A Comprehensive Assessment of Human Natural Killer Cell Phenotype and Function in Whole Blood

M Market[1*], G Tennakoon[2], J Ng[2], M Scaffidi[1], M A Kennedy[2], C Tanese de Souza[2], R C Auer[2,3]

[1]University of Ottawa, Ottawa, ON, Canada
[2]The Ottawa Hospital Research Institute, Ottawa, ON, Canada
[3]Department of Surgery, University of Ottawa, The Ottawa Hospital, Ottawa, ON, Canada

*** Correspondence:**
Marisa Market
mmark030@uottawa.ca

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Abstract

The majority of data on human Natural Killer (NK) cell phenotype and function has been generated using cryopreserved peripheral blood mononuclear cells (PBMCs). However, cryopreservation can have adverse effects on PBMCs. In contrast, investigating immune cells in whole blood can reduce the time, volume of blood required, and potential artifacts associated with manipulation of the cells. Whole blood collected from healthy donors and cancer patients was processed by three separate protocols that can be used independently or in parallel to assess extracellular receptors, intracellular signaling protein phosphorylation, and intracellular and extracellular cytokine production in human NK cells. To assess extracellular receptor expression, 200 µL of whole blood was incubated with an extracellular staining (ECS) mix and cells were subsequently fixed and RBCs lysed prior to analysis. The phosphorylation status of signaling proteins was assessed in 500 µL of whole blood following co-incubation with interleukin (IL)-2/12 and the ECS mix for 20 minutes prior to cell fixation and RBC lysis and subsequent permeabilization for staining with an intracellular staining (ICS) mix. Cytokine production (IFNγ) was similarly assessed by incubating 1 mL of whole blood with PMA-ionomycin or IL-2/12 prior to incubation with ECS and subsequent ICS antibodies. In addition, plasma was collected from stimulated samples prior to ECS for quantification of secreted IFNγ by ELISA. Results were consistent, despite inherent inter-patient variability. Although we did not investigate an exhaustive list of targets, this approach enabled quantification of representative ECS surface markers including activating (NKG2D and DNAM-1) and inhibitory (NKG2D, PD-1, TIGIT, and TIM-3) receptors, cytokine receptors (CD25, CD122, CD132, and CD212) and ICS markers associated with NK cell activation following stimulation, including signaling protein phosphorylation (p-STAT4, p-STAT5, p-p38 MAPK, p-S6K) and IFNγ in both healthy donors and cancer patients. In addition, we compared extracellular receptor expression using whole blood versus cryopreserved PBMCs and observed a significant difference in the expression of almost all receptors. The methods presented permit a relatively rapid parallel assessment of immune cell receptor expression, signaling protein activity, and cytokine production in a minimal volume of whole blood from both healthy donors and cancer patients.

1 Introduction

Natural Killer (NK) cells, first identified by Kiessling et al. in 1975, are cytotoxic lymphocytes that play a critical role in the innate immune response through the destruction of stressed, infected, or cancerous cells(1). Defective NK cell function has been linked to autoimmune and infectious diseases as well as cancer(2–6). Our investigations focus on understanding the suppression of NK cells following surgery in cancer patients and the impact of immunosuppression on metastasis. Specifically, our lab and others have shown that postoperative defects in NK cell cytotoxicity and IFNγ production contribute to increased metastasis in models of surgical stress(7–9). Our initial observations of this suppressed phenotype were in cryopreserved PBMCs; however, we have also observed this phenomenon in whole blood. We then developed protocols that can be used in parallel to assess the phenotype, intracellular signaling following cytokine stimulation, and cytokine production of immune cells, and as an example, in this paper we highlight its implementation for our ongoing research investigating NK cells in cancer patients.

For practical reasons, the majority of the data on human NK cells has been generated using peripheral blood mononuclear cells (PBMCs). For instance, cryopreservation allows for running batched samples simultaneously as well as logistical flexibility for the storage and shipment of samples between research facilities(10). Using this approach, the study of cryopreserved PBMCs through functional and phenotypic assays has yielded a great deal of understanding about the role of NK cell function in disease. However, the use of cryopreserved PBMCs in immunologic studies is associated with adverse effects on cell populations/ certain cell markers and altered gene expression(11–13). As a result, our understanding of NK cells may benefit in certain circumstances from investigations of non-cryopreserved cells.

In trying to assess the mechanism of NK cell dysfunction in cancer patients in the context of surgery, we sought to assess key markers and intracellular pathways associated with this dysfunctional NK cell phenotype. We investigated upstream receptor expression and subsequent signaling protein phosphorylation in order to elucidate the mechanism of NK cell suppression. NK cells do not undergo clonal selection, they instead express a limited number of germline-encoded receptors(14). NK cell activating receptors recognize pathogen-derived antigens as well as stress-induced ligands in what is termed the "induced-self recognition model"(15–17). These activating signals are antagonized by inhibitory receptors that recognize constitutively expressed self-molecules or inhibitory checkpoint proteins(15,16). We sought to assess the expression levels of the activating receptors NKG2D and DNAM-1 and the inhibitory receptors NKG2A, PD-1, TIGIT, and TIM-3. In addition to these receptors, NK cells also express a plethora of cytokine receptors, including interleukin (IL)-2R and IL-12R(18). NK cell activity is thus regulated by the integration of activating and inhibitory ligands through these many receptors, which results in phosphorylation and transduction through signaling proteins such as STAT4, STAT5, p38 MAPK, and S6K(9,19–23). This culminates in the regulation of transcription factor activity that controls the transcription of cytokines such as IFNγ and cytotoxic proteins, including granzymes and perforin(24,25). In characterizing the perioperative NK cell phenotype, we found it challenging to assess phosphorylation status in cryopreserved PBMCs. As a solution, we considered the use of whole blood, which proved to be far superior. In the troubleshooting process we also discovered a discrepancy between the phenotypes observed in cryopreserved PBMCs versus whole blood staining. The successes we experienced by using whole blood samples, compared to cryopreserved PBMCs, prompted us to continue using whole blood samples for assessment of NK cell activity

and develop a series of easily implemented, standardized protocols that enable a comprehensive investigation of NK phenotype and function.

There is a paucity of studies investigating immune cell function from whole blood(26). We posit that such studies would avoid the adverse effects of cryopreservation and provide more biologically relevant results in some circumstances. For example, investigating protein phosphorylation states by flow cytometry is difficult in cryopreserved samples due to the low signal to noise ratio of the target protein compared to investigations in whole blood samples(27). Many of these limitations can be overcome by staining directly in whole blood, which also allows for simpler and faster protocols that require minimal manipulation of the cells of interest and therefore support the biological relevance of the results. A limitation of whole blood assays includes having to process patient samples immediately and therefore they cannot be tested simultaneously, which could lead to greater inter-assay variability. However, technical expertise, appropriate controls, and validated standard operating procedures can be implemented to help mitigate this limitation.

Comparisons of immunologic assays using cryopreserved PBMCs and whole blood samples have previously been reported and is not the focus of our report(24,25,28). Here we sought to highlight the feasibility and advantages of using whole blood samples as a strategy for phenotypic and functional assessments in NK cells. As a proof of concept, we show the utilization of these protocols in our ongoing research. We explored the differential expression of phenotypic receptors necessary for NK activity and phosphorylation of downstream signaling molecules in healthy donors and cancer patients using whole blood. Finally, NK cell function was investigated by quantifying intracellular and extracellular IFNγ by flow cytometry and ELISA following stimulation with IL-2/IL-12 or PMA/Ionomycin. We show that assaying cryopreserved cells results in altered NK cell phenotype in human patients as compared to whole blood analysis. In addition, we outline in detail novel whole blood protocols that can be used in parallel to assess immune cell receptor expression, signaling protein phosphorylation, and cytokine production. Although developed to assess NK cell activity in the perioperative period, these protocols could be used to assess other immune cell phenotypes in other pathological conditions.

2 Materials and Equipment

- 37°C incubator
- 37°C water bath
- Centrifuge
- Sodium-heparin tubes (BD Vacutainer® Cat #367878/ 367874)
- BD FACS Lyse/ Fix Buffer (Cat #558049)
- Deionized/ distilled H_2O
- BD Perm III Buffer (Cat #558050)
- 1% Paraformaldehyde
- Recombinant human IL-2 (Tecin Teceleukin)
- Recombinant human IL-12 (R&D System Cat #219-IL005)
- Phosphate Buffered Saline (PBS)
- Flow Buffer (PBS + 2.5g BSA + 0.5M EDTA)
- Biolegend Human Fc block (Cat #422302)
- BD Golgi plug (Brefaldin A) (Cat #51-2301K2)
- R&D Quantikine Human IFNγ ELISA (Cat #DIF50)

- Flow cytometer (LSR Fortessa)
- Antibodies: (**Table 1**)

3 Methods

This protocol was approved by the Ottawa Health Science Research Ethics Board. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Eligible patients were >18 years of age and had a planned surgical resection of the primary or metastatic tumour (cancer patients) or healthy donors who volunteered to participate. Exclusion criteria included a history of active viral or bacterial infection or known HIV or Hepatitis B or C, autoimmune diseases, or use of immunosuppressive medications.

