

Toll-like receptor stimulation in splenic marginal zone lymphoma can modulate cell signaling, activation and proliferation

Eleonora Fonte,^{1,2} Andreas Agathangelidis,¹ Daniele Reverberi,³ Stavroula Ntoufa,⁴ Lydia Scarfò,^{1,5,6} Pamela Ranghetti,¹ Giovanna Cutrona,³ Alessandra Tedeschi,⁷ Alikì Xochelli,⁴ Federico Caligaris-Cappio,^{1,5,6} Maurilio Ponzoni,⁵ Chrysoula Belessi,⁸ Zadié Davis,⁹ Miguel A. Piris,¹⁰ David Oscier,⁹ Paolo Ghia,^{1,5,6} Kostas Stamatopoulos^{4,11} and Marta Muzio¹

¹Division of Experimental Oncology, IRCCS San Raffaele Scientific Institute, Milano, Italy; ²Università degli studi di Pavia, Italy; ³UOC Patologia Molecolare, IRCCS AOU S. Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy; ⁴Institute of Applied Biosciences, Centre For Research and Technology Hellas, Thessaloniki, Greece; ⁵Department of Onco-Hematology, San Raffaele Hospital, Milano, Italy; ⁶Università Vita-Salute San Raffaele, Milano, Italy; ⁷Ospedale Niguarda, Milano, Italy; ⁸Hematology Department, Nikea General Hospital, Athens, Greece; ⁹Department of Haematology, Royal Bournemouth Hospital, UK; ¹⁰Hospital Universitario Marques de Valdecilla and Instituto de Formación e Investigación Marqués de Valdecilla, Santander, Spain; and ¹¹Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece

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Correspondence: ghia.paolo@hsr.it

Supplemental Methods

Tissue samples

Fifty-one samples from patients with SMZL from collaborating institutions in Italy, Greece and the United Kingdom were included in this study (**Supplementary Table 1**), all meeting established diagnostic criteria ¹. Splenic lymphoma/leukemia unclassifiable (SLLU) samples were precluded from the study either by splenic histology or by omission of cases with morphological features of SLLU. All patients had lymphocytosis and were either untreated or off therapy for at least 6 months prior to sampling. Peripheral blood and spleen samples were obtained after informed consent with the approval of the local Institutional Ethics Committees.

Buffy coats from healthy donors and spleen samples were used to isolate normal B lymphocytes.

Cell culture

Purified cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Bovine Serum, 2 mM L-glutamine and 15 µg/ml gentamicin at a concentration of 3×10^6 cells/ml. Cells were either left unstimulated or stimulated with TLR ligands. Briefly, Pam3CysSerLys4 (Pam3CSK4; Invivogen), a synthetic tripalmitoylated lipopeptide specific for TLR1/TLR2 heterodimer, was used at the concentration of 1 µg/ml. Macrophage-activating Lipopeptide-2 (MALP-2; Axxora Platform), a specific ligand for TLR2/TLR6 heterodimer, was used at the concentration of 0,2 µg/ml. CpG oligonucleotide type B-Human (ODN 2006; Invivogen), a specific ligand for TLR9, was used at the concentration of 2,5 µg/ml.

