Supplemental Data

Monocyte Notch2 expression predicts interferon-beta immunogenicity in multiple sclerosis patients

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Supplemental Tables

Supplemental Table 1: Markers retained for analysis in the different subsets

CD4$^+$ T cells		**Monocytes**		**B cells**	
beta2 microglobulin	CD41	beta2 microglobulin	CD45	beta2 microglobulin	CD49c
BTLA	CD43	C3AR	CD45RA	BTLA	CD49d
C3AR	CD44	C5L2	CD45RB	CCR10	CD49e
CCR10	CD45	CCR10	CD45RO	CD10	CD49f
CD100	CD45RA	CD10	CD46	CD100	CD5
CD101 (BB27)	CD45RB	CD100	CD47	CD102	CD50
CD102	CD45RO	CD101 (BB27)	CD48	CD105	CD51
CD107a	CD46	CD102	CD49a	CD107a	CD51-61
CD119 (IFNgR a chain)	CD47	CD103	CD49d	CD108	CD52
CD11a	CD48	CD105	CD49e	CD112 (Nectin-2)	CD53
CD11b	CD49a	CD107a	CD49f	CD116	CD54
CD126 (IL-6Ra)	CD49c	CD109	CD50	CD119 (IFNgR a chain)	CD55
CD127 (IL-7Ra)	CD49d	CD112 (Nectin-2)	CD51	CD11a	CD58
CD132	CD49e	CD114	CD51-61	CD11b	CD59
CD134	CD49f	CD115	CD52	CD11c	CD6
CD143	CD5	CD116	CD53	CD123	CD61
CD148	CD50	CD119 (IFNgR a chain)	CD54	CD124	CD62E
CD150 (SLAM)	CD51	CD11a	CD55	CD126 (IL-6Ra)	CD62L
CD154	CD52	CD11b	CD56	CD129 (IL-9R)	CD62P
CD156c (ADAM10)	CD53	CD11b (activated)	CD57	CD131	CD63
CD16	CD54	CD11c	CD58	CD132	CD66a-c-e
CD161	CD55	CD123	CD59	CD137L (4-1BBL)	CD69
CD162	CD57	CD126 (IL-6Ra)	CD6	CD144	CD7
CD164	CD58	CD129 (IL-	CD61	CD146	CD70

		9R)			
CD165	CD59	CD13	CD62E	CD148	CD71
CD166	CD6	CD131	CD62L	CD150 (SLAM)	CD73
CD172g	CD61	CD132	CD62P	CD156c (ADAM10)	CD74
CD18	CD62L	CD137L (4-1BBL)	CD63	CD158e	CD79b
CD183	CD63	CD138	CD64	CD16	CD80
CD184	CD69	CD140b	CD66a-c-e	CD162	CD81
CD195	CD7	CD141	CD69	CD164	CD82
CD196	CD71	CD144	CD7	CD165	CD83
CD197	CD73	CD146	CD71	CD166	CD84
CD2	CD81	CD148	CD73	CD167a	CD85j
CD200	CD82	CD15	CD74	CD170	CD86
CD200R	CD84	CD155 (PVR)	CD79b	CD172a	CD87
CD205	CD85a	CD156c (ADAM10)	CD80	CD172b	CD89
CD21	CD88	CD158d	CD81	CD18	CD8a
CD210	CD8a	CD16	CD82	CD180	CD9
CD218a	CD9	CD162	CD83	CD183	CD95
CD229	CD95	CD163	CD84	CD184	CD96
CD231	CD96	CD164	CD85a	CD193	CD97
CD235ab	CD97	CD165	CD85d	CD195	CD99
CD243	CD99	CD166	CD85h	CD196	CX3CR1
CD244	CLEC12A	CD167a	CD85j	CD197	CXCR7
CD245	CX3CR1	CD169	CD85k	CD1c	DLL4
CD25	HLA-A,B,C	CD170	CD86	CD1d	DR3
CD257	HLA-A2	CD172a	CD87	CD2	FcRL6
CD26	HLA-E	CD172b	CD88	CD20	HLA-A,B,C
CD27	Integrin beta7	CD179a	CD89	CD200	HLA-A2
CD270	MSC (W4A5)	CD18	CD8a	CD200R	HLA-E
CD274	Notch 2	CD180	CD9	CD202b	Ig light chain k
CD277	TCR a-b	CD181	CD93	CD205	Ig light chain lambda
CD278	TCR V beta8	CD182	CD94	CD21	IgD
CD279	TCR V beta9	CD183	CD95	CD210	IgM
CD28		CD184	CD96	CD213a2	Integrin beta7
CD29		CD195	CD97	CD218a	MSC (W4A5)

