $CD3^+$ cells. Anti-HLA-C,E, anti-CCR2, anti-CD11b, and anti-CD33 staining is shown on $CD3^-CD7^-CD14^+$ cells. Anti-CD14 staining is shown on $CD3^-CD7^-CD33^+$ cells and anti-CD56 staining is shown on $CD3^-CD7^+CD14^-HLA\text{-}DR^-$ cells. Histograms showing staining with the freshly prepared panel are in red. Histograms showing staining with the panel after storage at -80°C for approximately two months are in blue. Histograms showing staining with the panel after storage in liquid nitrogen (LN_2) for approximately two months are in green. Samples were stained and run on different days. Files were normalized and beads were removed using the premessa package.

Table 1: NK panel. Markers are ordered according to the isotopic mass of the metal to which they were conjugated. $^{191}Ir/^{193}Ir$ is the natural abundance of the nucleic acid intercalator. $^{194}Pt/^{195}Pt$ is the natural abundance of cisplatin.

Table 2: Ligand panel. Markers are ordered according to the isotopic mass of the metal to which they were conjugated. $^{191}Ir/^{193}Ir$ is the natural abundance of the nucleic acid intercalator. $^{194}Pt/^{195}Pt$ is the natural abundance of cisplatin.

Supplemental Table 1: Antibodies for NK cell panel that were tested, but not used.

Supplemental Table 2: Antibodies for NK cell panel that were tested with a different antibody/metal pairing.

DISCUSSION:

Here we describe the design and application of two complimentary CyTOF panels aimed at profiling the NK cell receptor-ligand repertoire. This protocol includes several steps that are critical to obtaining quality data. CyTOF uses heavy metal ions, rather than fluorochromes, as label probes for antibodies[19]. This technology is therefore subject to potential contaminating signals from environmental metals[20]. Potential sources of metal impurities include laboratory dish soap (barium) and lab buffers (mercury, lead, tin). For this reason, it is advised that all buffers be prepared with ultrapure water, and that all reagents be stored in plastic or glass containers that have never been washed with soap. Another critical step in this protocol is the viability stain, which uses a cisplatin-based method as described by Fienberg et al.[21]. This method includes a one-minute incubation step, during which cisplatin preferentially labels non-viable cells. Cisplatin staining must be performed in the absence of FBS. Consequently, cells must be thoroughly

washed with CyPBS prior to staining. Additionally, to avoid off-target staining of viable cells, the cisplatin stain needs to be quenched with FBS after precisely one minute. This protocol was optimized for maximum cell recovery and staining performance. Therefore, the fixation and permeabilization steps are also significant. Several fixation and permeabilization reagents are compatible with CyTOF. However, we found that fixation with 2% PFA, followed by permeabilization with a specific permeabilization buffer detailed in the **Table of Materials**, resulted in maximal cell recovery. As this is a transient permeabilization method, intracellular staining needs to be performed in the permeabilization buffer to ensure adequate antibody penetrance. Cells also need to be washed thoroughly with the permeabilization buffer following intracellular staining to remove unbound antibodies.

This protocol allows for several possible modifications. The CyTOF panels detailed here can be customized to include additional markers or substitute existing ones. In particular, the ligand panel was designed with several open channels to allow for flexibility in panel design. Any change or addition to the panel may require additional troubleshooting. In particular, any antibody/metal isotope pair should be thoroughly validated as described above, to avoid any issue of signal spillover to the existing channels. These panels were also designed to be compatible with sample barcoding. Barcoding decreases the possibility of batch effects and sample-to-sample carryover, while minimizing reagent consumption[22]. Although barcoding typically results in overall increased data quality, this step is not necessary for the acquisition of good quality CyTOF data and can be skipped entirely. Similarly, although we recommend NK cell purification prior to staining, the NK cell panel is compatible with the phenotyping of NK cells from whole PBMCs.

This method has some limitations. Due to the inherently low throughput nature of CyTOF, this method is not suitable for samples with low cell counts. Such samples are unlikely to yield data of sufficient quality for analysis. Additionally, given that these panels were specifically designed to interrogate NK cell-target cell interactions, they are limited in their ability to assess interactions between other cell types, such as $CD8^+$ T cells and myeloid cells. Similarly, these panels were designed for direct *ex vivo* immunophenotyping and were not tested or validated for use under activating conditions, such as cytokine stimulation. Moreover, although these panels cover a comprehensive list of NK cell receptors and ligands, they are not fully exhaustive, and several potentially important markers were not included due to space limitations. Some of these markers include, but are not limited to KLRG1, CRACC, TIM-3, LAIR-1 in the NK panel and PD-L1 in the ligand panel. Finally, this method is not suitable for use in fixed samples, given that the majority of NK cell markers' epitopes are affected by fixation.

The protocol described here has significant benefits compared to other methods. Other groups have described flow cytometry panels aimed at the study of NK cells[3–8,23]. Compared to flow cytometry, the use of CyTOF eliminates issues related to fluorophore compensation, allowing for the simultaneous detection of a high number of markers. Although others have also developed CyTOF panels to study NK cells[9–14], here we describe the use of two complementary CyTOF panels, which interrogate the expression of both NK cell receptors and their ligands, therefore providing a more detailed picture of NK cell function.

Our group has used this protocol and one or both of these panels to characterize the human NK cell response in healthy donors, and in a variety of disease settings including HIV infection and dengue virus infection[24–28]. Despite being designed for the purpose of studying viral infections, these panels lend themselves to the study of NK cells in other conditions given the breath of proteins they cover. In fact, our group also used these panels to characterize the NK cell receptor-ligand repertoire in patients with immunodeficiencies and multiple sclerosis[25,27,29,30], as well as in humanized mice[31,32]. As such, the use of these panels can be extended to other contexts. For example, many NK cell receptors and cognate ligands implicated in the setting of cancer are included in these panels, making these panels excellent tools for future studies on the role of NK cells in the anti-tumor response. More broadly, our protocol for mass production and storage of CyTOF panels as well as parallel processing of samples can be applied to the execution and application of any CyTOF panel.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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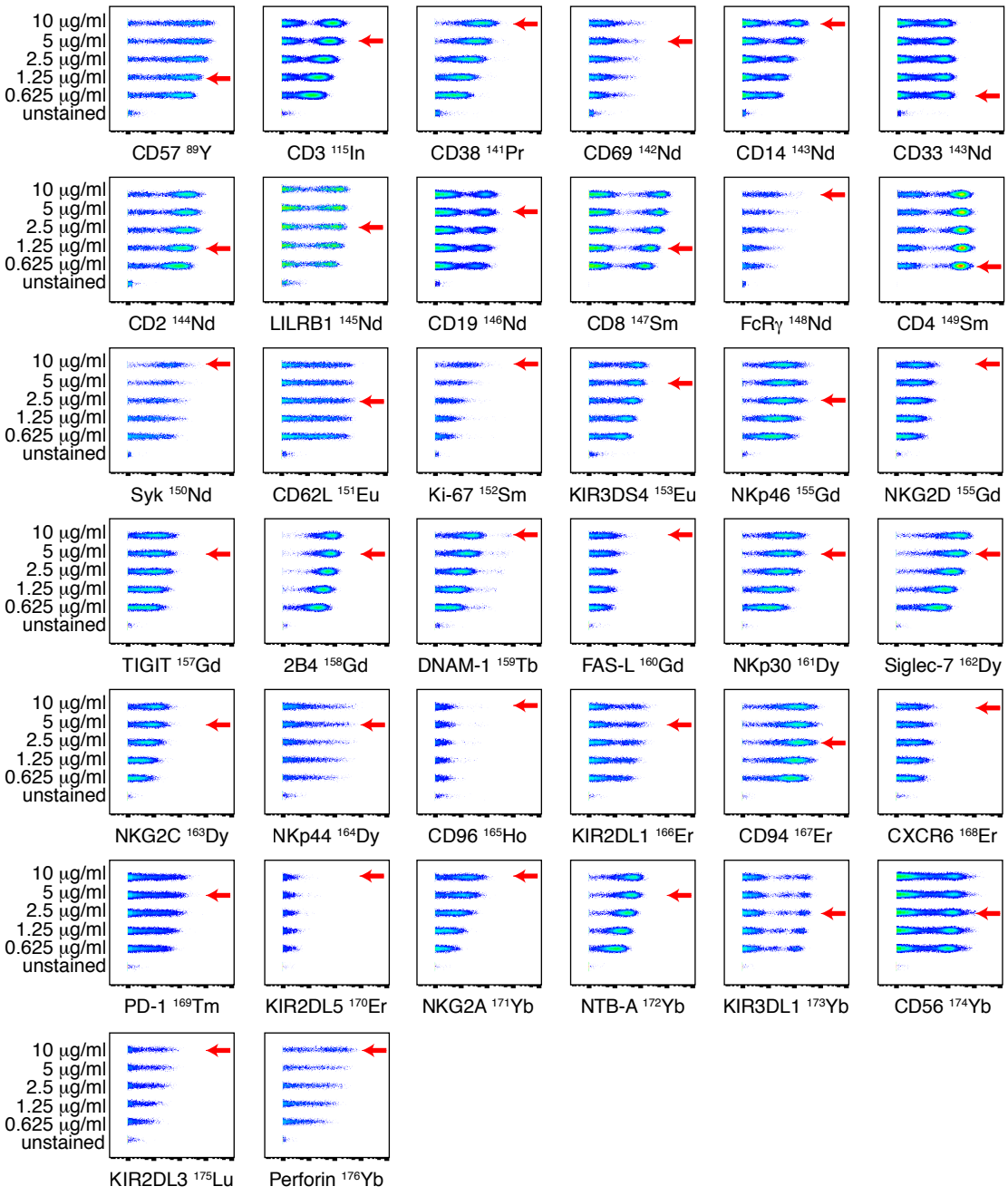