Statistical analysis

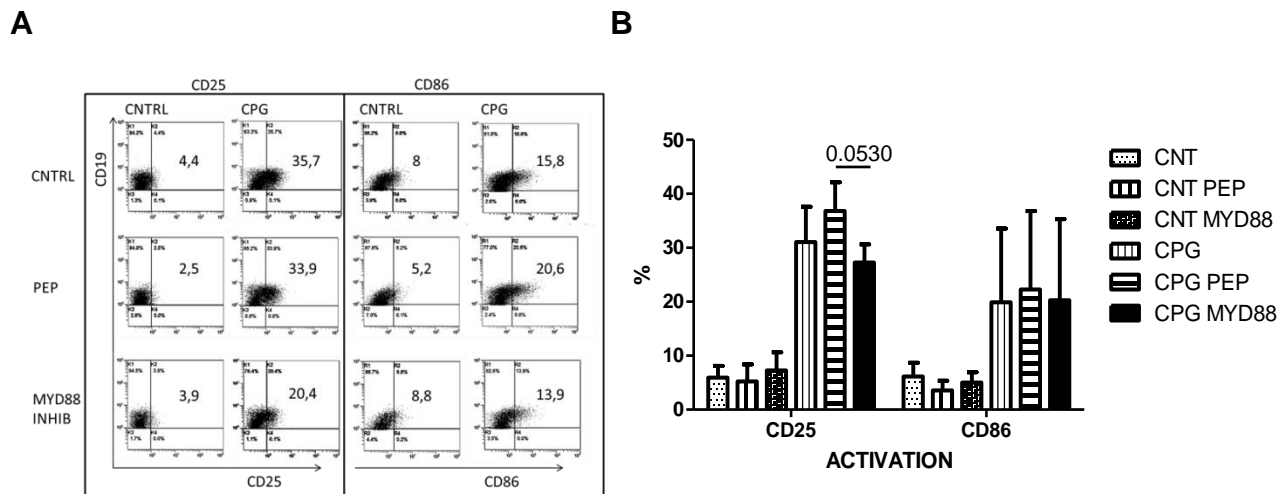
Data were analyzed with a Wilcoxon matched pairs test or Mann Whitney test (GraphPad Prism 5.01 software). The statistical analysis of the RT-PCR array results was performed according to the manufacturer's instructions, as previously reported ². All p values were two-sided and regarded as statistically significant if <0.05 .

References

1. Matutes E, Oscier D, Montalban C, et al. Splenic marginal zone lymphoma proposals for a revision of diagnostic, staging and therapeutic criteria. *Leukemia*. 2008;22(3):487-495
2. Arvaniti E, Ntoufa S, Papakonstantinou N, et al. Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenic significance in specific subsets of patients. *Haematologica*. 2011;96(11):1644-1652.

