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Supplementary Information for

MAPK pathway and B cells over-activation in Multiple Sclerosis: a phosphoproteomics – genomic analysis

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Other supplementary materials for this manuscript include the following:

Dataset S1

Supplementary Information Text

Supplementary Methods

Samples and processing

Unified standard operating procedures (SOPs) were defined to isolate, stimulate and lyse PBMCs, for storing and shipping samples, for conducting xMAP, genotyping and cytometry assays. In addition, a kit was produced centrally to process the samples that contained the necessary reagents and buffers. The reagents were prepared from a single batch and the plates were prepared from a single batch for each stimulus. QC checks were carried out to ensure that the reagents remained stable for 3 months.

xMAP assays

xMAP assays were performed blinded at ProtAtOnce (Athens, Greece) (technicians were unaware of the clinical characteristics and group of the samples). We optimized the assays from a list of 70 candidates based in QC checks and the signal to noise analysis and obtained a final list of 17 phosphoproteins with optimized assays: AKT1, CREB1, FAK1, GSK3A, HSPB1, IKBA, JUN, MK03, MK12, MP2K1, PTN11, STA5A, STAT1, STAT3, STAT6, TF65, WNK1 (**Table S2**). The assays were standardized to minimize the errors and be robust against freezing and storage process. We used a set of 19 stimuli that included: pro-inflammatory and pro-oxidant stimuli (Anti-CD3, ConA, H2O2, IFNG, IL1A, IL6, LPS, NaCl, PolyIC, TNFA); immunomodulatory stimuli (IFNB1a, S1P, vitD3); neuroprotectants and anti-oxidants (BDNF, EGCG, INS); and MS DMDs (DMF, FTY, Teriflunomide) (**Table S3**). Such stimuli are known to activate several pathways known being associated with MS pathogenesis (e.g. MAPK, NfKB or STAT) or trigger the activation of DMDs receptors (**Fig. 1**). The samples were collected at the baseline (time 0), and 5 and 25 min after stimulation. All the data was normalized after reading the signals.

Phosphoprotein Analysis

To normalize the phosphoproteomic datasets in order to perform protein-to-protein and condition-to-condition comparisons, the changes in phosphorylation for each protein and each patient were calculated with respect to the control conditions(1). The significance of the differential phosphorylation of each protein was used to determine the degree of correction needed to perform the analysis. Since the phosphorylation of different proteins was tested in several conditions (after stimulation and in control medium), the phosphorylation of each protein in response to stimulation was defined as the log2 of the response to the stimulus relative to the response to the medium. These values were normally distributed, allowing a T-test to be used for group comparisons.

Cytometry

Whole blood was collected in sodium-heparin tubes, lysed immediately and fixed with the BD Lys/Fix solution, and processed according to the manufacturer's instructions. Washed cells were permeabilized with ice cold 95% methanol at room temperature for 10 min and stored at -80 °C. Two hours after collection of the last sample, all the patient's samples were washed three times and stained with the antibodies (**Table S6**). All antibodies were titrated for optimal separation and staining, and the cells for each staining cocktail were obtained from an average of 200 µl whole blood. Samples were run on a BD Canto apparatus, and the BD Cytometry Setup and Tracking beads were used for standardization of the application, while BD compensation beads were used for electronic compensation. Compensation was evaluated by single and dual staining for optimal compensation parameters. Four subtypes of immune cells were identified and gated: $CD4^+$ cells (CD4); $CD8^+$ cells (CD8); B cells (CD19); and monocytes (CD33). An average of about 40,000 events were collected on lymphocyte gate.