Figure 2

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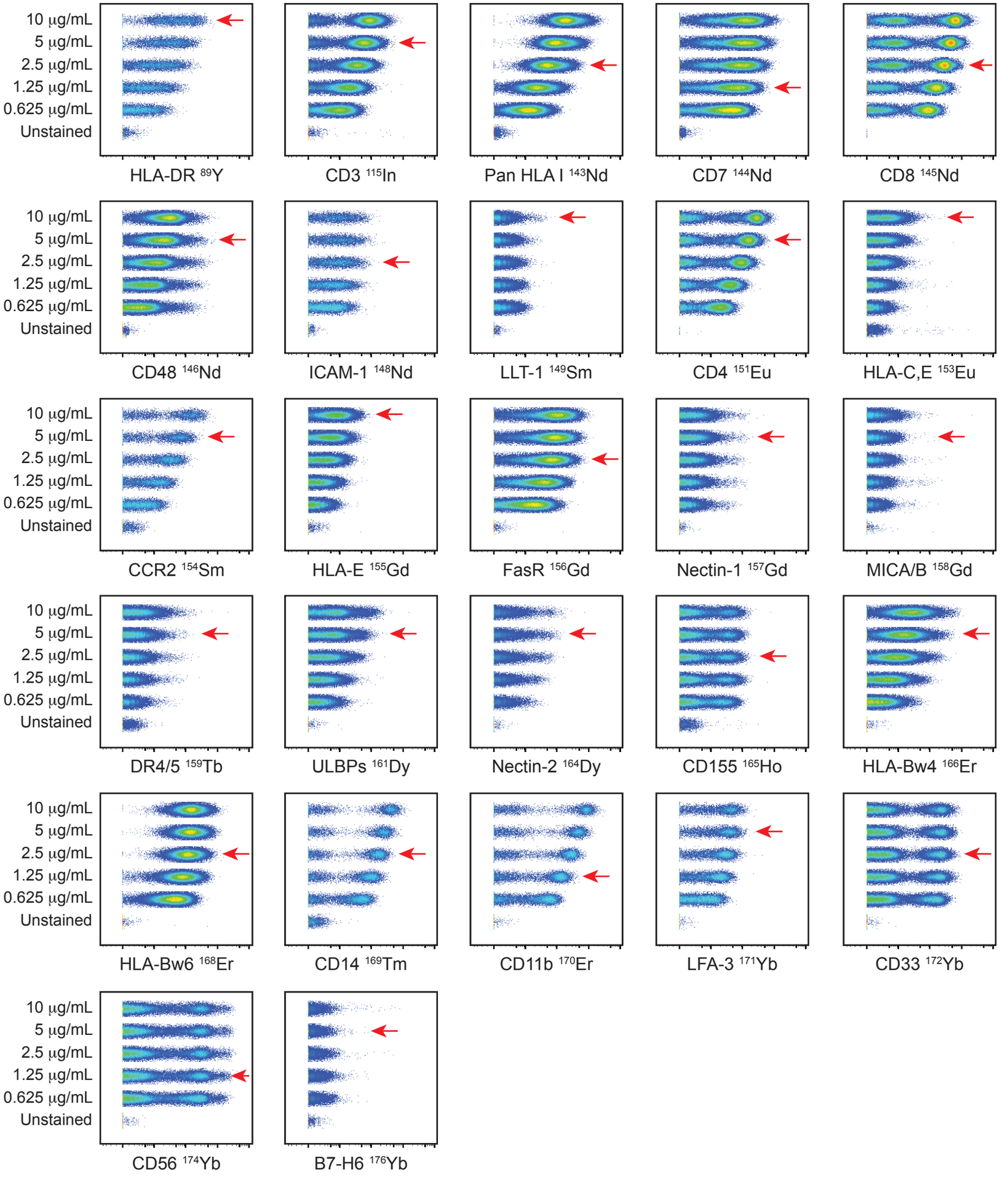


Figure 3

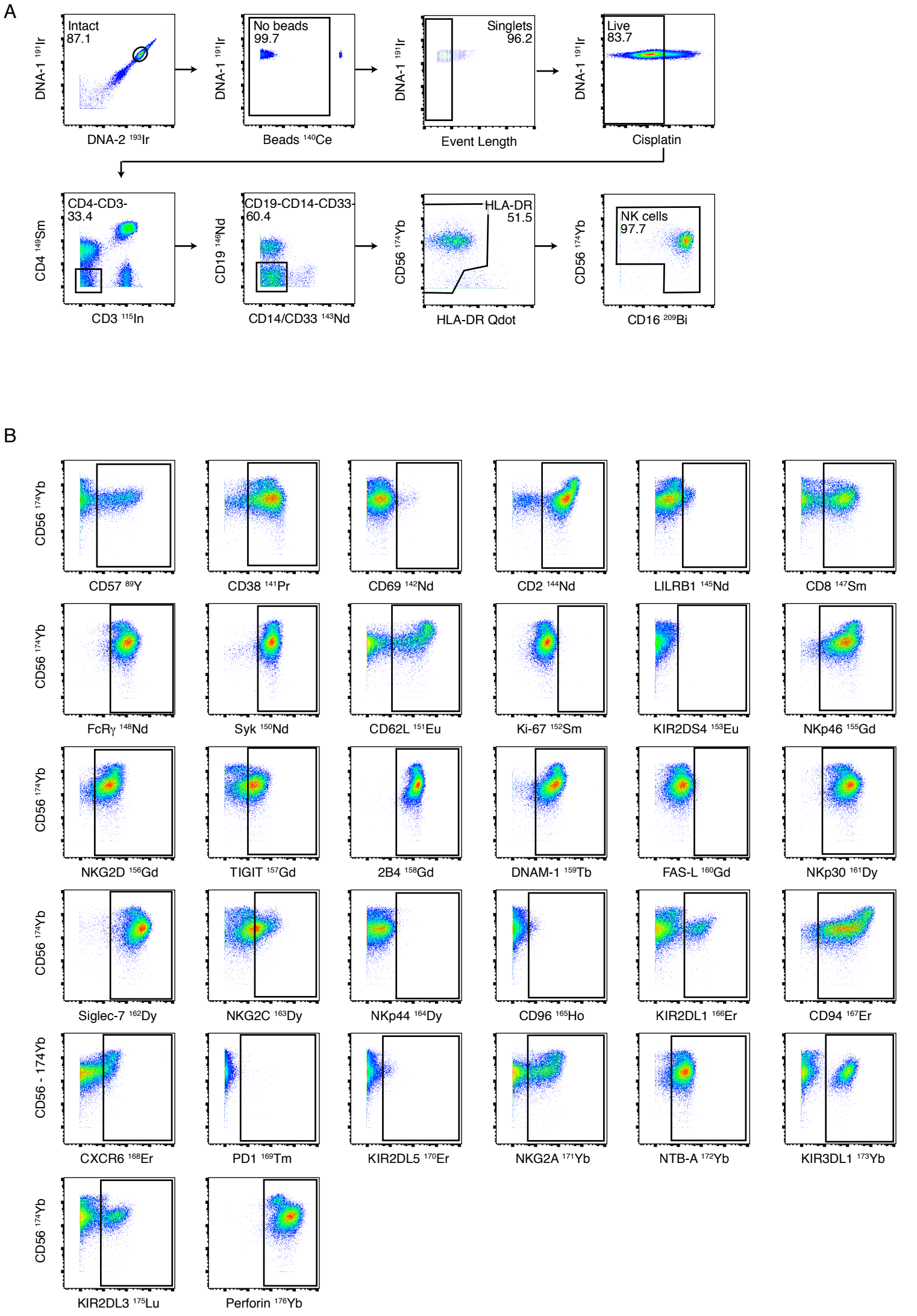
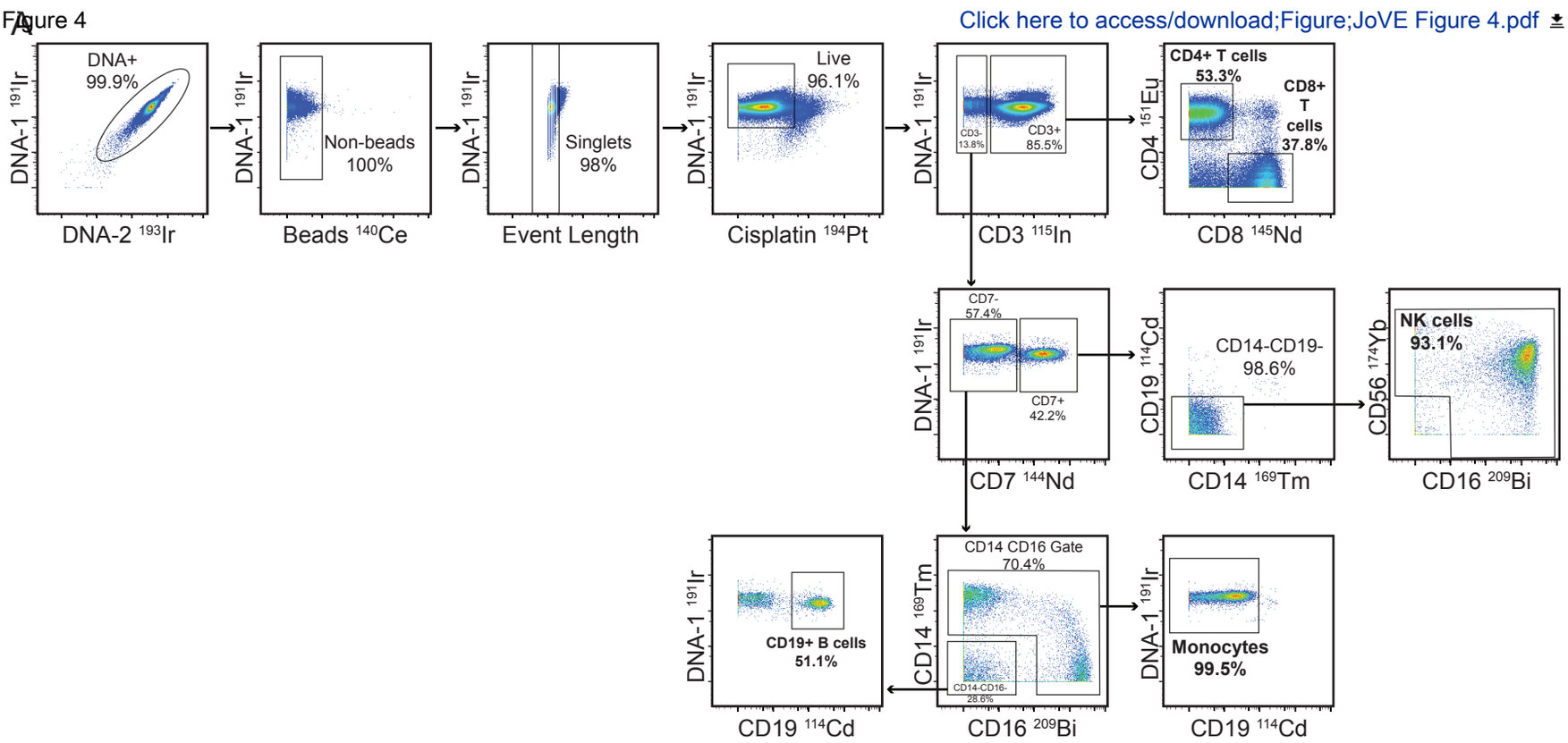


