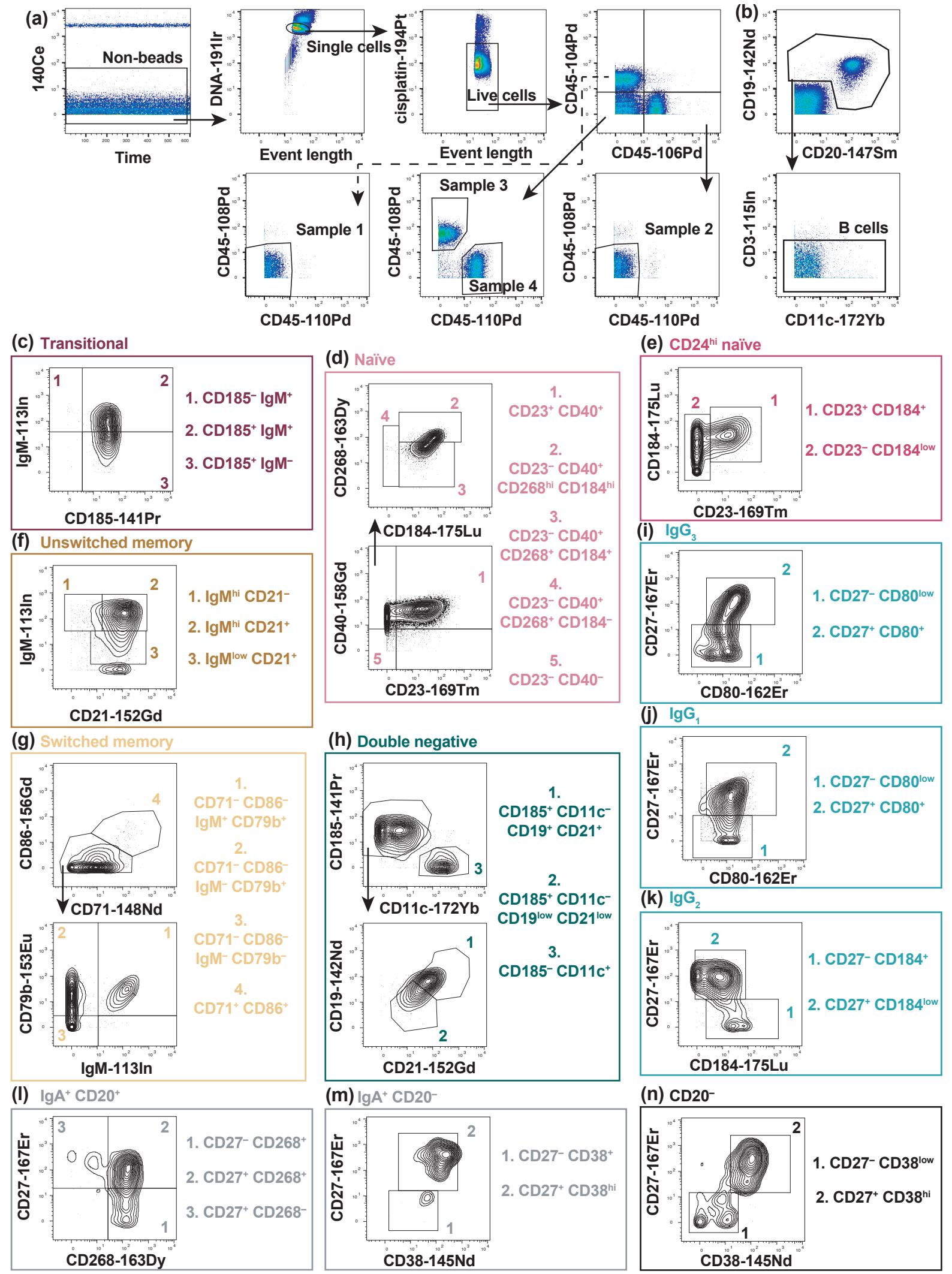

Supplementary figure 1. Single live B cell gating strategy. **(a-b)** Gating strategy for single live B cells from barcoded samples. Manual gating strategy of transitional **(c)**, naïve **(d)**, CD24^{hi} naïve **(e)**, unswitched memory **(f)**, switched memory **(g)**, double negative **(h)**, IgG$_3$ **(i)**, IgG$_1$ **(j)**, IgG$_2$ **(k)**, IgA$^+$ CD20$^+$ **(l)**, IgA$^+$ CD20$^-$ **(m)** and CD20$^-$ **(n)** B cell subsets.

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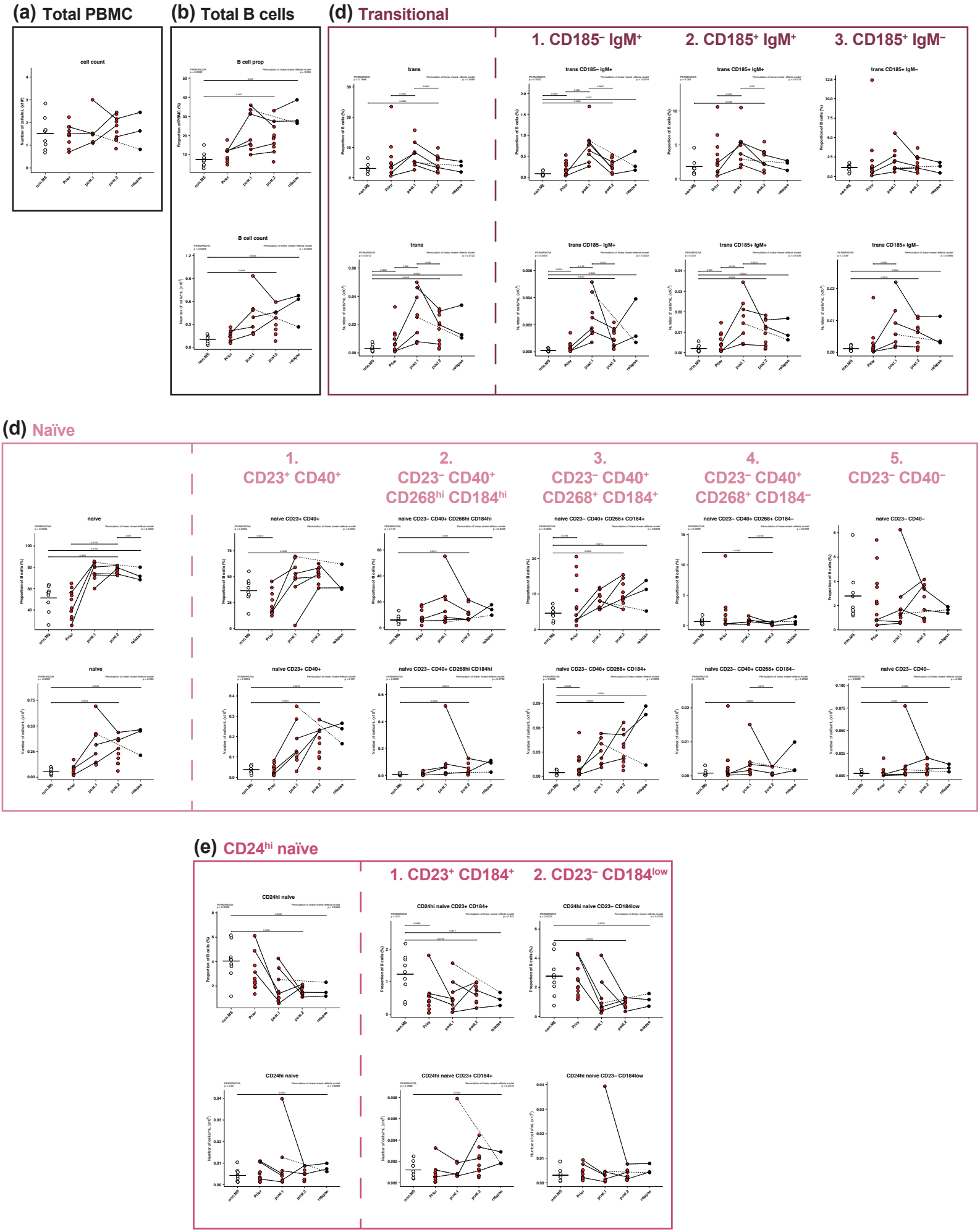

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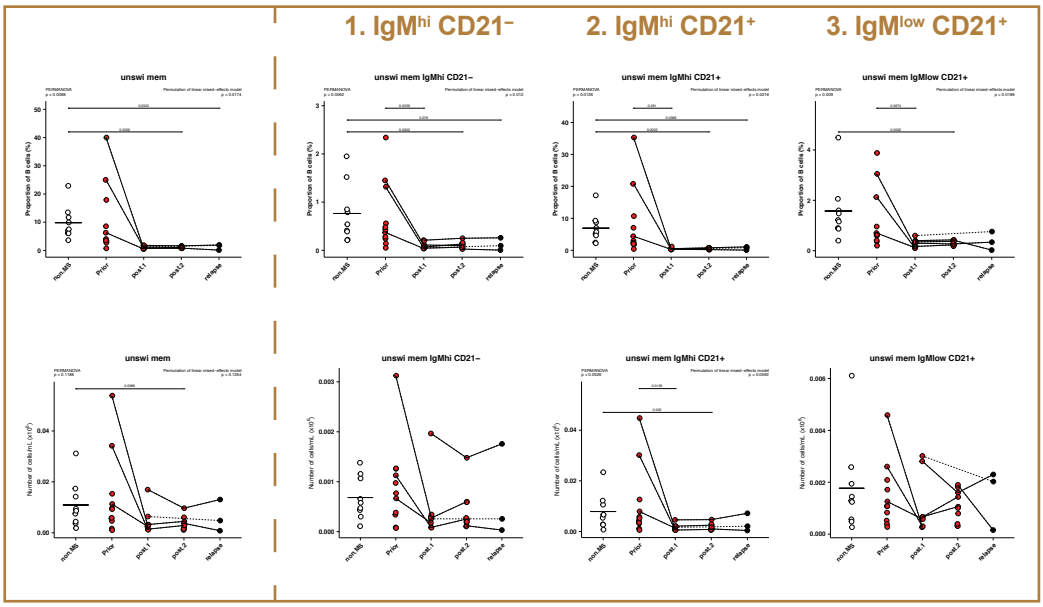
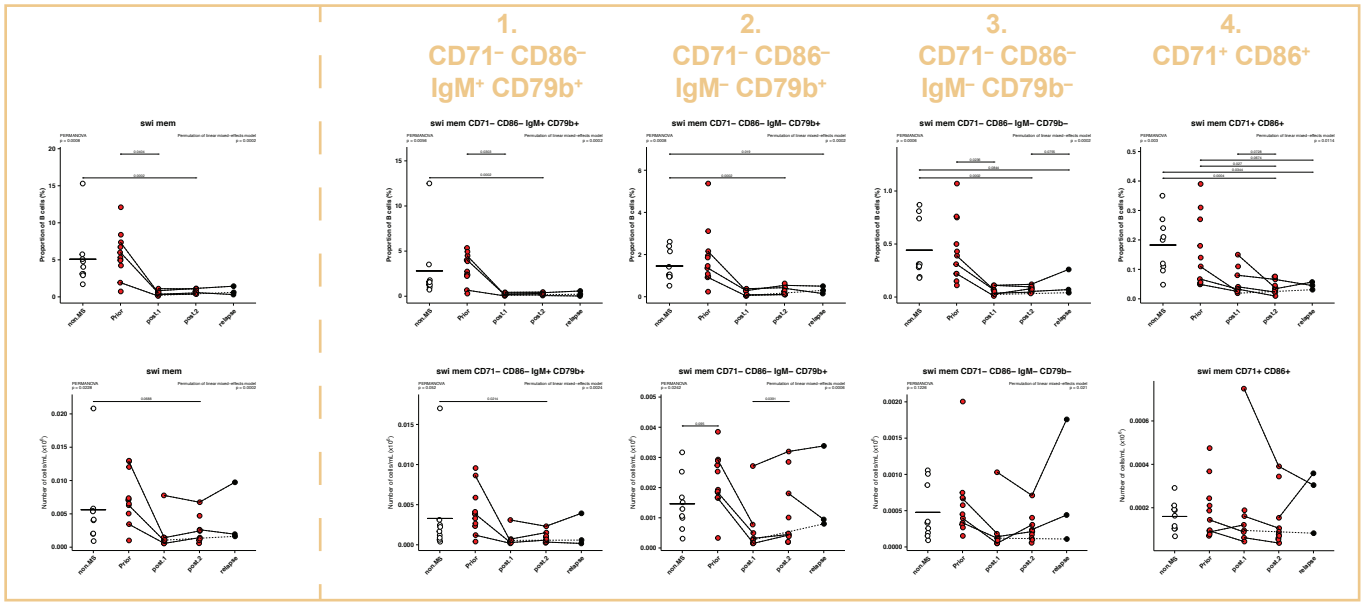
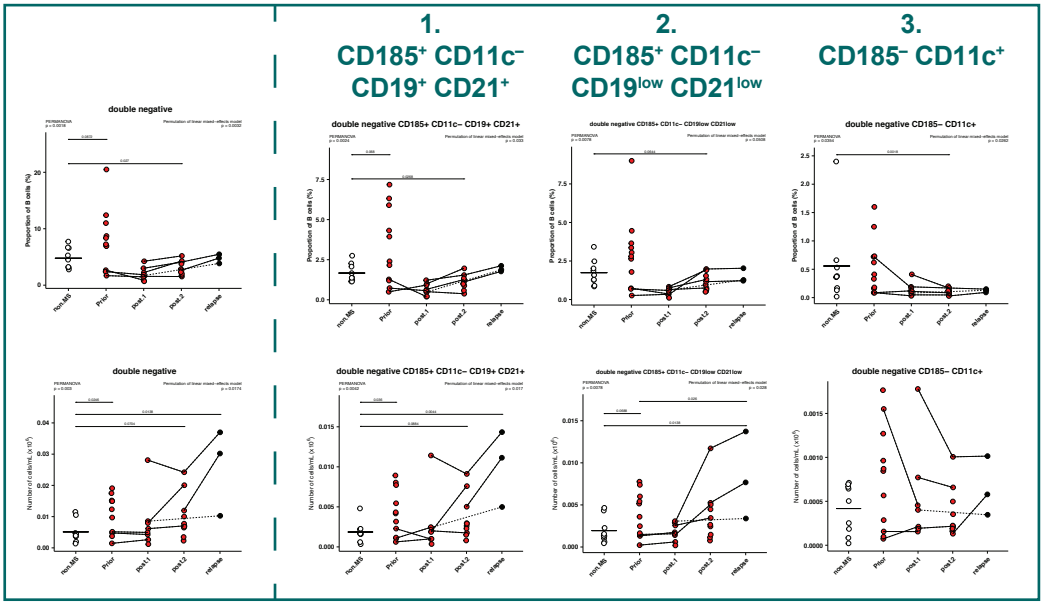
(f) Unswitched memory



