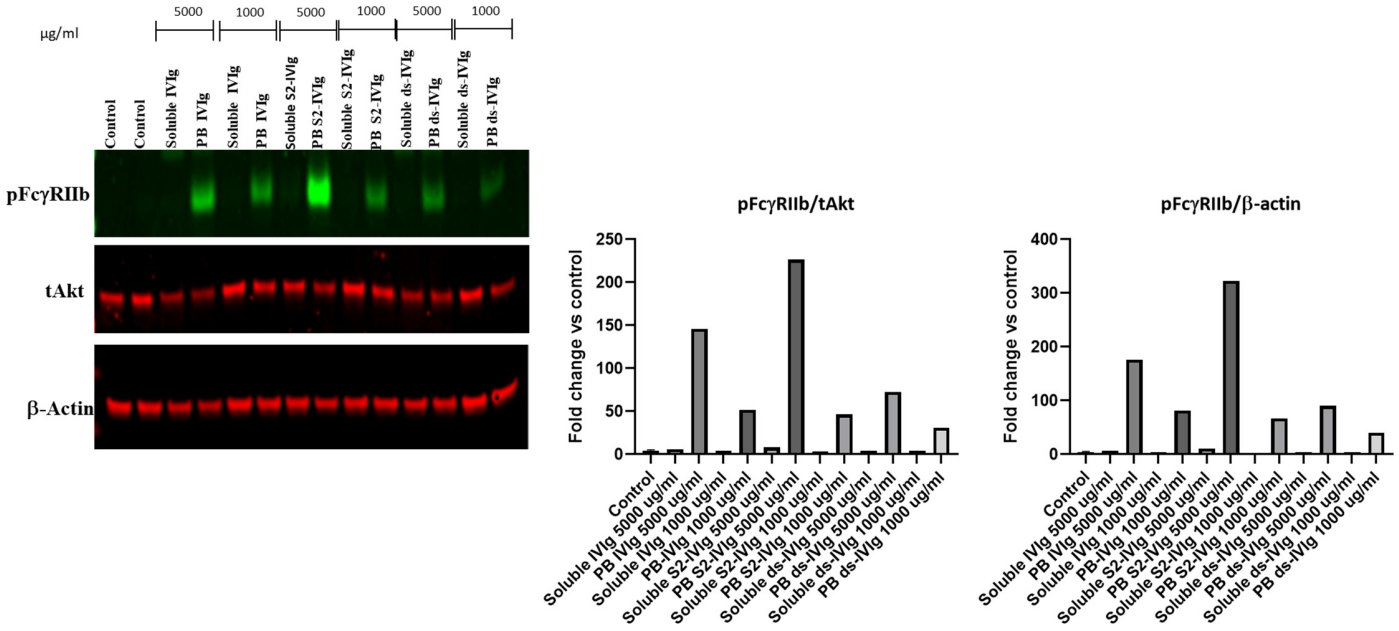
Inhibitory Fc-gamma IIb Receptor Signaling Induced by Multivalent IgG-Fc is Dependent on Sialylation

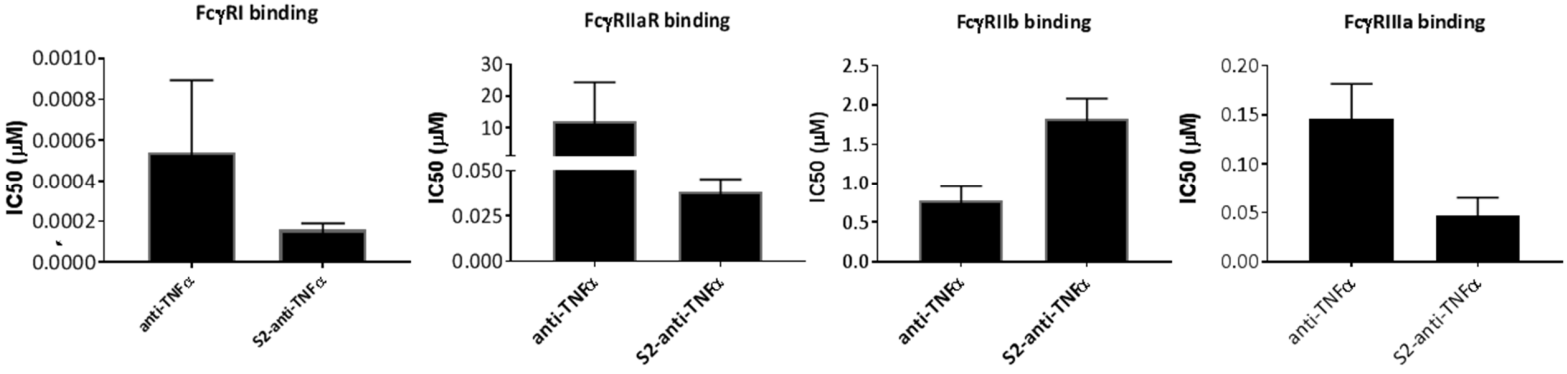
Christopher Beneduce[1,2], Stephanie Nguyen[1], Nathaniel Washburn[1,2], John Schaeck[1], Robin Meccariello[1,2], Kimberly Holte[1], Daniel Ortiz[1], Anthony M. Manning[1], Carlos J. Bosques[1] and Elma Kurtagic[1,2]*