Supplementary Figure 1

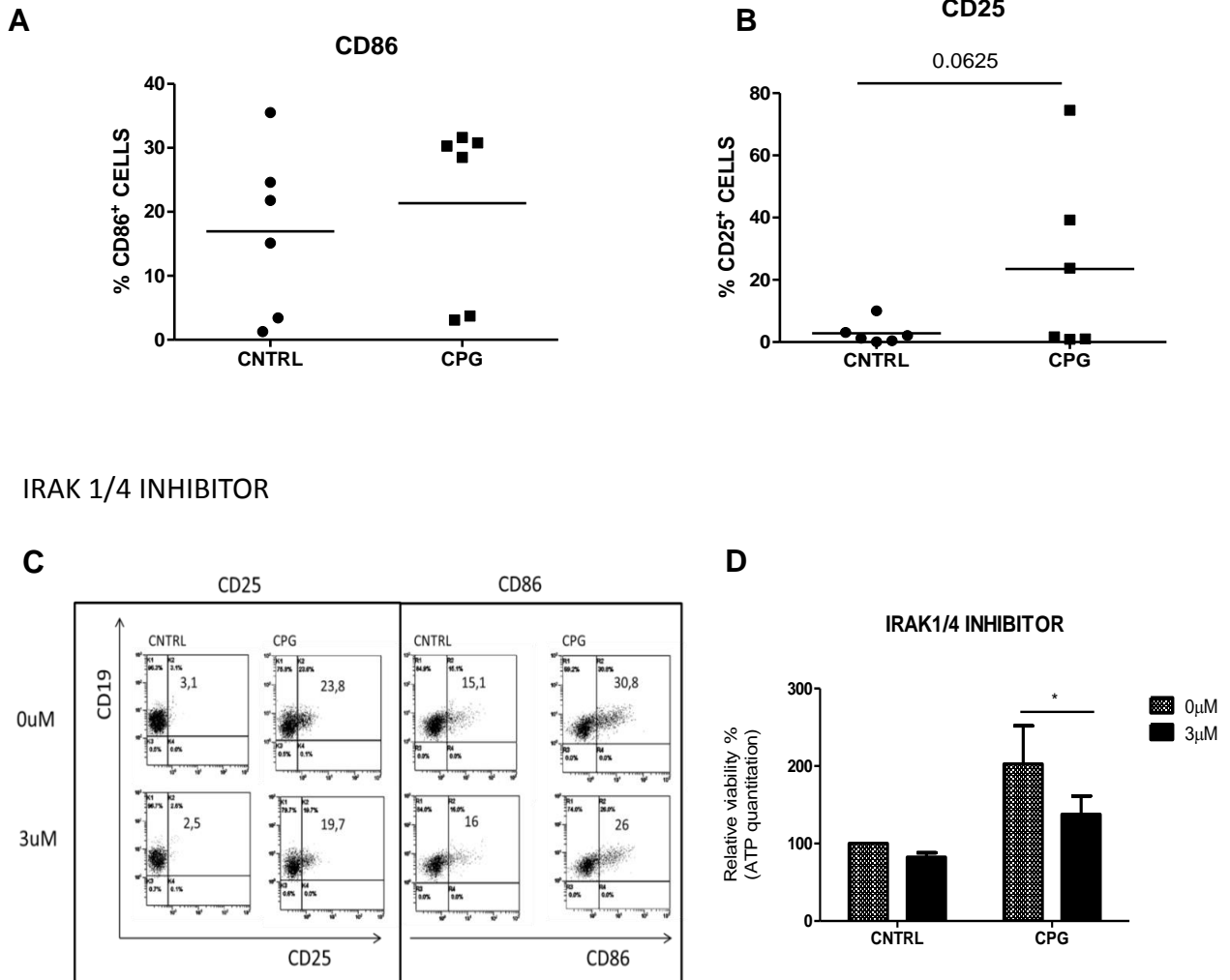
PERIPHERAL BLOOD SAMPLES



Supplementary Figure 1. MyD88 inhibitor reduced the expression of the CD25 and CD86 markers induced by TLR stimulation in peripheral blood SMZL cells. (A) SMZL cells from one representative sample were treated with 100µM of Myd88 inhibitor (MYD88) or with a control peptide (PEP) for 30 minutes and then stimulated with TLR ligands for 24h. CD25 and CD86 markers were then analyzed by flow cytometry. (B) Statistical analysis was performed on 4 samples and revealed a trend (p value 0,053) of CD25 downregulation upon Myd88 inhibition.

Supplementary Figure 2

SPLEEN SAMPLES



Supplementary figure 2. TLR-induced activation of CD25 marker in SMZL spleen samples. (A-B) SMZL cells from 6 spleen samples were stimulated with CpG (TLR9 ligand) for 24h and analyzed by flow cytometry. Only CD19⁺/7AAD⁻ cells were taken into account. (C) SMZL cells were also treated with 3µM of IRAK inhibitor for 30 minutes and then stimulated with TLR ligands for 24h. CD25 and CD86 markers were then analyzed by flow cytometry and (D) cell viability was measured by ATP concentration. Data were normalized with each control (unstimulated untreated cells were considered as 100%). * indicates a p value <0,05.