(g) Switched memory



(h) Double negative



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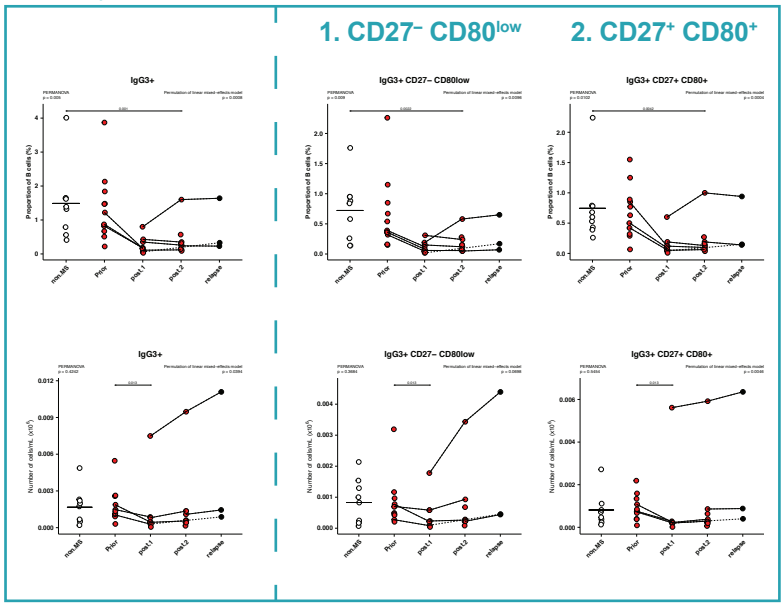
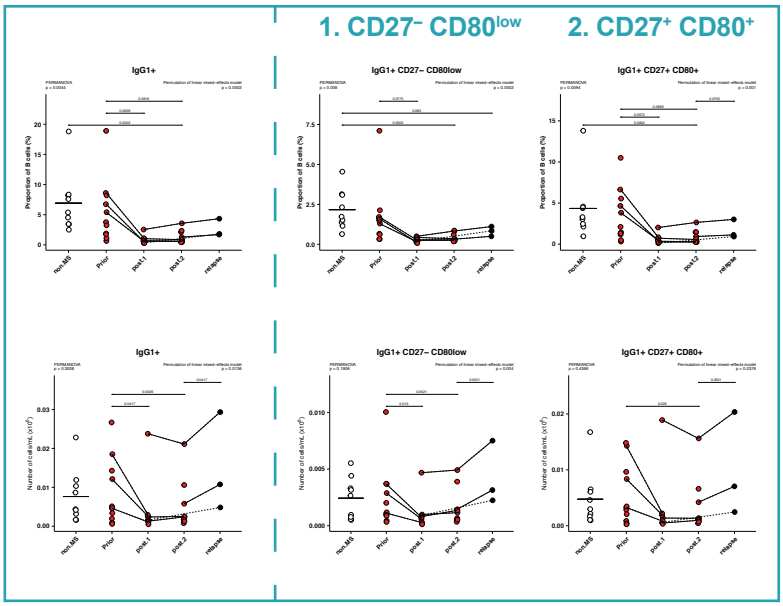
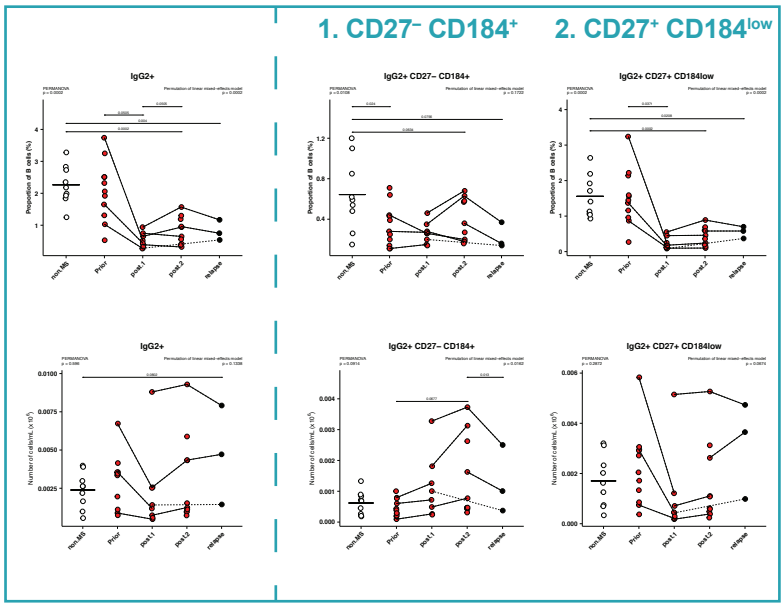
(i) IgG$_3$