Genotyping

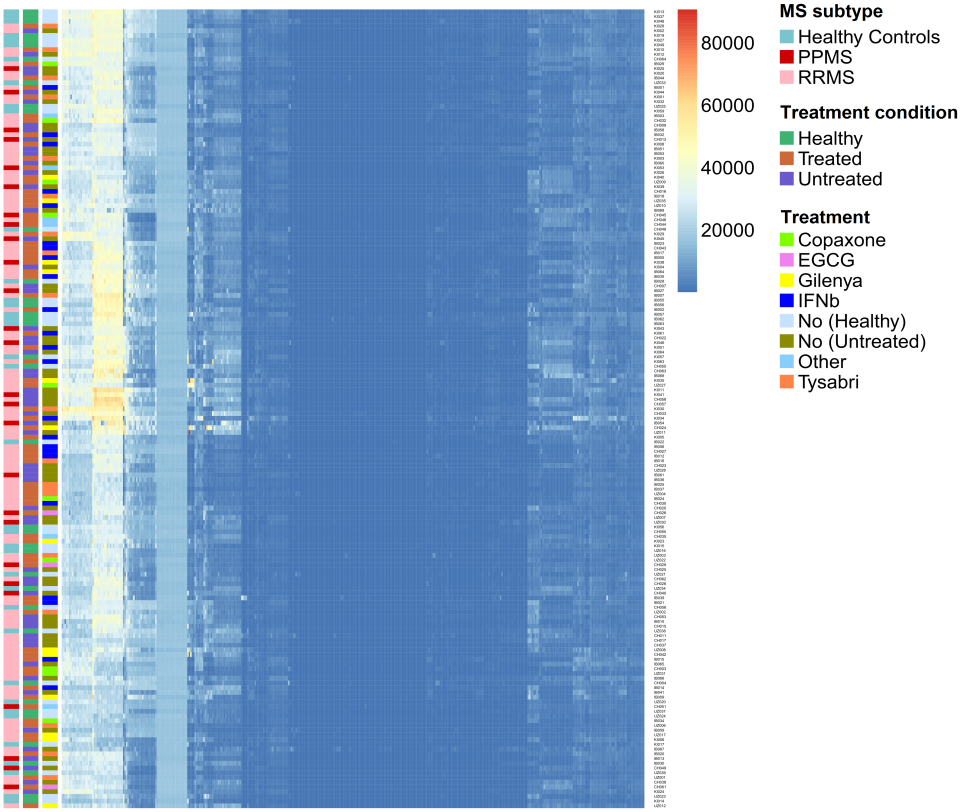
Genotyping was performed on DNA samples collected from the subjects, assessing SNPs previously validated as associated with MS(2). The final list includes 112 SNPs, including 1 SNP associated with HLA-DRB1*1501 (**Table S4**). The SNPs associated with MS were analyzed using a Sequenom MassArray system at the Spanish Center for Genotyping (http://www.usc.es/cegen).

Statistical and bioinformatic Analysis

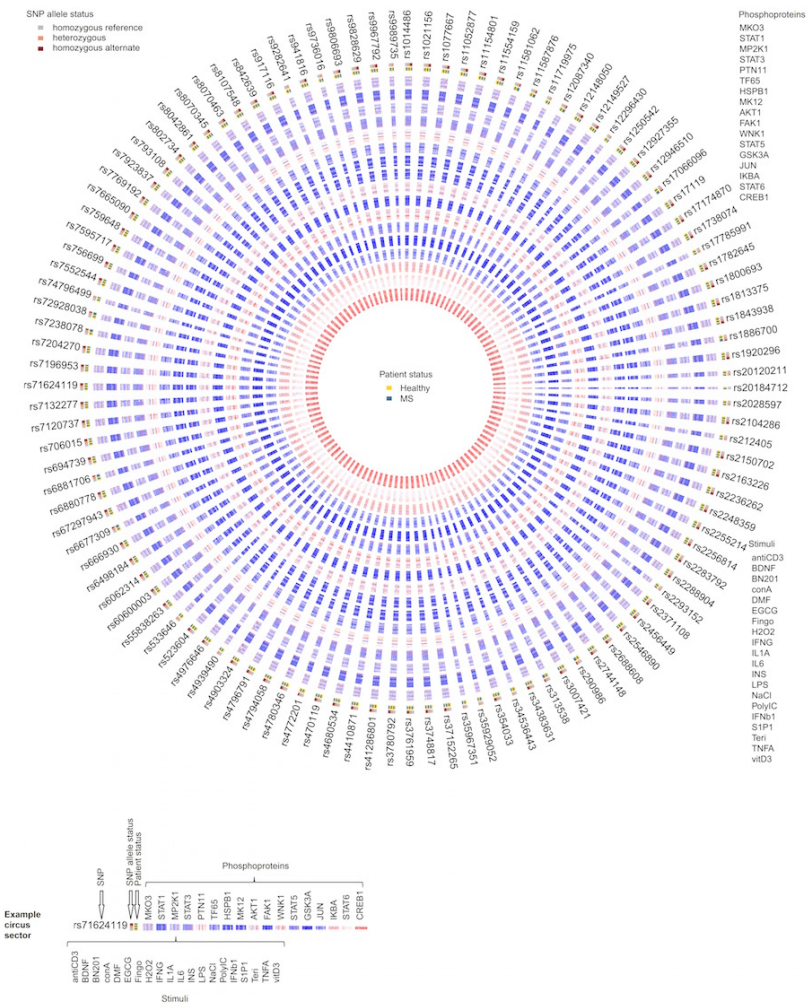
In order to test for significant differences between patient subgroups, we performed different types of statistical testing applied to both the normalized basaline phosphorylation as well as the responses to stimulation. First, we compared pairs of groups using a Wilcoxon test (for normalized phosphorylation at baseline) or a T-test (for responses to stimuli) using R software. We then tested if the genotype might affect the differences in phosphorylation between the distinct subgroups using two-way ANOVA, and with each SNP as a first independent factor and the patient group as a second factor. Patient subgroups and SNPs that were found to be significant using ANOVA were analyzed in detail by performing pairwise comparisons between patient groups for each SNP. The false discovery rate was always corrected using the Benjamini-Hochberg procedure (reported as significant when FDR <0.05).

