Impaired clearance of apoptotic cells leads to HMGB1 release in the bone marrow of patients with myelodysplastic syndromes and induces TLR4-mediated cytokine production

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Online Supplementary Design and Methods

Long-term bone marrow cultures

Long-term bone marrow cultures (LTBMCs) were grown according to a standard assay from 10^7 bone marrow mononuclear cells in 10 mL Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS; Gibco), 10% horse serum (Gibco), 2 mmol L-glutamine (Gibco), 100 IU/mL penicillin/streptomycin, and 10^{-6} mol hydrocortisone sodium succinate (Sigma-Aldrich), and incubated at 33°C in 5% CO_2 in a fully humidified atmosphere.[1,2] Cultures were fed weekly by demi-depopulation and on confluence (week 3), cell-free supernatants were harvested and kept at -80°C for HMGB1 measurement by means of an enzyme-linked immunosorbent assay (ELISA; Shino-Test Corporation, Japan). The adherent cell layers were trypsinized and assayed by flow-cytometry for Toll-like receptor (TLR) expression.

Flow-cytometric analysis of TLR expression

To evaluate surface TLR1, TLR2, TLR4 and intracellular TLR3 and TLR9 expression in the $CD14^+$ monocyte cell fraction of bone marrow cells, we performed three-color flow-cytometry using a panel of directly conjugated monoclonal antibodies in an Epics Elite flow-cytometer (Coulter, Miami, FL, USA). The surface and intracellular staining procedures, monoclonal antibody clones and gating strategy have been previously described.[3] Surface and intracellular TLR expression were also evaluated in the $CD45^+/CD14^+$ and $CD45^-$ cell fractions of trypsinized adherent cells of confluent LTBMCs, representing the monocytic and mesenchymal/stromal cell components of the marrow microenvironment, respectively.[3] Results are expressed as the proportion of TLR^+ cells in the above gates and also as the mean ratio of relative fluorescence intensity (MRFI) defined by the ratio of the mean fluorescence intensity (MFI) of the TLR staining/MFI of the isotype-matched monoclonal antibody staining.

Generation of bone marrow-derived macrophages

To generate bone marrow-derived macrophages, 10^7 BMMCs were cultured in 10 mL Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 in a fully humidified atmosphere for 24 h. The non-adherent cells were then harvested, transferred to a 75 cm^2 tissue culture flask containing 10 mL complete DMEM supplemented with 25 ng/mL recombinant human (rh) interleukin (IL)-3 (R&D Systems, Minneapolis, USA) and 2.5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems) and incubated for 4 days before adding another 10 mL of complete, growth factor-containing DMEM. After 15 days in culture, the purity of the adherent macrophages was more than 90% as determined by flow-cytometric determination of CD14 antigen expression (clone RMO52; Beckman Coulter, Marseille, France).

Phagocytosis assay

Autologous non-adherent BMMCs seeded in RPMI-1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin for 15 days, were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen) and incubated with 30 μg/mL etoposide[4] for 5 h so that more than 75% of cells became apoptotic as determined by flow-cytometry and staining with 7-amino-actinomycin D (7AAD) (Calbiochem-Novabiochem, La Jolla, CA, USA).[2] Titration experiments were performed to determine the dosage of the drug needed to achieve the desirable proportion of apoptosis. Apoptotic cells were then added to the macrophage monolayers at a 4:1 ratio (target cell: macrophage) and co-incubated for 35 min at 37°C in 5% CO_2 in a fully humidified atmosphere. At the end of the incubation period, macrophages were washed three times to remove non-internalized cells, fixed with 4% paraformaldehyde and stained with fluorescein isothiocyanate-conjugated anti-CD14 monoclonal antibody and 4',6-diamidino-2-phenylindole (DAPI, Abbott Laboratories, Illinois, USA). Under fluorescence microscopy, macrophages appear green while apoptotic cells show intense green fluorescence (*Online Supplementary Figure S1*). The phagocytic/efferocytic index was calculated after examining blindly 200 macrophages under the fluorescent microscope using the formula [(number of apoptotic bodies/200 total macrophages) x 100].[5] The examination was performed by two independent observers.