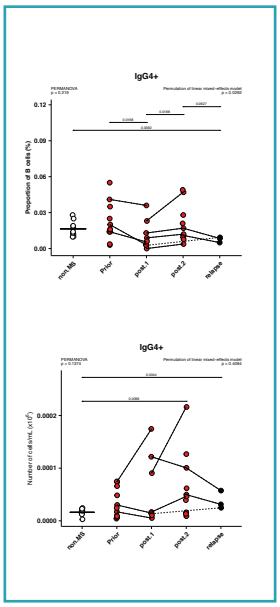
(j) IgG$_1$

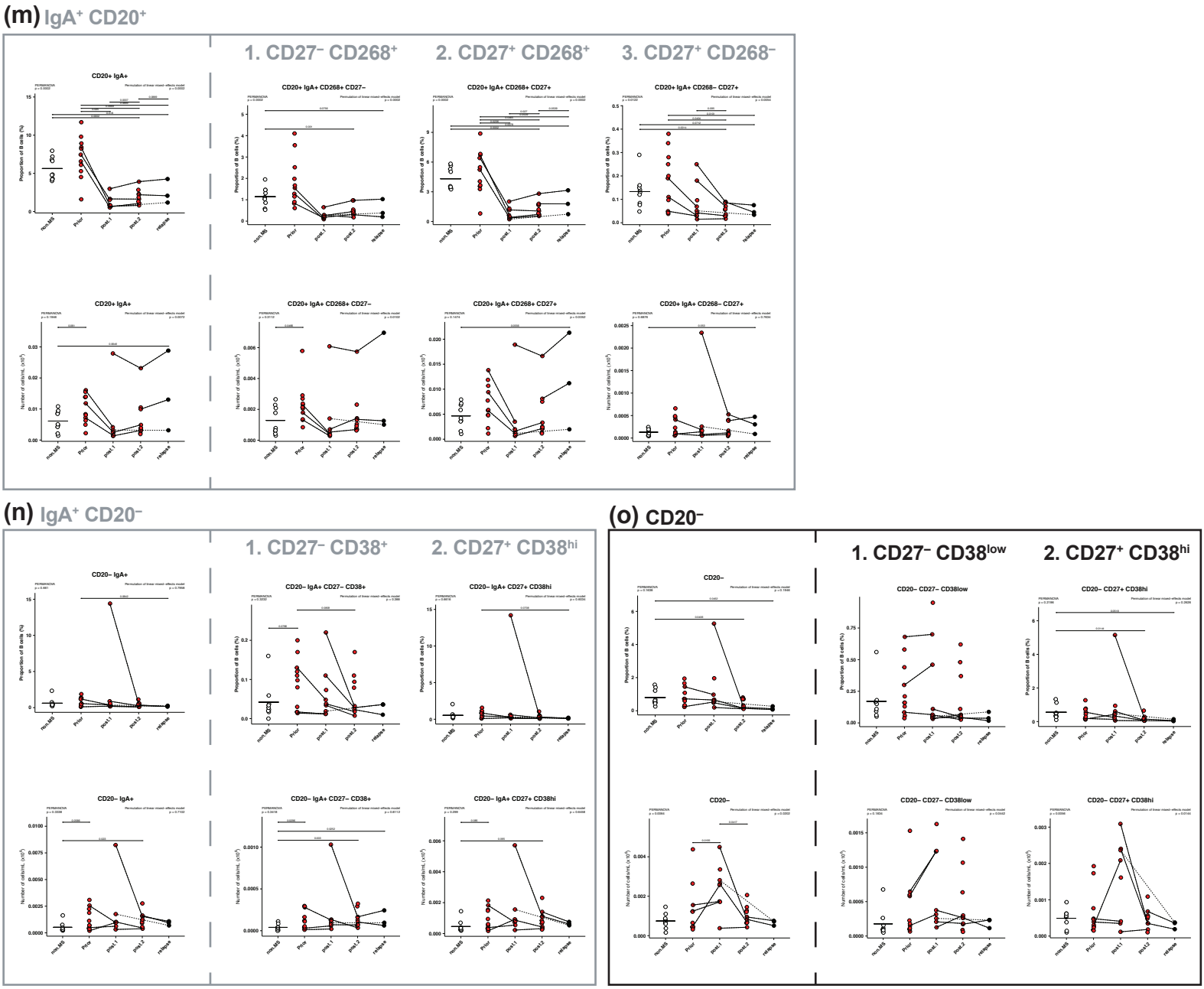


(k) IgG$_2$

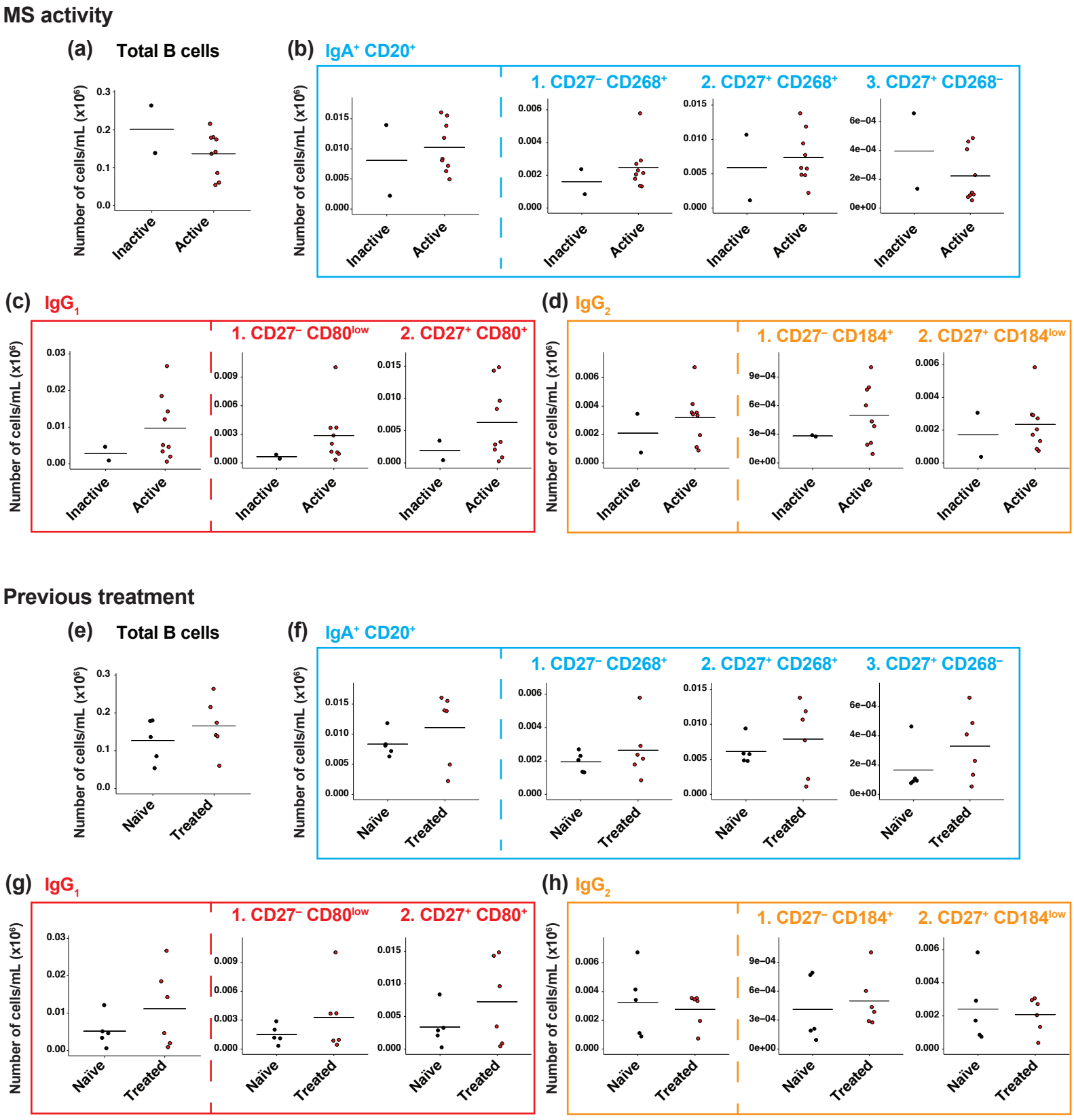


(l) IgG$_4$





Supplementary figure 2. B cell subset levels across groups. **(a)** Total PBMC count across groups. Proportion and count data for total B cells **(b)**, transitional **(c)**, naïve **(d)**, CD24^{hi} naïve **(e)**, unswitched memory **(f)**, switched memory **(g)**, double negative **(h)**, IgG_3 **(i)**, IgG_1 **(j)**, IgG_2 **(k)**, IgG_4 **(l)**, IgA$^+$ CD20$^+$ **(m)**, IgA$^+$ CD20$^-$ **(n)** and CD20$^-$ **(o)** B cell subsets. Solid lines signify data are available for adjacent timepoints, whilst dotted lines indicate patients with non-adjacent timepoints. For comparisons of B cell subset levels between all five groups (*non-MS* (multiple sclerosis) controls (n = 9), untreated MS patients (*prior*, n = 11), and MS patients *post-1* (up to twelve months after alemtuzumab dose, n = 9 proportion data and n = 8 count data), *post-2* (greater than twelve months, n = 10) alemtuzumab and *relapse* (n = 3)), a PERMANOVA was done followed pairwise comparisons with Holm's correction. *Prior*, *post-2* and *relapse* groups were compared to *non-MS* controls (for three comparisons). A linear mixed-effects model (LMM) was calculated when comparing between MS patients before and after treatment. 4999 permutations were then run to calculate *P*-values. Five multiple comparisons were made (*prior* to *post-1*, *post-2* and *relapse*, and *post-1* to *post-2*, and *post-2* to *relapse*) using a further 4999 permutations with Holm's correction. Mean is shown in *non-MS* controls, *P*-values < 0.1 are shown.



Supplementary figure 3. MS activity and prior treatments. **(a-d)** Comparions of inactive (n = 2) and active (n = 9) disease in multiple sclerosis (MS) patients prior to alemtuzumab treatment. **(e-h)** Comparions of untreated naïve (n = 5) and previously treated (n = 6) MS patients prior to alemtuzumab treatment. **(a,e)** total B cells, **(b,f)** IgA^+ $CD20^+$ B cell subsets, **(c,g)** IgG_1 B cell subsets, **(d,h)** IgG_2 B cell subsets. Permutation of *t*-test, mean shown.

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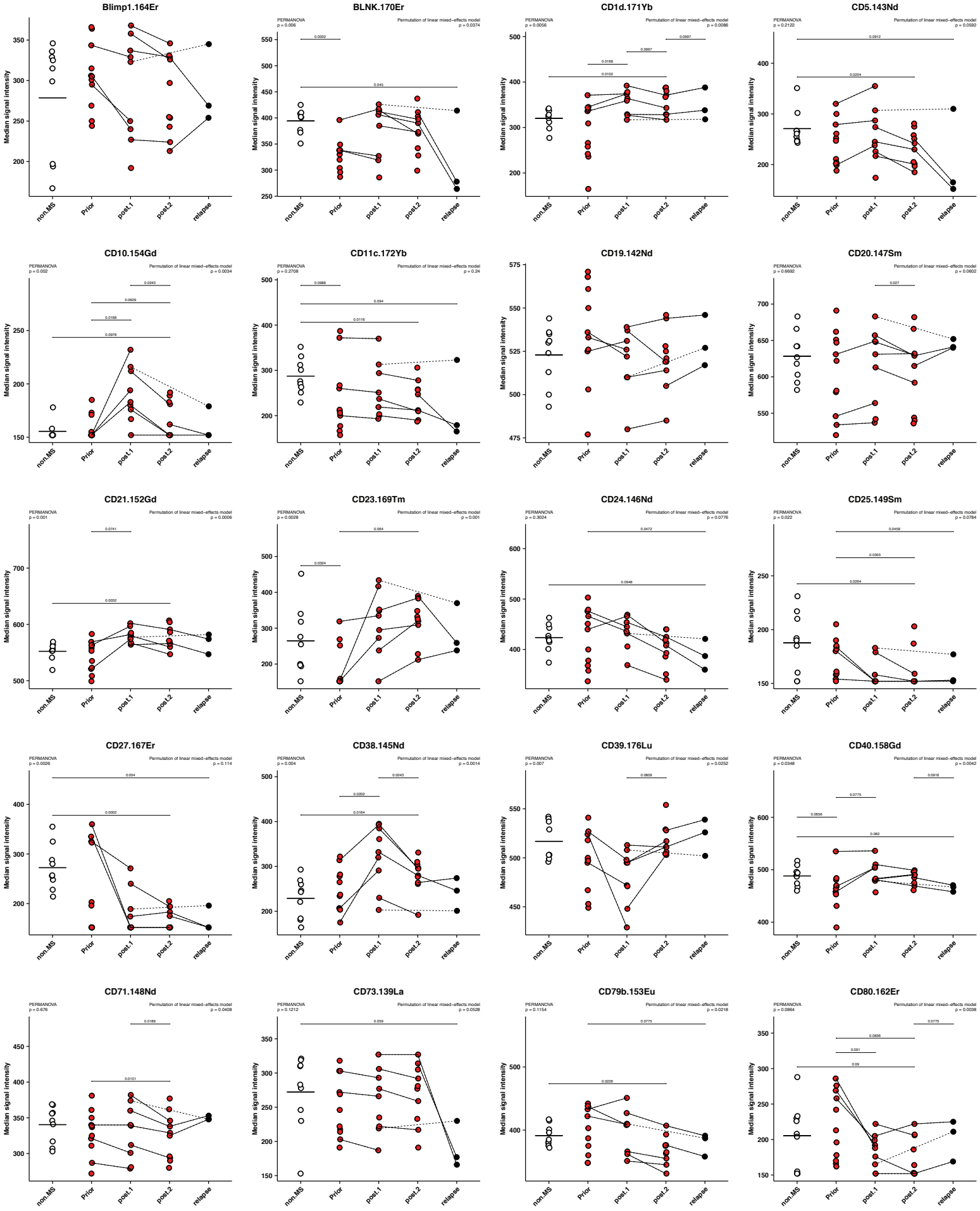

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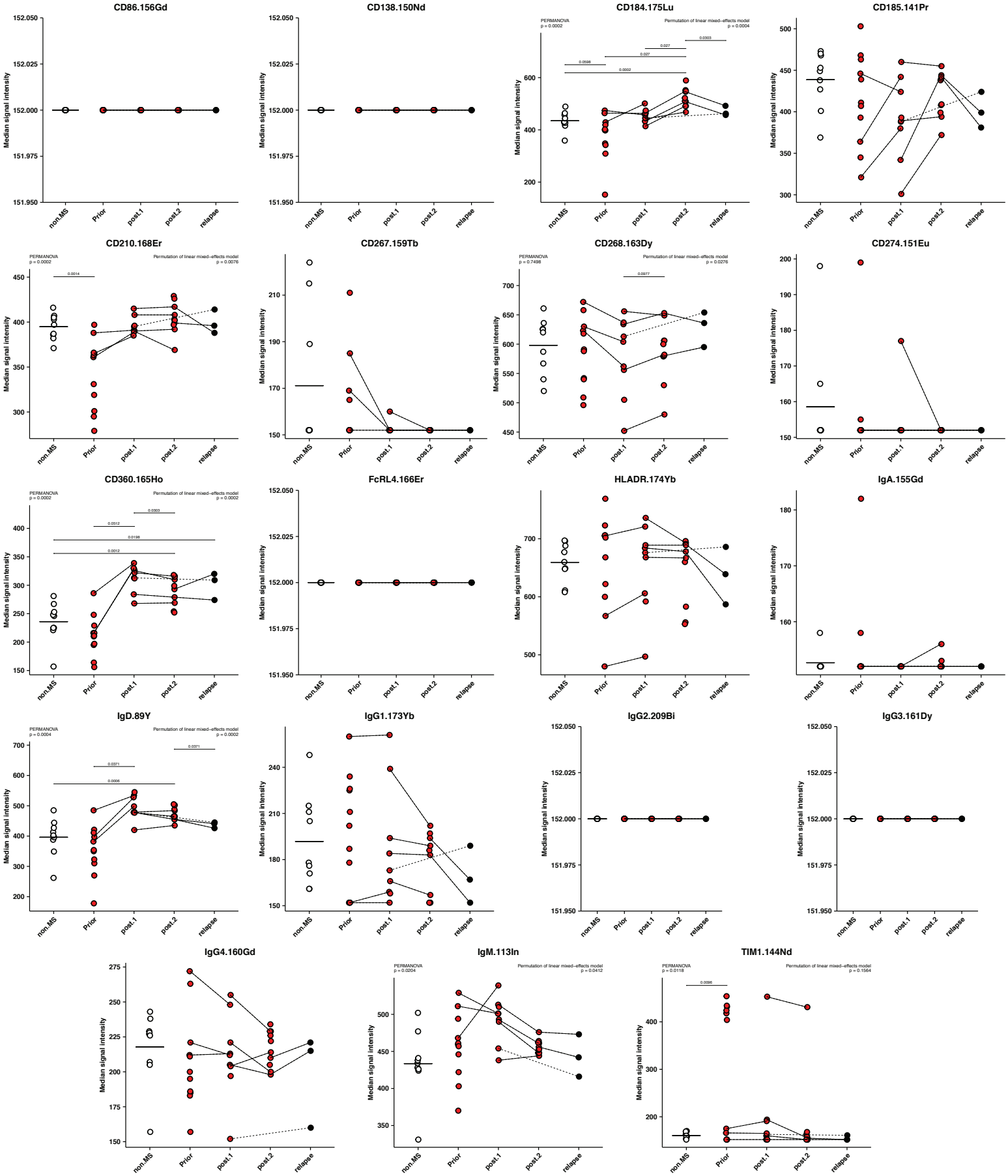
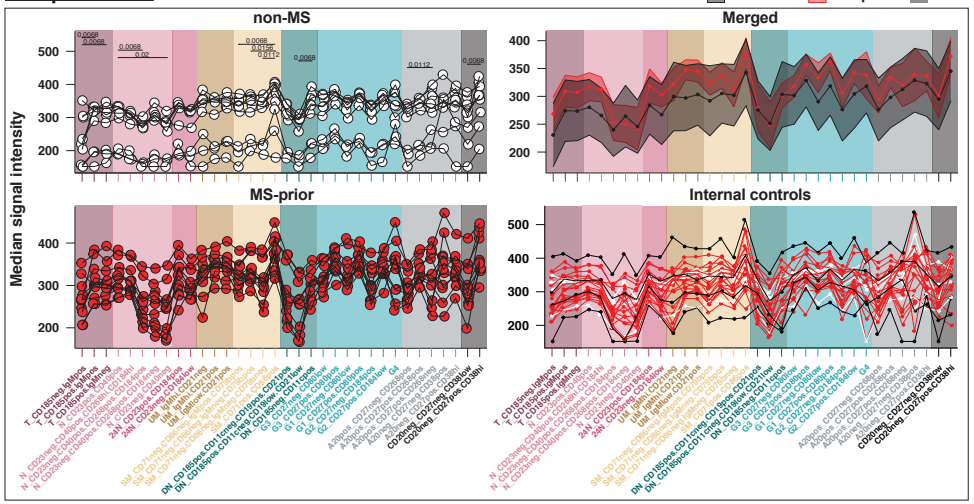
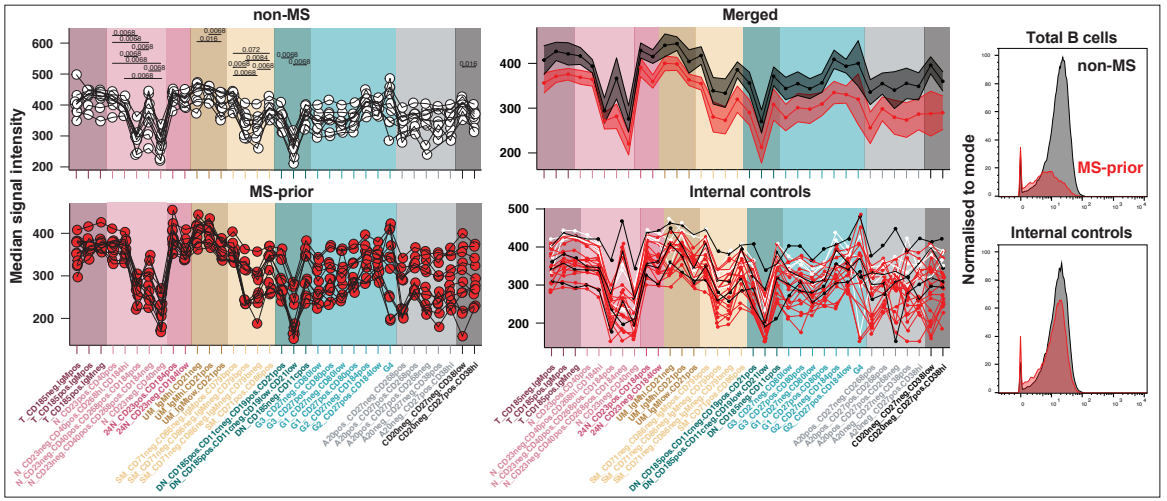
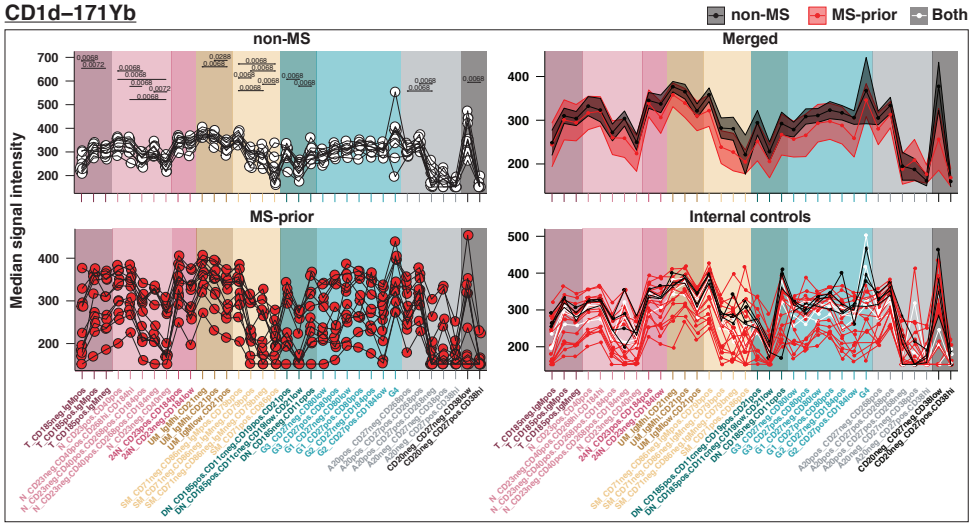
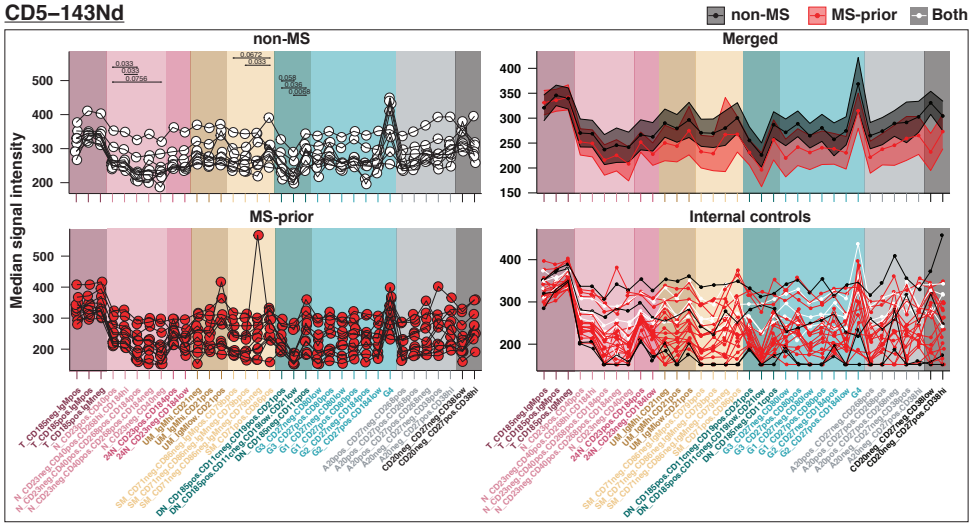