Protein network analysis was conducted using publicly available igraph and iRefR R packages to access iRefIndex physical interactions(3) as well as MetaCore/Metabase network (https://clarivate.com/products/metacore) to access additional types of interactions. We utilized TieDie algorithm(4) implemented in the Computational Biology Methods for Drug Discovery (CBDD) R package (https://cbdd.clarivate.com/cbdd/about) in order to find potentially causative directed pathway connecting SNPs with corresponding affected phosphoproteins. We used iRefIndex consolidated database of molecular interactions(3) and the MetaBase/MetaCore to get physical interactions connecting SNP-related genes of interest with the corresponding kinases. We also complemented these findings with potentially SNP-disturbed mechanisms of transcriptional regulation. To do that we have exploited the

MetaBase/MetaCore network, and TieDie algorithm that searches interconnecting genes on a background network using a diffusion strategy



Supplementary figure S1. Phosphoprotein levels in healthy controls and in patients with MS. Heat map of the raw phosphorylation levels (after normalization) annotated for different disease subtypes and treatments. Imputation of the missing values using knn: k=10 for each cpp 10 nearest patients/samples.



Supplementary figure S2. Allelic distribution of MS susceptibility SNPs in MS patients and controls, and their association with the phosphoprotein levels. For each SNP we show 6 sub-sectors: 3 for each allele variant for the two groups (MS and HC). The heat map shows the level of one of the 17 phosphoproteins in response to the 19 different stimuli for each radius. Each sector related to a given SNP at a specific radius could be considered as a separate heat map of the phosphorylation response of the selected kinase stratified by genetic variance at the position of this SNP. Circular tracks from out to in: 1) SNP name; 2) allele status of the SNP: reference homozygous (grey), heterozygous (orange), alternative homozygous (red); 3) disease status, MS (blue) or HC (yellow); 4) the combination of allele status and disease status defines the number of rows in the heat map for each specific phosphoprotein; 5) heat maps of the response of the specific phosphoproteins as a function of the overall variance in the data (from out to in): MKO3, STAT1, MP2K1, STAT3, PTN11, TF65, HSPB1, MK12, AKT1, FAK1, WNK1, STAT5, GSK3A, JUN, IKBA, STAT6, CREB1. Each phosphoprotein heat map has 4-6 rows (number of allele statuses * number of disease statuses) and 20 columns, related to the stimuli: anti-CD3, BDNF, ConA, DMF, EGCG, FTY, H_2O_2, IFNG, IL1A, IL6, INS, LPS, NaCl, PolyIC, IFNb1a, S1P1, Teriflunomide, TNFA, vitD3. The color represents the phosphorylation response (log2 [phosphorylation of the selected kinase after stimulation/ phosphorylation of the selected kinase in the medium]). Heat map: blue - lower values, red - higher values averaged over the cohort with the specific allele/disease status.

Supplementary Table S1. Demographics and clinical variables of MS patients and controls. Columns indicate the subjects recruited for the study, the subjects used for the xMAP study based in phosphoproteomic data that passed QC and the subjects used for the cytometry study.

	MS			HC		
	recruited	**xMAP study**	**cytometry study**	**recruited**	**xMAP study**	**cytometry study**
n	195	132	47	60	37	22
Sex (M/F)	66/129	46/86	14/33	21/39	11/26	8/14
Age	43.1+11.3	44.0+11.0	49.2+10.4	39.9+8.5*	39.5+7.8	35.3+7.3*
Disease duration (months)	104.9+93.2	111.9+96.7	163.4+109.5	--	--	--
Age at MS onset	34.5+10.3	34.6+9.9	35.4+9.8	--	--	--
Disease subtype						
CIS	24	10	0	--	--	--
RRMS	129	96	34	--	--	--
SPMS	6	2	0	--	--	--
PPMS	36	24	13	--	--	--
EDSS	2 (0-6.0)	2.7 (0-7.0)	2 (1-7.5)	--	--	--
DMD						
IFNB	37	23	10	--	--	--
GA	18	10	0	--	--	--
NTZ	22	19	0	--	--	--
FTY	20	13	11	--	--	--
Untreated	98	58	13	--	--	--

CIS: clinically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary-progressive multiple sclerosis; PPMS: primary-progressive multiple sclerosis; EDSS: expanded disability status scale; DMD: disease modifying drug; IFNB: interferon-beta1b); GA: glatiramer acetate; NTZ: natalizumab; FTY: fingolimod.