3.1 Protocol 1 – Extracellular Receptor Staining (Figure 1A)

Objective: Assess cell surface receptor expression in Natural Killer cells from whole blood.

1. Collect whole blood in sodium-heparin tubes (blood drawn by trained nurse/ phlebotomist)
2. Prepare FACS lyse/ fix buffer in advance (1:5 dilution with diH_2O; 20X blood volume per sample)
3. Transfer 200 µL of whole blood to 15 mL falcon tube
4. Add extracellular staining (ECS) mix (40 µL); Mix by pipetting up and down
5. Incubate for 15 mins at room temperature
6. Add FACS lyse/ fix buffer
7. Incubate for 10 minutes in 37°C water bath
8. Spin at 500 g for 8 minutes
9. Aspirate supernatant and resuspend in 1 mL flow buffer
10. Spin at 500 g for 5 mins
11. Resuspend in 200 µL flow buffer
12. Store at 4°C for up to 24 hours

3.2 Protocol 2 – Intracellular Signaling Protein Phosphorylation Staining (Figure 4A)

Objective: Assess signaling protein/ transcription factor phosphorylation in response to stimuli in Natural Killer cells from whole blood.

1. Collect whole blood in sodium-heparin tubes (blood drawn by trained nurse/ phlebotomist)
2. Prepare FACS lyse/ fix buffer in advance (1:5 dilution with diH_2O; 20X blood volume per sample)
3. Aliquot 500 µL blood into new sodium-heparin tubes
4. Add stimulation and ECS mix and incubate for 20 minutes in 37°C water bath
5. Transfer whole blood to 15 mL falcon tubes
6. Add FACS lyse/fix buffer
7. Incubate for 10 minutes in 37°C water bath
8. Spin at 500 g for 8 minutes
9. Aspirate supernatant and wash with 1 mL flow buffer

10. Spin at 500 g for 5 minutes
11. Aspirate supernatant and resuspend in 500 μL chilled BD Perm III buffer
12. Incubate on ice in the dark for 30 minutes
13. Spin at 300 g for 10 minutes
14. Aspirate supernatant and resuspend in 400 μL flow buffer
15. Transfer 200 μL/well into 96 well v-bottom plate
16. Spin at 500 g for 5 minutes
17. Empty plate and resuspend in appropriate intracellular staining (ICS) mix (200 μL)
18. Incubate at room temperature in the dark for 1 hour
19. Spin at 500 g for 5 mins
20. Empty plate and resuspend in 200 μL flow buffer
21. Store at 4°C for up to 24 hours

3.3 Protocol 3 – Intracellular IFNγ Staining (Figure 5A)

Objective: Quantify intracellular IFNγ production as a measure of activity in Natural Killer cells from whole blood.

1. Collect whole blood in sodium-heparin tubes (blood drawn by trained nurse/ phlebotomist)
2. Prepare FACS lyse/ fix buffer in advance (1:5 dilution with diH_2O; 20X blood volume per sample)
3. Aliquot 1 mL of whole blood into new sodium-heparin tubes
4. Incubate whole blood with PMA-ionomycin for 5 hours at 37°C and IL-2/IL-12 for 24 hours at 37°C
5. Add 10 ug/mL Golgi plug (Brefaldin A) per tube for the last 2 hours of each incubation
6. Invert tubes 10 times and incubate at 37°C for remaining 2 hours
7. Collect 600 μL whole blood in Eppendorf tube, spin at 13000 rpm for 1 minute, and store at -80°C for IFNγ ELISA
8. Transfer remaining 400 μL to 15mL falcon tubes
9. Incubate with Fc block (50 μL) for 5 mins at room temperature
10. Add ECS mix (40 μL); Mix by pipetting up and down
11. Incubate for 15 mins at room temperature
12. Add FACS lyse/ fix buffer
13. Incubate for 10 minutes in 37°C water bath
14. Spin at 500 g for 8 minutes
15. Aspirate supernatant and resuspend in 1 mL flow buffer
16. Spin at 500 g for 5 mins
17. Aspirate supernatant and resuspend in 500 μL chilled BD Perm III buffer
18. Incubate on ice in the dark for 30 minutes
19. Spin at 300 g for 10 minutes
20. Aspirate supernatant and resuspend in 400 μL flow buffer
21. Transfer 200 μL/ well into 96 well v-bottom plate
22. Spin at 500 g for 5 minutes
23. Empty plate and resuspend in appropriate ICS mix (200 μL)
24. Incubate at 4°C for 30 minutes

25. Empty plate and resuspend in 200 μL flow buffer
26. Store at 4°C for up to 24 hours

Extracellular IFNγ Quantification (Figure 6A)

Objective: Quantify extracellular IFNγ production as a measure of activity in plasma from whole blood.

1. Thaw plasma samples at room temperature
2. Prior to beginning the ELISA, dilute samples at 1:5 or 1:0 for optimal quantification
3. Follow R&D Quantikine Human IFNγ ELISA protocol for patient plasma

4 Data Analysis

Descriptive statistics were used to summarize data collected on extracellular receptors, phospho-signaling proteins, and IFNγ production (median with interquartile range (IQR)). Wilcoxon matched-pairs signed rank test was used to determine if there were significant changes in receptor expression (percentage and MFI) between cryopreserved and whole blood samples. The level for statistical significance was set a priori at ≤0.05 (*p≤0.05, **p≤0.005, ***p≤0.0005, ****p≤0.00005). All statistical analyses were performed using Prism 8.

5 Results

5.1 Protocol 1 – Extracellular Receptor Staining

Quantifying NK cell surface receptors in whole blood

Using a 10 colour flow cytometry panel, we assessed the surface expression of six NK cell receptors, which are known to activate (NKG2D and DNAM-1) or inhibit (NKG2A, PD-1, TIGIT, and TIM-3) NK cell effector functions in a cohort of 16 healthy donors and 20 cancer patients (**Table 2**). Using a 9 colour flow cytometry panel, we similarly assessed whether the expression of IL-2/12 receptor subunits (CD25 (α), CD122 (β), CD132 (γ), and CD212 (β1)) could be detected in 13 healthy donors and 11 cancer patients (**Table 2**). We assessed the percentage of positive cells as well as the relative expression level (median fluorescence intensity/ MFI) of both activating/ inhibitory and cytokine receptors in NK cells ($CD56^+$ $CD3^-$) using the indicated gating strategy (**Figure 1B and C**). We were able to assess both activating/ inhibitory and cytokine receptor expression using this whole blood protocol (**Figure 2A,B and Supplemental Table 1**).

Discrepancies between whole blood and cryopreserved NK cell surface receptors

In addition to whole blood we also assessed the expression of NKG2D, DNAM-1, PD-1, TIGIT, and TIM-3 (n=10) and CD25 and CD212 (n=14) in NK cells from cryopreserved PBMCs. After ficoll density centrifugation, PBMCs were isolated, washed, and stored in liquid nitrogen in 90% FBS 10% DMSO. We followed a standard protocol whereby PBMCs were thawed, rested overnight, and stained using a 10 colour (activating/ inhibitory receptors) or a 9 colour (cytokine receptors) flow cytometry panel(29–32). Consistent with previous publications, we found

significant differences between the percentage of positive cells and receptor MFI in cryopreserved versus whole blood NK cells(33,34). (**Figure 3, Supplemental Table 2**).

5.2 Protocol 2 – Intracellular Signaling Protein Phosphorylation Staining

Detecting NK cell cytokine signaling in whole blood

The phosphorylation of signaling proteins downstream of IL-2/12 receptors was assessed in 13 healthy donors and 9 cancer patients. Activity was assessed with two 7 colour flow cytometry panels that included phospho-specific antibodies against STAT5, STAT4, p38 MAPK, and S6K. The indicated gating strategy allowed for the assessment of the relative expression level (MFI) of phosphorylated protein in NK cells (**Figure 4B**). We were able to quantify the phosphorylation of these signaling molecules in response to IL-2/12 stimulation in both healthy donors and cancer patients (**Figure 4C, Supplemental Table 3**).