CD298		CD196	CD99	CD22	Notch 1
CD3		CD1c	CLEC12A	CD221	Notch 2
CD303		CD1d	CLEC9A	CD229	Siglec-10
CD304		CD2	CX3CR1	CD23	SSEA-1
CD31		CD20	CXCR7	CD231	SSEA-4
CD317		CD200R	erbB3	CD235ab	TLT-2
CD319		CD205	FceRIalpha	CD243	
CD324		CD21	FcRL6	CD245	
CD35		CD210	Galectin-9	CD25	
CD352		CD215	GARP	CD253	
CD36		CD220	HLA-A,B,C	CD255	
CD360		CD226	HLA-A2	CD257	
CD39		CD229	HLA-E	CD261	
		CD23	HLA-G	CD262	
		CD235ab	IFNg R b chain	CD267	
		CD244	Ig light chain k	CD268	
		CD245	Ig light chain lambda	CD27	
		CD252	IgM	CD270	
		CD254	Integrin alpha9 beta1	CD274	
		CD257	Integrin beta7	CD275	
		CD258	LAP	CD277	
		CD261	Lymphotoxin b receptor (LT-bR)	CD28	
		CD262	Mac-2 (galectin-3)	CD284	
		CD268	MAIR-II	CD29	
		CD270	MSC (W4A5)	CD290	
		CD274	Notch 1	CD298	
		CD275	Notch 2	CD3	
		CD277	NPC (57D2)	CD301	
		CD28	Siglec-10	CD307	
		CD282	Siglec-9	CD31	
		CD284	SSEA-1	CD317	
		CD286	Tim-3	CD319	
		CD29	TLT-2	CD32	
		CD298	TRA-1-60-R	CD344	
		CD3		CD35	
		CD30		CD352	
		CD300e		CD36	

		CD300f		CD360	
		CD301		CD39	
		CD31		CD40	
		CD317		CD41	
		CD319		CD42b	
		CD32		CD43	
		CD324		CD44	
		CD325		CD45	
		CD328		CD45RA	
		CD33		CD45RB	
		CD338		CD46	
		CD34		CD47	
		CD344		CD48	
		CD35			
		CD352			
		CD354			
		CD355			
		CD357			
		CD36			
		CD360			
		CD39			
		CD40			
		CD41			
		CD42b			
		CD43			
		CD44			

Supplemental Table 1: Legendscreen optimisation. Immune cell surface markers used to identify differentially expressed markers (DEMs) in multiple sclerosis (MS) patients with and without anti-drug antibodies to IFN-β. Peripheral blood mononuclear cells (PBMCs) from healthy controls (HCs) (n=10), and patients with MS (treated for 12 months, MS-T, n=28 and treatment naïve, MS-N, n=15) were analysed using the LegendScreen platform. The expression of the 332 cell surface markers in all groups were compared as described in Supplemental Figure 1. All cell surface markers that were negative in all HCs and MS patients (treated or not treated with IFNβ) were excluded from future analysis. This Table lists all the markers on monocytes, B-cells and T-cells that were included in future analysis.

Supplemental Table 2:

Cell subset	Markers	P value
$CD14^+$ Monocytes (% positive)	CD34	0.0357533
	FcRL6	0.0182166
	Notch 2	0.000387312
	CD138	0.0400212
	CD319	0.00225787
	CD200R	0.0327181
	CD284	0.0297907
	CD274	0.00389132
	CD129 (IL-9R)	0.0233099
	CD16	0.0287405
	CD169	0.0110785
	CD123	0.012337
	CD71	0.00318563
	Tim-3	0.0262036
	CD245	0.0166616
$CD14^+$ Monocytes (MFI)	CD262	6.162782e-005
	HLA.E	0.00171913
	Notch2	0.00216778
	CD317	0.00238299
	CX3CR1	0.00746864
	CD81	0.00824417
	CD82	0.0102909
	CD254	0.0105262
	CLEC9A	0.0108831
$CD19^+$ B-cells (MFI)	CD165	0.00324693
	CD183	0.00345526
	MSC_NPC	0.00450016

Supplemental Table 2: List of differentially expressed markers (DEMs) identified between neutralizing anti-drug antibody $(nADA)^-$ and $nADA^+$ multiple sclerosis (MS)

patients: Peripheral blood mononuclear cells **(**PBMCs) from the discovery cohort (Table 1) of MS patients treated with IFN-β for at least 12 months who had ($nADA^+$, n=16) or had not ($nADA^-$, n=12) developed neutralizing anti-drug antibodies (nADA) were analysed by Legendscreen and the results compared using unpaired t-tests and a false discovery rate (FDR) <0.2 (see Figure 2A). The majority of DEMs were on monocytes (n=21), with 3 B-cell markers and no $CD4^+$T-cell markers identified. MFI; mean fluorescence intensity.