Figure 4



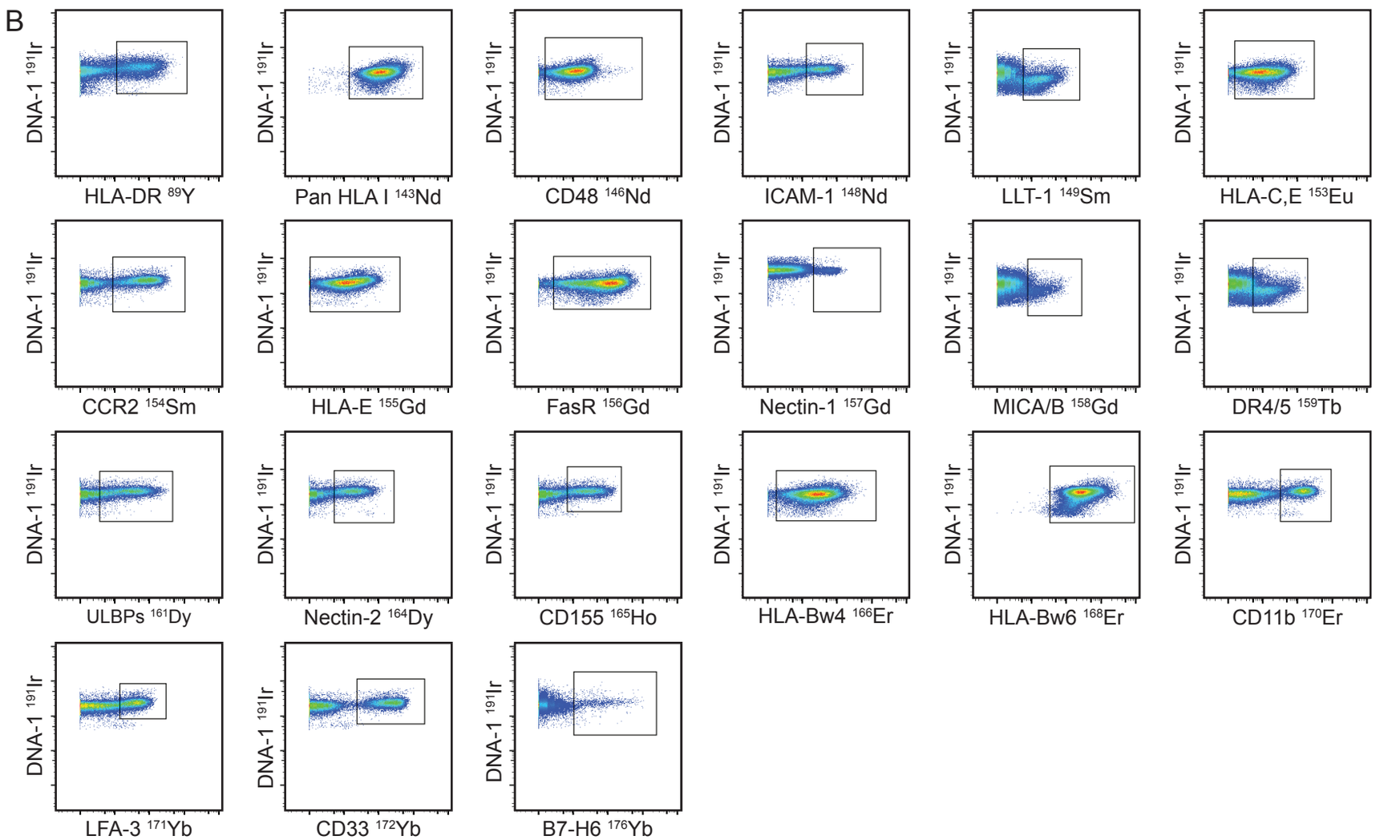


Table 1

Specificity	Clone	Isotope
CD57	HCD57	^{89}Y
CD45	HI30	^{102}Pd, ^{104}Pd, ^{106}Pd, ^{108}Pd
HLA-DR	Tü36	Qdot 655 (^{112}Cd-^{114}Cd)
CD3	UCHT	^{115}In
CD38	HIT2	^{141}Pr
CD69	FN50	^{142}Nd
CD33	WM53	^{143}Nd
CD14	M5E5	^{143}Nd
CD2	RPA-2.10	^{144}Nd
LILRB1	MAB20172	^{145}Nd
CD19	HIB19	^{146}Nd
CD8	SK1	^{147}Sm
FcRγ	polyclonal	^{148}Nd
CD4	SK3	^{149}Sm
Syk	4D10.2	^{150}Nd
CD62L	DREG-56	^{151}Eu
ki-67	Ki-67	^{152}Sm
KIR2DS4	179315	^{153}Eu
NKp46	9.00E+02	^{155}Gd
NKG2D	1D11	^{156}Gd
TIGIT	741182	^{157}Gd
2B4	C1.7	^{158}Gd
DNAM-1	DX11	^{159}Tb
FAS-L	NOK-1	^{160}Gd
NKp30	P30-15	^{161}Dy
Siglec-7	S7.7	^{162}Dy
NKG2C	134522	^{163}Dy
NKp44	P44-8	^{164}Dy
CD96	NK92.39	^{165}Ho
KIR2DL1/KIR2DS5	143211	^{166}Er
CD94	DX22	^{167}Er
CXCR6	K041E5	^{168}Er
PD1	EH12.2H7	^{169}Tm
KIR2DL5	UP-R1	^{170}Er
NKG2A	131411	^{171}Yb
NTB-A	NT-7	^{172}Tb
KIR3DL1	DX-9	^{173}Yb

CD56	NCAM16.2	^{174}Yb
KIR2DL3	180701	^{175}Lu
Perforin	B-D48	^{176}Yb
DNA-1/DNA-2	NA	$^{191}Ir/^{193}Ir$
Cisplatin	NA	$^{194}Pt/^{195}Pt$
CD16	3G8	^{209}Bi

Purpose	Surface/Intracellular
Maturity/Memory	surface
Barcoding	surface
Activation/Lineage	surface
T cell lineage	surface
Activation Marker	surface
Activation Marker	surface
Monocyte lineage	surface
Myeloid lineage	surface
Activation/Maturity	surface
Inhibitory Receptor	surface
B cell lineage	surface
T cell lineage and NK cell Activation/Maturity	surface
Maturity/Adaptive	intracellular
T cell lineage	surface
Signaling	intracellular
Activation	surface
Proliferation	intracellular
Activating Receptor	surface
Activating Receptor	surface
Activating Receptor	surface
Inhibitory Receptor	surface
Activating Receptor	surface
Activating Receptor	surface
Apoptosis	surface
Activating Receptor	surface
Inhibitory Receptor	surface
Maturity/Memory	surface
Activating Receptor	surface
NKG2 Co-receptor	surface
Inhibitory Receptor	surface
Activating Receptor	surface
Memory	surface
Inhibitory Receptor	surface
Inhibitory Receptor	surface
Inhibitory Receptor	surface
Activating Receptor	surface
Inhibitory Receptor	surface

NK cell lineage	surface
Inhibitory Receptor	surface
Cytolytic Protein	intracellular
Nucleated cells	surface
Viability	surface
FcgRIII receptor	surface

Table 2

Specificity	Clone	Isotope	Purpose
HLA-DR	L243	^{89}Y	Antigen presenting cells, activati
CD45	HI30	^{102}Pd, ^{104}Pd, ^{106}Pd, ^{108}Pd	Barcoding
CD19	SJ25-C1	Qdot 655 (^{112}Cd-^{114}Cd)	Lineage
CD3	UCHT1	^{115}In	Lineage
Pan HLA class I	W6/32	^{143}Nd	KIR ligands
CD7	CD7-6B7	^{144}Nd	Lineage
CD8	SK1	^{145}Nd	Lineage
CD48	BJ40	^{146}Nd	2B4 ligand
ICAM-1	HA58	^{148}Nd	LFA-1 ligand
LLT-1	402659	^{149}Sm	CD161 ligand
CD4	OKT4	^{151}Eu	Lineage
HLA-C,E	DT9	^{153}Eu	KIR ligands
CCR2	K036C2	^{154}Sm	Monocyte functional marker
HLA-E	3D12	^{155}Gd	NKG2A/CD94 and NKG2C/CD94 l
Fas (CD95)	DX2	^{156}Gd	FasL receptor
Nectin-1	R1.302	^{157}Gd	CD96 ligand
MICA/B	159227/236511	^{158}Gd	NKG2D ligands
DR4/5	DJR1/DJR2-2	^{159}Tb	TRAIL receptors
ULBP-1/2,5,6	170818/165903	^{161}Dy	NKG2D ligands
Nectin-2	TX31	^{164}Dy	DNAM-1, TIGIT, and CD96 ligand
CD155	SKII.4	^{165}Ho	DNAM-1, TIGIT, and CD96 ligand
HLA-Bw4	REA274	^{166}Er	KIR3DL1 ligand
HLA-Bw6	REA143	^{168}Er	KIR null allele
CD14	M5E2	^{169}Tm	Lineage
CD11b	ICRF44	^{170}Er	Lineage
LFA-3	TS2/9	^{171}Yb	CD2 ligand
CD33	WM53	^{172}Yb	Lineage
CD56	NCAM16.2	^{174}Yb	Lineage
B7-H6	875001	^{176}Yb	NKp30 ligand
DNA-1/DNA-2	NA	^{191}Ir/^{193}Ir	Nucleated cells
Cisplatin	NA	^{194}Pt/^{195}Pt	Viability
CD16	3G8	^{209}Bi	Lineage