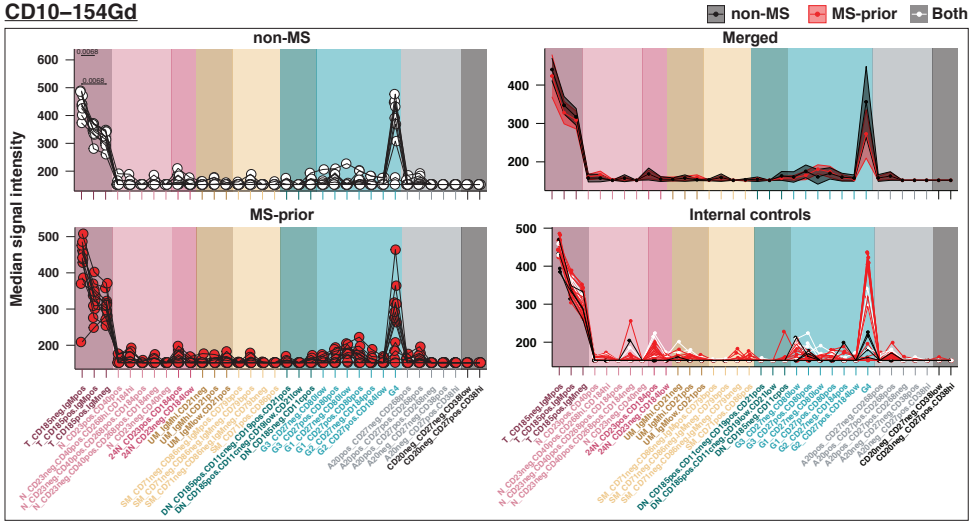
Supplementary figure 4. Median signal intensity of markers expressed by B cells across groups. Median signal intensity of marker expression by B cells across groups are shown. Solid lines signify data are available for adjacent timepoints, whilst dotted lines indicate patients with non-adjacent timepoints. For comparisons of B cell subset levels between all five groups (*non-MS* (multiple sclerosis) controls (n = 9), untreated MS patients (*prior*, n = 11), and MS patients *post-1* (up to twelve months after alemtuzumab dose, n = 9), *post-2* (greater than twelve months, n = 10) alemtuzumab and *relapse* (n = 3)), a PERMANOVA was done followed pairwise comparisons with Holm's correction. *Prior*, *post-2* and *relapse* groups were compared to *non-MS* controls (for three comparisons). A linear mixed-effects model (LMM) was calculated when comparing between MS patients before and after treatment. 4999 permutations were then run to calculate *P*-values. Five multiple comparisons were made (*prior* to *post-1*, *post-2* and *relapse*, and *post-1* to *post-2*, and *post-2* to *relapse*) using a further 4999 permutations with Holm's correction. Mean is shown in *non-MS* controls, *P*-values < 0.1 are shown.

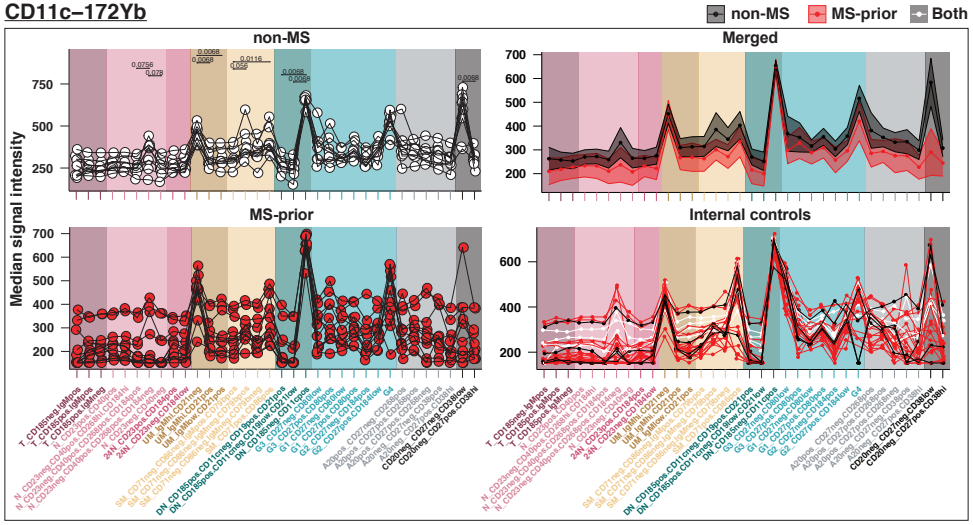

Blimp-1–164Er
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


BLNK–170Er
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity
Total B cells
non-MS
MS-prior
Internal controls
Normalised to mode


CD1d–171Yb
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


CD5–143Nd
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


CD10–154Gd
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


CD11c–172Yb
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity

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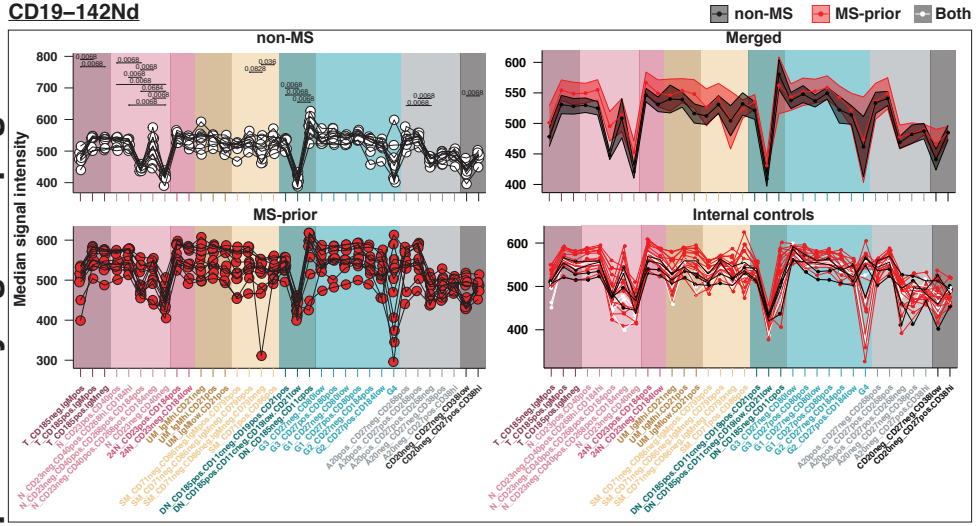