Supplemental Table 3: DEMs correlation with change in brain lesion size in the previous 12 months assessed by QMRI T1 and T2 scanning

Cell subset	Markers	T1 lesions		T2 lesions	
		r	p	r	p
CD14$^+$ Monocytes (% positive)	CD34	0.1204	0.6236	-0.3788	0.1098
	FcRL6	-0.06549	0.7900	-0.1789	0.4638
	Notch 2	-0.3940	0.0951	-0.4011	0.0888
	CD138	-0.2105	0.3870	-0.6825	**0.0013**
	CD319	-0.1649	0.4999	-0.1088	0.6576
	CD200R	0.04035	0.8697	0.1737	0.4770
	CD284	-0.1649	0.4999	-0.3263	0.1727
	CD274	-0.1333	0.5863	-0.2193	0.3670
	CD129 (IL-9R)	-0.2912	0.2264	-0.06491	0.7918
	CD16	0.06667	0.7863	0.007018	0.9773
	CD169	0.01492	0.9517	0.2756	0.2535
	CD123	0.1847	0.4631	-0.05470	0.8293
	CD71	-0.2807	0.2444	-0.1035	0.6733
	Tim-3	-0.1455	0.5645	-0.5191	**0.0273**
	CD245	0.04912	0.8417	-0.2982	0.2149
CD14$^+$ Monocytes (MFI)	CD262	0.4228	0.0713	0.1579	0.5185
	HLA.E	0.1421	0.5617	-0.00702	0.9773
	Notch2	-0.3509	0.1408	-0.1807	0.4591
	CD317	0.06667	0.7863	-0.09649	0.6943
	CX3CR1	0.2333	0.3364	-0.06316	0.7973
	CD81	0.2667	0.2698	0.07018	0.7753
	CD82	0.3860	0.1027	-0.2404	0.3216
	CD254	0.4368	0.0615	0.5140	**0.0244**
	CLEC9A	0.2386	0.3253	0.5684	**0.0111**
CD19$^+$ B-cells (MFI)	CD165	0.1912	0.4329	0.1737	0.4770
	CD183	0.2035	0.4034	0.0	> 0.9999
	MSC_NPC	0.5474	**0.0153**	-0.02807	0.9092

Supplemental Table 3: Differentially expressed markers (DEMs) correlation with disease activity. DEMs identified in Figure 2A and Supplemental Table 2 were correlated with changes in brain lesion inflammation detected by QMRI T1 and T2 scanning. Brain lesions at the time of peripheral blood mononuclear cell (PBMC) isolation were compared to MRI scans performed 12 months previously. Markers found to be associated with disease activity are highlighted in bold and the correlation shown in Figure 2A.

Supplemental Table 4: Differentially expressed markers (DEMs) associated with neutralizing anti-drug antibodies (nADA)-Discovery cohort

Cell subsets	Markers	P value
$CD14^+$ Monocytes (% positive)	Notch 2	0.000387312
	CD200R	0.0327181
	CD284	0.0297907
	CD169	0.0110785
	CD245	0.0166616
$CD14^+$ Monocytes (MFI)	CD262	6.162782e-005
	Notch2	0.00216778
	CD317	0.00238299

Supplemental Table 4: Differentially expressed markers (DEMs) associated with neutralizing anti-drug antibodies (nADA)- Discovery cohort. Seven markers expressed on monocytes were associated exclusively with the development of nADA in multiple sclerosis (MS) patients treated for at least 12 month with IFN-β after excluding those markers whose expression was influenced by disease progression and IFN-β drug itself (see also figure 2B-F and fig, S2A-C). Analysed by unpaired T-tests and a false discovery rate (FDR) <0.2.

Supplemental Table 5: "Gold Standard" markers used for the generation of mixture model

CD4+ Th cells		Monocytes		CD19+ BCells	
Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
TCR a/b	CD19	CD11a	TCR Vb13.	CD20	TCR g/d
CD3	CD22	CD11b	TCR Vb13.2	CD21	TCR Vb13.
CD2	CD23	CD11c	TCR Vb13.2	CD22	TCR Vb13.2
CD28	CD13	CD13	TCR Vb13.2	BTLA	TCR Vb13.2
CD5	CD14	CD15	TCR Vb23	CD11a	TCR Vb13.2
CD6	CD14	CD18	TCR Vb8	CD18	TCR Vb23
CD7	CD15	CD31	TCR Vb9	CD48	TCR Vb8
CD27	CD33	CD32	TCR Vg9	CD32	TCR Vb9
CD44	CD66b	CD33	TCR Va24-Ja18	CD40	TCR Vg9
CD45	CD138	CD35	TCR Va7.2	CD180	TCR Va24-Ja18
CD45RO		CD36	CD90	CD27	TCR Va7.2
CD45RA		CLEC12a	CD22	CD23	CD90
CD127		CD298		CD24	

Supplemental Table 6: Discovery and validation patients nADA titres

Cohort	nADA$^+$ Patients	ADA Titre	Cohort	nADA$^+$ Patients	ADA Titre
Discovery	Pt1	320	Validation	Pt1	20480
	Pt2	10240		Pt2	160
	Pt3	640		Pt3	640
	Pt3	80		Pt4	5120
	Pt4	20480		Pt5	1280
	Pt5	1280		Pt6	2560
	Pt6	20480		Pt7	640
	Pt7	320		Pt8	1280
	Pt8	160		Pt9	1280
	Pt9	160		Pt10	10240
	Pt10	160		Pt11	40
	Pt11	160		Pt12	1280
	Pt12	80		Pt13	1280
	Pt13	1280			
	Pt14	320			
	Pt15	640			
	Pt16	320			

Supplemental Table 6: Neutralizing anti-drug antibody (nADA) titres of discovery and validation cohort nADA$^+$ patients. Serum samples collected 12 mouths after the first IFN-β administration were tested for nADA using a luciferase-based bioassay (LUC) (1). nADA titres were determined in all positive samples. Immuno-depletion was performed for all samples screened positive to assess assay specificity.