*Control group was matched with the RRMS group.

Supplementary Table S2. List of phosphoproteins tested by xMAP.

Uniprot ID Name	Entrez-gene identifier	HGNC symbol	Uniprot Recommended Name	Uniprot Alternative Name	Gene Names	Antibody company catalog	Pathway	Biological role and Association with MS
AKT1	207	AKT1	RAC-alpha serine/threonine-protein kinase	Protein kinase B	AKT1, PKB, RAC	PAO P-AKT1-A01	PI3K/AKT/mTOR	key mediator of PI3K and mTOR signaling pathways, all involved in cell survival
CREB1	1385	CREB1	Cyclic AMP-responsive element-binding protein 1	-	CREB1	PAO: P-CREB1-A01	AP-1/MAPK	Participates in PI3K, MAPKinase pathways, promoting cell survival, neuronal activity, synaptic plasticity and expression of HLA molecules
FAK1	5747	PTK2	Focal adhesion kinase 1	Protein phosphatase 1 regulatory subunit 71	PTK2, FAK, FAK1	PAO: P-FAK1-A01	Integrin/src	key signaling mediator of integrin receptors and axon guidance as well as growth factor receptors (PI3K pathway)
GSK3A	2931	GSK3A	Glycogen synthase kinase-3 alpha	Serine/threonine-protein kinase GSK3A	GSK3A	PAO: P-GSK3A-A01	PI3K/AKT/mTOR	chemokine signaling pathway, being downstream AKT
HSPB1	3315	HSPB1	Heat shock protein beta-1	28 kDa heat shock protein	HSPB1, HSP27, HSP28	PAO: P-HSPB1-A01	p38/MAPK	Part of MAPKinase pathway, downstream p38, and promotes actin reorganization supporing cell migratio. Marked elevation in HSP27 levels during the relapse phase of MS.
IKBA	4792	NFKBIA	NF-kappa-B inhibitor alpha	I-kappa-B-alpha	NFKBIA, IKBA, MAD3, NFKBI	PAO: P-IKBA-A01	NFkB	NFkB signaling
JUN	3725	JUN	Transcription factor AP-1	Proto-oncogene c-Jun	JUN	PAO: P-JUN-A01	JNK/MAPK	JUN is part of the immediate early gene responses, being downstream JNK and mediating apoptosis

MP2K1	5604	MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1	MEK1	MAP2K1, MEK1, PRKMK1	PAO: P-MP2K1-A01	AP-1/MAPK	Mediates signaling of PDGFR pathway, promoting cell survival
PTPN11	5781	PTPN11	Tyrosine-protein phosphatase non-receptor type 11	SHP2	PTPN11, PTP2C, SHPTP2	PAO: P-PTN11-A01	AP-1/MAPK	Member of the MAPKinase pathway, involved in proliferation, differentiation, cell cycle progression, cell transmigration, NK cytotxicity and axonal guidance
STA5A	6776	STAT5A	Signal transducer and activator of transcription 5A	-	STAT5A, STAT5	PAO: P-STAT5-A01	JAK/STAT	Mediates IL-2, chemokines, NGR3-ErbB signaling and promoting cell survival. Upon IL-7 stimulation, MS patients experience stronger STAT5 activation in CD8-EM compared with HC.
STAT1	6772	STAT1	Signal transducer and activator of transcription 1-alpha/beta	Transcription factor ISGF-3 components p91/p84	STAT1	PAO: P-STAT1-A01	JAK/STAT	Mediates interferon gamma and beta signaling. Macrophages from MS patients displayed enhanced STAT1, STAT6 and NF-kB activity.
STAT3	6774	STAT3	Signal transducer and activator of transcription 3	Acute-phase response factor	STAT3, APRF	PAO: P-STAT3-A01	JAK/STAT	Mediates IL-6, IL-10, neurocytokines (LIF) signaling. STAT3 is required for IL-17 production by Th17.
STAT6	6778	STAT6	Signal transducer and activator of transcription 6	IL-4 Stat	STAT6	PAO: P-STAT6-A01	JAK/STAT	JAK-STAT pathway mediates Il-4 signaling. PBMCs from MS patients have significantly elevated constitutive phosphorylation of STAT6 compared to PBMCs from normal subjects.
TF65	5970	RELA	Transcription factor p65	Nuclear factor NF-kappa-B p65 subunit	RELA, NFKB3	PAO: P-TF65-A01	NFkB	Key member of NfKB pathway, meadiating inflammatory signals and cell survival. Macrophages from MS patients displayed enhanced STAT6, STAT1 and NF-kB activity.
WNK1	65125	WNK1	Serine/threonine-protein kinase WNK1	Erythrocyte 65 kDa protein	WNK1, HSN2, KDP, KIAA0344, PRKWNK1	PAO: P-WNK1-A01	ERK5/MAPK	EGF pathway and participate in the regulation of ion homeostasis and iron uptake

Supplementary Table S3. Stimulus used in the in vitro assays.