Supplemental Table 1

Clinical and biological parameters of SMZL patients

N°	SAMPLE ORIGIN	% CD19 ⁺ CELLS in the sample for analysis	IGHV GENE	IGHV IDENTITY (%)	CD25 ⁺ cells (CPG-CN)	CD86 ⁺ cells (CPG-CN)	MYD88 MUTATION
1	GREECE	PURIFIED	nd	nd	40,3	55,2	NOT TESTED
2	GREECE	PURIFIED	IGHV3-21	100	-0,9	-0,7	NEGATIVE
3	GREECE	PURIFIED	IGHV4-34	100	-0,2	0,1	NEGATIVE
4	GREECE	PURIFIED	IGHV3-7	95,8	1,3	23,5	L265P mutation
5	GREECE	PURIFIED	IGHV3-23	97,6	2,3	0,2	NEGATIVE
6	GREECE	PURIFIED	IGHV5-51	97	0,8	20,8	NEGATIVE
7	GREECE	PURIFIED	IGHV3-30-3	94	11,7	9,8	L265P mutation
8	ITALY	PURIFIED	IGHV4-4*02	98,11	-4,2	-0,3	0 Haloplex
9	ITALY	PURIFIED	IGHV5-51*03	97,92	20,7	25,6	0 Haloplex
10	ITALY	PURIFIED	IGHV1-18	87,7	3,6	28,7	NEGATIVE
11	ITALY	PURIFIED	IGHV3-7	98,9	1,2	2,2	NEGATIVE
12	ITALY	40%	IGHV3-23*01	90,62	0,5	-1	NEGATIVE
13	ITALY	PURIFIED	IGHV4-59*01	100	11,2	25,4	0 Haloplex
14	ITALY	PURIFIED	IGHV3-23*01	96,88	0,7	0,6	0 Haloplex
15	ITALY	PURIFIED	IGHV3-23	9,4	28,5	0,2	NEGATIVE
16	ITALY	PURIFIED	IGHV3-73	92,3	1,7	33,2	NEGATIVE
17	ITALY	PURIFIED	IGHV3-74*02	94,37	0,2	80,4	NEGATIVE
18	ITALY	PURIFIED	IGHV3-7*01	93,06	59,5	63,7	1 Haloplex
19	UK	85	IGHV4-34	94,1	20,3	1	0 (MLPA) T:T genotyping
20	UK	92,1	IGHV3-23	94	53,2	67,7	T:T genotyping
21	UK	75	IGHV4-34	100	-0,7	0,1	T:T genotyping
22	UK	73	IGHV3-7	98,2	11,8	1,8	0 (MLPA) T:T genotyping
23	UK	89,3	IGHV3-72	89,7	38,9	51,2	0 (MLPA) T:T genotyping
24	UK	83,5	IGHV3-74	98,3	2,7	-0,2	T:T genotyping
25	UK	89,4	IGHV5-51	96,6	-0,5	-0,1	T:T genotyping
26	UK	92,2	IGHV3-64	92,3	17,2	29,5	T:T genotyping
27	UK	67,2	IGHV4-59	97,5	3,9	21,3	NOT TESTED
28	UK	74,8	IGHV5-51	89,7	26,8	8,4	NOT TESTED
29	UK	69,5	IGHV1-2	97,8	5,8	15,1	NOT TESTED
30	UK	51	IGHV4-30	92	-10,1	6,7	NOT TESTED
31*	UK	65,7	IGHV3-30	95,49	48,7	31,4	haloplex MYD88 positive
32	GREECE	86	IGHV6-1	94,06	-0,2	0,8	NOT TESTED
33	GREECE	87	IGHV3-23	100	0,1	1,4	NEGATIVE
34	GREECE	75	IGHV3-23	91	-1	-0,6	NEGATIVE
35	GREECE	91	IGHV3-30	94,7	2,9	2,1	NEGATIVE
36	GREECE	75	IGHV4-34	100	8,7	0	NEGATIVE
37	ITALY	PURIFIED	IGHV3-30-3*01	95,47	3,2	12,9	0 Haloplex
38	ITALY	PURIFIED	IGHV4-34*01	98,95	16,56	20,68	0 Haloplex
39	GREECE	65	IGHV1-2*04	99,65	0,1	-1,5	NEGATIVE
40	GREECE	90	IGHV1-2*04	94,1	-0,1	-1,8	NEGATIVE
41	GREECE	25	IGHV1-2*04	98,3	0	-0,1	0 Haloplex
42	GREECE	91	IGHV1-2*02	100	16,6	5,8	0 Haloplex
43*	UK				38,6	9,9	NOT TESTED
44*	UK	75 (3/5/12)	IGHV1-2	97,38	3,1	46,6	0 (MLPA)
45	UK	51 (19/6/14)	IGHV1-2	98,13	21,7	16,1	0 (MLPA)

SPLEEN SAMPLES

46*	UK	92	IGHV4-39	87,78	1,3	6,7	NOT TESTED
47	UK	48			0,9	-5,2	NOT TESTED
48*	UK	80	IGHV3-30	95,49	64,5	1,8	haloplex MYD88 positive
49*	UK	77			20,7	15,7	NOT TESTED
50	UK	20	IGHV1-2	98,5	-0,3	7	NOT TESTED
51	ITALY	85	IGHV4-34	98,5	37,1	0,3	NEGATIVE

LEGEND

*indicate peripheral blood and spleen from the same patient (31 and 48; 43 and 46; 44 and 49)
gray line: underline MyD88 mutation