5.3 Protocol 3 – Intracellular IFNγ Staining

Quantifying NK cell responsiveness to cytokine stimulation

Finally, NK cell activity was quantified by intracellular and extracellular IFNγ production in response to two stimuli: PMA-ionomycin (a receptor-independent stimulator of cytokine production), and IL-2/12. Intracellular IFNγ was assessed in 11 healthy donors and 9 cancer patients and extracellular IFNγ was assessed in 13 healthy donors and 10 cancer patients. A 6 colour flow cytometry panel and the indicated gating strategy was used to assess the percentage of NK cells producing IFNγ (**Figure 5B**). The healthy donor and cancer patient populations produced intracellular IFNγ in response to both stimuli (**Figure 5C, Supplemental Table 4**). In addition, extracellular IFNγ was quantified in 11 healthy donors and 10 cancer patients from plasma collected prior to intracellular staining. (**Figure 6B, Supplemental Table 4**).

6 Advantages

These assays require minimal volumes of blood (200 µL - 1 mL per sample) as compared to methods employing cryopreserved PBMCs (~30-40 mL). This allows for the simultaneous assessment of many targets and the use of whole blood from one patient for multiple assays. In addition, patients may be more likely to consent to blood draws for research that requires minimal blood volumes. These assays can be used to effectively measure extracellular and intracellular targets in healthy as well as disease states (cancer). Here we have assessed NK cell phenotype and function, however, the use of whole blood allows for the assessment of any immune cell, including neutrophils which would otherwise the excluded in PBMC isolation protocols. Finally, a discrepancy can be seen between extracellular targets measured on NK cells using whole blood versus cryopreserved PBMCs. We believe that assessing immune cell phenotypes using whole blood may be more biologically relevant as these protocols minimize the time between blood draw and cell staining and reduce the manipulation of cells that may otherwise impact target expression.

7 Limitations

There is potential for inter-assay variability as whole blood samples must be processed immediately, and therefore a large cohort cannot be assessed simultaneously without multiple consenting patients and researchers.

8 Troubleshooting

- Antibodies need to be titrated using whole blood to ensure appropriate staining
- May have to adjust incubation times for signaling proteins depending on target (15-30 minutes is a good range to test)
- Mix FACS lyse/fix and blood vigorously to ensure lysis of RBCs; if many RBCs left in pellet, repeat lyse/ fix a second time
- May want to test different Golgi plug timepoints - 4 hours vs. 2 hours
- Intracellular staining can be done in falcon tubes instead of 96 well v-bottom plate, but the pellet is easier to visualize in the plate
- Have also resuspended in PFA instead of flow buffer for longer storage at 4°C.
- CD45 and CD14 staining make the CD56+ CD3- population cleaner but are not necessary
- ELISA: Will need to test different dilutions to prevent OVERFLOW wells - 5x and 10x dilutions are usually acceptable

9 Discussion

The workflow of some immune cell studies may be more compatible with protocols utilizing cryopreserved samples, for example multi-institute studies, however, due to the advantages reported here we suggest that some assays may be significantly improved through the implementation of whole blood protocols. These assays circumvent the limitations associated with the use of cryopreserved PBMCs, namely manipulation of cells and the thawing process which may alter cell phenotype and function. Although comparisons have previously been made in the literature between cryopreserved and whole blood PBMC assays, we show that cryopreservation results in an aberrant NK cell phenotype and this is in keeping with what has been previously shown in the literature(24,33,34). We suggest that this discrepancy may lead to misinterpreted conclusions about altered immune cell phenotype and function. As a possible solution, we present methodologies for parallel assessment of immune cell receptor expression, signaling protein activity, and cytokine production in whole blood-derived NK cells. We have demonstrated the feasibility of these assays through the detection of target protein expression in both healthy and disease states (namely solid malignancies), the reproducibility of these assays in patient cohorts despite inherent inter-patient heterogeneity, and the validity of these assays in that our results are comparable to those previously described in the literature(2,12,32,34–39). They are simple, time-efficient, and allow for the assessment of any peripheral immune cell population using a minimal volume of whole blood. Finally, we suggest that they could be used to assess immune cell phenotype and function in any pathological condition, provided sufficient blood volumes.

10 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

11 Author Contributions

MM and GT performed the experiments and data analysis and were responsible for manuscript preparation. JN and MS screened and consented all patients and performed blood processing on cryopreserved samples. MM, GT, CD, MK, and RA oversaw experimental design and data interpretation.

12 Funding

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13 Data Availability Statement

The datasets for this study can be made available upon reasonable request.

Table 1. Antibodies used in whole blood panels.

Antibody	Vendor	Cat #	Clone
CD3 FITC	Invitrogen	11-0039-41	HIT3a
CD56 BV421	BD	562751	NCAM16.2
CD16 BV650	BD	563692	3G8
CD14 APC-Cy7	BD	557831	MφP9
CD45 AF700	BD	560566	HI30
Fixable viability dye BV510	BD	564406	-
IFNγ APC	Invitrogen	17-7319-82	4S.B3
APC IgG1	Biolegend	400119	MOPC-21
CD25 PE-Cy7	BD	557741	M-A251
PE-Cy7 IgG1	BD	557872	MOPC-21
p-STAT5 PE-Cy7	BD	560117	47/Stat5
CD212 BV786	BD	744207	2400000
BV786 IgG1	BD	563330	X40
p-STAT4 PE	BD	558249	38/p-Stat4
PE IgG2b (mouse)	Invitrogen	12-4732-41	eBMG2b
CD122 PE	BD	554522	Mik-β2
PE IgG2a (mouse)	Biolegend	400214	MOPC-173
NKG2D BV650	BD	563408	1D11
BV650 IgG1	BD	563231	X40
NKG2A PE	R&D Systems	FAB1059P-025	131411
PE IgG2A	BioLegend	400214	MOPC-173
BV786 TIM-3	BD	742857	7D3
PE-Cy7 DNAM-1	BioLegend	338315	11A8
APC TIGIT	BioLegend	372705	A15153G
APC IgG2A	BioLegend	400219	MOPC-173
PD-1 PerCP-Cy5.5	BioLegend	329913	EH12.2H7
PerCP-Cy5.5 IgG1	BioLegend	400149	MOPC-21

Table 2. Whole blood patient demographics. Summary of patient information including sex, age, cancer type, and cancer staging for patient samples used to quantify receptor expression, phosphorylation status, and IFNγ production using whole blood.

Category	**Subcategory**	**Healthy Donors**	**Patients**
Total (n)		40	39
Sex	Male	16	21
	Female	24	18
Patient Age	< 60 years	24	11
	60-69 years	9	19
	> 70 years	7	9
Cancer Type	Prostate	-	11
	Lung	-	7
	Colorectal	-	6
	Renal	-	3
	Ovarian	-	3
	Sarcoma	-	3
	Pancreatic	-	1
	Parathyroid	-	1
	Neuroendocrine	-	1
	Endometrial	-	1
	Duodenal	-	1
	Uterine	-	1
Staging	I	-	14
	II	-	7
	III	-	15
	IV	-	3

Table 3. Cryopreserved PBMC patient demographics. Summary of patient information including sex, age, cancer type, and cancer staging for patient samples used to quantify activating/ inhibitory/ cytokine receptors using cryopreserved PBMCs.

Category	Subcategory	Patients
Total (n)		22
Gender	Male	19
	Female	6
Patient Age	< 60 years	7
	60-69 years	13
	> 70 years	5
Cancer Type	Prostate	13
	Lung	6
	Colorectal	2
	Esophageal	1
Staging	I	4
	II	4
	III	10
	IV	1

Figure 1. Whole blood receptor methodology and gating strategy.
Whole blood was collected from patients and 200 μL was aliquoted per receptor panel. After a 15-minute incubation with the receptor panel, lyse/fix buffer was added and incubated for 10 minutes before blood was spun down, cells were washed and resuspended to be assessed by flow cytometry (**A**). The lymphocyte population was gated on before excluding doublets and dead cells. $CD45^+$ $CD14^-$ $CD56^+$ $CD3^-$ cells were gated on to assess activating/ inhibitory receptor (**B**) and cytokine receptor (**C**) expression based on isotype staining.

Figure 2. Whole blood extracellular surface receptor expression.
The percentage of CD56+ CD3- cells expressing activating receptors (NKG2D and DNAM-1), inhibitory receptors (NKG2A, PD-1, TIGIT, and TIM-3), and cytokine receptor subunits (CD25, CD122, CD132, and CD212) was assessed in healthy donors (n=29) and cancer patients (n=31) (**A**). The relative level of expression (MFI) of the same activating/ inhibitory/ cytokine receptors was also assessed in $CD56^+$ $CD3^-$ cells (**B**). Shown are the median values ± IQR.