Supplemental Figures

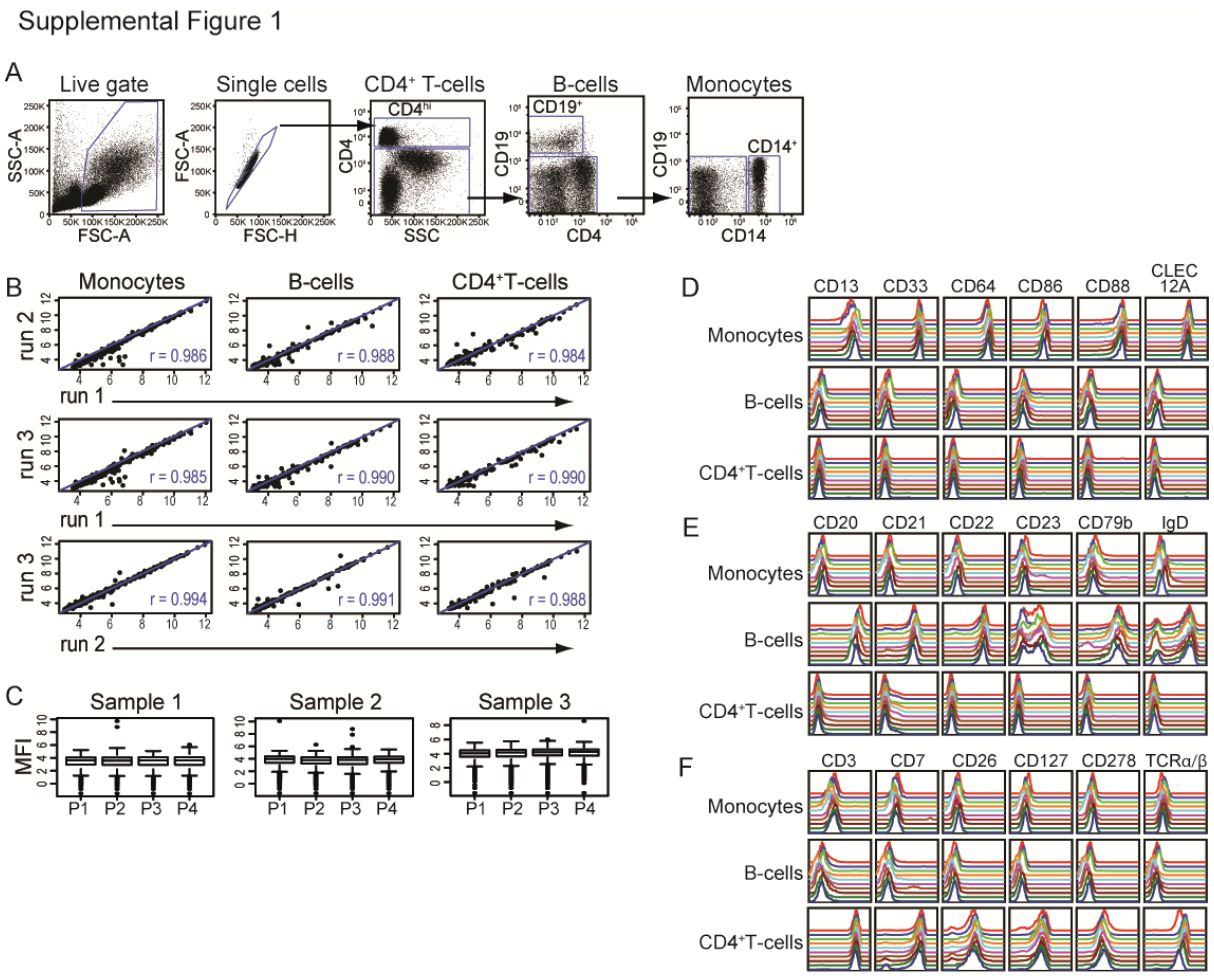