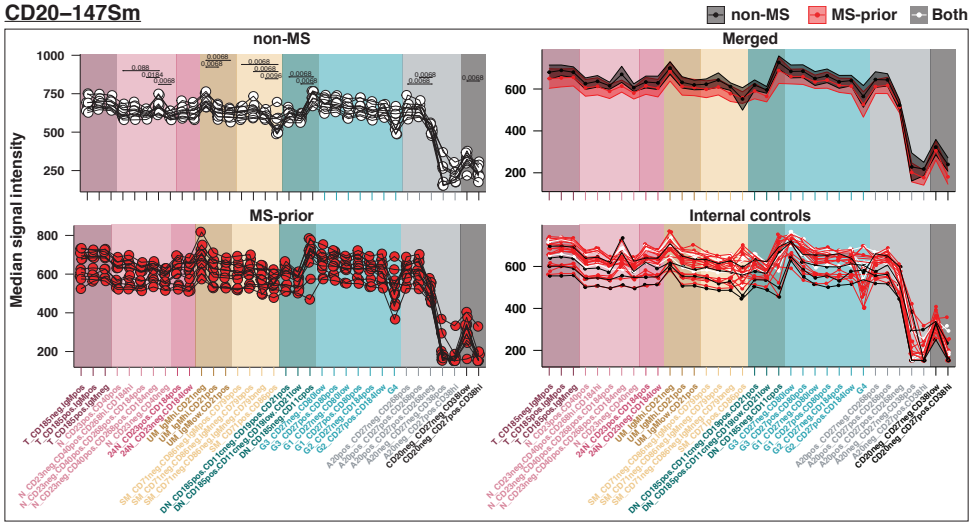

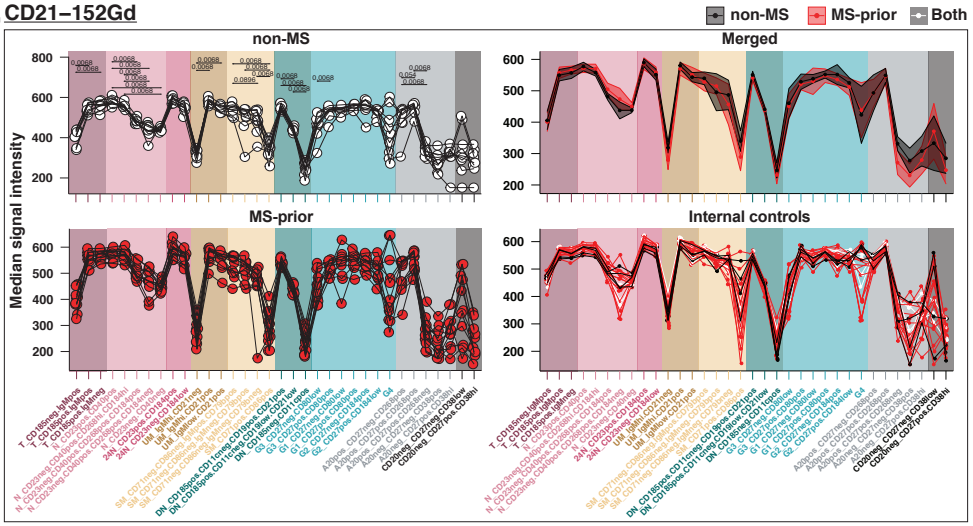

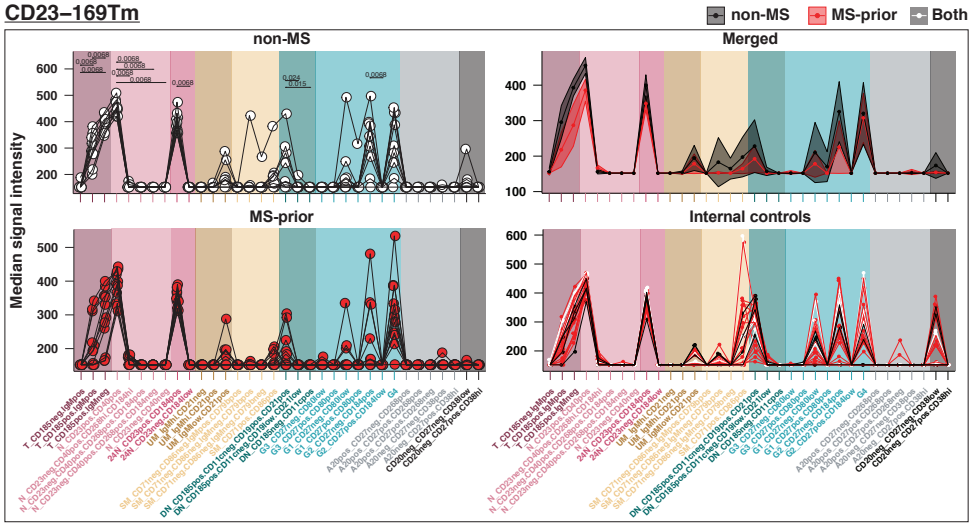

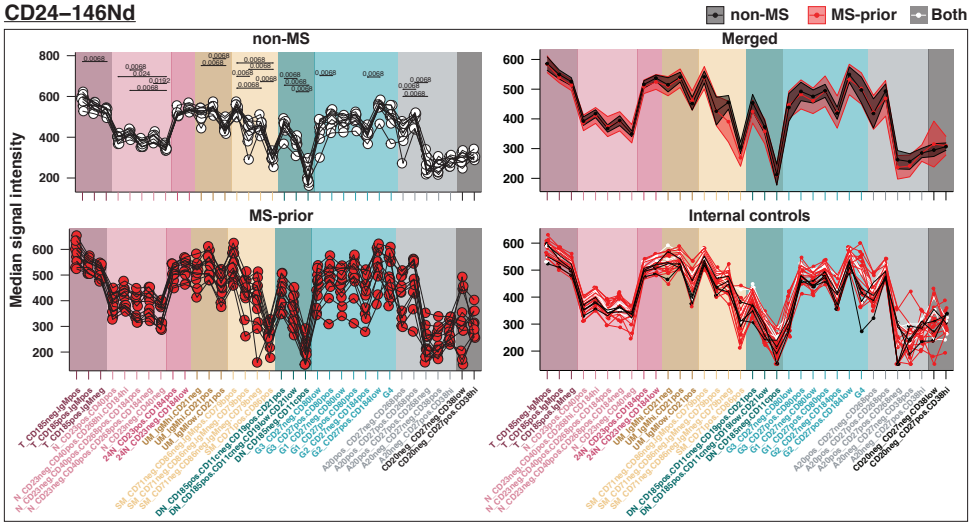

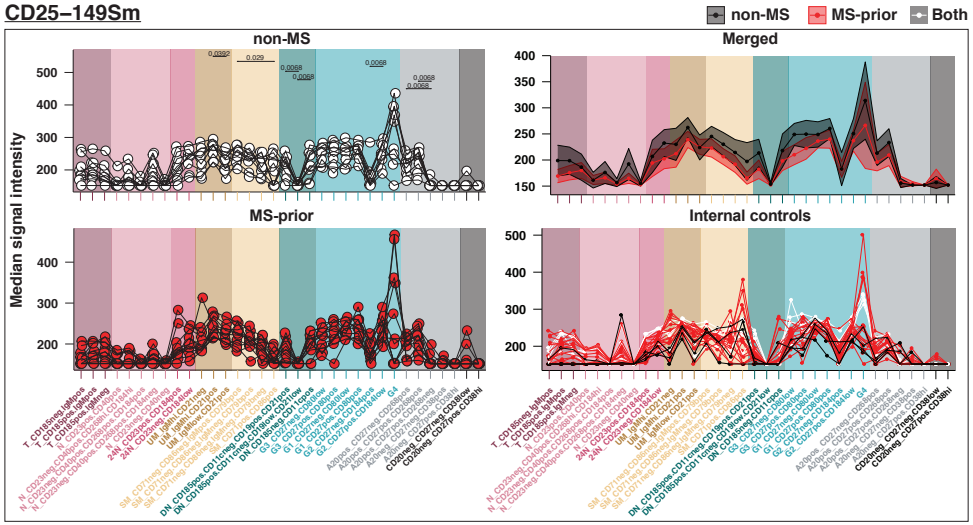

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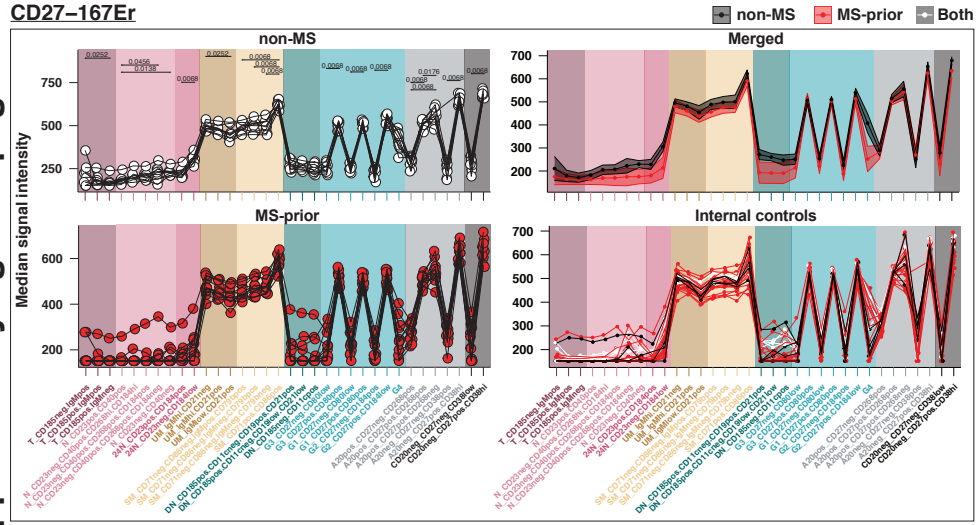