Surface/Intracellular
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Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
^{89}Y	Sigma-Aldrich	204919	
102-Palladium nitrate	Trace Sciences International	Special Order	
104-Palladium nitrate	Trace Sciences International	Special Order	
106-Palladium nitrate	Trace Sciences International	Special Order	
108-Palladium nitrate	Trace Sciences International	Special Order	
^{115}In	Trace Sciences International	Special Order	
^{141}Pr	Fluidigm	201141A	
^{142}Nd	Fluidigm	201142A	
^{143}Nd	Fluidigm	201143A	
^{144}Nd	Fluidigm	201144A	
^{145}Nd	Fluidigm	201145A	
^{146}Nd	Fluidigm	201146A	
^{147}Sm	Fluidigm	201147A	
^{148}Nd	Fluidigm	201148A	
^{149}Sm	Fluidigm	201149A	
^{150}Nd	Fluidigm	201150A	
^{151}Eu	Fluidigm	201151A	
^{152}Sm	Fluidigm	201152A	
^{153}Eu	Fluidigm	201153A	
^{154}Sm	Fluidigm	201154A	
^{155}Gd	Fluidigm	201155A	
^{156}Gd	Fluidigm	201156A	

^{157}Gd	Trace Sciences International	N/A	
^{158}Gd	Fluidigm	201158A	
^{159}Tb	Fluidigm	201159A	
^{160}Gd	Fluidigm	201160A	
^{161}Dy	Fluidigm	201161A	
^{162}Dy	Fluidigm	201162A	
^{163}Dy	Fluidigm	201163A	
^{164}Dy	Fluidigm	201164A	
^{165}Ho	Fluidigm	201165A	
^{166}Er	Fluidigm	201166A	
^{167}Er	Fluidigm	201167A	
^{168}Er	Fluidigm	201168A	
^{169}Tm	Fluidigm	201169A	
^{170}Er	Fluidigm	201170A	
^{171}Yb	Fluidigm	201171A	
^{172}Yb	Fluidigm	201172A	
^{173}Yb	Fluidigm	201173A	
^{174}Yb	Fluidigm	201174A	
^{175}Lu	Fluidigm	201175A	
^{176}Yb	Fluidigm	201176A	
^{209}Bi anti-CD16	Fluidigm	3209002B	Clone 3G8. Used at a 1:50 dilution.
697 cells	Creative Bioarray	CSC-C0217	
Amicon Ultra Centrifugal Filter Units 0.5 with Ultracel-30 Membrane, 30 kDa	Millipore	UFC503096	
Anhydrous acetonitrile	Fisher Scientific	BP1165-50	
anti-2B4	Biolegend	329502	Clone C1.7.

anti-B7-H6	R&D Systems	MAB7144	Clone 875001.
anti-CCR2	Biolegend	357202	Clone K036C2.
anti-CD2	Biolegend	300202	Clone RPA-2.10.
anti-CD3	Biolegend	300402	Clone UCHT1.
anti-CD4	Biolegend	317402	Clone OKT4.
anti-CD4	Biolegend	344602	Clone SK3.
anti-CD7	Biolegend	343102	Clone CD7-6B7.
anti-CD8	Biolegend	344702	Clone SK1.
anti-CD11b	Biolegend	301302	Clone ICRF44.
anti-CD14	Biolegend	301802	Clone M5E2.
anti-CD19	Biolegend	302202	Clone HIB19.
anti-CD33	Biolegend	303402	Clone WM53.
anti-CD38	Biolegend	303502	Clone HIT2.
anti-CD48	Biolegend	336702	Clone BJ40.
anti-CD56	BD Pharmingen	559043	Clone NCAM16.2.
anti-CD57	Biolegend	322302	Clone HCD57.
anti-CD62L	Biolegend	304802	Clone DREG-56.
anti-CD69	Biolegend	310902	Clone FN50.
anti-CD94	Biolegend	305502	Clone DX22.
anti-CD95	Biolegend	305602	Clone DX2.
anti-CD155	Biolegend	337602	Clone SKII.4.
anti-CXCR6	Biolegend	356002	Clone K041E5.
anti-DNAM-1	BD Biosciences	559787	Clone DX11.
anti-DR4	Biolegend	307202	Clone DJR1.
anti-DR5	Biolegend	307302	Clone DJR2-2.
anti-FAS-L	Biolegend	306402	Clone NOK-1.
anti-FcRg	Millipore	06-727	Polyclonal antibody.
anti-HLA-C,E	Millipore	MABF233	Clone DT9.
anti-HLA-Bw4	Miltenyi Biotec	Special Order	Clone REA274.
anti-HLA-Bw6	Miltenyi Biotec	130-124-530	Clone REA143.
anti-HLA-DR	Biolegend	307602	Clone L243.
anti-HLA-E	Biolegend	342602	Clone 3D12.
anti-ICAM-1	Biolegend	353102	Clone HA58.

anti-Ki-67	Biolegend	350502	Clone Ki-67.
anti-KIR2DL1/KIR2DS5	R&D Systems	MAB1844	Clone 143211.
anti-KIR2DL3	R&D Systems	MAB2014	Clone 180701.
anti-KIR2DL5	Miltenyi Biotec	130-096-200	Clone UP-R1.
anti-KIR2DS4	R&D Systems	MAB1847	Clone 179315.
anti-KIR3DL1	BD Biosciences	555964	Clone DX-9.
anti-LFA-3	Biolegend	330902	Clone TS2/9.
anti-LILRB1	R&D Systems	292319	Clone MAB20172.
anti-LLT-1	R&D Systems	AF3480	Clone 402659.
anti-MICA	R&D Systems	MAB1300-100	Clone 159227.
anti-MICB	R&D Systems	MAB1599-100	Clone 236511.
anti-Nectin-1	Biolegend	340402	Clone R1.302.
anti-Nectin-2	Biolegend	337402	Clone TX31.
anti-NKG2A	R&D Systems	MAB1059	Clone 131411.
anti-NKG2C	R&D Systems	MAB1381	Clone 134522.
anti-NKG2D	Biolegend	320802	Clone 1D11.
anti-NKp30	Biolegend	325202	Clone P30-15.
anti-NKp44	Biolegend	325102	Clone P44-8.
anti-NKp46	Biolegend	331902	Clone 9E2.
anti-NTB-A	Biolegend	317202	Clone NT-7.
anti-Pan HLA class I	Biolegend	311402	Clone W6/32.
anti-PD1	Biolegend	329902	Clone EH12.2H7.
anti-Perforin	Abcam	ab47225	Clone B-D48.
anti-Siglec-7	Biolegend	347702	Clone S7.7.
anti-Syk	Biolegend	644302	Clone 4D10.2.
anti-TACTILE	Biolegend	338402	Clone NK92.39.
anti-TIGIT	R&D Systems	MAB7898	Clone 741182.
anti-ULBP-1	R&D Systems	MAB1380-100	Clone 170818.
anti-ULBP-2, 5, 6	R&D Systems	MAB1298-100	Clone 165903.
Antibody Stabilizer	Candor Bioscience	131 050	
Benzonase Nuclease	Millipore	70664	
Bond-Breaker TCEP Solution	Thermo Fisher Scientific	77720	
Bovine Serum Albumin solution	Sigma-Aldrich	A9576	

Calcium chloride dihydrate ($CaCl_2$+2H_2O)	Sigma-Aldrich	223506-25G	
Cis-Platinum(II)diamine dichloride (cisplatin)	Enzo Life Sciences	ALX-400-040-M250	A 100 mM stock solution was prepared in DMSO and divided into 25 µL aliquots. Used at a 25 µM dilution for live/dead stain. Signal appears in 194Pt and 195Pt channels.
DMSO	Sigma-Aldrich	D2650	
eBioscience Permeabilization Buffer	Thermo Fisher Scientific	00-8333-56	

EDTA (0.5 M)	Hoefer	GR123-100	A double-concentrated HEPES buffer with EDTA was made according to the following recipe: 1.3 g NaCl (Thermo Fisher Scientific), 27 mg $CaCl_2$+$2H_2O$ (Sigma-Aldrich), 23 mg $MgCl_2$ (Sigma-Aldrich), 83.6 mg KH_2PO_4 (Thermo Fisher Scientific), 4 mL of 1M HEPES (Thermo Fisher Scientific), 2 mL of 0.5M EDTA (Hoefer, Holliston, MA, USA), and 100mL H_2O. The pH of this double-concentrated HEPES buffer was adjusted to a pH of 7.3 using 1M HCl and 1M NaOH.
EQ Four Element Calibration Beads	Fluidigm	201078	
Fetal Bovine Serum	Thermo Fisher Scientific	N/A	
Helios mass cytometer	Fluidigm	N/A	
HEPES (1M)	Thermo Fisher Scientific	15630080	
HyClone Antibiotic/Antimycotic Solution (Pen/Strep/Fungiezone) solution	Fisher Scientific	SV3007901	
Iridium - 191Ir/193Ir intercalator	DVS Sciences (Fluidigm)	201192B	Used at a 1:10000 dilution.