Figure 3. Expression of extracellular surface receptors differs between cryopreserved PBMCs and whole blood samples.
A significant difference was observed between the percentage of $CD56^+$ $CD3^-$ cells expressing all activating and inhibitory receptors (cryopreserved n=10, whole blood n=20), as well as the cytokine receptor subunit CD25 (cryopreserved n=14, whole blood n=11) (**A**). In addition, a significant difference was also observed in the relative expression levels of DNAM-1, all inhibitory receptors, and both cytokine receptor subunits (**B**). Shown are the median values ± IQR. The Mann-Whitney test was used to assess statistical significance. $p \leq 0.05$ (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p \leq 0.00005$).

Figure 4. Whole blood signaling protein methodology, gating strategy, and quantification.
Whole blood was collected from patients and 200 μL was aliquoted per receptor panel. After a 15-minute incubation with IL-2/12 stimulation and the desired receptor panel, lyse/fix buffer was added and incubated for 10 minutes before blood was spun down. Cells were washed, resuspended in Perm III Buffer, and incubated on ice for 30 minutes before being spun down and resuspended in an intracellular staining mix. Cells were then incubated for an additional hour at room temperature prior to being resuspended and assessed by flow cytometry (**A**). The lymphocyte population was gated on before excluding doublets and dead cells. $CD45^+$ $CD14^-$ $CD56^+$ $CD3^-$ cells were gated on to assess signaling protein phosphorylation based on unstimulated controls (**B**). The relative level of expression (MFI) of phospho-proteins STAT5, STAT4, p38 MAPK, and S6K was assessed in healthy donor (n=13) and cancer patient (n=9) $CD56^+$ $CD3^-$ cells (**C**). Shown are the median values ± IQR.

Figure 5. Whole blood secretory cytokine methodology, gating strategy, and quantification.
Whole blood was collected from patients and 200 μL was aliquoted per stimulatory condition. Whole blood was stimulated for 24 hours in the presence of PMA-ionomycin, or IL-2/12. At 22 hours post-stimulation, Golgi plug was added to whole blood and incubated for an additional 2 hours. At 24 hours post-stimulation, whole blood was incubated for 15 minutes with an extracellular staining mix. Lyse/fix buffer was then added and incubated for 10 minutes before blood was spun down. Cells were washed, resuspended in Perm III Buffer, and incubated on ice for 30 minutes before being spun down and resuspended in an intracellular staining mix. Cells

were then incubated for an additional 30 minutes at 4°C prior to being resuspended and assessed by flow cytometry (**A**). The lymphocyte population was gated on before excluding doublets and dead cells. $CD45^+$ $CD14^-$ $CD56^+$ $CD3^-$ cells were gated on to assess intracellular IFNγ based on unstimulated controls (**B**). The percentage of $CD56^+$ $CD3^-$ cells expressing INFγ after stimulation was assessed in healthy donors (n=11) and cancer patients (n=9) (**C**). Shown are the median values ± IQR.

Figure 6. Plasma collection methodology for secretory cytokine quantification by ELISA. After incubation in the absence of stimulation or with either PMA-ionomycin or IL-2/12 stimulation for 24 hours, 600 μL of whole blood was collected and spun down prior to being collected and stored at -80°C for subsequent use in a human IFNγ ELISA (**A**). Extracellular IFNγ was quantified in response to stimuli in healthy donors (n=13) and cancer patients (n=10) (**B**). Shown are the median values ± IQR.

14 References

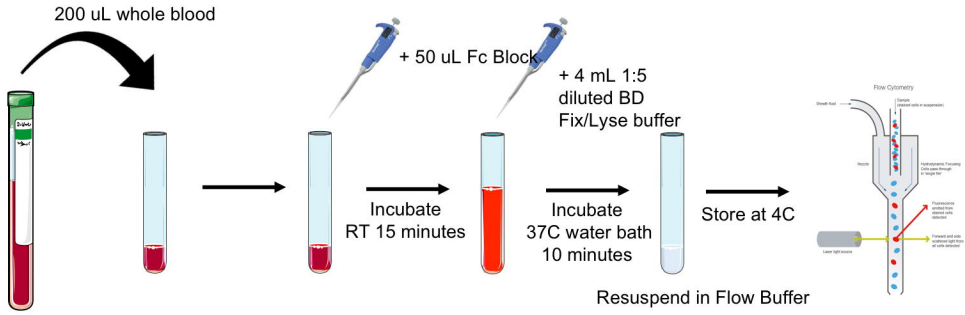
1. Kiessling R, Klein E, Pross H, Wigzell H. „Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* (1975) doi:10.1002/eji.1830050209
2. Angka L, Martel AB, Kilgour M, Jeong A, Sadiq M, de Souza CT, Baker L, Kennedy MA, Kekre N, Auer RC. Natural Killer Cell IFNγ Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery. *Ann Surg Oncol* (2018) doi:10.1245/s10434-018-6691-3
3. Weinberg A, Song LY, Wilkening C, Sevin A, Blais B, Louzao R, Stein D, Defechereux P, Durand D, Riedel E, et al. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol* (2009) doi:10.1128/CVI.00342-08
4. Zhang W, Nilles TL, Johnson JR, Margolick JB. The effect of cellular isolation and cryopreservation on the expression of markers identifying subsets of regulatory T cells. *J Immunol Methods* (2016) doi:10.1016/j.jim.2016.02.004
5. Ruitenberg JJ, Mulder CB, Maino VC, Landay AL, Ghanekar SA. VACUTAINER® CPT™ and Ficoll density gradient separation perform equivalently in maintaining the quality and function of PBMC from HIV seropositive blood samples. *BMC Immunol* (2006) doi:10.1186/1471-2172-7-11
6. Yang J, Diaz N, Adelsberger J, Zhou X, Stevens R, Rupert A, Metcalf JA, Baseler M, Barbon C, Imamichi T, et al. The effects of storage temperature on PBMC gene expression. *BMC Immunol* (2016) doi:10.1186/s12865-016-0144-1
7. Paust S, Senman B, Von Andrian UH. Adaptive immune responses mediated by natural killer cells. *Immunol Rev* (2010) **235**:286–296. doi:10.1111/j.0105-2896.2010.00906.x
8. Long EO, Sik Kim H, Liu D, Peterson ME, Rajagopalan S. Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annu Rev Immunol* (2013) doi:10.1146/annurev-immunol-020711-075005
9. Jonjić S, Babić M, Polić B, Krmpotić A. Immune evasion of natural killer cells by viruses. *Curr Opin Immunol* (2008) doi:10.1016/j.coi.2007.11.002
10. Fogel LA, Yokoyama WM, French AR. Natural killer cells in human autoimmune disorders. *Arthritis Res Ther* (2013) doi:10.1186/ar4232
11. Orange JS, Ballas ZK. Natural killer cells in human health and disease. *Clin Immunol* (2006) doi:10.1016/j.clim.2005.10.011
12. Tai LH, Alkayyal AA, Leslie AL, Sahi S, Bennett S, Tanese de Souza C, Baxter K, Angka L, Xu R, Kennedy MA, et al. Phosphodiesterase-5 inhibition reduces postoperative metastatic disease by targeting surgery-induced myeloid derived suppressor cell-dependent inhibition of Natural Killer cell cytotoxicity. *Oncoimmunology* (2018) doi:10.1080/2162402X.2018.1431082
13. Tai LH, De Souza CT, Bélanger S, Ly L, Alkayyal AA, Zhang J, Rintoul JL, Ananth AA, Lam T, Breitbach CJ, et al. Preventing postoperative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. *Cancer Res* (2013) **73**:97–107. doi:10.1158/0008-5472.CAN-12-1993
14. Zhang S, Kaplan MH. The p38 Mitogen-Activated Protein Kinase Is Required for IL-12-Induced IFN- Expression. *J Immunol* (2000) doi:10.4049/jimmunol.165.3.1374
15. Chester C, Fritsch K, Kohrt HE. Natural killer cell immunomodulation: Targeting

activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy. *Front Immunol* (2015) **6**: doi:10.3389/fimmu.2015.00601
16. Marçais A, Cherfils-Vicini J, Viant C, Degouve S, Viel S, Fenis A, Rabilloud J, Mayol K, Tavares A, Bienvenu J, et al. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nat Immunol* (2014) **15**:749–757. doi:10.1038/ni.2936
17. Kasahara T, Hooks JJ, Dougherty SF, Oppenheim JJ. Interleukin 2-mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. *J Immunol* (1983) **130**:1784–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6403613
18. Bachmann MF, Oxenius A. Interleukin 2: from immunostimulation to immunoregulation and back again. *EMBO Rep* (2007) **8**:1142–1148. doi:10.1038/sj.embor.7401099
19. Ouyang W, Jacobson NG, Bhattacharya D, Gorham JD, Fenoglio D, Sha WC, Murphy TL, Murphy KM. The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway. *Proc Natl Acad Sci U S A* (1999) **96**:3888–3893. doi:10.1073/pnas.96.7.3888
20. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. *Nat Rev Immunol* (2013) **13**:777–789. doi:10.1038/nri3536
21. Chan CJ, Smyth MJ, Martinet L. Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death Differ* (2014) **21**: doi:10.1038/cdd.2013.26
22. Mavropoulos A, Sully G, Cope AP, Clark AR. Stabilization of IFN-γ mRNA by MAPK p38 in IL-12- and IL-18-stimulated human NK cells. *Blood* (2005) doi:10.1182/blood-2004-07-2782
23. Posevitz-Fejfár A, Posevitz V, Gross CC, Bhatia U, Kurth F, Schütte V, Bar-Or A, Meuth SG, Wiendl H. Effects of blood transportation on human peripheral mononuclear cell yield, phenotype and function: Implications for immune cell biobanking. *PLoS One* (2014) doi:10.1371/journal.pone.0115920
24. Dominguez E, Lowdell MW, Perez-Cruz I, Madrigal A, Cohen SBA. Natural killer cell function is altered by freezing in DMSO. in *Biochemical Society Transactions* doi:10.1042/bst025175s
25. Mata MM, Mahmood F, Sowell RT, Baum LL. Effects of cryopreservation on effector cells for antibody dependent cell-mediated cytotoxicity (ADCC) and natural killer (NK) cell activity in 51Cr-release and CD107a assays. *J Immunol Methods* (2014) doi:10.1016/j.jim.2014.01.017
26. Claus M, Greil J, Watzl C. Comprehensive analysis of NK cell function in whole blood samples. *J Immunol Methods* (2009) doi:10.1016/j.jim.2008.11.006
27. Ebbo M, Gérard L, Carpentier S, Vély F, Cypowyj S, Farnarier C, Vince N, Malphettes M, Fieschi C, Oksenhendler E, et al. Low Circulating Natural Killer Cell Counts are Associated With Severe Disease in Patients With Common Variable Immunodeficiency. *EBioMedicine* (2016) doi:10.1016/j.ebiom.2016.02.025
28. Domogala A, Alejandro Madrigal J, Saudemont A. Cryopreservation has no effect on function of natural killer cells differentiated in vitro from umbilical cord blood CD34+ cells. *Cytotherapy* (2016) doi:10.1016/j.jcyt.2016.02.008
29. Ramachandran H, Laux J, Moldovan I, Caspell R, Lehmann P V., Subbramanian RA. Optimal Thawing of Cryopreserved Peripheral Blood Mononuclear Cells for Use in High-Throughput Human Immune Monitoring Studies. *Cells* (2012) doi:10.3390/cells1030313
30. Kutscher S, Dembek CJ, Deckert S, Russo C, Körber N, Bogner JR, Geisler F, Umgelter

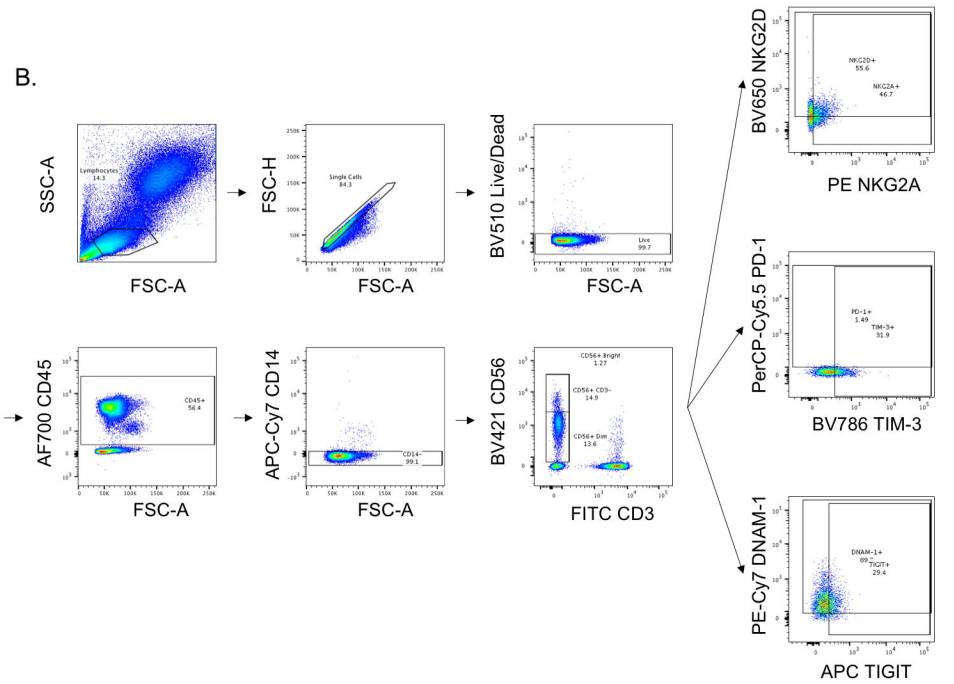
A, Neuenhahn M, Albrecht J, et al. Overnight Resting of PBMC Changes Functional Signatures of Antigen Specific T- Cell Responses: Impact for Immune Monitoring within Clinical Trials. *PLoS One* (2013) doi:10.1371/journal.pone.0076215

31. Wang L, Hückelhoven A, Hong J, Jin N, Mani J, Chen B an, Schmitt M, Schmitt A. Standardization of cryopreserved peripheral blood mononuclear cells through a resting process for clinical immunomonitoring--Development of an algorithm. *Cytometry A* (2016) doi:10.1002/cyto.a.22813
32. Dunne J, Lynch S, O'Farrelly C, Todryk S, Hegarty JE, Feighery C, Doherty DG. Selective Expansion and Partial Activation of Human NK Cells and NK Receptor-Positive T Cells by IL-2 and IL-15. *J Immunol* (2001) doi:10.4049/jimmunol.167.6.3129
33. Miralles A, Peiró M, Amill B, de Dalmases C, Piñol G, Rueda F, García J. Differential effect of cryopreservation on natural killer cell and lymphokine-activated killer cell activities. *Transfusion* (1993) doi:10.1046/j.1537-2995.1993.33893342746.x
34. Naranbhai V, Bartman P, Ndlovu D, Ramkalawon P, Ndung'u T, Wilson D, Altfeld M, Carr WH. Impact of blood processing variations on natural killer cell frequency, activation, chemokine receptor expression and function. *J Immunol Methods* (2011) doi:10.1016/j.jim.2011.01.001
35. Saito H, Osaki T, Ikeguchi M. Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer. *Gastric Cancer* (2012) doi:10.1007/s10120-011-0059-8
36. Yin X, Liu T, Wang Z, Ma M, Lei J, Zhang Z, Fu S, Fu Y, Hu Q, Ding H, et al. Expression of the Inhibitory Receptor TIGIT Is Up-Regulated Specifically on NK Cells With CD226 Activating Receptor From HIV-Infected Individuals. *Front Immunol* doi:10.3389/fimmu.2018.02341
37. Han B, Mao F, Zhao Y, Lv Y, Teng Y, Duan M, Chen W, Cheng P, Wang T, Liang Z, et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 Expression on Circulating NK Cells Is Associated with Tumor Progression in Human Gastric Cancer. *J Immunol Res* (2018) doi:10.1155/2018/6248590
38. Hatton O, Strauss-Albee DM, Zhao NQ, Haggadone MD, Pelpola JS, Krams SM, Martinez OM, Blish CA. NKG2A-expressing natural killer cells dominate the response to autologous lymphoblastoid cells infected with epstein-barr virus. *Front Immunol* (2016) doi:10.3389/fimmu.2016.00607
39. Agaugué S, Marcenaro E, Ferranti B, Moretta L, Moretta A. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. *Blood* (2008) **112**:1776–1783. doi:10.1182/blood-2008-02-135871