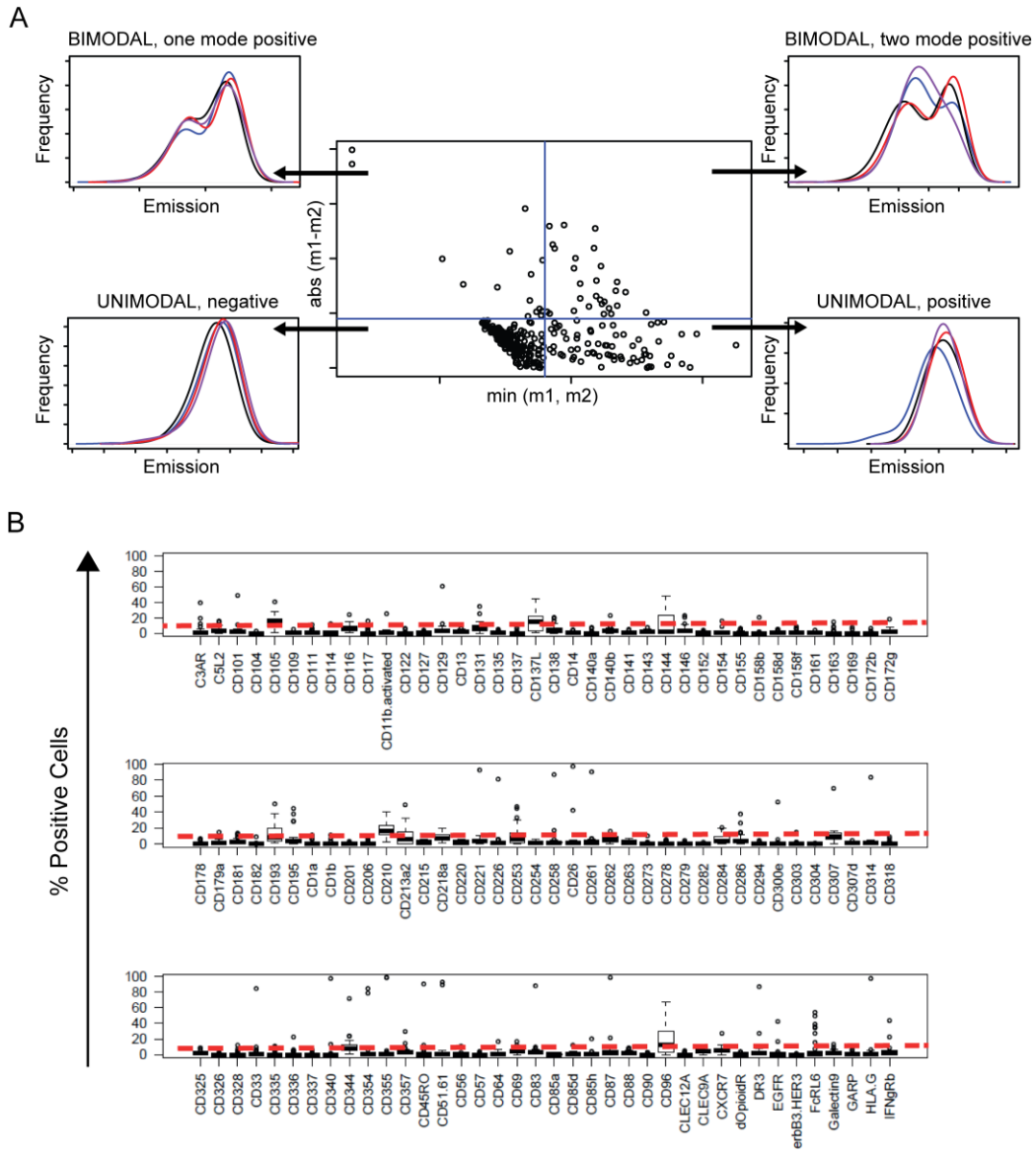