Isothiocyanobenzyl-EDTA (ITCB-EDTA)	Dojindo Molecular Technologies, Inc.	M030-10	Diluted to 1.25 mg/mL in anhydrous acetonitrile.
K562 cells	American Type Culture Collection (ATCC)	ATCC CCL-243	
L-Glutamine (200 mM)	Thermo Fisher Scientific	SH30034	
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	208337-100G	
Maxpar X8 Antibody Labeling Kits	Fluidigm	N/A	No catalog number as kits come with metals.
Millex-VV Syringe Filter Unit, 0.1 µm	Millipore	SLVV033RS	
Milli-Q Advantage A10 Water Purification System	Millipore	Z00Q0V0WW	
MS Columns	Miltenyi Biotec		
NALM6 cells	American Type Culture Collection (ATCC)	ATCC CRL-3273	
Nanosep Centrifugal Devices with Omega Membrane 3K	Pall Corporation	OD003C35	
NK Cell Isolation Kit, human	Miltenyi Biotec	130-092-657	
Paraformaldehyde (16%)	Electron Microscopy Sciences	15710	
PBS	Thermo Fisher Scientific	10010023	
Potassium Phosphate Monobasic (KH_2PO_4)	Fisher Scientific	MP021954531	
Qdot 655 anti-CD19	Thermo Fisher Scientific	Q10179	Clone SJ25-C1. Used at a 1:50 dilution. Signal appears in 112Cd-114Cd channels.
Qdot 655 anti-HLA-DR	Thermo Fisher Scientific	Q22158	Clone Tü36. Used at a 1:200 dilution.

Rockland PBS	Rockland Immunochemicals, Inc.	MB-008	Used to make CyPBS (10X Rockland PBS diluted to 1X in Milli-Q water) and CyFACS buffers (10X Rockland PBS diluted to 1X in Milli-Q water with 0.1% BSA and 0.05% sodium azide). Buffers were sterile-filtered through a 0.22 µM filter and sotred at 4°C in Stericup bottles.
RPMI 1640	Thermo Fisher Scientific	21870092	
Sodium azide (NaN_3)	Sigma-Aldrich	S2002	
Sodium chloride (NaCl)	Fisher Scientific	S271-500	
Stericup Quick Release-GP Sterile Vacuum Filtration System	Millipore Sigma	S2GPU10RE	
Tuning solution	Fluidigm	201072	
Washing solution	Fluidigm	201070	

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October 16, 2020

Dear Dr. Nguyen,

Thank you for the opportunity to submit our revised manuscript, “Profiling of the human natural killer cell receptor-ligand repertoire”, to JoVE. We appreciate the reviewers’ thorough evaluation of our method and thoughtful comments, and we hope you now find it suitable for publication. Please find attached our point-by-point responses to the specific comments that were raised by the editor and each reviewer.

Please let us know if further information is needed. Thank you for your consideration.

Sincerely,

Catherine A. Blish, M.D., Ph.D., FIDSA
Associate Professor, Department of Medicine and Stanford Immunology
Associate Director, Stanford Medical Scientist Training Program (MSTP)
Chan Zuckerberg Investigator, Chan Zuckerberg Biohub
Tashia and John Morgridge Faculty Scholar, Stanford Child Health Research Institute Fellow,
Stanford Center for Innovation in Global Health (CIGH)

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have gone through the manuscript and made these corrections.

2. Please provide at least 6 keywords or phrases.

This edit has been made.

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

This edit has been made.

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol details were verified, and the protocol contains everything we would like shown in the video. However, please note that due to COVID-19 related restrictions of access to Stanford University campus we may not be able to complete video production at this time.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: NanoDrop, Maxpar® XB antibody labeling kits, "We have worked with Biolyph,…" Miltenyi Biotec's NK cell Isolation Kit etc

Any references to commercial products were removed from the protocol.

6. 1: did you purchase or synthesize the antibodies? Which antibodies did you use in this study?

Antibodies were purchased for this protocol. This is now clarified in step 1 of the protocol.

7. 5: Please describe the procedure for antibody titration, and add data in the figures (cite them also) and show the signal intensity and separation of populations in the video.

More specific details are now provided for antibody titrations, including a citation to the figures.

8. 14: What do the MS columns contain? What are their dimensions and volume?

MS columns are commercially available through Miltenyi and contain a matrix composed of superparamagnetic spheres. Given the term “MS columns” is specific to Miltenyi, it was substituted with a more generic “elution column”.

9. The Discussion already has information that covers most of the points listed below. However, there is some material in the Representative Results that can be moved to the Discussion to further describe these points. Hence, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations.
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

All of these points are now included in the discussion

10. After you revise the protocol and add some details, please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

This edit has been made and protocol text to be included in the video has been highlighted in yellow.

11. Please sort the Materials Table alphabetically by the name of the material.

This edit has been made.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this paper, Vendrame et al. describe in detail two panels for mass cytometry (Cytof), one targeted at natural killer (NK) cell receptors, and one targeted at the ligands of these receptors. This combination of panels is an excellent approach, as usually scientists in the field are more focusing on the NK cell side. The protocol is well described and quite clear, so that it should be reproducible in other labs, ideally already familiar with mass cytometry. The figures presented give a good overview of what to expect as a readout of the method. The authors also warn the

reader against some potential problems, such as metal impurities from the environment or antibodies that do not perform well.

Major Concerns:
The authors heavily insist on the usefulness of their panels for the assessment of viral infections. This is fine, but the great potential of this approach for cancer research is, although mentioned, not emphasized enough. Indeed, knowing the NK cell phenotype to such an extent together with the ligands could be of major interest for cancer immunotherapy (example: PD-1/PD-L1). This aspect might be discussed more in depth.

We agree that this protocol could be applied to the setting of cancer. In order to emphasize this more we added the following sentence to the introduction: “This interest in NK cell biology extends to the field of cancer immunotherapy where researchers are investigating the role of NK cells tumor immunosurveillance and in the tumor microenvironment [1]”. We also mention in the discussion that PD-L1 would be a good addition to the ligand panel.

Furthermore, some markers are missing in the NK cell panel, such as KLRG1, CRACC, TIM-3, LAIR-1, and, in the ligand panel, PD-L1. However, the authors mention that the panel can be customized, so this is not really a major problem.

We agree with the reviewer that the panels are not fully exhaustive. The following sentence has been added to the discussion to address this concern: “Although these panels cover a comprehensive list of NK cell receptors and ligands, they are not fully exhaustive, and several potentially important markers were not included due to space limitations. Some of these markers include, but are not limited to KLRG1, CRACC, TIM-3, LAIR-1 in the NK panel and PD-L1 in the ligand panel.”

Some cell lines are used: the histology (for example: chronic myeloid leukemia for K562) and sources of these lines, as well as their culture conditions, might be mentioned.

Cell line information was added to the text and pertinent figure legends. These cell lines have also been added to the Materials Table along with their sources.

Next, it would be better to indicate already in the protocol why benzonase, cisplatin and DNA intercalator are used.

These clarifications were added on steps 5, 8 and 13.

Minor Concerns:
Introduction: page 2, NK cells are not that rare among PBMC, as they represent 5% - 20% of peripheral blood lymphocytes. The author's argument that it is better to purify NK cells is valid.

This sentence has been changed to no longer state that NK cells are a rare immune cell subset. It now states that, “Given that NK cells only make up 5-20% of peripheral blood mononuclear

cells (PBMCs), staining whole PBMCs rather than isolated NK cells means that a majority of the collected events will not be NK cells". We agree with the reviewer that our argument for purifying NK cells is valid.

Protocol: if healthy pediatric donors were investigated, they should not be called patients.

This edit has been made. The sentence in question now reads: "PBMCs from de-identified healthy pediatric donors and pediatric acute dengue patients were obtained from Gorgas Memorial Institute of Health Studies in Panama City, Panama and hospitals belonging to the Ministry of Health, the Social Security System in Panama City, and suburban areas".

Page 3, line 9: the word "using" appears twice.

This edit has been made.

Also on page 3, is it allowed to indicate a given company as supplier for the lyophilization?

The reference to the company who performed the lyophilization has been removed.

The abbreviation "PFA" should be explained already in the protocol.

This edit has been made.

Figure 2 and Suppl. Figure 7: an anti-HLA-B/C antibody is mentioned in the legends, but in the figures there is no such antibody but an anti-HLA-C,E antibody. Could this point be clarified?

This edit has been made. The figure legends now correctly refer to the anti-HLA-C,E antibody.

Reviewer #2:

Manuscript Summary:

Vendrame et al. describe and characterize use of CyTOF to phenotype NK receptors on NK cells and NK ligands on non-NK hematopoietic cells. Their protocol is very detailed and shows multiple steps in the process of protocol development, including clones used, performance after freeze-thaw, and antibody titration. The staining is comprehensive and covers multiple relevant markers on effectors versus targets/ regulatory cells. The results and protocol is likely to be of significant interest to NK investigators across a diversity of fields.

Major Concerns:

1) The text describes assessment of samples after dengue fever and HIV infection, but it is unclear if these data are shown or if the representative data are all from healthy donors. Please clarify.

The text has been clarified to state the following: “Figure 4A illustrates the gating strategy used to identify $CD4^+$ T cells, $CD8^+$ T cells, NK cells, monocytes, and $CD19^+$ B cells in PBMCs from a healthy donor”. The following sentence explicitly states that staining examples for each ligand are shown using PBMCs “from acute dengue patients and HIV-infected individuals who were virologically suppressed”. The figure legends were also clarified and the origin of the samples has now been added to each legend.

2) If data are available from pathogen-infected patients, would be informative to show to compare and contrast with healthy donor state.

We agree with the reviewer that comparing pathogen-infected patients and healthy patients is interesting and these panels have been in fact designed specifically for that purpose. In fact, such data has been previously published by our group and referenced in the discussion [2–4]. Here, our goal was to provide detailed protocols, a request that we have received frequently at meetings and in other discussions with colleagues. Due to the amount of data and markers assessed (80+ markers, in 3 or more disease states), it would be impractical to add these comparisons to this protocol, which is why we referenced the relevant publications in the discussion.

3) How many times were data repeated? This is not clear to me. How representative are these data given how heterogeneous NK cells are depending on genetic and epigenetic factors?

Titrations and validation of the panels were performed using 1-3 donors. Representative data are shown in the figures. The validated panels have been extensively used with samples from both in healthy and virus-infected individuals. Relevant publications were cited in the discussion.

4) Have authors assessed same panels under activating conditions (e.g. cytokine stimulation)? Do the panels remain as detailed and high fidelity?

