Supplemental Methods

Flow Cytometry

The following flow cytometry antibodies were utilized: CD4 FITC (eBiosciences, Cat: 11-0048, RRID:AB_1633391), CD3 ECD (Beckman Coulter, Cat: IM2705U, RRID:AB_130860), CD56 PeCy7 (Beckman Coulter, Cat: A51078, RRID:AB_10641223), CD25 APC (BD Pharmingen, Cat: 555434, RRID:AB_398598), CD8 APC-H7 (BD Pharmingen, Cat: 561423, RRID:AB_10682894), CD45 KO (Beckman Coulter, Cat: A96416, RRID: AB_2888654), CD16 PB (BD Biosciences, Cat: 558122, RRID:AB_397042), Ki67 PE (BD Pharmingen, Cat: 556027, RRID:AB_2266296), granzyme B PeCy5.5 (Molecular Probes, Cat: GRB18, RRID:AB_2536541), Foxp3 BV450 (eBiosciences, Cat: 48-4776-42, RRID:AB_1834364).

Mass Cytometry and Data Analysis

For all samples, EQ Four Element calibration beads were used during collection according to the manufacturer's instructions (Fluidigm) and analyzed using Cytobank (RRID:SCR_014043). The data were normalized using Fluidigm CyTOF2 Bead Normalization Tool. Metal-tagged antibodies were purchased from Fluidigm or custom-conjugated using the Maxpar X8 Antibody Labeling Kit according to manufacturer's instructions (Fluidigm). All custom conjugated antibodies were titrated. Staining was comparable to flow cytometry (Beckman Coulter Gallios, Kaluza 2.0). Samples were barcoded prior to collection, normalized using bead-normalization (GitHub, Nolan lab), and then de-barcoded using the Single Cell De-barcoder (GitHub, Nolan lab). For staining $0.25\text{x}10^6$ sorted NK cells or $0.5\text{-}1.0\text{x}10^6$ PBMCs were stained with surface antibodies for 45 min at $4^{o}C$ in CyFACS buffer (0.1% BSA, 0.02% NaN2, 2mM EDTA in CyPBS, Rockland). Surface antibodies were washed away using CyPBS. Cells were then stained for viability with 2.5 μM cisplatin (Enzo life sciences) according to a standard protocol. The cells were then permeabilized using eBiosciences Cytofix/Cytoperm according to the manufacturer's instructions. Intracellular staining was performed in eBiosciences Permeablization Wash buffer for 45 minutes at $4^{o}C$. Cells

were washed. Cells were barcoded (20-plex Barcode, Fluidigm) according to manufacturer's instructions and pooled. Pooled samples were washed 3 times and stained with Cell-ID intercalator according the manufacturer's instructions (Fluidigm). CD8+ T cell metacluster subsets were then annotated based on median expression and percentage positive of CCR7 and CD45RO (1).

CITE-seq Sample Staining & Sequencing

Cells were blocked with 5 μl of Human Fc block (Biolegend, Cat: 422301, RRID:AB_2818986) and 0.1% dextran-sulfate (MP Biomedicals, Cat: 101516) in Cell Staining Buffer (Biolegend, Cat: 420201) for 10 minutes at 4ºC. Antibody cocktails were prepared fresh with 0.5 μg of each antibody (Table S2) in 50 μl of Cell Staining Buffer. Prior to adding to cells, antibody cocktails were spun at 14,000g for 10 minutes twice to remove oligonucleotide aggregates with the antibody supernatant transferred to a new tube after each spin. Cell staining was performed with the antibody supernatant in 100 μl final volume for 30 minutes at 4ºC. Following staining, Cells were washed in Cell Staining Buffer, filtered, and resuspended in 0.04% BSA in PBS. Cells were loaded onto a 10x Chromium Instrument, where individual cells were barcoded, cDNA libraries constructed, and sequenced on an Illumina NovaSeq6000.

CITE-seq Data Processing Details

Murine cells were removed for combined analysis, and low-quality, dying, or doublet cells were removed by removing cells expressing less than 200 features and greater than 3500 features or ≥ 12% mitochondrial gene from downstream analysis. Gene expression was log-normalized with a scale factor of 10^4, and the top 2000 variable genes were selected for each patient and applied in batch correction using Seurat's canonical correlation analysis (CCA) batch correction implementation (dimensions = 1:30) (2). Cell cycle effects were regressed out during gene expression data scaling.

Significant Principle components (PC) for downstream analysis were selected based off of the Elbow Plot inflection, Jackstraw analysis, and clarity and significance of PC Heatmaps (final PC = 30). Unsupervised graph-based clustering (resolution=2.2) and *t*SNE were used to visualize the data. Clustering resolution was selected based on the minimum resolution necessary to distinguish established immune cell types (e.g. MAIT from $\gamma\delta$ T cells). For antibody data (ADT), we removed CCR7, DNAM-1, and KLRG1 due to poor and non-specific staining. The resulting ADT data was normalized using centered log ratio and scaled.