Stimulus	Full Name	pathway	cat number	Vendor	stock conc	units	target conc	units
antiCD3	Anti-Human CD3	TCR	16-0037-85	eBioscience	1	mg/ml	5000	ng/ml
BDNF	Brain-Derived Neurotrophic Factor human	TrkB/p75	B3795-5ug	Sigma	0.1	mg/ml	100	ng/ml
conA	Concanavalin A	TCR	C5275-5MG	Sigma	5	mg/ml	2500	ng/ml
DMF	Dimethyl fumarate	Nrf2	242926	Sigma	5	mg/ml	10000	ng/ml
EGCG	Epigallocatechin-3-gallate	anti-oxidative stress	sc-200802	Santa Cruz	10	mg/ml	45.84	ug/ml
FTY	Fingolimod	S1PR		Novartis	1	mg/ml	3000	ng/ml
H2O2	Hydrogen peroxide	oxidative stress	H3410	Sigma	330	mg/ml	17005	ng/ml
IFNB1a	Interferon beta 1a	Type I IFNR	101322	Merck	0.088	mg/ml	50	ng/ml
IFNG	Interferon gamma	Type II IFNR	I3265	Sigma	0.1	mg/ml	100	ng/ml
IL1A	Interleukin-1 alpha	IL1R pathway	200-01A	PeproTech	0.1	mg/ml	50	ng/ml
IL6	Interleukin-6	IL6R	200-06	PeproTech	0.1	mg/ml	100	ng/ml
INS	Insulin	InsulinR	I9278	Sigma	1.722	mg/ml	1722	ng/ml
LPS	Lipopolysaccharide	TLR4	L4391	Sigma	1	mg/ml	10000	ng/ml
NaCl	Sodium Chloride	PI3K	S5886	Sigma	11.7	mg/ml	2.34	mg/ml
PolyIC	Polyinosinic-Polycytidylic acid	TLR3	p0913-10mg	Sigma	10	mg/ml	10000	ng/ml
S1P	Sphingosine 1-phosphate	S1P	S9666	Sigma	0.125	mg/ml	100	ng/ml
Teriflu	Teriflunomide	pyrimidin synthesis NFkB inhibitor	A77 1726	Calbiochem	10	mg/ml	13510	ng/ml
TNFA	Tumor necrosis factor alpha	TNF	300-01A	PeproTech	0.1	mg/ml	100	ng/ml
vitD3	vitamin D3	B-catenin	C9756	Sigma	1	mg/ml	500	ng/ml

Supplementary Table S4. SNPs associated with MS tested.

rsID	chr	position	RefSeq_name	RefSeq_distance
rs371522651	5	142126999	NDFIP1	0
rs11581062	1	101407519	SLC30A7	0
rs11587876	1	85915183	DDAH1	0
rs12087340	1	85746993	BCL10	4405
rs3007421	1	6530189	PLEKHG5	0
rs35967351	1	160711804	SLAMF7	0
rs3748817	1	2525665	MMEL1	0
rs3761959	1	157669278	FCRL3	0
rs41286801	1	92975464	EVI5	0
rs55838263	1	200874728	C1orf106	0
rs666930	1	120258970	PHGDH	0
rs6677309	1	117080166	CD58	0
rs7552544	1	101240893	VCAM1	36291
rs17174870	2	112665201	MERTK	0
rs2163226	2	43361256	ZFP36L2	88283
rs7595717	2	68587477	PLEK	4843
rs842639	2	61095245	FLJ16341	0
rs9967792	2	191974435	STAT4	0
rs9989735	2	231115454	SP140	0
rs1014486	3	159691112	IL12A	15509
rs11719975	3	18785585	SATB1	305319
rs1813375	3	28078571	CMC1	204551
rs1920296	3	121543577	IQCB1	0
rs2028597	3	105558837	CBLB	0
rs2255214	3	121770539	CD86	3668
rs2371108	3	27757018	EOMES	866
rs4680534	3	159698945	IL12A	7676
rs9282641	3	121796768	CD86	0
rs9828629	3	71530346	FOXP1	0
rs313538	4	25264754	PI4K2B	0
rs7665090	4	103551603	MANBA	1038
rs2546890	5	158759900	LOC285626	0
rs4976646	5	176788570	RGS14	0
rs6880778	5	40399096	PTGER4	280934
rs6881706	5	35879156	IL7R	2232
rs71624119	5	55440730	ANKRD55	0
rs756699	5	133446575	TCF7	3825
rs11154801	6	135739355	AHI1	0
rs17066096	6	137452908	IL22RA2	12047