Figure 1: Validation of high-throughput flow cytometry methodology to detect immune signatures in healthy donor lymphocytes and monocytes.

(A) PBMC gating strategy identifying $CD4^+$ T-cells, B-cells and monocytes analysed by the LegendScreen™ platform (see Figure 1A).

Analysis of inter and intra-assay stability: **(B)** $10x10^7$ PBMCs from one healthy donor (HC) were isolated and frozen in liquid nitrogen. $30x10^6$ PBMCs were thawed on three separate days and analysed using LegendScreen. Expression levels of the 332 markers were assessed on each cell population on Day 1, Day 2 and Day 3 (runs 1-3) and the results were correlated using Spearman's correlation coefficients (r). **(C)** Intra-assay stability was measured by assessing mean fluorescence emissions from each of the 4 plates of the platform (P1-P4). Box plots, Mean ±SD, One-way ANOVA. **(D-F)** The specificity of the antibodies used for monocytes (D), $CD19^+$ B-cells (E) and $CD4^+$ T-cells (F) were validated using a panel of markers widely reported to be specific for each cell population.

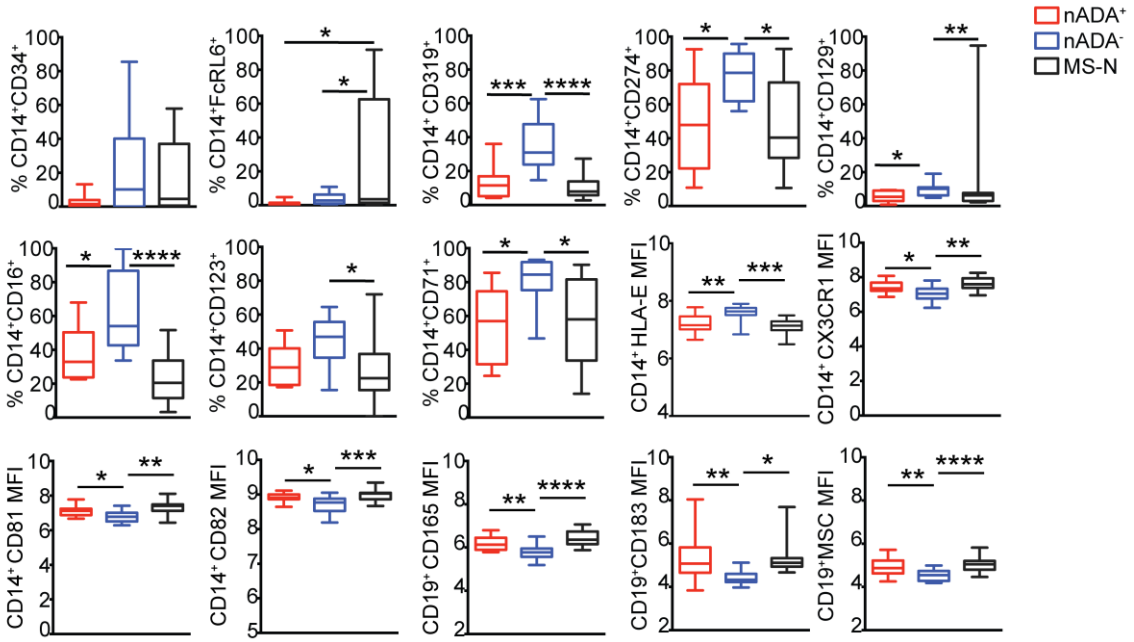
Supplemental Figure 2



Supplemental Figure 2: Statistical analysis strategy to improve the power of the cell surface marker analyses.

(A) Since many markers were negative in all samples tested (Figure 1C) to increase the statistical power of the analysis a mixture model with two components was fitted on to each of the 332 markers analyzed followed by 4 class clustering for each cell subpopulation. **(B)** Markers that clustered with known negative gold standard markers and were expressed by less than 5% of each cell subsets in at least 90% of the samples (Total sample number=55) were removed from the analysis. Refer to materials and Methods and supplemental table 5 for a comprehensive explanation.