[1] Momenta Pharmaceuticals Inc, Cambridge, MA, USA
[2] Janssen Pharmaceutical Companies of Johnson & Johnson, Cambridge, MA, USA
* Correspondence: ekurtagi@its.jnj.com.com

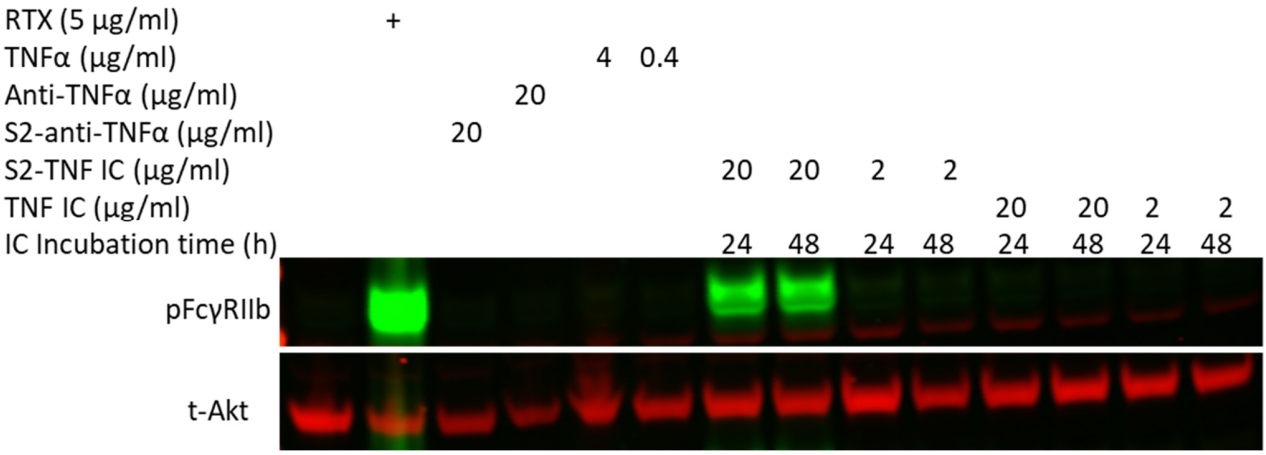
Supplemental Figures:



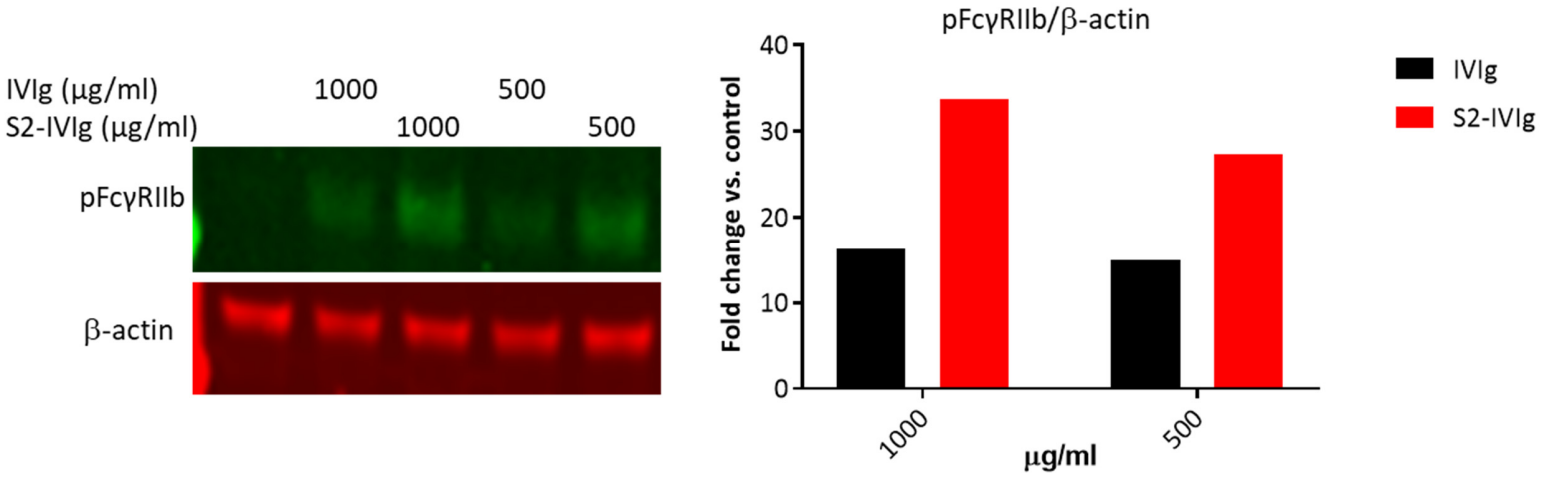
Supplementary Figure S1. S2-IVIg induces FcγRIIb signaling in Daudi cells when presented in polymeric fashion. Left**:** Western blot of phospho FcγRIIb, tAkt and β-actin after 30 min stimulation of Daudi cells with soluble or plate-bound IVIg, S2-IVIg or ds-IVIg. Right panel: densitometry of the western blots showing pFcγRIIb signal over tAkt or β-actin, resulting in the same relative fold changes.



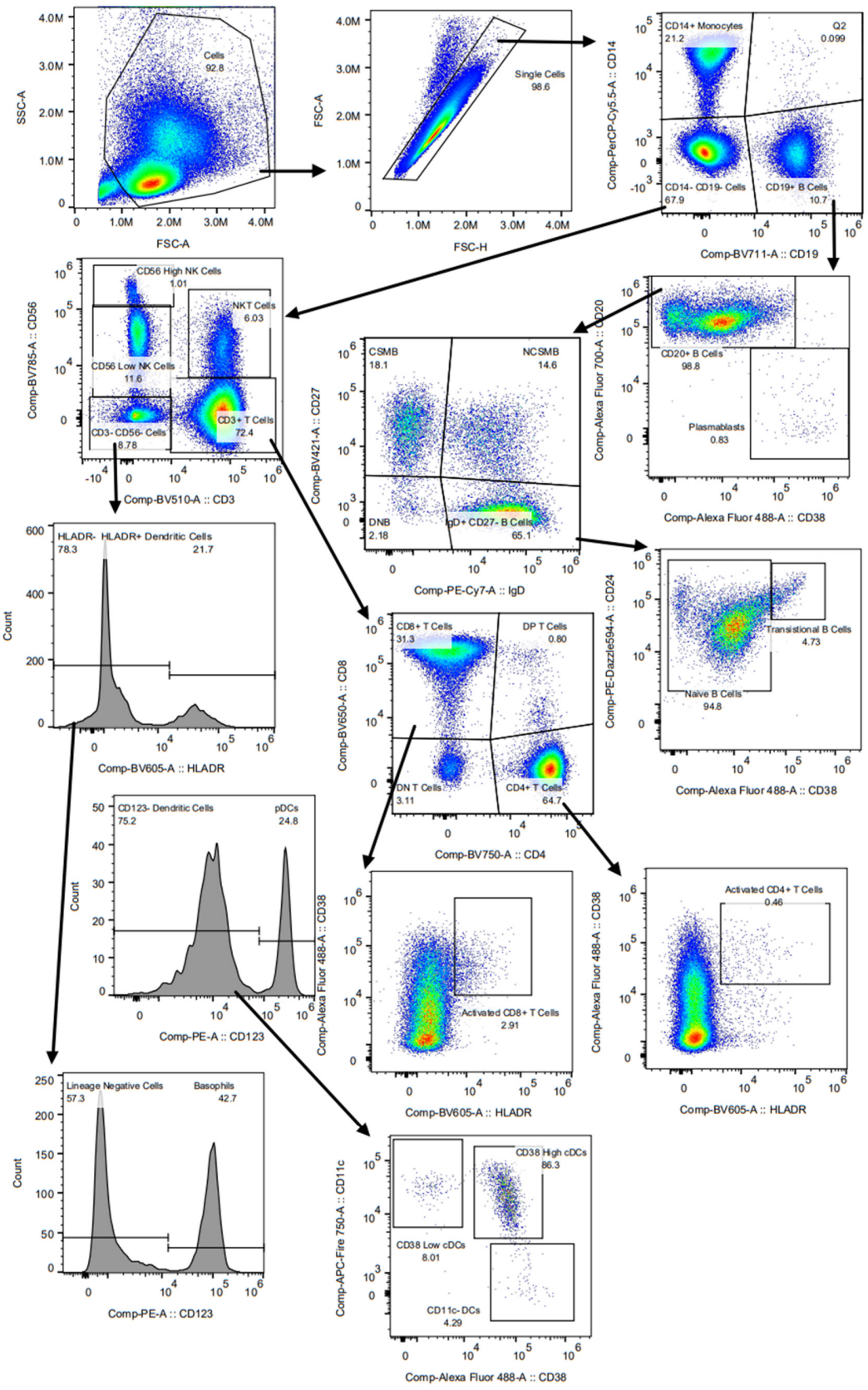
Supplementary Figure S2. Relative binding of anti-TNF*α*/S2-anti-TNF*α* to FcγRs measured using cell-based homogeneous TR-FRET CISBIO.