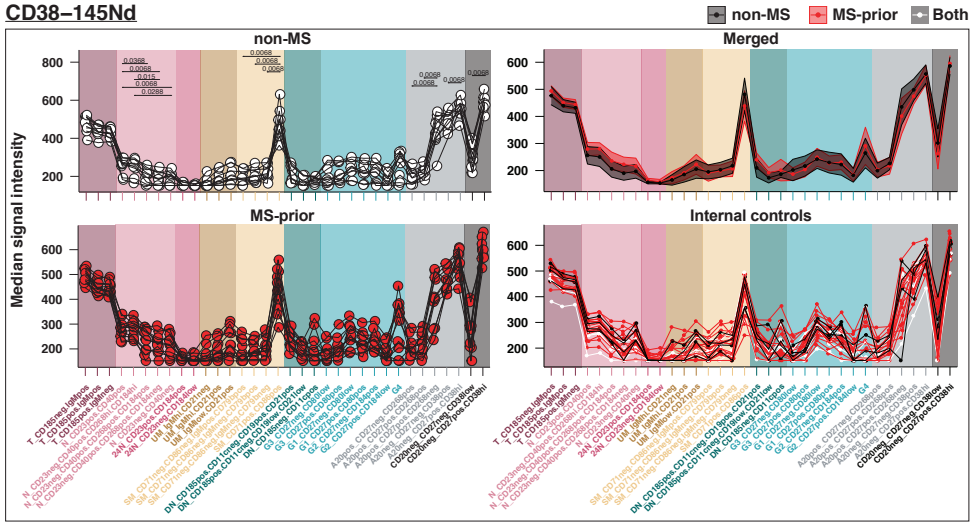

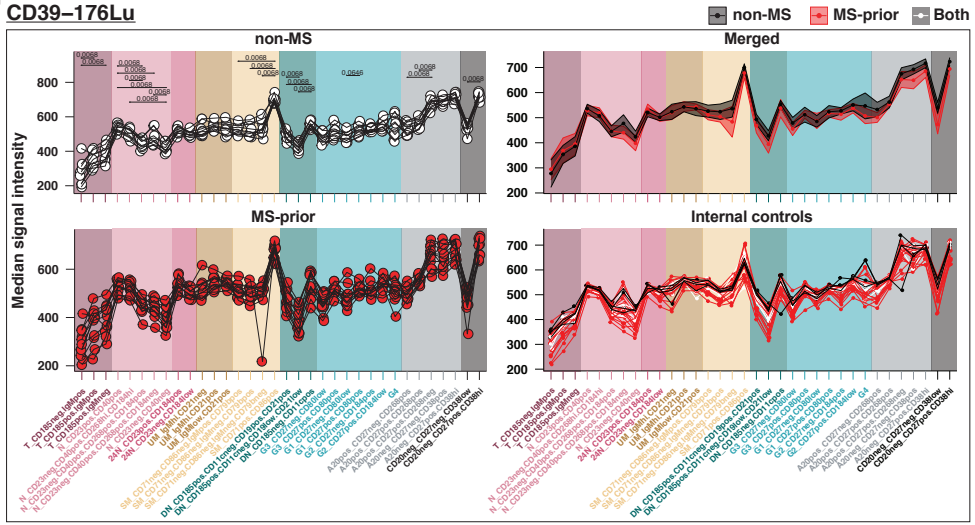

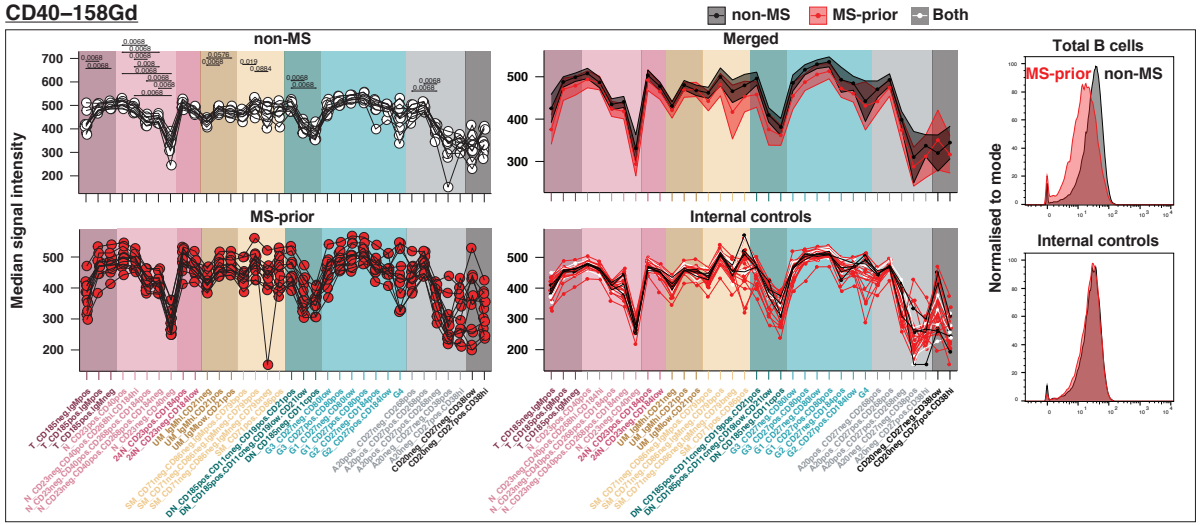

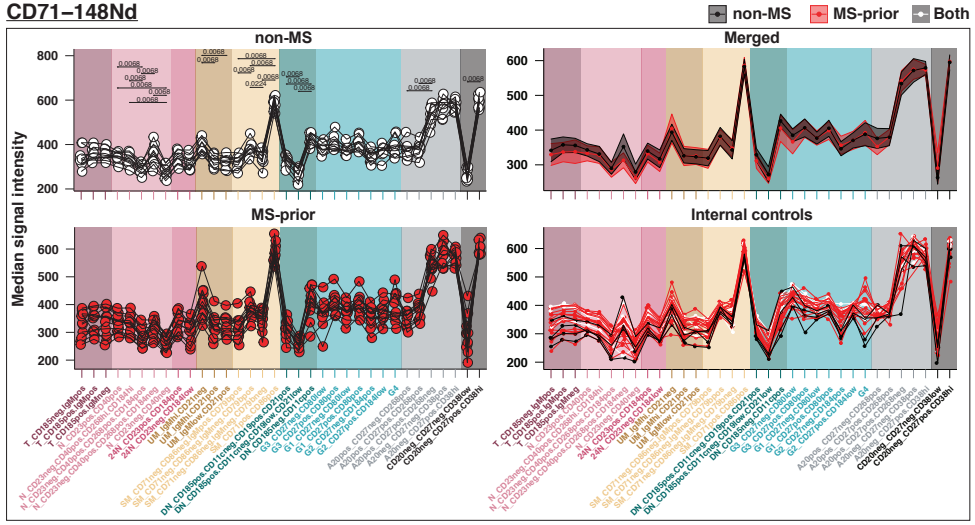

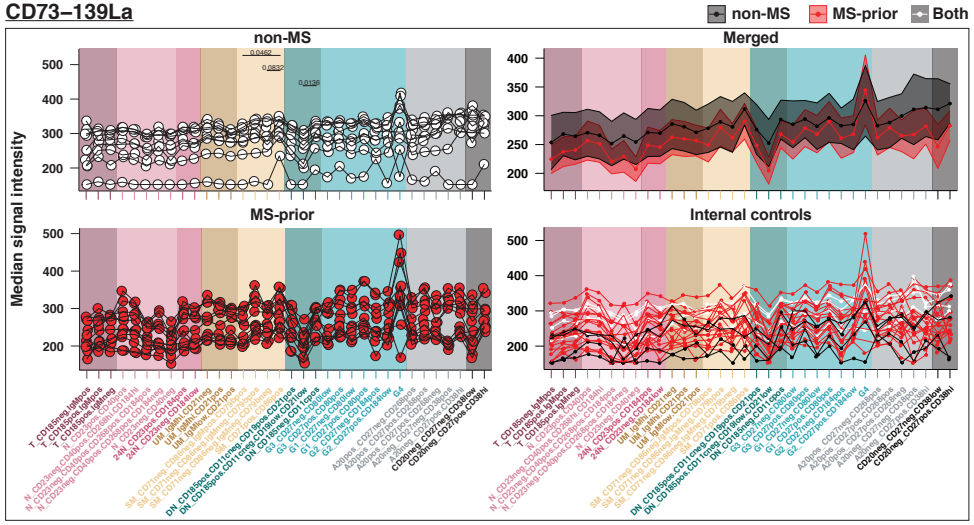

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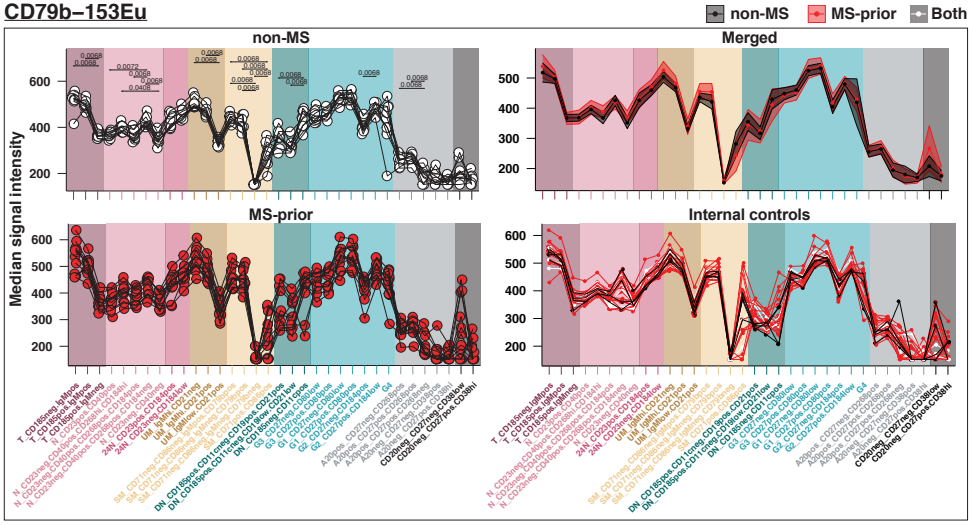