Supplemental Table 2

List of the primers used

Gene	5' Primer	3' Primer	Reference
TLR1			(18)
TLR2			(18)
TLR3	GGCTAGCAGTCATCCAACAGA	GTAGATCATCGGGTACCTGAG	
TLR4	AGTGAGGATGATGCCAGGATG	TTGAGATGTCCAATGGGGAAG	
TLR5	ACTAGAAGTCCCTTCTGCTAG	GCAGTGACTIONCTGATATAG	
TLR6			(18)
TLR7	CTCTTCAACCAGACCTCTACA	TTCGTGGTGTTTCGTGGGAATA	
TLR8			(37)
TLR9	CTCGAGTGTGAAGCATCCTTC	GTTCCACTTGAGGTTGAGATG	
TLR10	GATGGTTGGATGGTCAGATTC	GAATCGGACATTTCTCTTGAG	
TIR8	CTTCAGTCCAGTGGCTGAAAG	TGAAGGAGGAGAAGCTGATGT	
β -actin			(18)

Supplemental Table 3 Part 1/2

List of the genes analyzed

Symbol	Unigene	GeneBank	Description	Gene Name
BTK	Hs.733206	NM_000061	Bruton agammaglobulinemia tyrosine kinase	AGMX1, AT, ATK, BPK, IMD1, PSCTK1, XLA
CASP8	Hs.599762	NM_001228	Caspase 8, apoptosis-related cysteine peptidase	ALPS2B, CAP4, Casp-8, FLICE, MACH, MCH5
CCL2	Hs.303649	NM_002982	Chemokine (C-C motif) ligand 2	GDCF-2, HC11, HSMCR30, MCAF, MCP-1, MCP1, SCYA2, SMC-CF
CD14	Hs.163867	NM_000591	CD14 molecule	-
CD80	Hs.838	NM_005191	CD80 molecule	B7, B7-1, B7.1, BB1, CD28LG, CD28LG1, LAB7
CD86	Hs.171182	NM_006889	CD86 molecule	B7-2, B7.2, B70, CD28LG2, LAB72
CHUK	Hs.198998	NM_001278	Conserved helix-loop-helix ubiquitous kinase	IKBKA, IKK-alpha, IKK1, IKKA, NFKBIKA, TCF16
CLEC4E	Hs.236516	NM_014358	C-type lectin domain family 4, member E	CLECSF9, MINCLE
CSF2	Hs.1349	NM_000758	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF
CSF3	Hs.2233	NM_000759	Colony stimulating factor 3 (granulocyte)	C17orf33, CSF3OS, GCSF
CXCL10	Hs.632586	NM_001565	Chemokine (C-X-C motif) ligand 10	C7, IFI10, INP10, IP-10, SCYB10, crg-2, gIP-10, mob-1
EIF2AK2	Hs.131431	NM_002759	Eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK1, PKR, PRKR
ELK1	Hs.715039	NM_005229	ELK1, member of ETS oncogene family	-
FADD	Hs.86131	NM_003824	Fas (TNFRSF6)-associated via death domain	MORT1
FOS	Hs.25647	NM_005252	FBJ murine osteosarcoma viral oncogene homolog	AP-1, C-FOS, p55
HMG81	Hs.434102	NM_002128	High mobility group box 1	HMG1, HMG3, SBP-1
HRAS	Hs.37003	NM_005343	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	C-BAS, HAS, C-H-RAS, C-HA-RAS1, CTLO, H-RASIDX, HAMSV, HRAS1, K-RAS, N-RAS, RASH1
HSPA1A	Hs.702139	NM_005345	Heat shock 70kDa protein 1A	HEL-S-103, HSP70-1, HSP70-1A, HSP70I, HSP72, HSPA1
HSPD1	Hs.727543	NM_002156	Heat shock 60kDa protein 1 (chaperonin)	CPN60, GROEL, HLD4, HSP-60, HSP60, HSP65, HUCHA60, SPG13