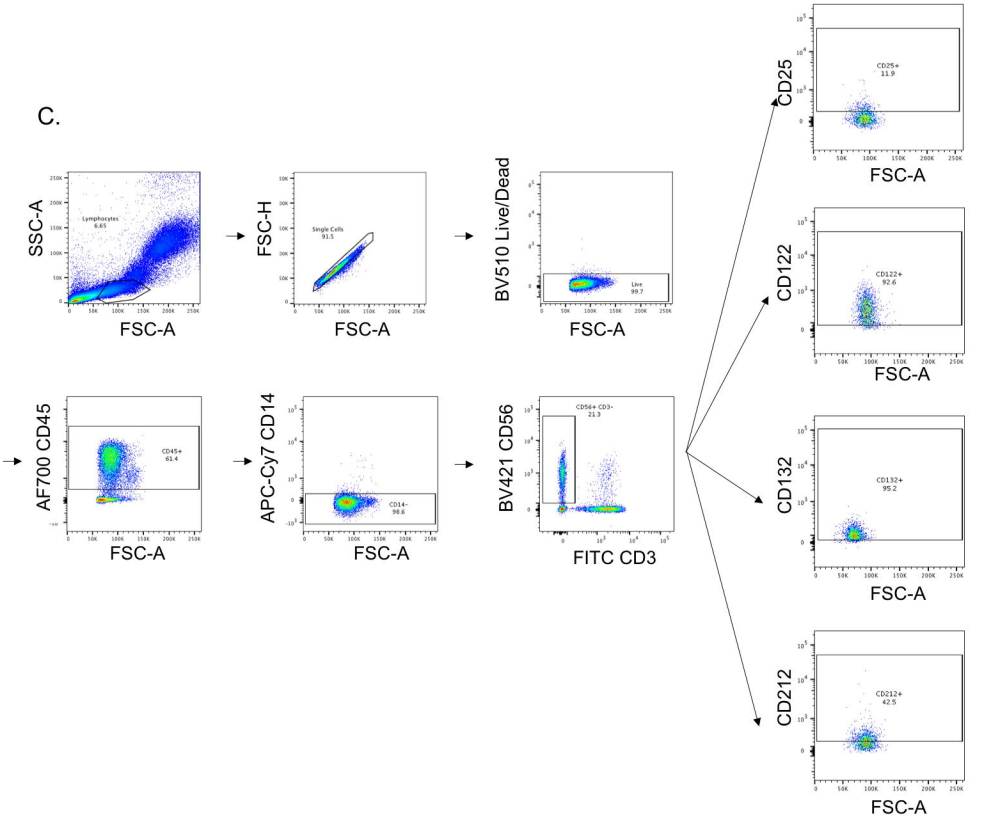
A.

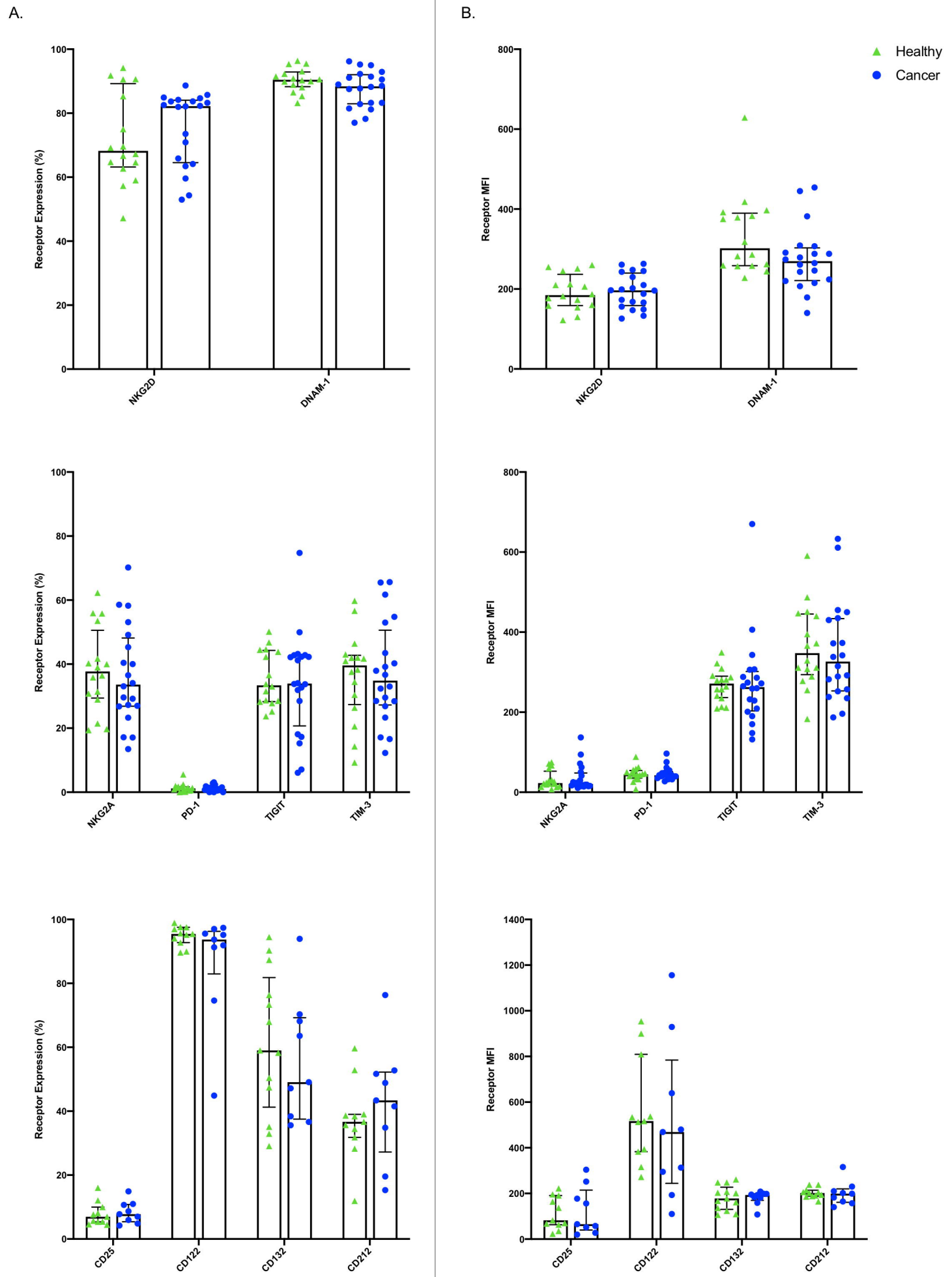

200 uL whole blood
+ 50 uL Fc Block
+ 4 mL 1:5 diluted BD Fix/Lyse buffer
Incubate RT 15 minutes
Incubate 37C water bath 10 minutes
Store at 4C
Resuspend in Flow Buffer
Flow Cytometry

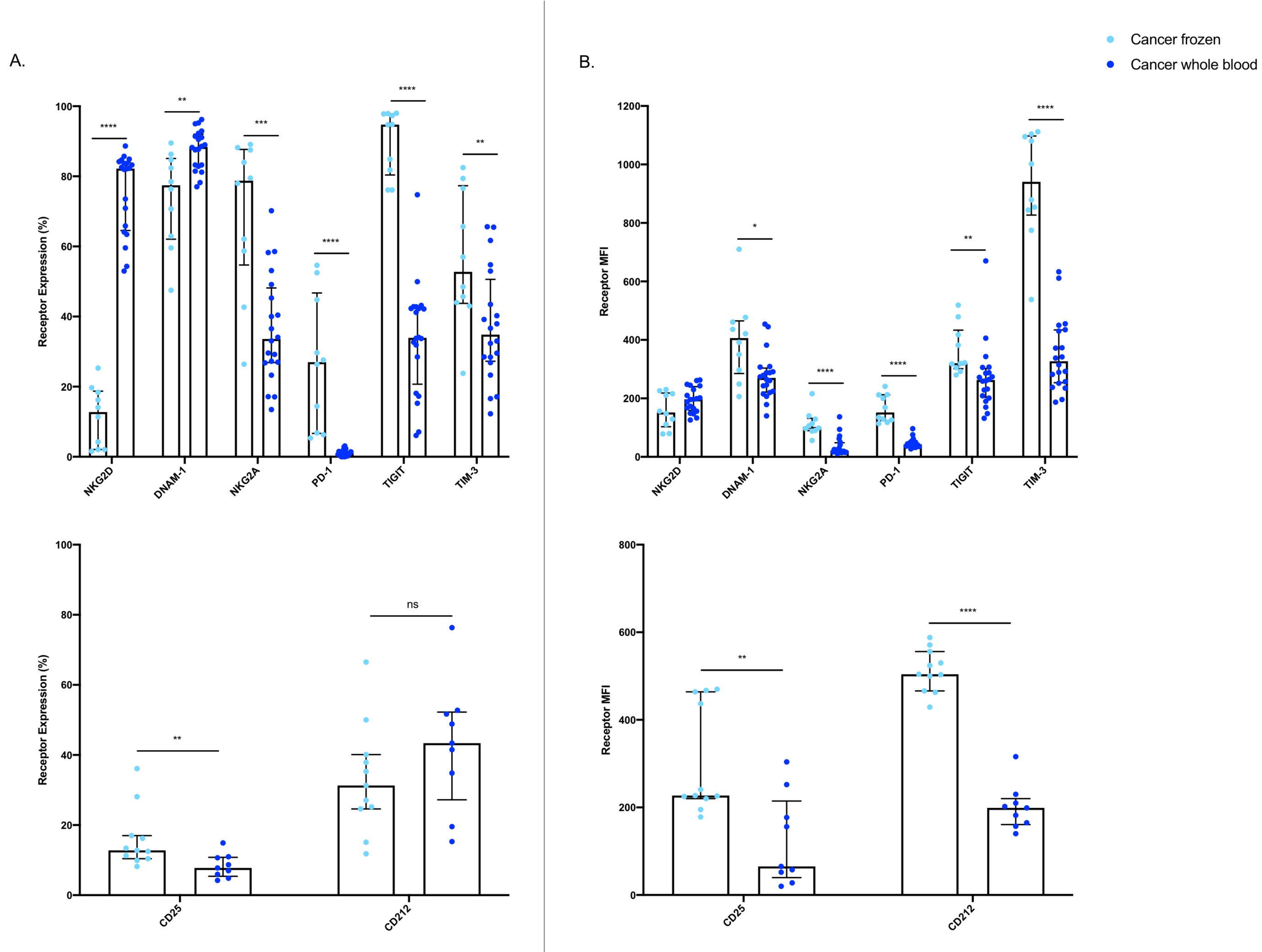
B.