rs17119	6	14719496	JARID2	527029
rs1738074	6	159465977	TAGAP	0
rs212405	6	159470559	TAGAP	4374
rs67297943	6	138244816	TNFAIP3	40366
rs72928038	6	90976768	BACH2	0
rs7769192	6	137962655	OLIG3	147123
rs802734	6	128278798	PTPRK	11124
rs941816	6	36375304	PXT1	0
rs1843938	7	3113034	CARD11	29524
rs201847125	7	50325567	IKZF1	18809
rs354033	7	149289464	ZNF767	0
rs60600003	7	37382465	ELMO1	0
rs706015	7	27014988	SKAP2	110646
rs917116	7	28172739	JAZF1	0
rs1021156	8	79575804	FAM164A	2476
rs2456449	8	128192981	POU5F1B	234874
rs4410871	8	128815029	PVT1	0
rs759648	8	129158945	MIR1208	3415
rs2150702	9	5893861	MLANA	0
rs290986	9	93563536	SYK	474
rs3780792	9	136835343	VAV2	0
rs1250542	10	81034670	ZMIZ1	0
rs1782645	10	81048611	ZMIZ1	0
rs2104286	10	6099045	IL2RA	0
rs2688608	10	75658349	C10orf55	11376
rs7923837	10	94481917	HHEX	26508
rs793108	10	31415106	ZNF438	94239
rs34383631	11	60793330	CD6	5481
rs4939490	11	60793651	CD6	5802
rs523604	11	118755738	CXCR5	0
rs533646	11	118566746	TREH	16364
rs694739	11	64097233	PRDX5	7937
rs7120737	11	47702395	AGBL2	0
rs9736016	11	118724894	CXCR5	29645
rs11052877	12	9905690	CD69	0
rs12296430	12	6503500	LTBR	2767
rs1800693	12	6440009	TNFRSF1A	0
rs201202118	12	58182062	TSFM	0
rs7132277	12	123593382	PITPNM2	0
rs4772201	13	100086259	MIR548AN	27704
rs12148050	14	103263788	TRAF3	0

rs2236262	14	69261472	ZFP36L1	1686
rs4903324	14	75961511	JDP2	22106
rs74796499	14	88432328	GALC	0
rs8042861	15	90977333	IQGAP1	0
rs9806693	15	79184387	MORF4L1	0
rs12149527	16	79110596	WWOX	0
rs12927355	16	11194771	CLEC16A	0
rs1886700	16	68685905	CDH3	0
rs2744148	16	1073552	SOX8	36572
rs35929052	16	85994484	IRF8	38272
rs4780346	16	11288806	CLEC16A	12759
rs6498184	16	11435990	RMI2	3319
rs7196953	16	79649394	MAF	14771
rs7204270	16	30156963	MAPK3	22332
rs12946510	17	37912377	IKZF3	8820
rs2293152	17	40481529	STAT3	0
rs4794058	17	45597098	NPEPPS	11344
rs4796791	17	40530763	STAT3	0
rs8070345	17	57816757	VMP1	0
rs8070463	17	45768836	TBKBP1	3792
rs7238078	18	56384192	MALT1	0
rs1077667	19	6668972	TNFSF14	0
rs11554159	19	18285944	IFI30	0
rs2288904	19	10742170	SLC44A2	0
rs34536443	19	10463118	TYK2	0
rs8107548	19	49870643	DKKL1	0
rs17785991	20	48438761	SLC9A8	0
rs2248359	20	52791518	CYP24A1	1001
rs2256814	20	62373983	SLC2A4RG	0
rs6062314	20	62409713	ZBTB46	0
rs2283792	22	22131125	MAPK1	0
rs470119	22	50966914	TYMP	0

Supplementary Table S5. MKO3, GSK3A, JUN and STAT3 phosphorylation levels at baseline