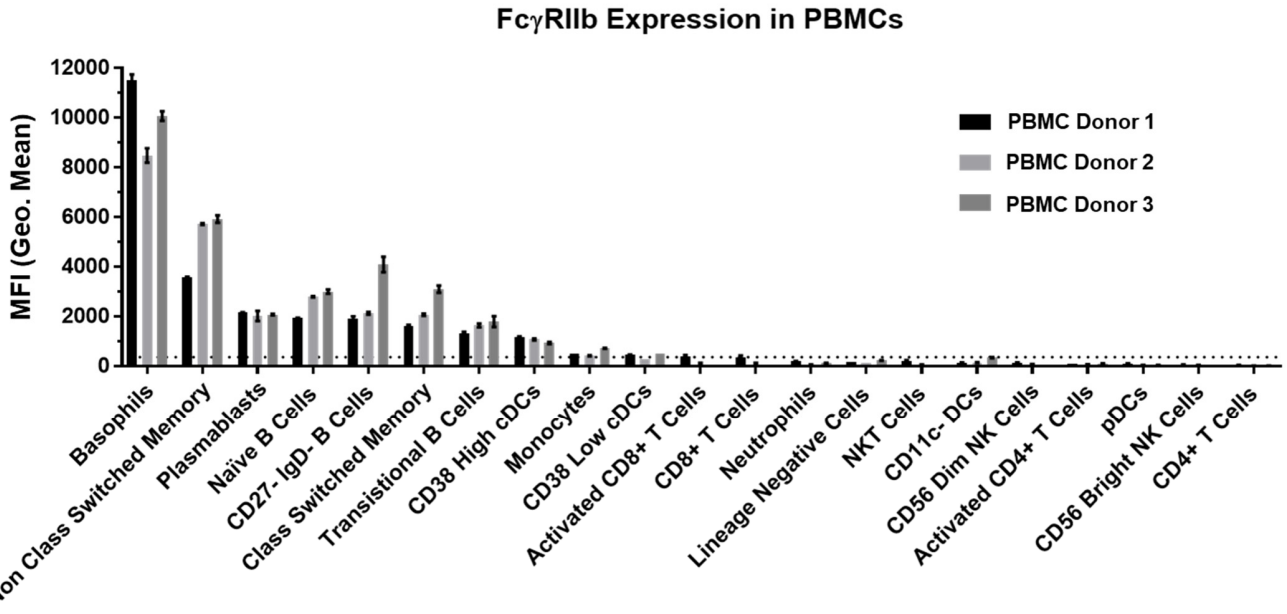
Supplementary Figure S3. TNFα-anti TNFα IC when sialylated, induced FcγRIIb signaling. Western blot of phospho FcγRIIb and β-actin after 30 min stimulation of Daudi cells with rituximab (5 µg/ml), TNFα (4 and 0.4 µg/ml), 20 µg/ml anti-TNFα and S2-anti-TNFα, TNFα-anti TNFα immune complex (TNF IC) or sialylated anti-TNFα-TNFα IC (S2-TNF IC) formed over 24 or 48 hr incubation time, at doses 20 and 2 µg/ml.