These panels were not tested or validated for use under activating conditions, such as cytokine stimulation. We have no reason to believe that other activating conditions would affect the performance of the panel, however this point was added as a limitation to the discussion.

5) Are the selections for optimal antibody titrations based on mathematical calculations or a visual assessment? If visual assessment, some of the selections appear arbitrary. Please clarify.

Titration selection was based on visual assessment considering that standard mathematical methods, such as staining indices, are not applicable to CyTOF data [5, 6]. The rationale for titer selection is now mentioned both in the protocol and the discussion. Briefly, we selected the lowest antibody titer which resulted in the highest signal intensity and the best separation between positive and negative populations (Figures 1 and 2). If a clear distinction between positive and negative populations was not identified, the most accurate working dilution for

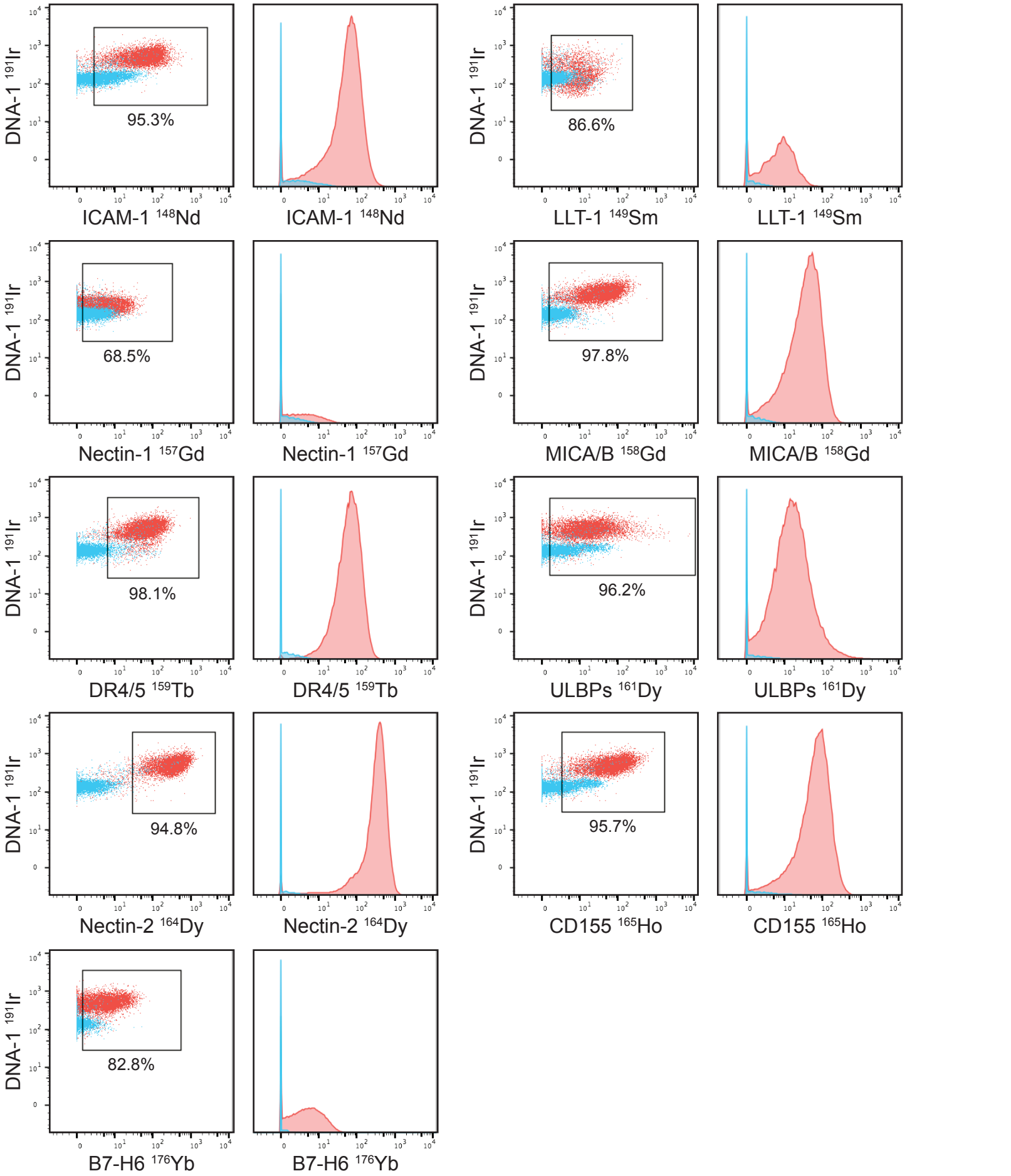
these antibodies was determined by assessing the stain on multiple cell types or on cell lines (Supplemental Figure 4).

References

1. Shimasaki, N., Jain, A., Campana, D. NK cells for cancer immunotherapy. *Nature reviews. Drug discovery*. **19** (3), 200–218 (2020).
2. McKechnie, J.L. *et al.* HLA Upregulation During Dengue Virus Infection Suppresses the Natural Killer Cell Response. *Frontiers in cellular and infection microbiology*. **9**, 268 (2019).
3. Vendrame, E. *et al.* TIGIT is upregulated by HIV-1 infection and marks a highly functional adaptive and mature subset of natural killer cells. *AIDS* . **34** (6), 801–813 (2020).
4. Zhao, N.Q. *et al.* Natural killer cell phenotype is altered in HIV-exposed seronegative women. *PloS one*. **15** (9), e0238347 (2020).
5. Leipold, M.D., Newell, E.W., Maecker, H.T. Multiparameter Phenotyping of Human PBMCs Using Mass Cytometry. *Methods in molecular biology* . **1343**, 81–95 (2015).
6. Baumgart, S., Peddinghaus, A., Schulte-Wrede, U., Mei, H.E., Grützkau, A. OMIP-034: Comprehensive immune phenotyping of human peripheral leukocytes by mass cytometry for monitoring immunomodulatory therapies. *Cytometry. Part A: the journal of the International Society for Analytical Cytology*. **91** (1), 34–38 (2017).

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE Sup Figure 1.pdf



Supplemental Figure 2

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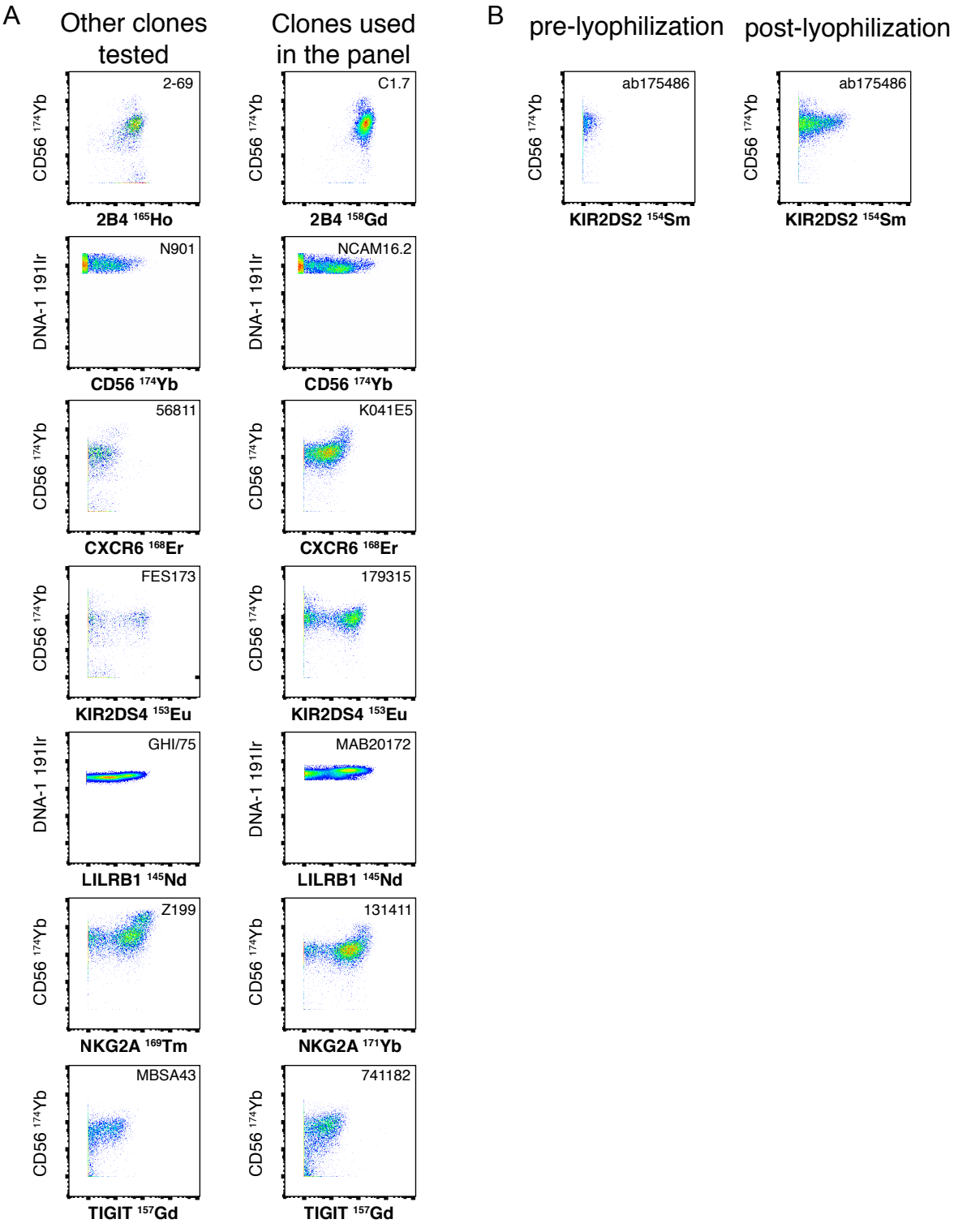