Compared Groups	CPP	Mean.Group 1	Mean.Group 2	Wilcoxon.pval	Adj.Wilc.pval
Healthy_PPMS	medium_STAT3	895.7162162	752.0096154	0.006569561	0.062410833
Healthy_MS	medium_MP2K1	2729.918919	3256.140152	0.00343999	0.065359817
Healthy_RRMS	**medium_MP2K1**	**2729.918919**	**3378.974057**	**0.001578621**	**0.029993804**
Healthy_Untreated MS	medium_MP2K1	2729.918919	3219.047414	0.018548558	0.199119244
Healthy_PPMS	medium_JUN	748.0743243	628.9423077	0.040138118	0.254208078
Healthy_MS	medium_GSK3A	390.3581081	415.592803	0.020288343	0.192739262
Healthy_PPMS	medium_GSK3A	390.3581081	436.4326923	0.002876038	0.054644714
Healthy_Untreated MS	medium_GSK3A	390.3581081	425.3706897	0.02095992	0.199119244

Supplementary Table S6. Differential phosphorylation and associated with SNPs in PBMCs from MS patients compared to controls. Table shows significantly different phosphorylated proteins between MS patients and controls, based in the stimulus for the in vitro assays and the associated MS susceptibility SNPs (2-way ANOVA with Benjamin correction for multiple testing).

Phospho	Stimulus	SNP	P-value	Adjusted P-value
JUN	BDNF	rs74796499	1.11E-06	0.000129158
	PolyIC	rs74796499	0.001141	0.027100989
	DMF	rs666930	0.000102	0.038705172
	INS	rs11554159	0.000113	0.04305268
	medium	rs7238078	0.00054	0.010257557
AKT1	BDNF	rs74796499	1.36E-06	0.000129158
	IFNB1a	rs35929052	0.000122	0.016999819
FAK1	BDNF	rs74796499	3.89E-06	0.000246682
	conA	rs74796499	0.00095	0.024074738
	IL1A	rs74796499	0.000798	0.021671934
	PolyIC	rs74796499	0.000138	0.004871523
	INS	rs12087340	0.000309	0.029377875
	INS	rs793108	7.60E-05	0.028871647
GSK3A	BDNF	rs74796499	6.50E-08	2.47E-05
	conA	rs74796499	6.02E-05	0.002857279
	IL1A	rs74796499	0.002312	0.048802245
	PolyIC	rs74796499	2.46E-06	0.000187152
	Teriflu	rs7552544	0.000109	0.0413861
MKO3	medium	rs666930	0.000201	0.003819853
MP2K1	IFNB1a	rs2456449	2.09E-05	0.007948691
	medium	rs666930	0.001424	0.013524132
	vitD3	rs35929052	5.38E-05	0.016999819
STAT1	BDNF	rs74796499	5.00E-07	9.50E-05
	PolyIC	rs74796499	1.34E-05	0.000730121
	INS	rs12087340	0.00015	0.028588328
STAT3	BDNF	rs12087340	0.000283	0.029377875
STAT5	BDNF	rs74796499	0.000116	0.004871523
	DMF	rs1250542	5.33E-05	0.020245562
	DMF	rs4680534	8.49E-05	0.032274089
	medium	rs666930	0.007292	0.034637287
STAT6	medium	rs666930	0.009487	0.036049416
TF65	IFNB1a	rs12087340	0.000578	0.043897593
	medium	rs3007421	0.002425	0.04607475
WNK1	medium	rs666930	0.003471	0.021985849
	vitD3	rs35929052	0.000134	0.016999819

BDNF: brain derived nerve factor; conA: concanavalin A; DMF: Dymethyl-fumarate; IFNB1a: interferon-beta; IL1A: interleukin 1A; INS: insulin; PolyIC: Polyinosinic:polycytidylic acid; vitD3: vitamin D3; Teriflu: Teriflunomide

Table S7. Differential kinase phosphorylation in fingolimod-treated and untreated MS patients. A) The table shows differentially phosphorylated targets between FTY-treated and untreated RRMS patients (Benjamin correction for multiple testing). B) Differential phosphorylation in PBMCs from RRMS patients treated with FTY or untreated, adjusted by MS susceptibility SNPs. Unstimulated phosphoprotein levels were compared using a Wilcoxon test, whereas the after stimulation phosphoproteins were normalized and compared using a T-test (mean ± SD values).

A	**Stimulus**	**Phosphorylation levels**		**p**	**Adjusted p**
		FTY	**Untreated RRMS**		
MKO3	ConA	2.84 ± 0.66	1.74 ± 1.07	3E-04	0.029
PTN11	IL6	0.93 ± 0.49	1.67 ± 0.78	9E-05	0.017
	IFNB1a	0.20 ± 0.19	0.45 ± 0.22	2E-04	0.029
STAT1	IL6	0.29 ± 0.56	1.12 ± 0.69	5E-05	0.017