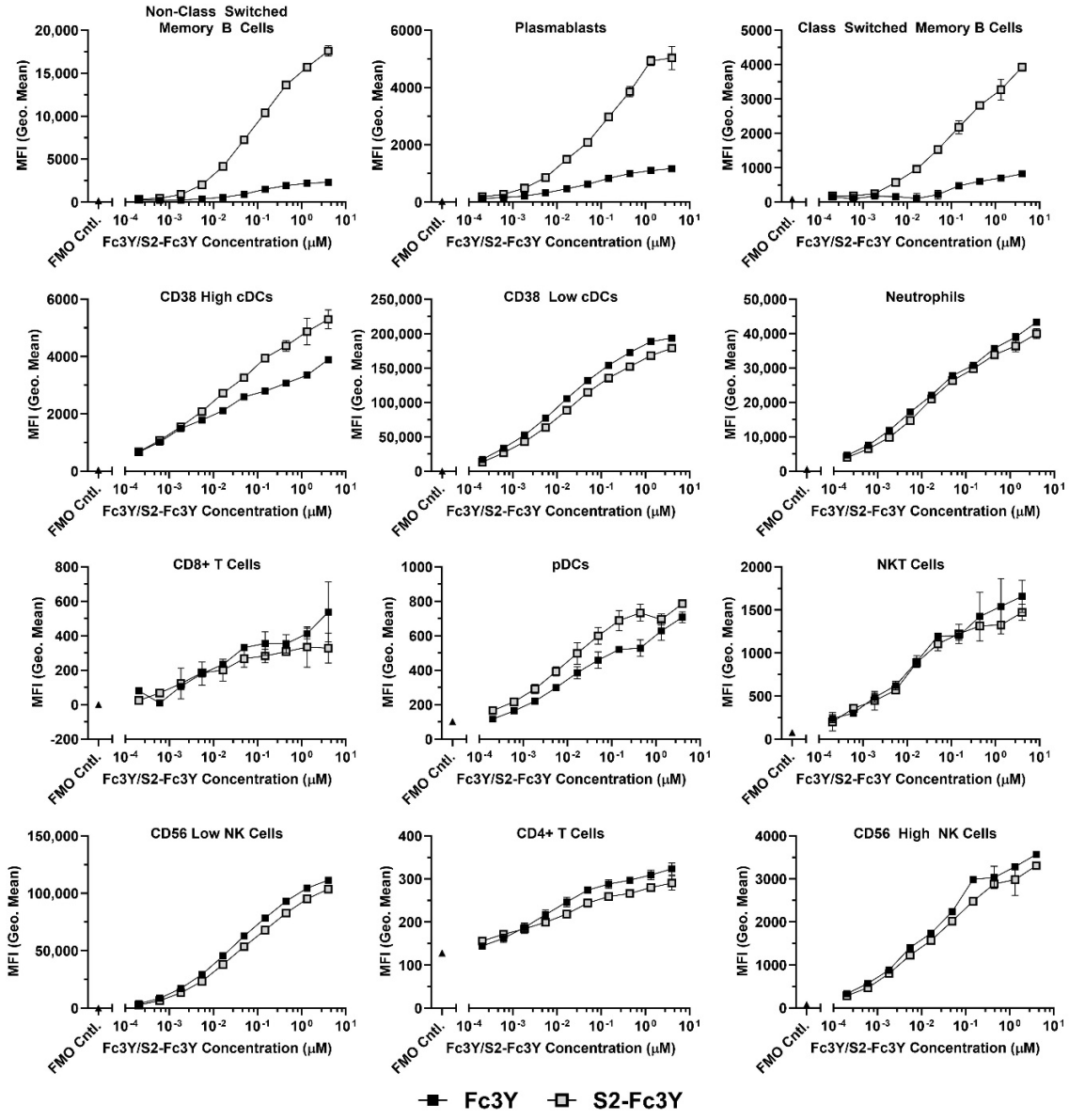
Supplementary Figure S4: S2-IVIg induces FcγRIIb signaling in PBMCs when presented in polymeric fashion. Left**:** Western blot of phospho FcγRIIb and β-actin after 30 min stimulation of freshly isolated PBMC cells with plate-bound IVIg or S2-IVIg (1000, 500 µg/ml). Right: densitometry of Western blot image.