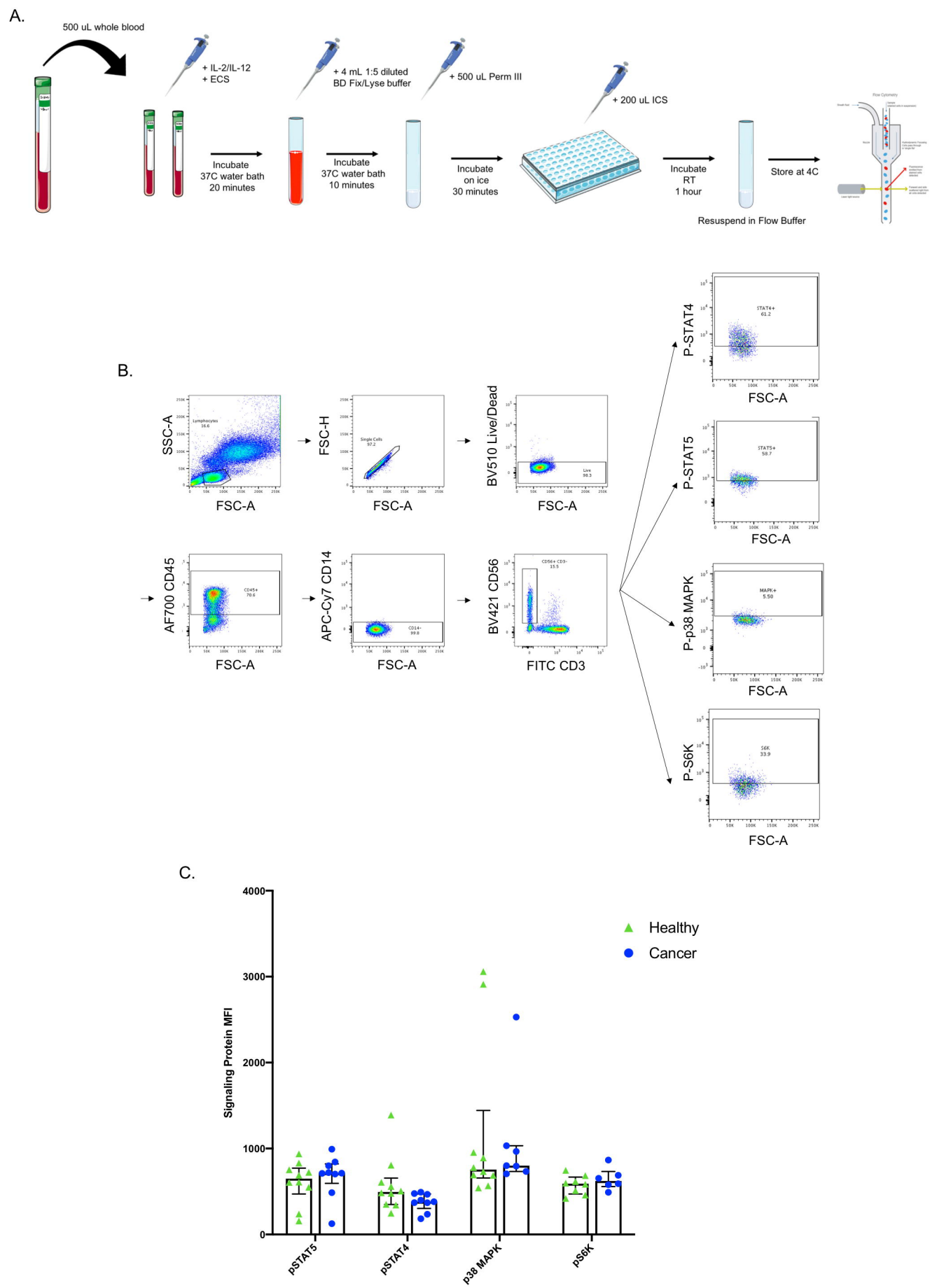

SSC-A
FSC-A
Lymphocytes 14.3
FSC-H
FSC-A
Single Cells 84.3
BV510 Live/Dead
FSC-A
Live 99.7
AF700 CD45
FSC-A
CD45+ 56.4
APC-Cy7 CD14
FSC-A
CD14- 99.1
BV421 CD56
FITC CD3
CD56+ Bright 1.27
CD56+ CD3- 14.9
CD56+ Dim 13.6
BV650 NKG2D
PE NKG2A
NKG2D+ 55.6
NKG2A+ 46.7
PerCP-Cy5.5 PD-1
BV786 TIM-3
PD-1+ 1.49
TIM-3+ 31.9
PE-Cy7 DNAM-1
APC TIGIT
DNAM-1+ 89.
TIGIT+ 29.4

C.

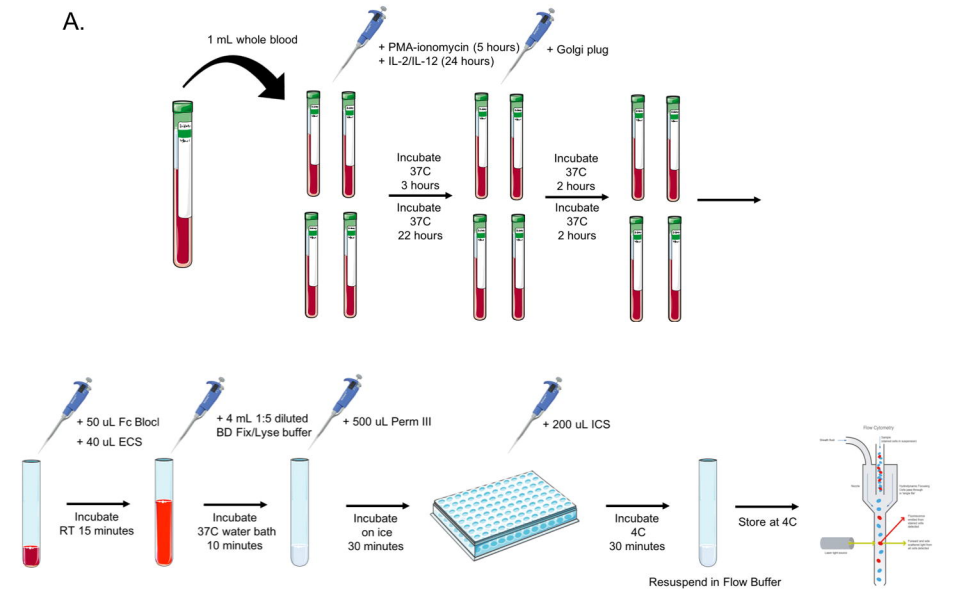

SSC-A
FSC-A
Lymphocytes 6.65
FSC-H
FSC-A
Single Cells 91.5
BV510 Live/Dead
FSC-A
Live 99.7
AF700 CD45
FSC-A
CD45+ 61.4
APC-Cy7 CD14
FSC-A
CD14- 98.6
BV421 CD56
FITC CD3
CD56+ CD3- 21.3
CD25
FSC-A
CD25+ 11.9
CD122
FSC-A
CD122+ 92.6
CD132
FSC-A
CD132+ 95.2
CD212
FSC-A
CD212+ 42.5