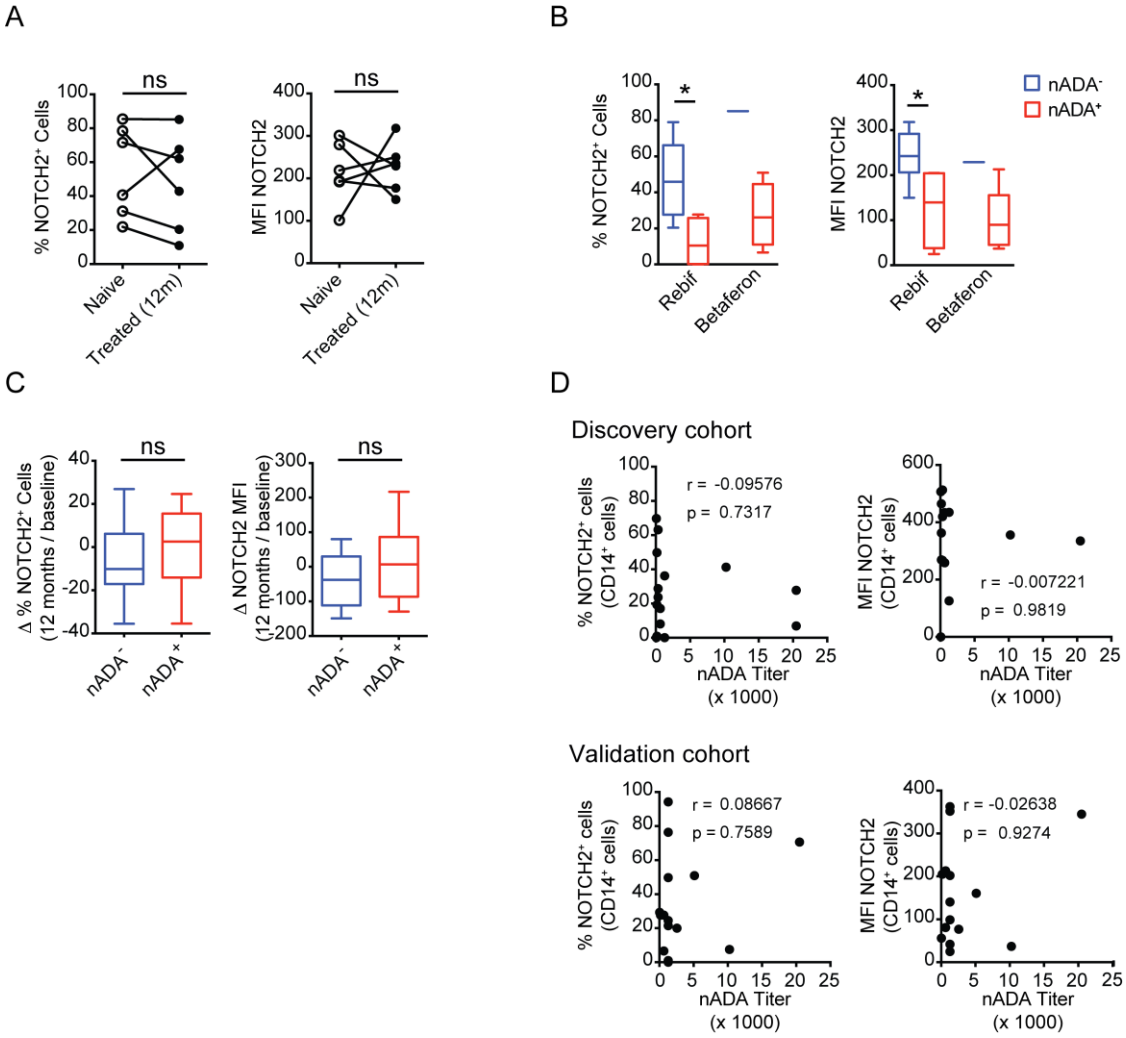
Supplemental Figure 3



Supplemental Figure 3. Differentially expressed markers (DEMs) associated with responses to IFN-β were excluded from further analysis.

Markers excluded from further analysis because their expression was affected by IFN-β. Expression of DEMs identified in Figure 2A and Supplemental Table 2 were compared in nADA$^+$ (n=11), nADA$^-$ (n=9) and MS naïve (MS-N; n=15) patients using the scheme shown in Figure 2E. DEMs found to be associated with IFN-β activity were excluded from further analysis (CD34, CDFcRL6, CD319, CD274, CD129, CD16, CD123, CD71 HLA-E on monocytes and CD165, CD183 and MSC on B-cells). ANOVA with Turkey's multiple comparison test. The box plot shows the median and 25th and 75th percentiles, and the whiskers represent minimum and maximum values.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Supplemental Figure 4

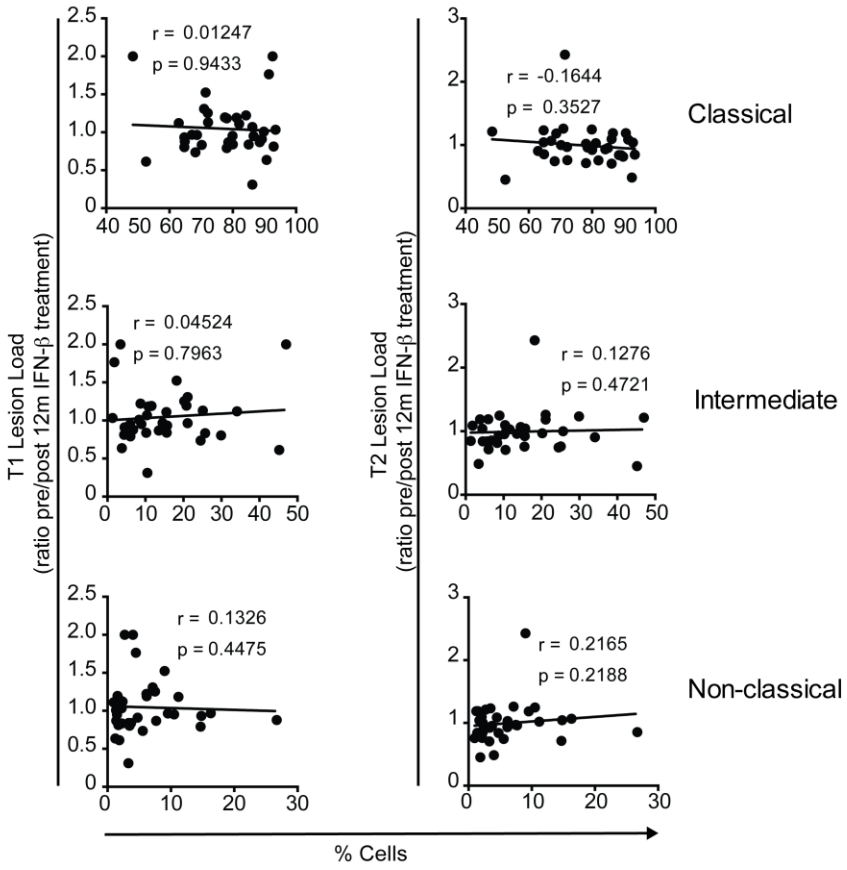


Supplemental Figure 4. Effect of IFN-β treatment, drug preparations and ADA titre on NOTCH2 expression.