SSC-A
FSC-A
Cells
92.8
FSC-A
FSC-H
Single Cells
98.6
Comp-PerCP-Cy5.5-A :: CD14
Comp-BV711-A :: CD19
CD14+ Monocytes
21.2
Q2
0.099
CD14- CD19- Cells
67.9
CD19+ B Cells
10.7
Comp-BV785-A :: CD56
Comp-BV510-A :: CD3
CD56 High NK Cells
1.01
NKT Cells
6.03
CD56 Low NK Cells
11.6
CD3+ T Cells
72.4
CD3- CD56- Cells
8.78
Comp-BV421-A :: CD27
Comp-PE-Cy7-A :: IgD
CSMB
18.1
NCSMB
14.6
DNB
2.18
IgD+ CD27- B Cells
65.1
Comp-Alexa Fluor 700-A :: CD20
Comp-Alexa Fluor 488-A :: CD38
CD20+ B Cells
98.8
Plasmablasts
0.83
Count
Comp-BV605-A :: HLADR
HLADR- HLADR+ Dendritic Cells
78.3
21.7
Comp-BV650-A :: CD8
Comp-BV750-A :: CD4
CD8+ T Cells
31.3
DP T Cells
0.80
DN T Cells
3.11
CD4+ T Cells
64.7
Comp-PE-Dazzle594-A :: CD24
Comp-Alexa Fluor 488-A :: CD38
Transitional B Cells
4.73
Naive B Cells
94.8
Comp-PE-A :: CD123
Comp-Alexa Fluor 488-A :: CD38
CD123- Dendritic Cells
75.2
pDCs
24.8
Activated CD8+ T Cells
2.91
Activated CD4+ T Cells
0.46
Lineage Negative Cells
57.3
Basophils
42.7
Comp-APC-Fire 750-A :: CD11c
CD38 High cDCs
86.3
CD38 Low cDCs
8.01
CD11c- DCs
4.29

Supplementary Figure S5: Gating strategy for PBMC flow cytometry experiments. Gating strategy used to identify major PBMC subsets. FSC-A = Forward Scatter-Area, FSC-H = Forward Scatter-Height, SSC-A = Side Scatter-Area. DNB = Double Negative B Cells. pDCs = Plasmacytoid Dendritic Cells. DN T cells = Double Negative T Cells. DP T Cells = Double Positive T Cells. CSMB = Class Switched Memory B Cells. NCSMB = Non-Class Switched Memory B Cells. Antibody panel information and cell type specific markers are described in Supplementary Tables S1 and S2, respectively.



Supplementary Figure S6: FcγRIIb expression in human PBMCs from healthy donors. FcγRIIb expression from healthy human donors analyzed using flow cytometry with the Anti-FcγRIIb/c antibody Clone 4F5. Each donor was analyzed with n=2 technical replicates, with a total of n = 3 PBMC donors analyzed. Dotted line represents the MFI of the APC-labeled Mouse IgG2a isotype control antibody.



Supplementary Figure S7: Dose response staining of human PBMCs with VivoTag 645 labeled Fc3Y and S2-Fc3Y. Extended dose responses of Fc3Y and S2-Fc3Y to major PBMC subsets identified using the antibody panel described in Supplementary Tables S1 and S2, and the gating strategy is Supplementary Figure S5. Results are reported in the geometric mean fluorescence intensity (MFI – Geo. Mean) of VivoTag 645. Results for one donor is shown with $n = 2$ technical replicates. Data is representative of $n = 3$ PBMC donors analyzed.