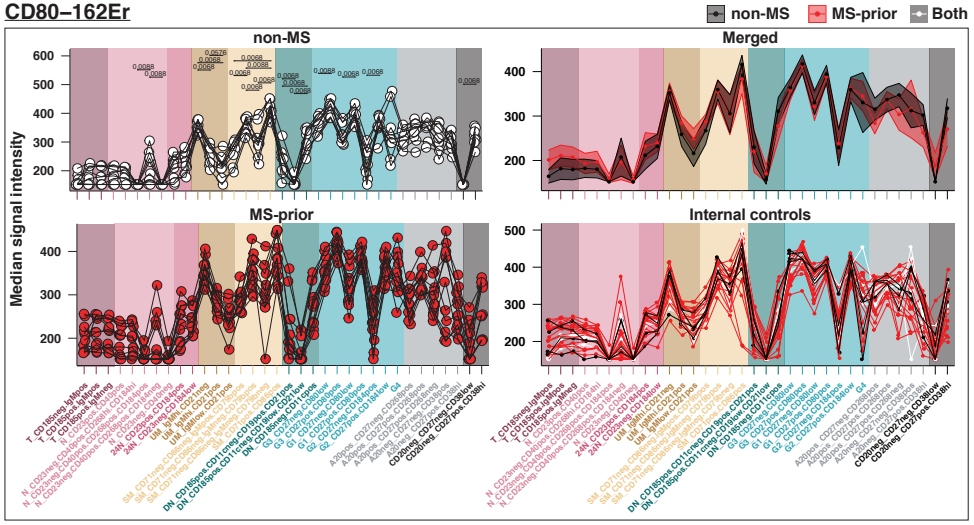

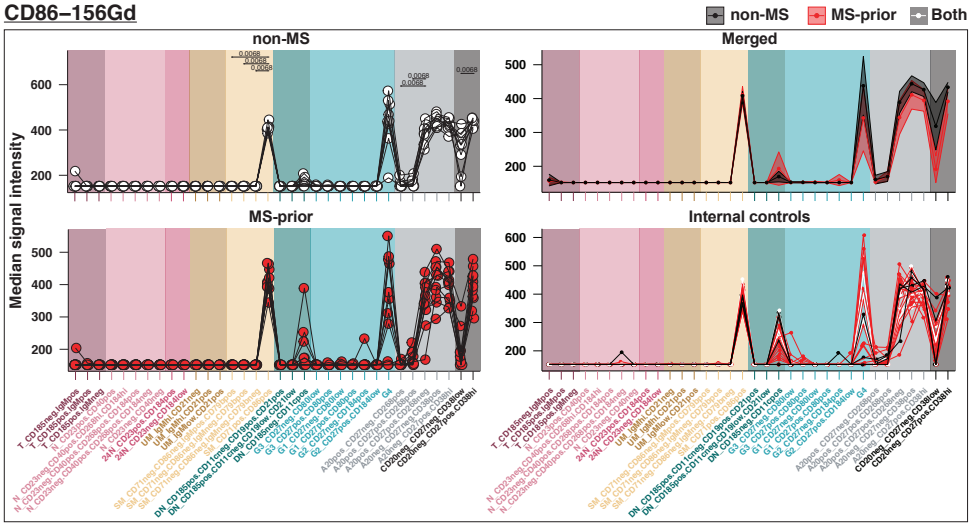

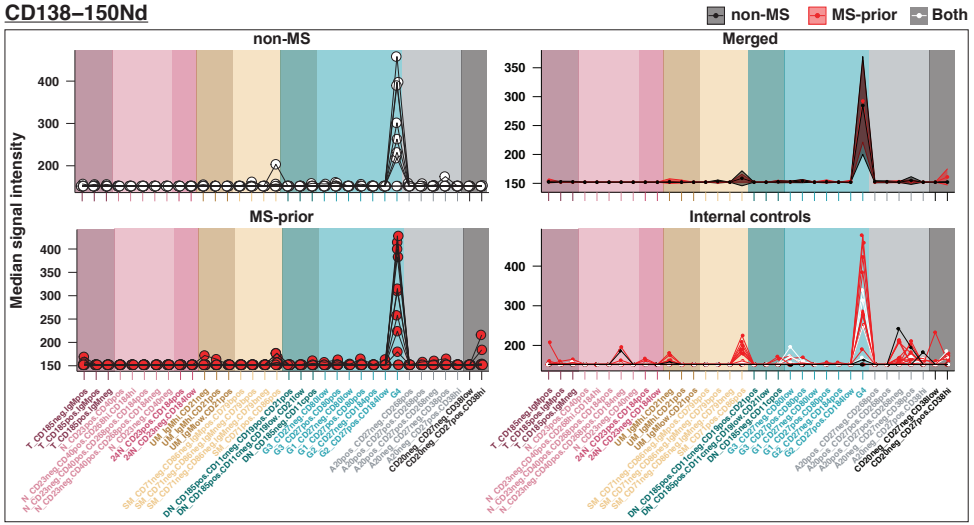

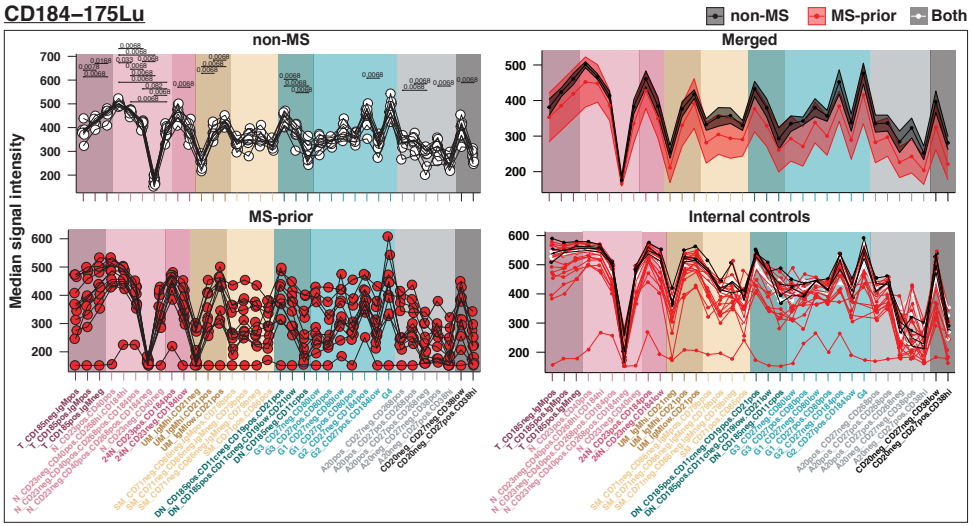

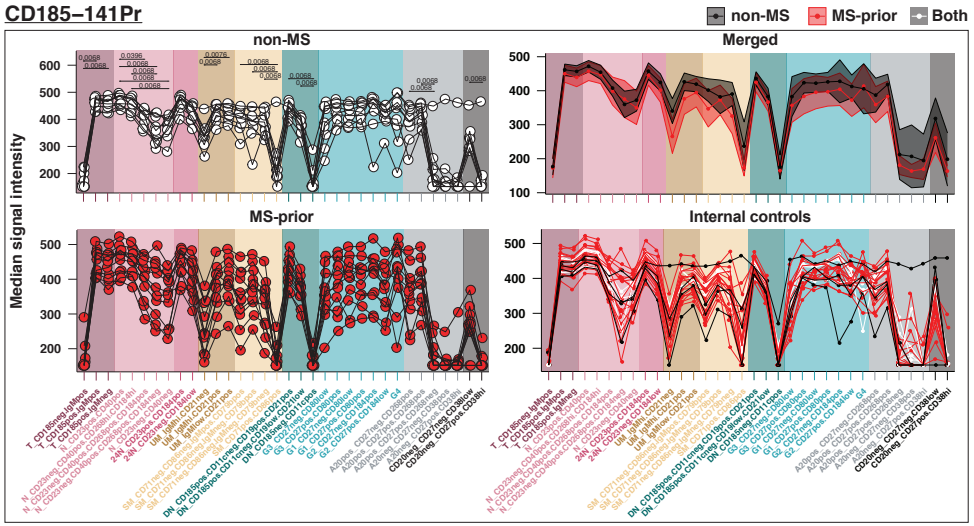

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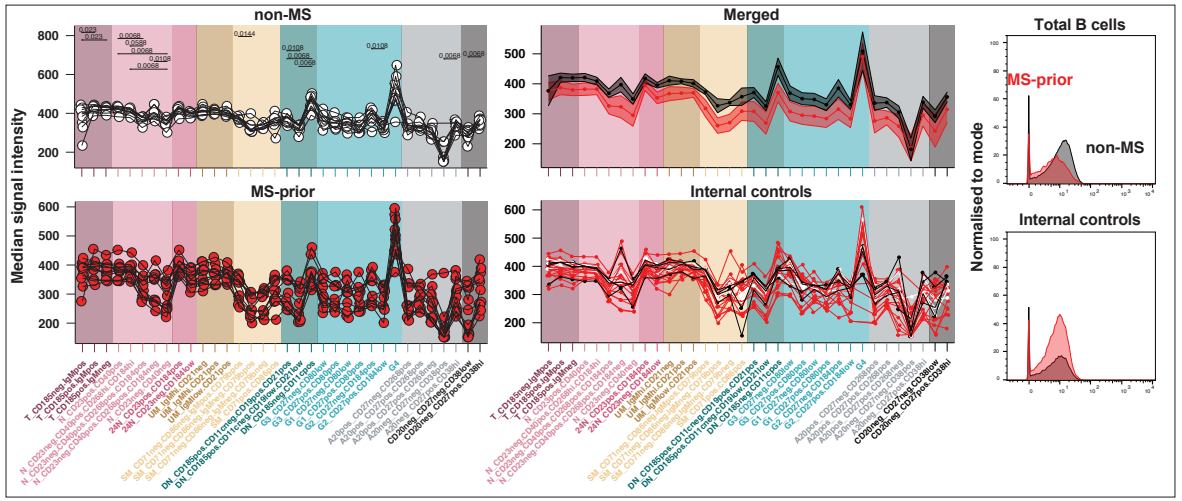
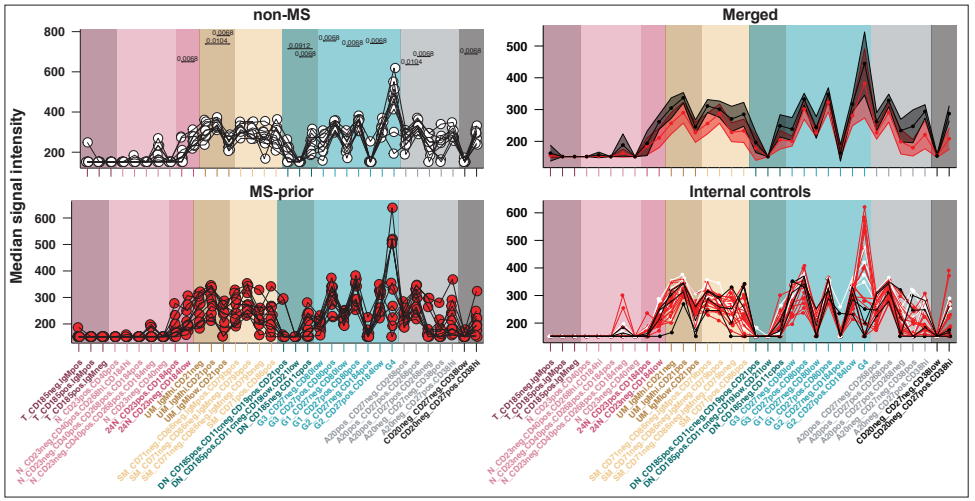
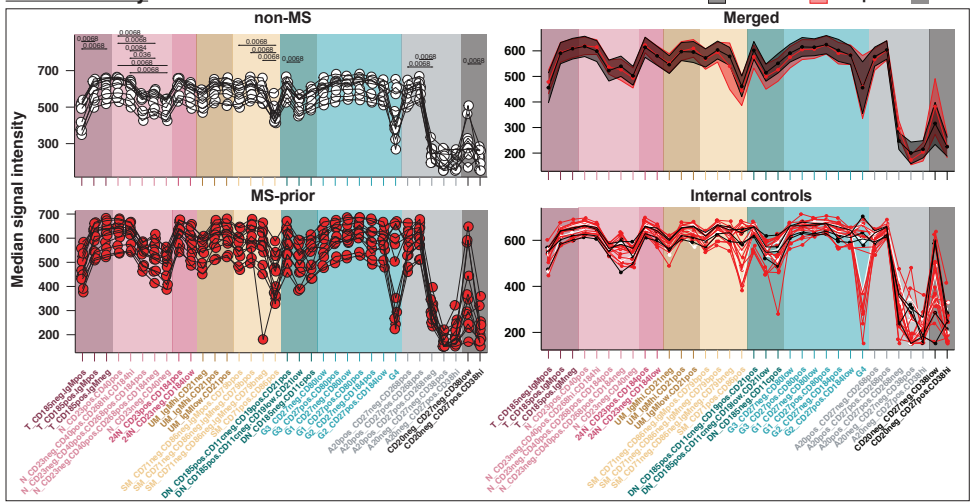
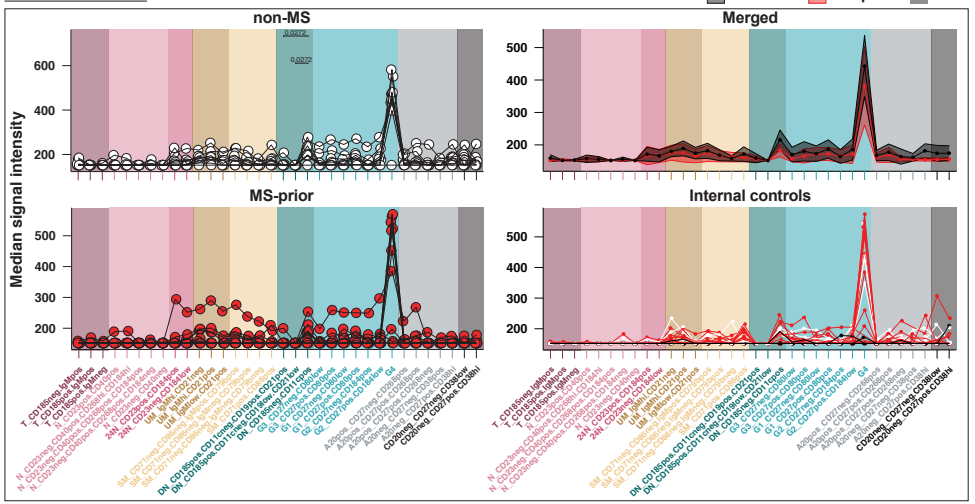
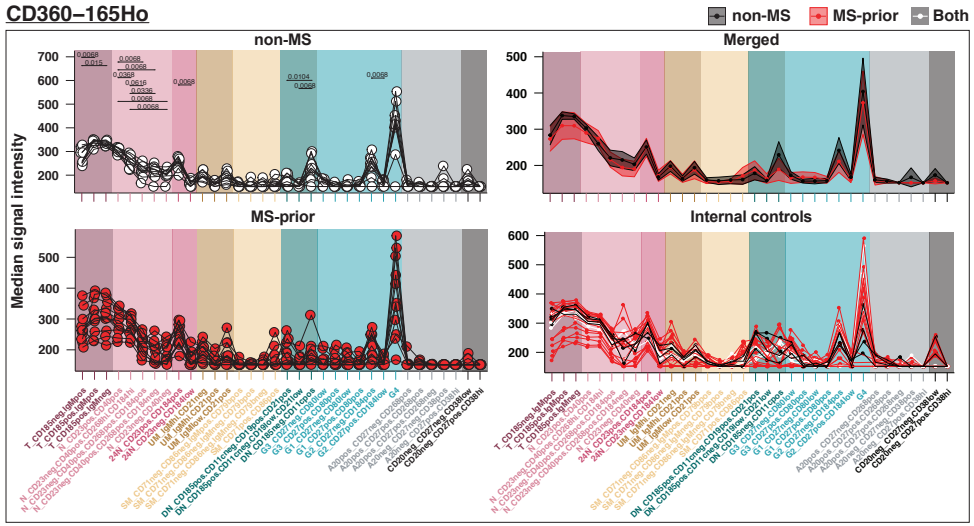
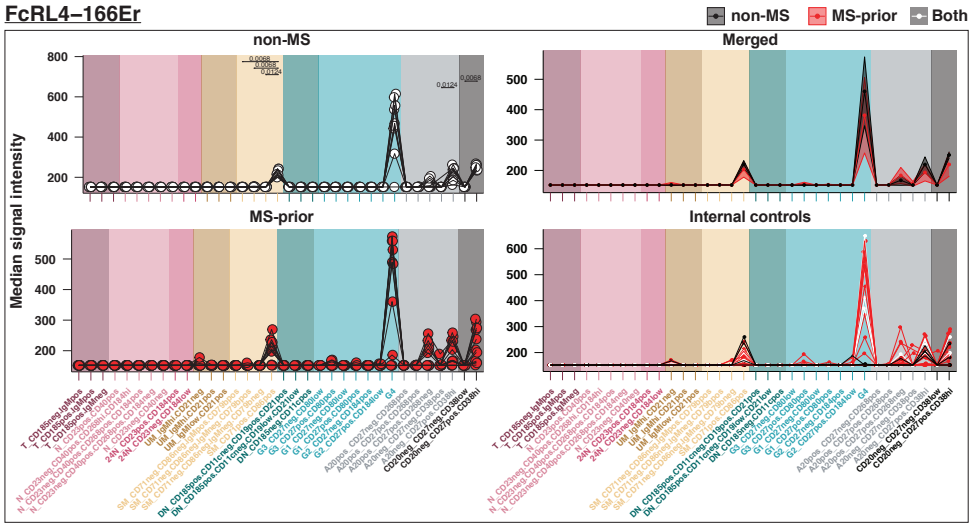