Supplemental Figure 3

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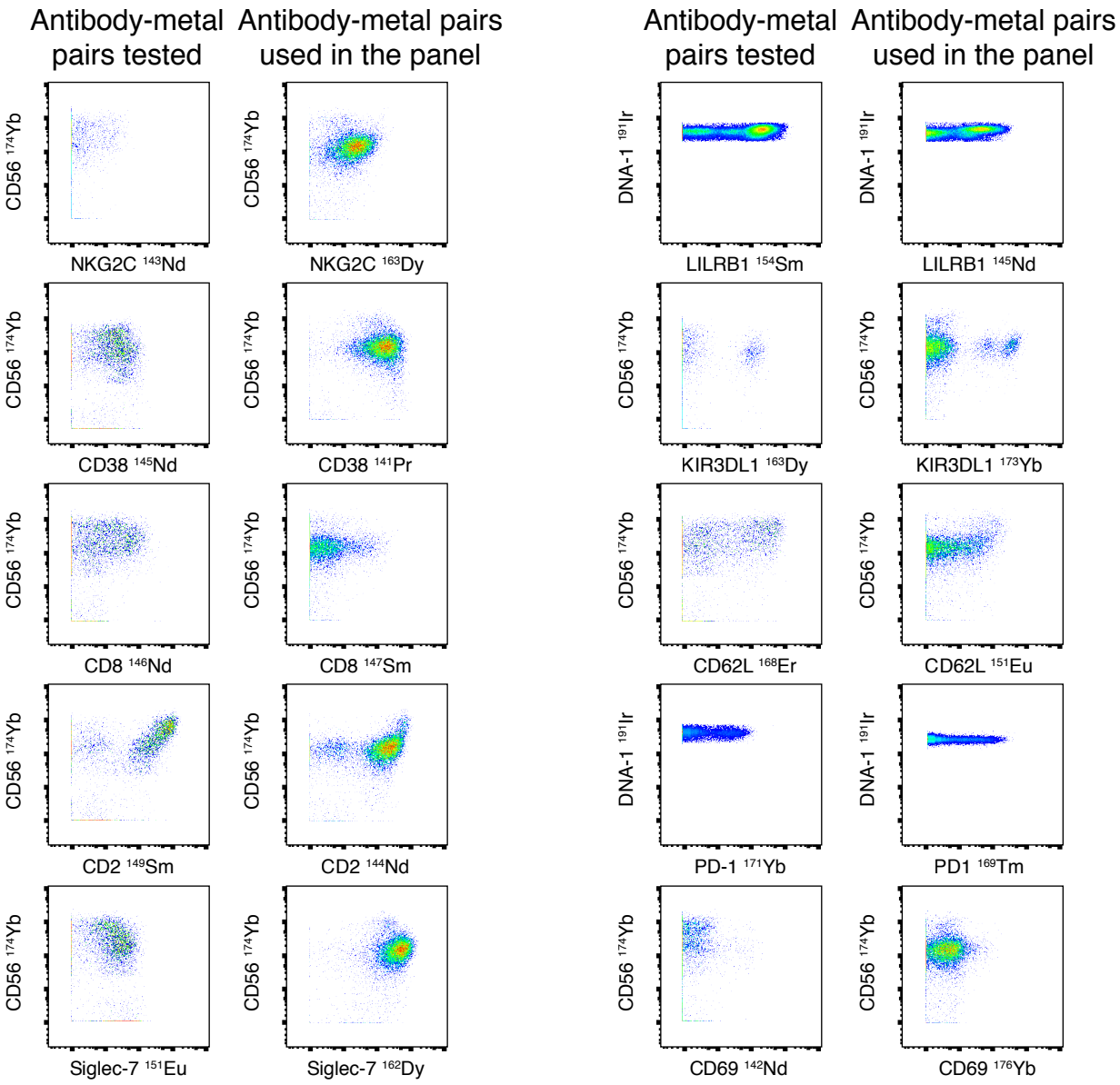

Supplemental Figure 4

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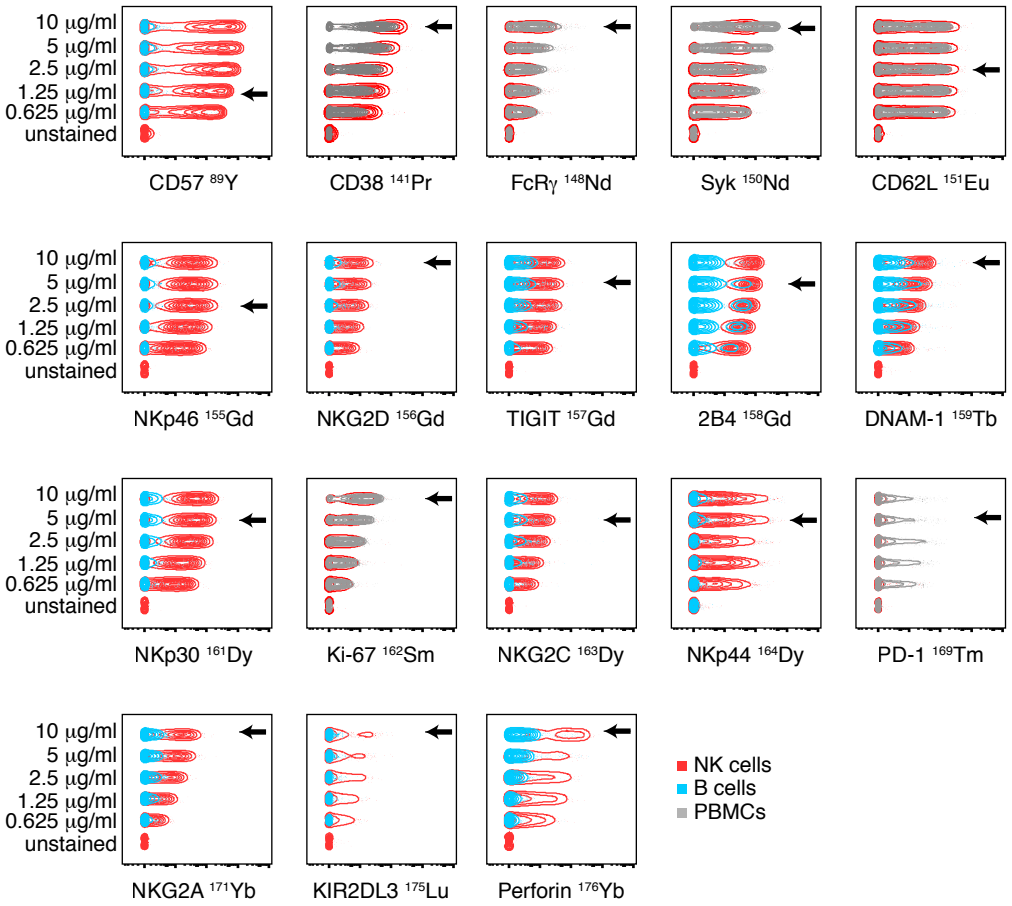
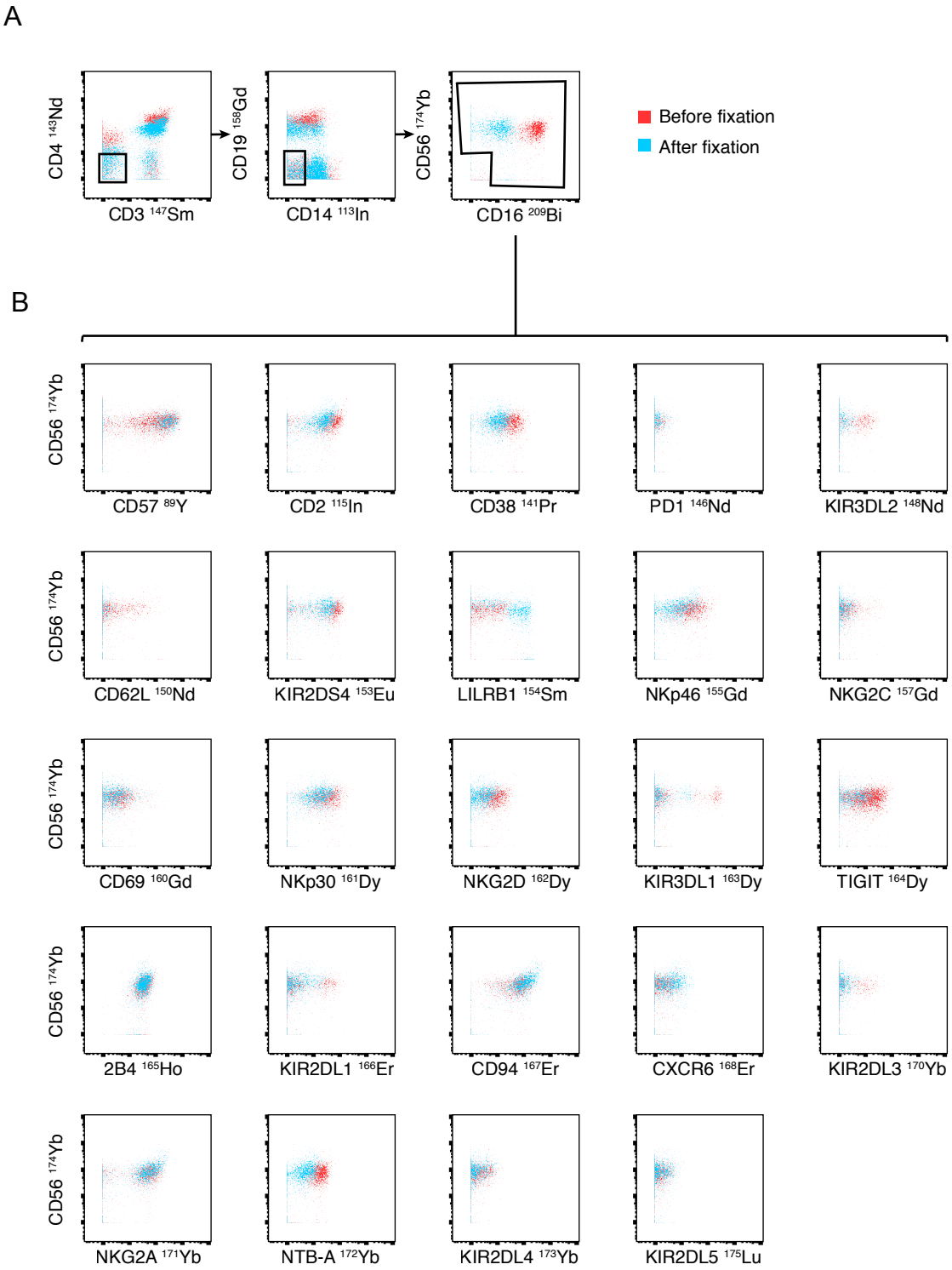
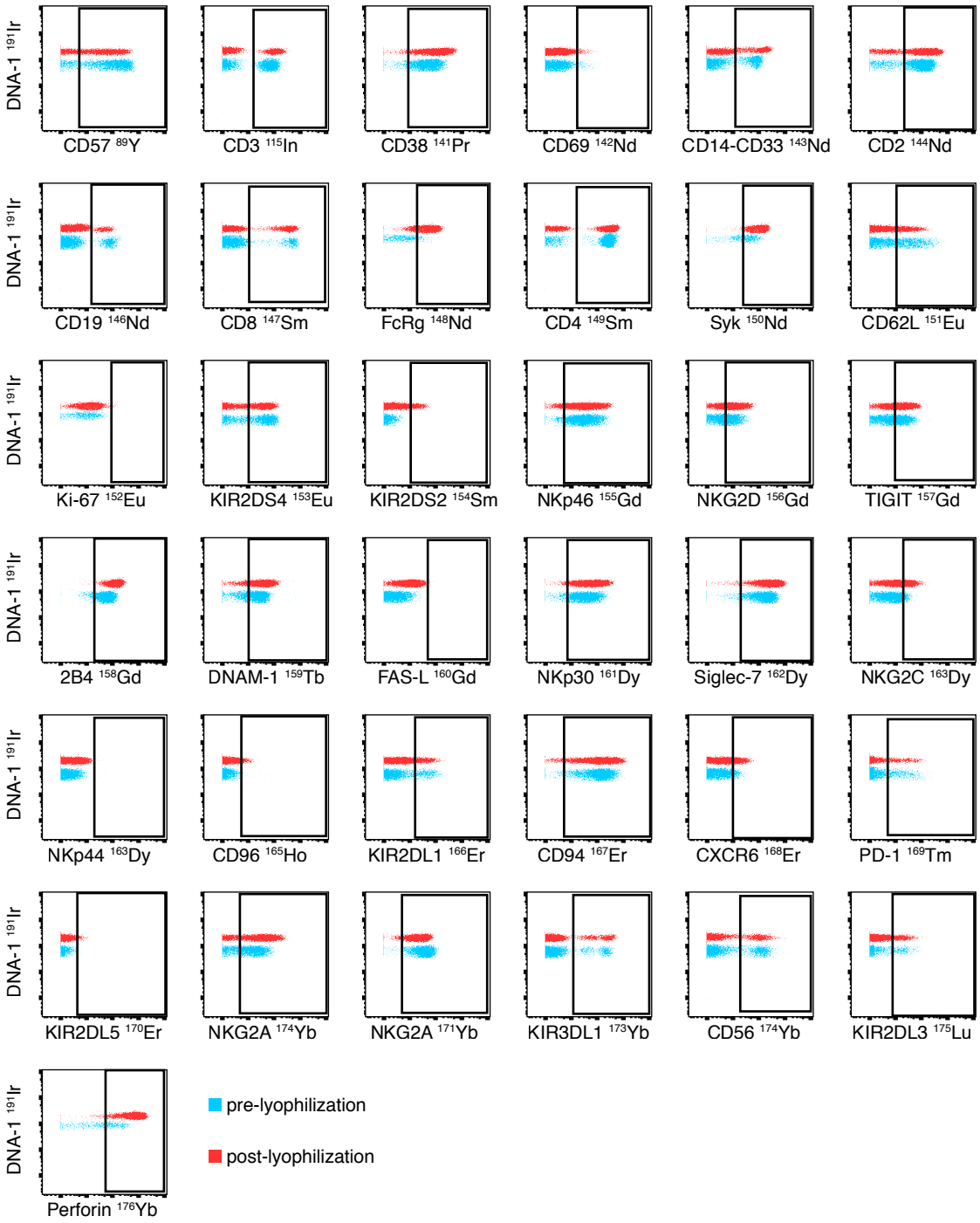