A) NOTCH2 monocyte membrane expression was evaluated in 6 MS patients before (open circles) and after IFN-β treatment (full circles). Paired T test; ns = not significant. **(B)** To evaluate the effect of different IFN-β preparations on NOTCH2 expression, patients were stratified accordingly the type of IFN-β taken (mammalian derived Rebif: $nADA^{+}$n=5, $nADA^{-}$ n=8; E.coli derived Betaferon: $nADA^{+}$n=8, $nADA^{-}$ n=1). One patient ($nADA^{-}$) was treated with Avonex (mammalian derived) in our cohorts and could not be included in the figure. The same trend for NOTCH2 expression was observed in the 2 groups analysed. The box plot shows the median and 25th and 75th percentiles, and the whiskers represent minimum and maximum values. Mann Whitney test *P<0.05. **(C)** The presence of nADA did not induce changes in NOTCH2 expression. There was no difference between $nADA^{+}$ and $nADA^{-}$ patients in the delta NOTCH2 percentage positive monocytes (p=0.5282) nor in the delta NOTCH2 MFI (p=0.315). The box plot shows the median and 25th and 75th

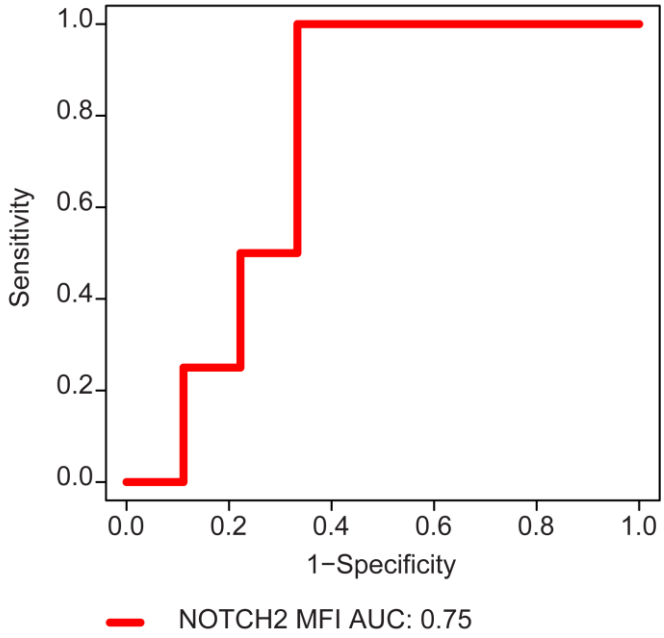
percentiles, and the whiskers represent minimum and maximum values. Mann Whitney test. **(D)** The titre of nADA in all analysed patients was correlated with the expression of NOTCH2. Spearman's correlation coefficient (r) and P value (p) are reported in figure.

Supplemental Figure 5



Supplemental Figure 5. Monocyte subsets correlation with disease progression.
The frequency of classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{low}CD16^{+}$) monocytes subsets was correlated with disease progression assessed by MRI measurements T1 and T2. Changes in brain lesions detected by comparing MRI scanning at the time of PBMC isolation to MRI scans performed 12 months before sampling. n=34. Spearman's correlation coefficient (r) and the associated P value (p) are reported in figure.

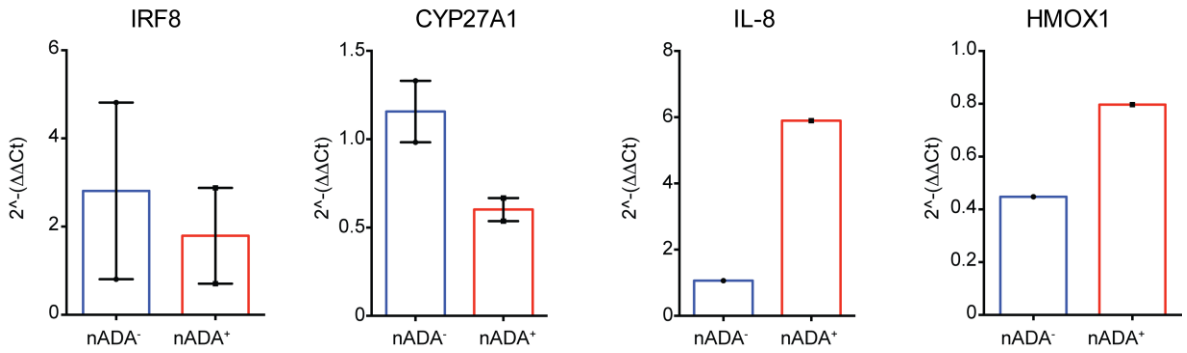
Supplemental Figure 6



Supplemental Figure 6. ROC Analysis of MS baseline samples screened with LegendScreen

We repeated the ROC curve analyses using the patients of the prospective cohort whose baseline samples had been run on a miniplate of selected markers to build the predictor (a logistic regression model) and applying the predictor on another subset of patients from the prospective cohort whose baseline samples had been run with the complete Legendscreen panel of 332 markers (patients description in Table 1 Discovery cohort). We obtained a good discriminatory ability of nADA status (AUC=0.75) with the predictor using NOTCH2 MFI, in spite of different fluorescence levels of the subsets of patients due to the different technologies used

Supplemental Figure 7



Supplemental Figure 7. Markers associated with non-classical monocyte differentiation after culture with nADA$^+$ or nADA$^-$ patient serum.

Healthy donor PBMCs were cultured with nADA$^-$ or nADA$^+$ patient serum. CD14$^+$ monocytes were isolated and the expression of markers previously reported to be differently expressed in monocyte subsets was assessed. IRF8 and CYP27A1 were previously reported to be most highly expressed in classical monocytes (2, 3); IL8 and HMOX1 are highly expressed in non-classical monocytes (2, 4).

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