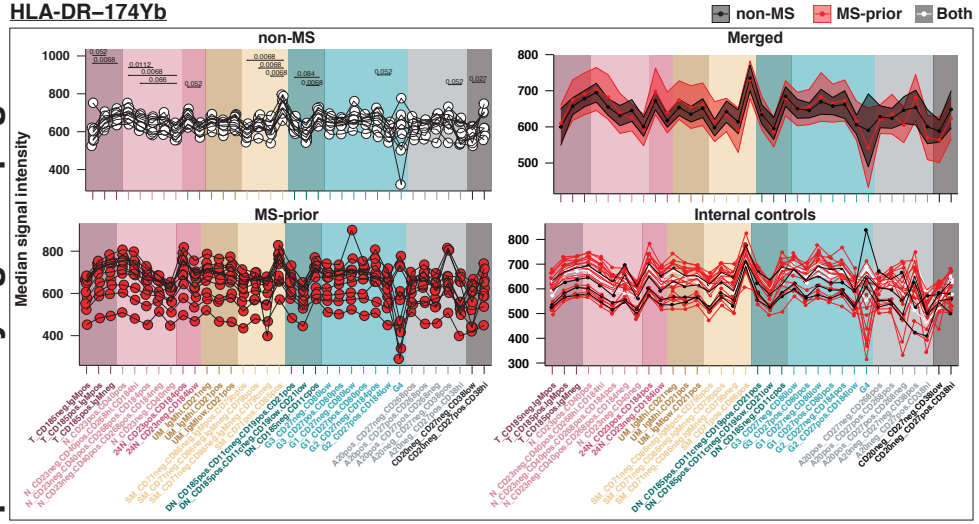


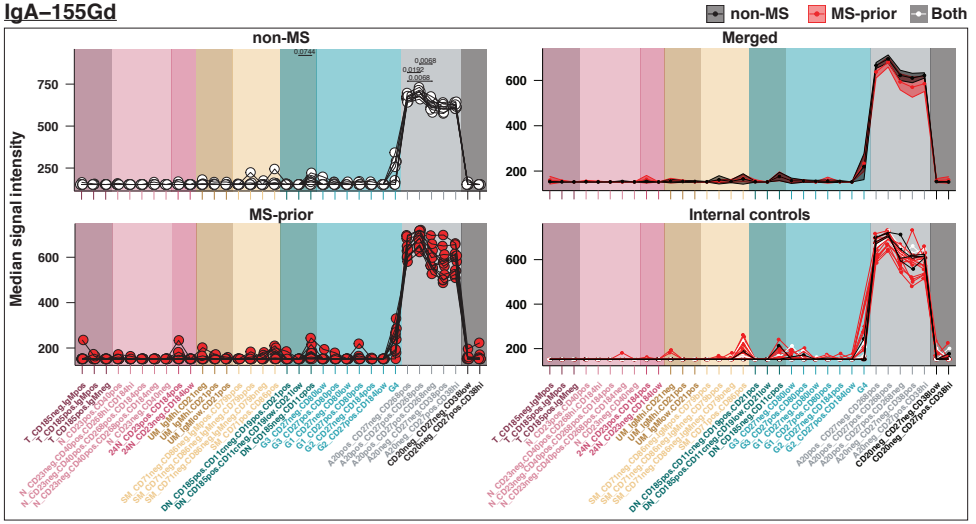


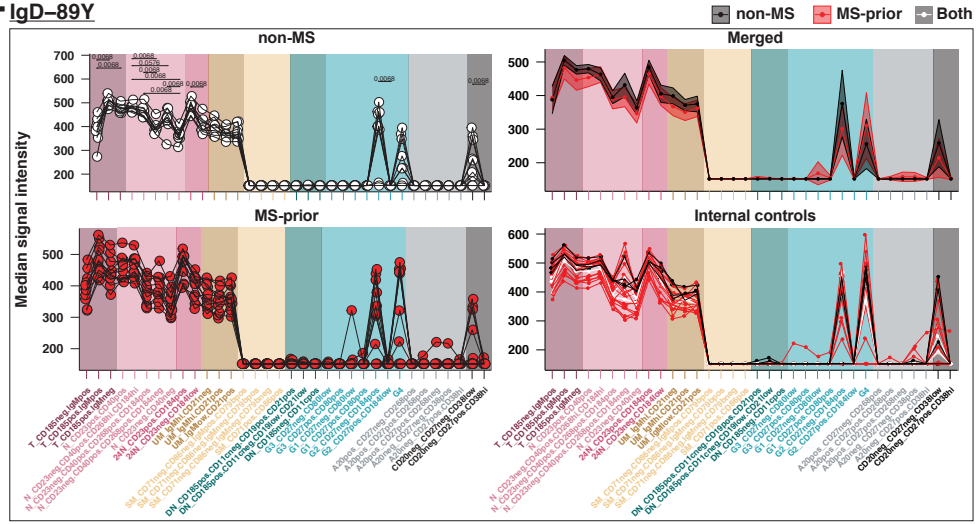


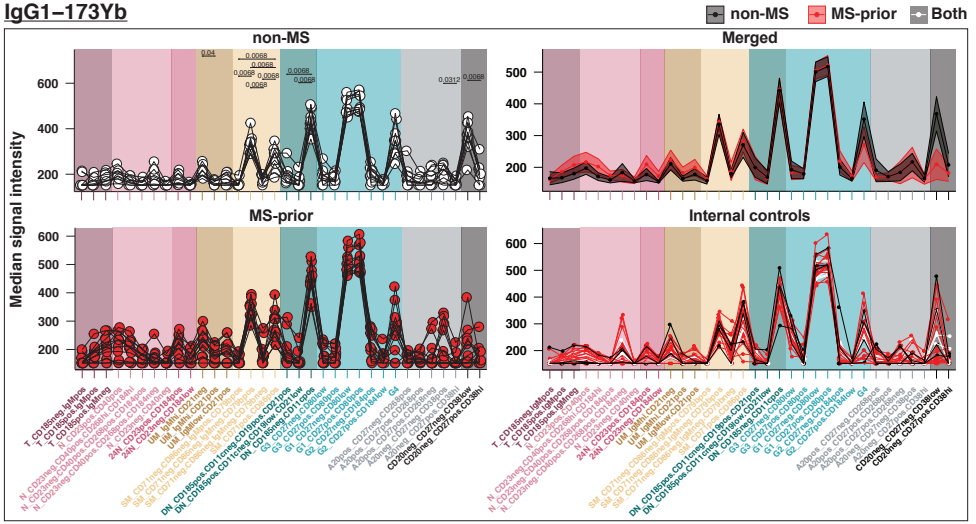


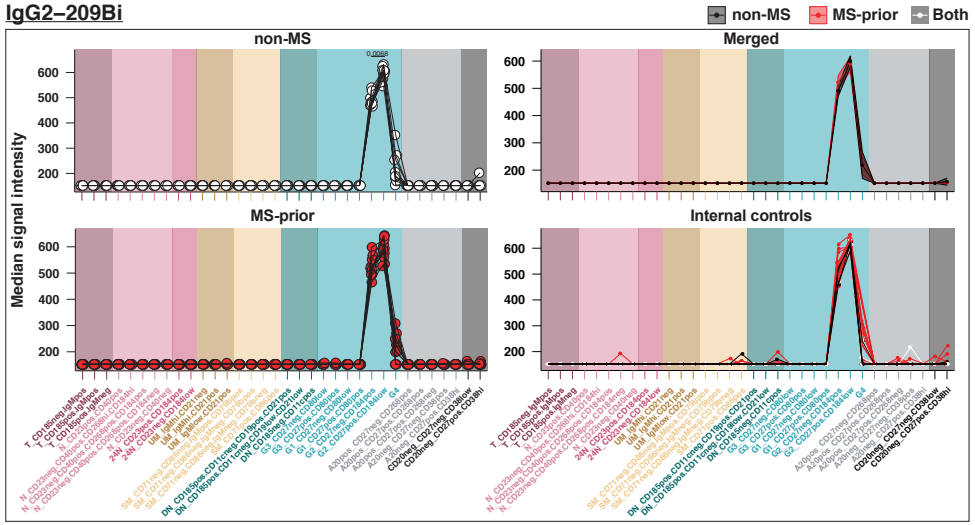


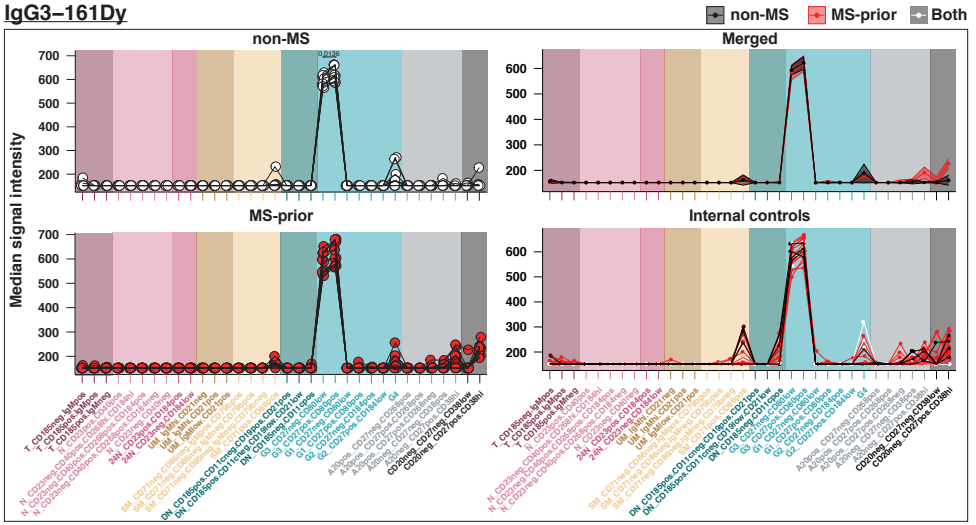

HLA-DR–174Yb
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity

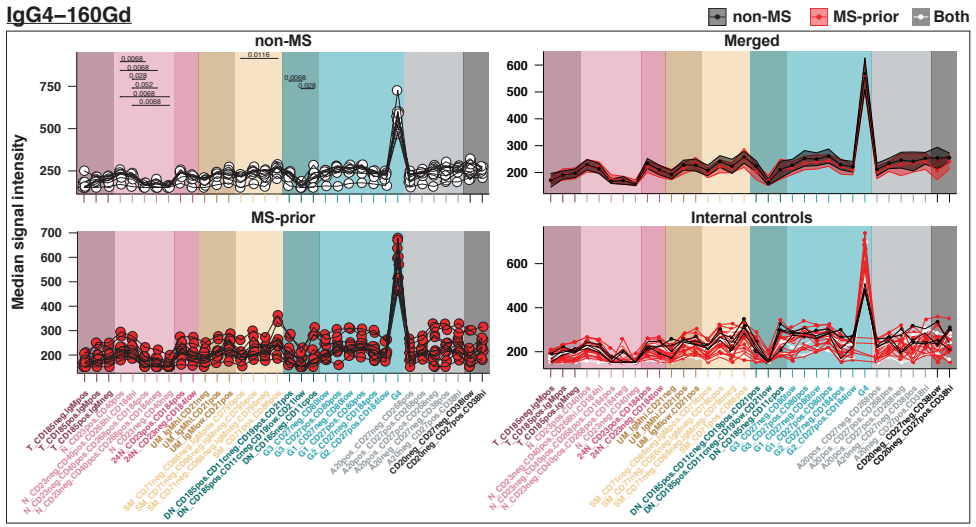

IgA–155Gd
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity

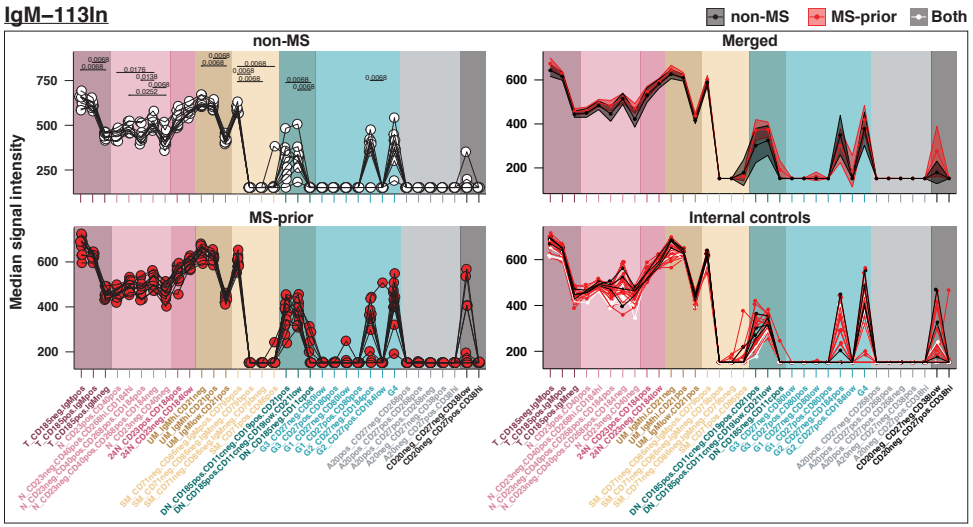

IgD–89Y
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity

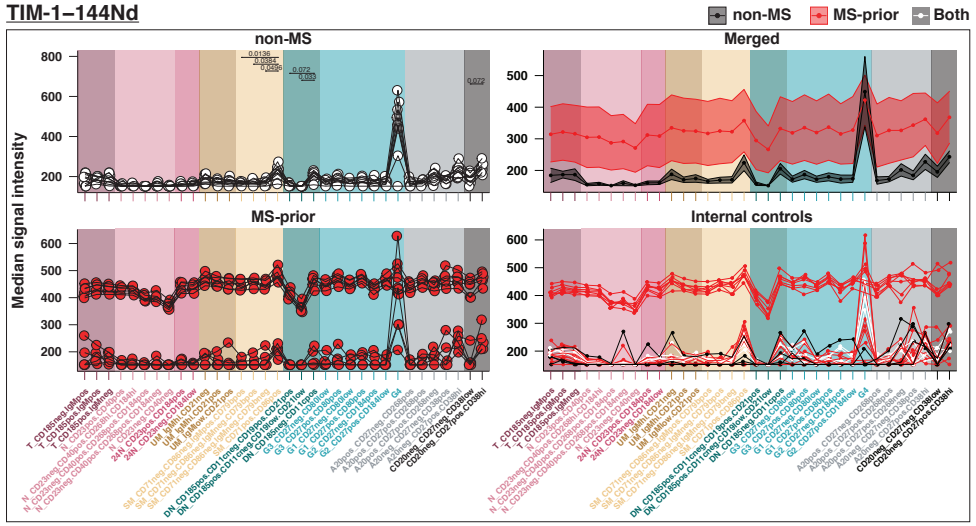

IgG1–173Yb
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


IgG2–209Bi
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity


IgG3–161Dy
non-MS
MS-prior
Both
non-MS
Merged
MS-prior
Internal controls
Median signal intensity







TIM-1 was incorrectly used intracellularly at first which is why it's so high in MS-prior

Supplementary figure 5. Median signal intensity of markers across defined B cell subsets. The median signal intensity of each defined B cell subset was calculated for *non-MS* (multiple sclerosis, n = 9, white circles) and untreated MS patients (*prior*, n = 11, red circles). For the *non-MS* controls, a linear mixed-effects model was calculated with permutations (4999 run). Multiple comparisons were made between subsets of the same conventional B cell subset (34 comparisons in total) with 4999 permutations and Holm's correction. Reported are *P*-values < 0.1. A combined plot was also generated, with mean and 95 % confidence interval shown for *non-MS* controls (black) and MS-*prior* (red). Internal controls from 25 batches, either alongside *non-MS* controls (black n = 4), MS-*prior* (red, n = 17) or a combination of both (white, n = 4) are shown to represent batch variability. BLNK, CD40 and CD210 have representative density plots for their expression between a *non-MS* control and MS-*prior*, and their internal controls. *T* transitional B cells, *N* naïve B cells, *24N* $CD24^{hi}$ B cells, *UM* unswitched memory B cells, *SM* switched memory B cells, *DN* double negative B cells, *G3* IgG_3^+ B cells, *G1* IgG_1^+ B cells, *G2* IgG_2^+ B cells, *G4* IgG_4^+ B cells, *A20*+ IgA^+ $CD20^+$ B cells, *A20*– IgA^+ $CD20^-$ B cells, *CD20*– $CD20^-$ B cells.

Supplementary Table 1. Panel used for identifying B cells.

Target	Isotope	Clone	Company	Algorithm calculations*	Conventional subset†	Defined subset‡
Blimp-1#	164Er§	ROS195G	BioLegend	✓		
BLNK#	170Er	REA240	Miltenyi Biotec	✓		
CD1d	171Yb	51.1	BioLegend	✓		
CD3	115In	UCHT1	BioLegend			
CD5	143Nd§	UCHT2	BioLegend	✓		
CD10	154Sm§	HI10a	BioLegend	✓		
CD11c	172Yb	Bu15	BioLegend	✓		✓
CD19	142Nd§	HIB19	BD Biosciences	✓		✓
CD20	147Sm§	2H7	BioLegend	✓	✓	
CD21	152Sm§	BU32	BioLegend	✓		✓
CD23	169Tm§	EBVCS-5	BioLegend	✓		✓
CD24	146Nd§	ML5	BioLegend	✓	✓	
CD25	149Sm§	2A3	BioLegend	✓		
CD27	167Er§	M-T271	BD Biosciences	✓	✓	✓
CD38	145Nd	HIT2	BioLegend	✓	✓	✓
CD39	176Yb	A1	BioLegend	✓		
CD40	158Gd	5C3	BioLegend	✓		✓
CD45¶	104Pd§	HI30	BioLegend			
	106Pd§		BioLegend			
	108Pd§		BioLegend			
	110Pd§		BioLegend			
CD71	148Nd§	CY1G4	BioLegend	✓		✓
CD73	139La	AD2	BioLegend	✓		
CD79b	153Eu	CB3-1	BioLegend	✓		✓
CD80	162Er§	L307.4	BD Biosciences	✓		✓
CD86	156Gd§	IT2.2	BD Biosciences	✓		✓
CD138	150Nd§	DL-101	BioLegend	✓		
CD184 (CXCR4)	175Lu§	12G5	BD Biosciences	✓		✓
CD185 (CXCR5)	141Pr	J252D4	BioLegend	✓		✓
CD210 (IL-10-R)	168Er	REA239	Miltenyi Biotec	✓		
CD267 (TACI)	159Tb	1A1	BioLegend	✓		
CD268 (BAFF-R)	163Dy§	11C1	BioLegend	✓		✓
CD274 (PD-L1)	151Eu	29E.2A3	BioLegend	✓		
CD360 (IL-21-R)	165Ho	REA233	Miltenyi Biotec	✓		
FcRL4 (CD307d)	166Er	413D12	BioLegend	✓		
HLA-DR	174Yb§	L243	BioLegend	✓		
IgA	155Gd	IS11-8E10	Miltenyi Biotec	✓	✓	
IgD	89Y§	IA6-2	BD Biosciences	✓	✓	
IgG1	173Yb	12G8G11	BioLegend	✓	✓	
IgG2	209Bi	HP6002	BioLegend	✓	✓	
IgG3	161Dy	HP6047	BioLegend	✓	✓	
IgG4	160Gd	IGHG4/1345	Novus Biologicals	✓	✓	
IgM	113In	MHM-88	BioLegend	✓		✓
TIM-1	144Nd	REA384	Miltenyi Biotec			

*Markers used for algorithms (including clustering and dimensionality reduction)

†Markers used for conventional B cell subsets

‡Markers used for defined B cell subsets

§Isotopes conjugated by the Ramaciotti Facility for Human Systems Biology, The University of Sydney, Australia.

¶CD45 was used for barcoding to allow up to three samples to be stained together.

#Markers stained intracellularly