Reverse transcription and quantitative polymerase chain reaction analysis

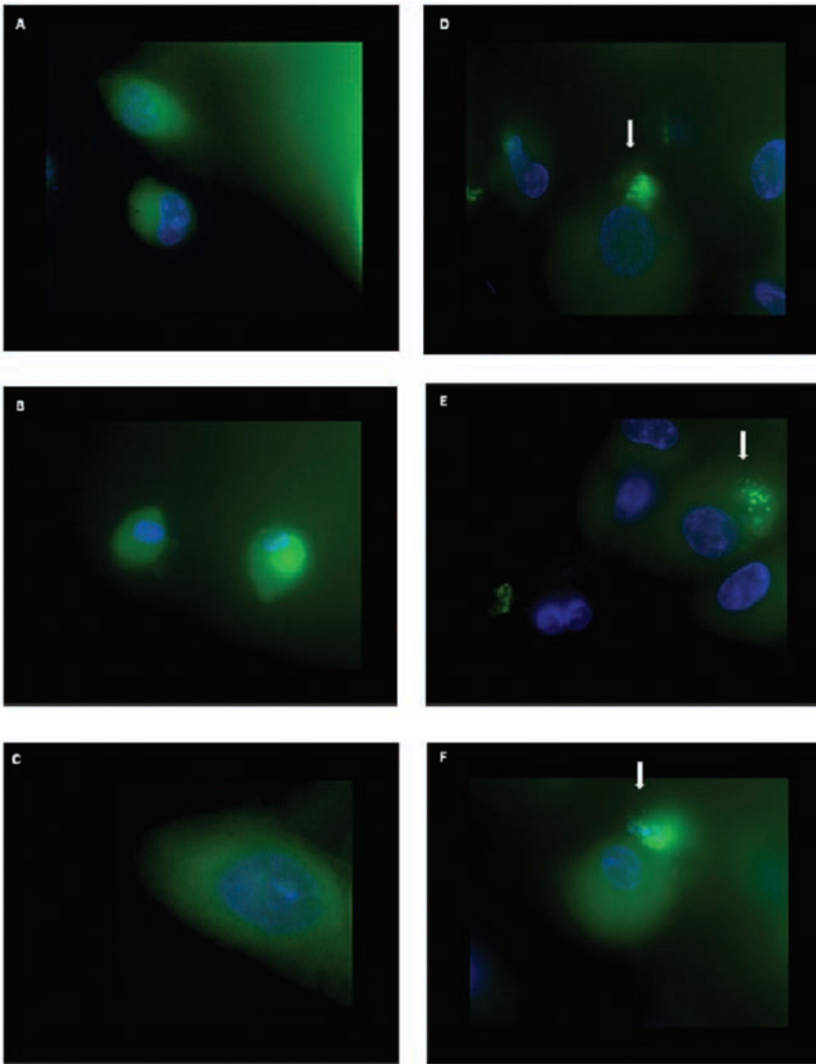
Total RNA was extracted from immunomagnetically sorted CD14+ cells (Mitenyi Biotec GmbH, Bergisch Gladbach, Germany) derived from the BMMC cell fraction using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The concentration of the extracted RNA was calculated by the Qubit Fluorometer (Invitrogen). RNA was reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen).

Quantitative SYBR green-based real-time polymerase chain reaction (PCR) analysis of 84 genes related to TLR-mediated signal transduction was performed using the human Toll-Like Receptor Signaling Pathway RT^2 Profiler™ PCR Array (SABiosciences, Qiagen) in an ABI Prism 7000 System (Applied Biosystems, Foster City, CA, USA). The expression of each gene was calculated according to the threshold cycle (Ct) relative quantification $2^{-\Delta CT}$ method, using the ribosomal protein L13a (RPL13A) as a housekeeping gene for normalization. For the genes showing at least a 4-fold difference in gene expression between test and control groups we performed a gene set enrichment analysis using the tool GOrilla[6]. This tool uses the Gene Ontology database to obtain sets of genes which are significantly enriched in specific molecular functions, biological processes and cellular components.

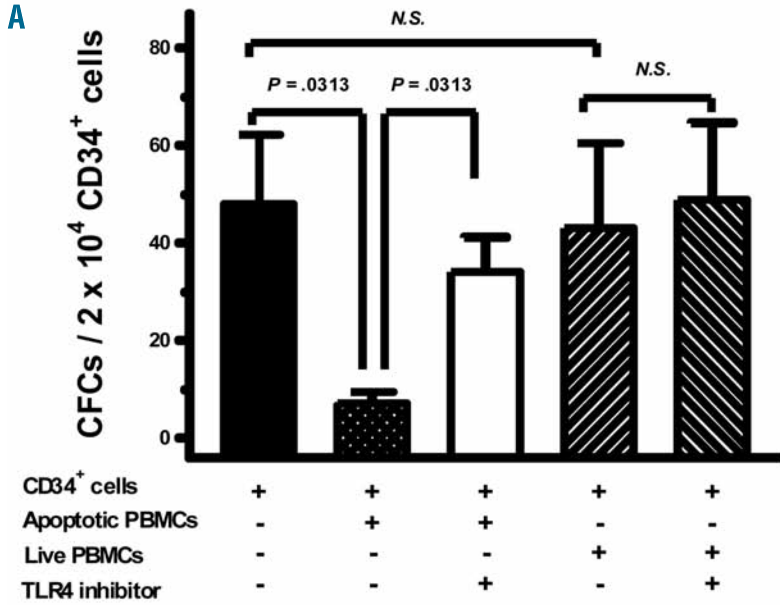
To confirm the results obtained from the PCR array, a number of significantly over-expressed genes were tested separately by quantitative reverse transcription PCR using primers purchased from SABiosciences. We also examined the expression of interleukin-1 receptor-associated kinase (IRAK)-M and SH2 domain containing polyinositol-5-phosphatase 1 (SHIP1) genes that negatively affect TLR signaling[7,8] and were not included in the array. For IRAKM the sense primer was 5'-TTTGAATGCAGCCAGTCTGA-3' and the antisense 5'-GCATTGCTTATGGAGCCAAT-3'; for SHIP1 the sense primer was 5'-CGACAAGAAGCTGAGTCCCTTT-3' and the antisense 5'-GGTAGTTAAGATCCCCAAACCAGAA-3'. All samples were run in triplicate. A 2-fold serial dilution of cDNA from a healthy individual was selected as a calibrator for quantification of the selected genes according to the $2^{-\Delta CT}$ method.