CITE-seq Cell Type Identification

Immune cell types were identified using a Wilcoxon Rank Sum test with a minimum absolute natural log FC of 0.25, and expressed in at least 25% of cells. The following marker genes or proteins were used - $CD56^{bright}$ NK ($CD56^{hi}/CD16^{lo}$ ADT), $CD56^{dim}$ NK ($CD56^{+}/CD16^{hi}$ ADT), B-cells (*CD79A*), CD16+ Monocytes ($CD16^{hi}$ ADT, *LYZ*), CD14+ Monocytes (*CD14*, *LYZ*, $CD16^{lo}$ ADT), myeloid Dendritic Cells (*FCER1A*, *CST3*), CD4+ T cells (*CD3D*, *CD4*), CD8+ T cells (*CD3D*, *CD8A*), $\gamma\delta$ T cells (*TRDV2*, *TRGV9*), mucosal-associated invariant T-cells (MAIT) (*TRAV1-2*), regulatory T cells (*FOXP3*, CD25-ADT). Naïve, Central Memory, Effector Memory, and Terminal Effector Memory RA T cell subsets were defined by their expression of *IL7R*, *SELL*, CD62L-ADT, CD57-ADT, *CCR7*, *CD27*, *TCF7*, CD45RA-ADT, and CD45RO-ADT (Supp. Fig. 3C). Cells with the following identities were removed from final analysis and visualization: Proliferating (*MKI67*, 465 cells), Doublets/Multiplets/unknown (mixed lineage markers, 1694 cells), Platelets (*PPBP*, 250 cells), and an unknown T cell subset with high ribosomal gene expression and predominantly abundant in patient 16 only (660/708 cells), without high expression of any T cell lineage markers (Fig. S3C). After exclusion of these cell types, there were a total of 45,102 cells (Patient 15: 9451, 16: 16503, 17: 19148).

Combined ADT & RNA CITE-seq NK Analysis

For clustering with the combined NK transcriptome and antibody-derived tags for each cell, we first pre-processed aggregated CellRanger matrices of patients 16 & 17 in Seurat with the same thresholds as used previously to identify NK cells with the following changes: 1) cells expressing greater than 90% UMIs from human or mouse were assigned to the corresponding species and cells expressing less than 90% UMIs from a given lineage were designated as 'mixed' species and removed from downstream analysis, 3) subsetting by feature count and mitochondrial percent was only conducted on cells identified as "human", and 4) PC = 21, clustering resolution = 1.1. The specificity of CITE-seq antibody staining was confirmed by spiking in mouse spleen cells as negative controls (Fig. S4B). Patient 16 & 17 NK cells were subsetted and imported into the R-based package, CiteFuse (3). To prepare the data for creating the correlation matrices, RNA expression was $\log_2$ transformed, and a similarity network fusion (SNF) matrix was constructed off of all ADT counts with default parameters(4). UMAP visualization and Louvain clustering were performed in CiteFuse on the resulting SNF matrix. Clusters were labeled using ADTs known to be relevant to NK cell maturation and top differentially expressed ADTs. Clusters were annotated by ADT expression. KIR- clusters are negative for KIR2DL2/3, KIR3DL1, and KIR2DL1/S1/S3/S5. We observed inconsistent normalized staining patterns of KIR2DL5 and NKp44 ADTs, so these markers were excluded from cluster annotation. In addition, we identified one cluster ("None", Fig. 4D.) with no ADT markers which was excluded from downstream analysis.

Gene Ontology Analysis

Gene lists for input into *clusterprofiler*(5) were obtained using the Wilcoxon Rank Sum test implemented in Seurat's *FindMarkers* function. Enriched genes were with default settings (minimum percent of cells expressing gene: 10%) with the exception of log fold-change threshold set to 0 to capture all genes expressed. The gene universe was the entire gene output of the statistical test for each cluster. The enriched gene list consisted of significant genes with ≥ 0.5 absolute $\log_2$ FC. Examination of gene fold change by each donor identified a single donor driving

differential gene expression in a small subset of genes. These genes were removed from the GO gene enriched input list to identify a N-803 and rituximab enrichment signature across all donors (CD14 Mono: *BAG3*, *HSPB1*; CD16 Mono: *TNFSF10*, *HSPA1B*; $CD56^{bright}$: *HSPA1A*, *HSPA1B*, *HSP90AA1*, *ANKRD37*; $CD56^{dim}$: *TUBA1A*).

References

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