IFNA1	Hs.37026	NM_024013	Interferon, alpha 1	IFL, IFN, IFN-ALPHA, IFN-alphaD, IFNA13, IFNA@
IFNB1	Hs.93177	NM_002176	Interferon, beta 1, fibroblast	IFB, IFF, IFNB
IFNG	Hs.856	NM_000619	Interferon, gamma	IFG, IFI
IKKBK	Hs.597664	NM_001556	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	IKK-beta, IKK2, IKKB, IMD15, NFKBIKB
IL10	Hs.193717	NM_000572	Interleukin 10	CSIF, GVHDS, IL-10, IL10A, TGIF
IL12A	Hs.673	NM_000882	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	CLMF, IL-12A, NFSK, NKSF1, P35
IL1A	Hs.1722	NM_000575	Interleukin 1, alpha	IL-1A, IL1, IL1-ALPHA, IL1F1
IL1B	Hs.126256	NM_000576	Interleukin 1, beta	IL-1, IL1-BETA, IL1F2
IL2	Hs.89679	NM_000586	Interleukin 2	IL-2, TCGF, lymphokine
IL6	Hs.654458	NM_000600	Interleukin 6 (interferon, beta 2)	BSF2, HGF, HSF, IFNB2, IL-6
IL8	Hs.624	NM_000584	Interleukin 8	CXCL8, GCP-1, GCP1, LECT, LUCT, LYNAP, MDNCF, MONAP, NAF, NAP-1, NAP1
IRAK1	Hs.522819	NM_001569	Interleukin-1 receptor-associated kinase 1	IRAK, pelle
IRAK2	Hs.449207	NM_001570	Interleukin-1 receptor-associated kinase 2	IRAK-2
IRF1	Hs.436061	NM_002198	Interferon regulatory factor 1	IRF-1, MAR
IRF3	Hs.289052	NM_001571	Interferon regulatory factor 3	-
JUN	Hs.696684	NM_002228	Jun proto-oncogene	AP-1, AP1, c-Jun
LTA	Hs.36	NM_000595	Lymphotoxin alpha (TNF superfamily, member 1)	LT, TNFB, TNFSF1
CD180	Hs.87205	NM_005582	CD180 molecule	LY64, Ly78, RP105
LY86	Hs.653138	NM_004271	Lymphocyte antigen 86	MD-1, MMD-1, dJ80N2.1
LY96	Hs.726603	NM_015364	Lymphocyte antigen 96	ESOP-1, MD-2, MD2, ly-96
MAP2K3	Hs.514012	NM_002756	Mitogen-activated protein kinase kinase 3	MAPKK3, MEK3, MKK3, PRKMK3, SAPKK-2, SAPKK2
MAP2K4	Hs.514681	NM_003010	Mitogen-activated protein kinase kinase 4	JNKK, JNKK1, MAPKK4, MEK4, MKK4, PRKMK4, SAPKK-1, SAPKK1, SEK1, SERK1, SKK1
MAP3K1	Hs.653654	NM_005921	Mitogen-activated protein kinase kinase kinase 1	MAPKKK1, MEKK, MEKK1, MEKK1, SRXY6
MAP3K7	Hs.594838	NM_003188	Mitogen-activated protein kinase kinase kinase 7	MEKK7, TAK1, TGF1a
TAB1	Hs.729080	NM_006116	TGF-beta activated kinase 1/MAP3K7 binding protein 1	3'-Tab1, MAP3K7IP1
MAP4K4	Hs.701013	NM_004834	Mitogen-activated protein kinase kinase kinase kinase 4	FLH21957, HEL-S-31, HGK, MEKKK4, NIK
MAPK8	Hs.522924	NM_002750	Mitogen-activated protein kinase 8	JNK, JNK-46, JNK1, JNK1A2, JNK21B1, 2, PRKM8, SAPK1, SAPK1c
MAPK8IP3	Hs.207763	NM_015133	Mitogen-activated protein kinase 8 interacting protein 3	JIP3, JSAP1, SYD2, syd