B	**Stimulus**	**SNP**	**Allele**	**Phosphorylation levels**		**p**	**Adjusted p**
				FTY	**untreated RRMS**		
CREB1	IL1A	rs6498184	CT	0.5792 (0.0543)	0.2033 (0.1830)	2.70E-05	0.0032
IKBA	antiCD3	rs11581062	AG	-0.1404 (0.0617)	0.1127 (0.1352)	0.00017	0.0165
TF65	PolyIC	rs2293152	GG	1.9980 (0.2358)	0.7934 (0.6810)	0.00027	0.0330

Table S8. Differential phosphorylation in PBMCs from Natalizumab-treated and untreated MS patients. Differentially phosphorylated targets in NTZ-treated and untreated RRMS patients (Benjamin correction for multiple testing). The unstimulated phosphoprotein levels were compared using a Wilcoxon test (median ± IQR), whereas phosphoprotein levels after stimulation were normalized and compared using a T-test (mean ± SD).

	Stimulus	**Phosphorylation levels**		**p**	**Adjusted p**
		NTZ	**untreated RRMS**		
MP2K1	LPS	1.37 ± 0.49	1.88 ± 0.68	2E-04	0.043
STAT3	INS	0.08 ± 0.14	0.24 ± 0.26	4E-04	0.049
STAT5	IFNB1a	1.53 ± 0.512	0.98 ± 0.44	1E-04	0.043
STAT6	baseline	1940 ± 806	3058 ± 3059	0.002	0.038

Supplementary Table S9. Antibodies used for flow cytometry assays.

Antibody	Company	Catalog	dilution (µl in 100µl)
CD4	B&D	560649	2.5
CD8	B&D	555369	10
CD33	B&D	345799	5
CD19	B&D	332780	5
pCREB1	B&D	558435	20
pHSPB1	Cell signaling	12172	2
pIKBA	B&D	560817	5
pMK03	B&D	612592	20
pMK12	B&D	612594	20
pSTAT1	B&D	612596	20
pSTAT3	B&D	557814	20
pSTAT5	B&D	612598	20
pWNK1	B&D	558421	20

Supplementary Table S10. Phosphoproteins assessed by flow cytometry. Table indicates patient's subgroup, phosphoprotein and stimuli used in the ex vivo assays.

Group	Kinase	Stimuli
HC	STAT3	IFNG, IFNB1a, IL6
	CREB1	PolyIC, FTY, DMF, INS
	HSPB1	TNFa, IL6, BDNF
	MK12	Anti-CD3, TNFA, BDNF, IFNB1a
	WNK1	INS, IL1, BDNF, LPS
	STAT5	DMF, IFNB1a, polyC, ConA
MS untreated	STAT3	IFNG, IFNB1a, IL6
	CREB1	PolyIC, BN201, FTY, DMF
	HSPB1	NaCl
	MK03	TNFa
RRMS untreated	STAT1	ECGC, anti-CD3
	IKBA	BDNF, INS polyIC
	CREB1	INS
	HSPB1	TNFa, IL6, BDNF
	MK12	Anti-CD3, TNFA, BDNF, IFNB1a
PPMS	STAT1	ECGC, antiCD3
	IKBA	BDNF, INS, polyIC
	STAT3	IFNG, IFNB1a, IL6
	WNK1	INS, IL1, BDNF, LPS
	STAT5	DMF, IFNB1a, polyIC, ConA
FTY treated	HSPB1	NaCl
IFNB treated	MK03	TNFa

Table S11. Differential kinase phosphorylation in PBMCs from MS patients and controls assessed by flow cytometry. The table shows the targets phosphorylated in MS patients relative to the healthy controls, indicating the stimuli used in the in vitro assays and the immune cell subpopulation (Benjamin correction for multiple tests). Unstimulated (baseline) phosphoprotein levels were compared using a Wilcoxon test (median ± IQR values), whereas the phosphoprotein levels after stimulation were normalized and compared using a T-test (mean ± SD).

		Stimulus	Cell type	Kinase phosphorylation		P	Adjusted p
				HC	MS		
HC vs MS	HSPB1	BDNF	$CD33^+$	-0.0802 ± 0.1299	0.2117 ±0.1909	0.0224	0.044
	STAT3	baseline	$CD19^+$	495 ± 103	561 ±101	0.0003	0.0119
HC vs PPMS	STAT1	baseline	$CD19^+$	459 ± 50	530 ± 54.5	0.0059	0.0436
	STAT3	baseline	$CD19^+$	495 ± 103	561 ± 55.2	0.0035	0.0436
	TF65	baseline	$CD19^+$	406 ± 41	476.5 ± 84.5	0.0044	0.0436
				FTY	Untreated MS		
FTY vs Untreated RRMS	HSPB1	NaCl	$CD19^+$	0.3155 ± 0.5195	-0.1422 ± 0.2014	0.0291	0.0291

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