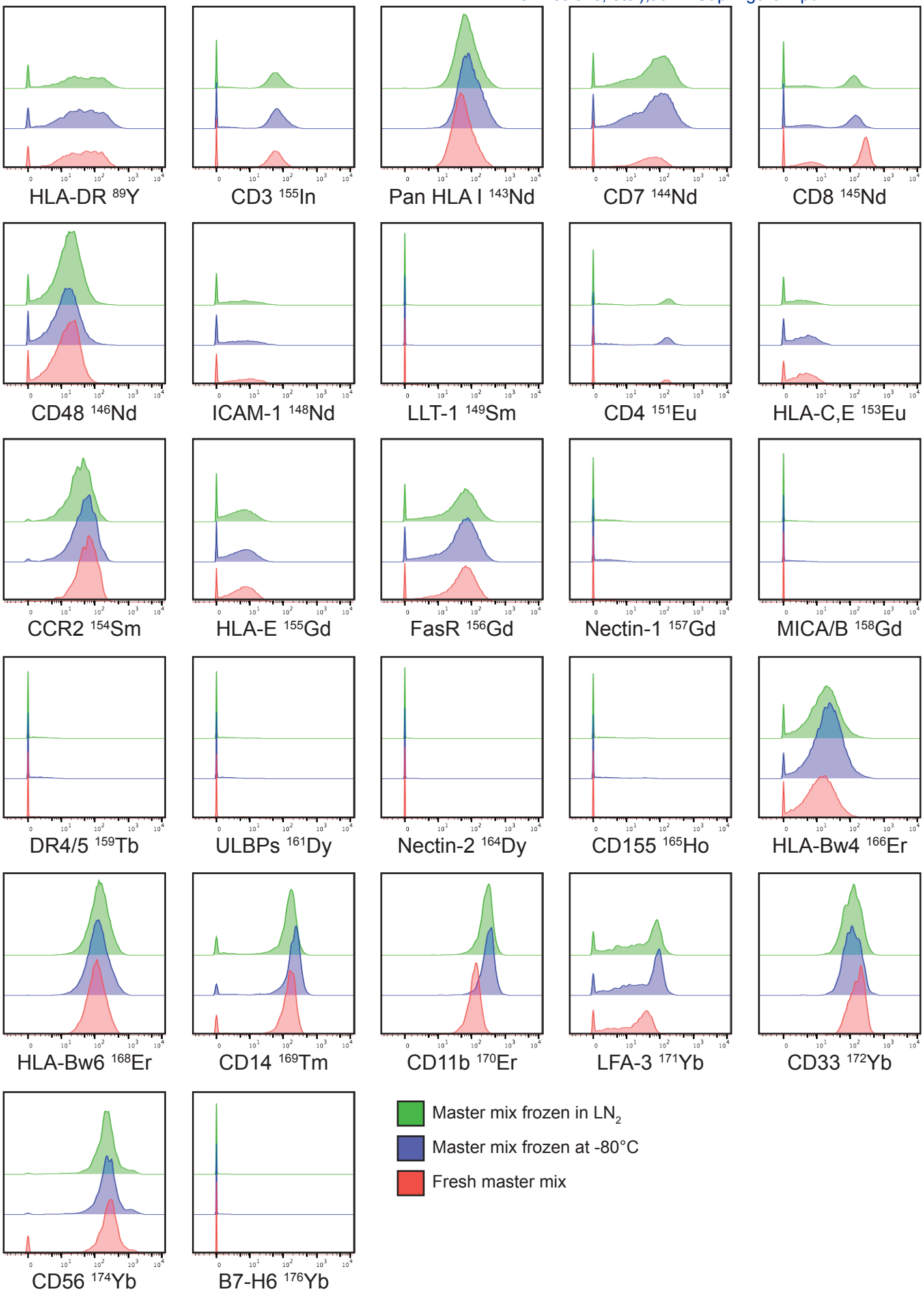
Supplemental Figure 6

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE Sup Figure 6.pdf



Supplemental Figure 7

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE Sup Figure 7.pdf



Supplemental Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVE Sup Table 1.xlsx

Specificity	Clone	Vendor	Catalog Number
2B4	2-69	BD Biosciences	550814
CD56	N901	Beckman Coulter	6602705
CXCR6	56811	R&D Systems	MAB699
KIR2DS2	polyclonal	Abcam	ab175486
KIR2DS4	FES172	Beckman Coulter	A60796
LILRB1	GHI/75	Biolegend	333702
NKG2A	Z199	Beckman Coulter	IM2750
TIGIT	MBSA43	Thermo Fisher Scientific	16-9500-82

Surface/ Intracellular	Notes
surface	new clone validated with improved staining
surface	new clone validated with improved staining
surface	new clone validated with lower cost
surface	non specific staining noted after lyophilization - not used for analyses
surface	new clone validated with improved staining/lower cost
surface	new clone validated with improved staining
surface	new clone validated with lower cost
surface	new clone validated

Supplemental Table 2

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Isotope	Specificity	Clone	Vendor
^{143}Nd	NKG2C	134522	R&D systems
^{145}Nd	CD38	HIT2	Biolegend
^{146}Nd	CD8	SK1	Biolegend
^{149}Sm	CD2	RPA-2.10	Biolegend
^{151}Eu	Siglec-7	S7.7	Biolegend
^{154}Sm	LILRB1	292319	R&D systems
^{163}Dy	KIR3DL1	DX-9	BD biosciences
^{168}Er	CD62L	DREG-56	Biolegend
^{171}Yb	PD1	EH12.2H7	Biolegend
^{176}Yb	CD69	EH12.2H7	Biolegend

Catalog Number	Surface/ Intracellular
MAB1381	surface
303502	surface
344702	surface
300202	surface
347702	surface
MAB20172	surface
555964	surface
304802	surface
329902	surface
329902	surface