Supplemental Table 3 Part 2/2

Symbol	Unigene	GeneBank	Description	Gene Name
MYD88	Hs.82116	NM_002468	Myeloid differentiation primary response gene (88)	MYD88D
NFKB1	Hs.618430	NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1, KBF1, NF-kB1, NF-kappa-B, NF-kappaB, NFKB-p105, NFKB-p50, NfkappaB, p105, p50
NFKB2	Hs.73090	NM_002502	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	CVID10, H2TF1, LYT-10, LYT10, NF-kB2, p105, p52
NFKBIA	Hs.81328	NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IKBA, MAD-3, NFKBI
NFKBIL1	Hs.2764	NM_005007	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	IKBL, LST1, NFKBIL
NFRKB	Hs.530539	NM_006165	Nuclear factor related to kappaB binding protein	INO80G
NR2C2	Hs.555973	NM_003298	Nuclear receptor subfamily 2, group C, member 2	TAK1, TR2R1, TR4, hTAK1
PEL1	Hs.7886	NM_020651	Pellino homolog 1 (Drosophila)	-
PPARA	Hs.592209	NM_005036	Peroxisome proliferator-activated receptor alpha	NR1C1, PPAR, PPARalpha, hPPAR
PRKRA	Hs.570274	NM_003690	Protein kinase, interferon-inducible double stranded RNA dependent activator	DYT16, PACT, RAX
PTGS2	Hs.196384	NM_000963	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	COX-2, COX2, GRIPGHS, PGG, HS, PGHS-2, PHS-2, hCox-2
REL	Hs.631886	NM_002908	V-rel reticuloendotheliosis viral oncogene homolog (avian)	C-Rel
RELA	Hs.502875	NM_021975	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	NFKB3, p65
RIPK2	Hs.103755	NM_003821	Receptor-interacting serine-threonine kinase 2	CARD3, CARDIAK, CCK, GIG30, RICK, RIP2
SARM1	Hs.743510	NM_015077	Sterile alpha and TIR motif containing 1	MyD88-5, SAMD2, SARM
SIGIRR	Hs.501624	NM_021805	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	TIR8
ECSIT	Hs.515146	NM_016581	ECSIT homolog (Drosophila)	SITPEC
TBK1	Hs.505874	NM_013254	TANK-binding kinase 1	NAK, T2K
TICAM2	Hs.718838	NM_021649	Toll-like receptor adaptor molecule 2	MyD88-4, TICAM-2, TIRAP3, TIRP, TRAM
TIRAP	Hs.537126	NM_001039661	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	BACTS1, Mal, MyD88-2, wyatt
TLR1	Hs.621817	NM_003263	Toll-like receptor 1	CD281, TIL, TIL. LPRS5, rsc786
TLR10	Hs.730896	NM_030956	Toll-like receptor 10	CD290
TLR2	Hs.519033	NM_003264	Toll-like receptor 2	CD282, TIL4
TLR3	Hs.657724	NM_003265	Toll-like receptor 3	CD283, IIAE2
TLR4	Hs.174312	NM_138554	Toll-like receptor 4	ARMED10, CD284, TLR-4, TOLL
TLR5	Hs.604542	NM_003268	Toll-like receptor 5	MELIOS, SLEB1, TIL3
TLR6	Hs.743572	NM_006068	Toll-like receptor 6	CD286
TLR7	Hs.659215	NM_016562	Toll-like receptor 7	TLR7-like
TLR8	Hs.660543	NM_138636	Toll-like receptor 8	CD288
TLR9	Hs.87968	NM_017442	Toll-like receptor 9	CD289
TNF	Hs.241570	NM_000594	Tumor necrosis factor	DIF, TNF-alpha, TNFA, TNFSF2
TNFRSF1A	Hs.279594	NM_001065	Tumor necrosis factor receptor superfamily, member 1A	CD120a, FPF, MS5, TBP1, TNF-R, TNF-R-I, TNF-R55, TNFAR, TNFR1, TNFR1-d2, TNFR55, TNFR60, p55, p55-R, p60
TOLLIP	Hs.368527	NM_019009	Toll interacting protein	IL-1RAcPIP
TRAF6	Hs.444172	NM_004620	TNF receptor-associated factor 6	MGC:3310, RNF85
TICAM1	Hs.29344	NM_182919	Toll-like receptor adaptor molecule 1	IIAE6, MyD88-3, PRVTIRB, TICAM-1, TRIF
UBE2N	Hs.524630	NM_003348	Ubiquitin-conjugating enzyme E2N	HEL-S-71, UBC13, Ubch-ben, Ubch13
UBE2V1	Hs.744839	NM_021988	Ubiquitin-conjugating enzyme E2 variant 1	CIR1, CROC-1, CROC1, UBE2V, UEV-1, UEV1, UEV1A
B2M	Hs.534255	NM_004048	Beta-2-microglobulin	-
HPRT1	Hs.412707	NM_000194	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
RPL13A	Hs.523185	NM_012423	Ribosomal protein L13a	L13A, TSTA1
GAPDH	Hs.544577	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD
ACTB	Hs.520640	NM_001101	Actin, beta	BRWS1, PS1TP5BP1