Supplemental Tables:

Antibody Staining Panel for PBMC Flow Cytometry					
Antibody	**Label**	**Antibody Clone**	**Vendor**	**Catalog #**	**Fold Dilution**
Anti-CD3	Brilliant Violet 510	OKT3	BioLegend	317332	100
Anti-CD4	Brilliant Violet 750	SK3	BioLegend	344644	500
Anti-CD8	Brilliant Violet 650	SK1	BioLegend	344730	250
Anti-CD14	PerCP-Cy5.5	HCD14	BioLegend	325622	100
Anti-CD19	Brilliant Violet 711	HIB19	BioLegend	302246	100
Anti-CD20	Alexa Fluor 700	2H7	BioLegend	302322	300
Anti-CD24	PE-Dazzle 594	ML5	BioLegend	311134	100
Anti-CD27	Brilliant Violet 421	M-T271	BioLegend	356418	100
Anti-CD123	PE	6H6	BioLegend	306006	100
Anti-CD56	Brilliant Violet 785	5.1H11	BioLegend	362550	100
Anti-IgD	PE-Cy7	IA6-2	BioLegend	348210	300
Anti-HLA-DR	Brilliant Violet 605	L243	BioLegend	307640	100
Anti-CD11c	APC Fire 750	Bu15	BioLegend	337240	100
Anti-CD38	Alexa Fluor 488	HB7	BioLegend	356634	100
Anti-FcγRIIb/c	APC	4F5	BioLegend	398406	100

Supplementary Table S1: Antibody panel used for PBMC flow cytometry experiments. All antibodies were purchased from BioLegend. Label refers to the specific fluorophore conjugated to the antibody. Fold Dilution refers to the dilution of the antibody stock into a 100µL final staining volume.

PBMC Flow Cytometry Gating Strategy	
Cell Type	**Gating Strategy**
CD19+ B Cells	CD19+, CD14-
CD14+ Monocytes	CD14+, CD19-
CD20+ B Cells	CD19+, CD14-, CD20+
Plasmablasts	CD19+, CD14-, CD20 low, CD38+
Naïve B-Cells	CD19+, CD14-, CD20+, IgD+, CD27-
Class Switched Memory B Cells (CSMB)	CD19+, CD14-, CD20+, IgD-, CD27+
Non-Class Switched Memory B Cells (NCSMB)	CD19+, CD14-, CD20+, IgD+, CD27+
Double Negative B-Cells	CD19+, CD14-, CD20+, IgD-, CD27-
Transitional B Cells	CD19+, CD14-, CD20+, IgD+, CD27-, CD24 High, CD38 High
CD3+ T Cells	CD19-, CD14-, CD3+, CD56-
CD8+ T Cells	CD19-, CD14-, CD3+, CD56-, CD8+, CD4-
Activated CD8+ T Cells	CD19-, CD14-, CD3+, CD56-, CD8+, CD4-, HLA-DR+, CD38+
CD4+ T Cells	CD19-, CD14-, CD3+, CD56-, CD8-, CD4+
Activated CD4+ T Cells	CD19-, CD14-, CD3+, CD56-, CD8-, CD4+, HLA-DR+, CD38+
Double Positive T Cells	CD19-, CD14-, CD3+, CD56-, CD8+, CD4+
Double Negative T Cells	CD19-, CD14-, CD3+, CD56-, CD8-, CD4-
CD56 Dim NK Cells	CD19-, CD14-, CD3-, CD56 low
CD56 Bright NK Cells	CD19-, CD14-, CD3-, CD56 high
NKT Cells	CD19-, CD14-, CD3+, CD56+
HLA-DR+ Dendritic Cells	CD19-, CD14-, CD3-, CD56-, HLA-DR+
pDCs	CD19-, CD14-, CD3-, CD56-, HLA-DR+, CD123+
CD11c- DCs	CD19-, CD14-, CD3-, CD56-, HLA-DR+, CD123-, CD38+, CD11c-
CD38 High cDCs	CD19-, CD14-, CD3-, CD56-, HLA-DR+, CD123-, CD38+, CD11c+
CD38 Low cDCs	CD19-, CD14-, CD3-, CD56-, HLA-DR+, CD123-, CD38 low, CD11c+
Basophils	CD19-, CD14-, CD3-, CD56-, HLA-DR-, CD123+
Lineage Negative Cells	CD19-, CD14-, CD3-, CD56-, HLA-DR-, CD123-

Supplementary Table S2: Gating strategy and cell type markers for PBMC flow cytometry experiments. "Gating Strategy" column refers to the specific marker expression for each cell type. "+" refers to positive expression, "-" refers to absence of expression. Where noted, "high" and "low" correspond to marker expression where populations express varying levels of a given marker. Abbreviations: pDCs = Plasmacytoid Dendritic Cells, DCs = Dendritic Cells, cDCs = Conventional Dendritic Cells.