A.
B.
Healthy
Cancer
Receptor Expression (%)
Receptor MFI
NKG2D
DNAM-1
NKG2A
PD-1
TIGIT
TIM-3
CD25
CD122
CD132
CD212

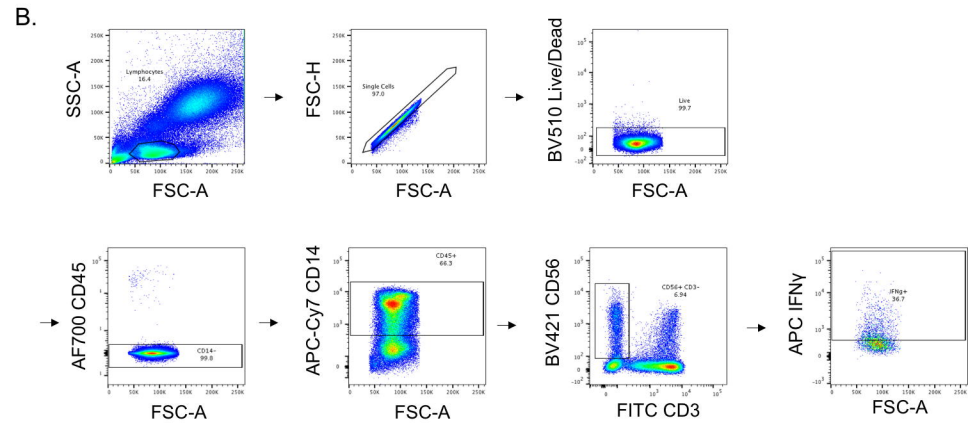

A.
B.
Cancer frozen
Cancer whole blood
Receptor Expression (%)
Receptor MFI
NKG2D
DNAM-1
NKG2A
PD-1
TIGIT
TIM-3
CD25
CD212
ns


A.
500 uL whole blood
+ IL-2/IL-12
+ ECS
Incubate
37C water bath
20 minutes
+ 4 mL 1:5 diluted
BD Fix/Lyse buffer
Incubate
37C water bath
10 minutes
+ 500 uL Perm III
Incubate
on ice
30 minutes
+ 200 uL ICS
Incubate
RT
1 hour
Resuspend in Flow Buffer
Store at 4C
B.
SSC-A
FSC-A
FSC-H
BV510 Live/Dead
AF700 CD45
APC-Cy7 CD14
BV421 CD56
FITC CD3
P-STAT4
P-STAT5
P-p38 MAPK
P-S6K
C.
Signaling Protein MFI
Healthy
Cancer
pSTAT5
pSTAT4
p38 MAPK
pS6K

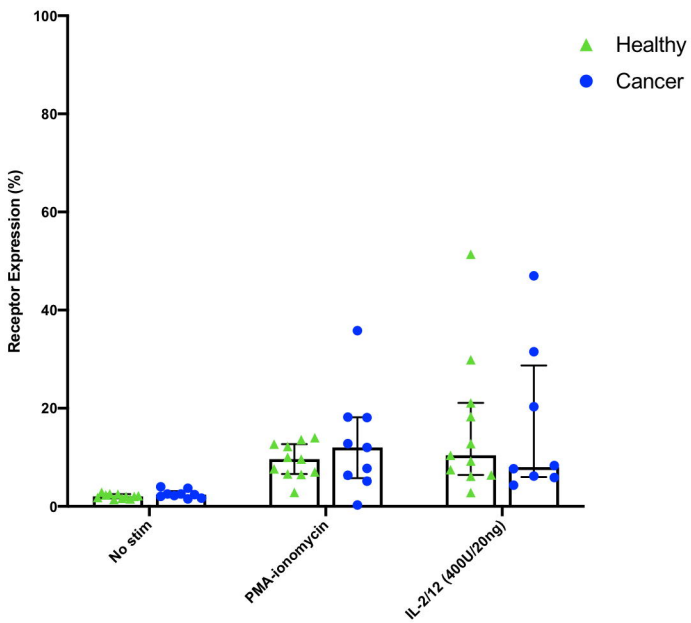
A.


1 mL whole blood
+ PMA-ionomycin (5 hours)
+ IL-2/IL-12 (24 hours)
+ Golgi plug
Incubate 37C 3 hours
Incubate 37C 22 hours
Incubate 37C 2 hours
Incubate 37C 2 hours
+ 50 uL Fc Block
+ 40 uL ECS
+ 4 mL 1:5 diluted BD Fix/Lyse buffer
+ 500 uL Perm III
+ 200 uL ICS
Incubate RT 15 minutes
Incubate 37C water bath 10 minutes
Incubate on ice 30 minutes
Incubate 4C 30 minutes
Store at 4C
Resuspend in Flow Buffer

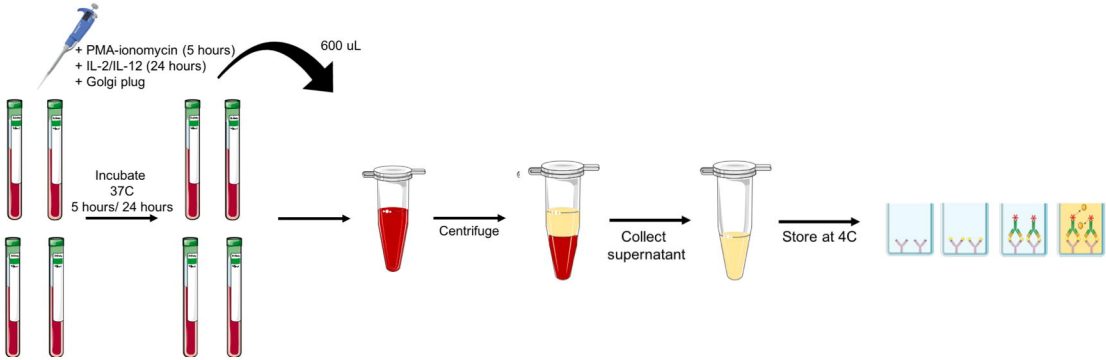
B.


SSC-A
FSC-A
Lymphocytes 16.4
FSC-H
FSC-A
Single Cells 97.0
BV510 Live/Dead
FSC-A
Live 99.7
AF700 CD45
FSC-A
CD14- 99.8
APC-Cy7 CD14
FSC-A
CD45+ 66.3
BV421 CD56
FITC CD3
CD56+CD3- 6.94
APC IFNγ
FSC-A
IFNg+ 36.7

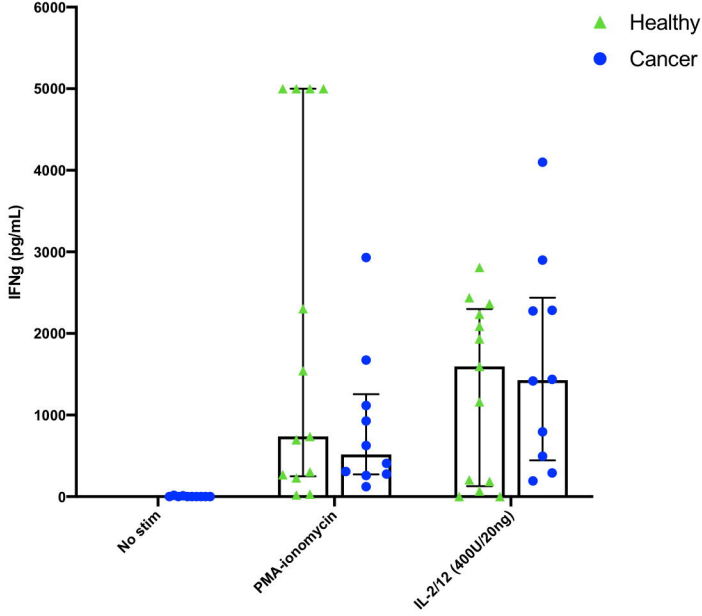
C.


Receptor Expression (%)
100
80
60
40
20
0
Healthy
Cancer
No stim
PMA-ionomycin
IL-2/12 (400U/20ng)

A.


+ PMA-ionomycin (5 hours)
+ IL-2/IL-12 (24 hours)
+ Golgi plug
600 uL
Incubate
37C
5 hours/ 24 hours
Centrifuge
Collect
supernatant
Store at 4C

B.


Healthy
Cancer
IFNg (pg/mL)
6000
5000
4000
3000
2000
1000
0
No stim
PMA-ionomycin
IL-2/12 (400U/20ng)