References

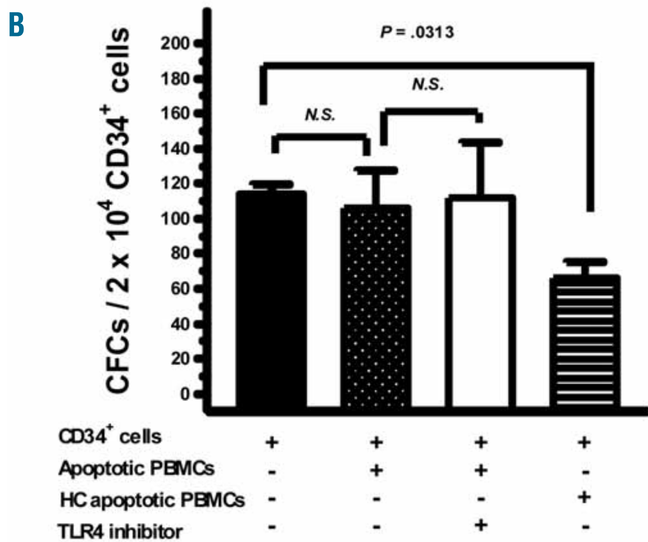
1. Coutinho LH, Gilleece MH, De Wynter EA, Will A, Testa NG. Clonal and long-term cultures using human bone marrow. In: Testa NG, Molineux G, eds. Haemopoiesis: A Practical Approach. Oxford, Unired Kingdom: Oxford University Press; 1993:75-106.
2. Papadaki HA, Eliopoulos AG, Kosteas T, Gemetzi C, Damianaki A, Koutala H, et al. Impaired granulocytopoiesis in patients with chronic idiopathic neutropenia is associated with increased apoptosis of bone marrow myeloid progenitor cells. Blood. 2003;101(7): 2591-600.
3. Velegraki M, Koutala H, Tsatsanis C, Papadaki HA. Increased levels of the high mobility group box 1 protein sustain the inflammatory bone marrow microenvironment in patients with chronic idiopathic neutropenia via activation of toll-like receptor 4. J Clin Immunol. 2012;32 (2):312-22.
4. Bell CW, Jiang W, Reich CF III, Pisetsky DS. The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol. 2006;291(6):C1318-25.
5. Fernandez-Boyanapalli RF, Frasch SC, McPhillips K, Vandivier RW, Harry BL, Riches DW, et al. Impaired apoptotic cell clearance in CGD due to altered macrophage programming is reversed by phosphatidylserine-dependent production of IL-4. Blood. 2009;113(9):2047-55.
6. Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 2009;10:48-55.
7. Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell. 2002;110(2):191-202.
8. An H, Xu H, Zhang M, Zhou J, Feng T, Qian C, et al. Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1) negatively regulates TLR4-mediated LPS response primarily through a phosphatase activity- and PI-3K-independent mechanism. Blood. 2005;105(12): 4685-92.

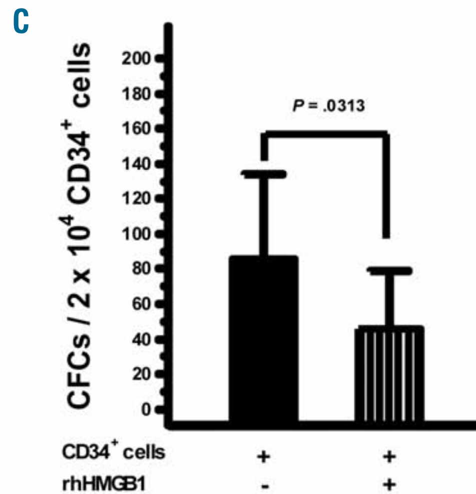


Online Supplementary Figure S1. Fluorescent microscopy-based phagocytic assay for the evaluation of the apoptotic cell clearance rate of BM macrophages. BM-derived macrophages from five MDS patients and five healthy donors were incubated with CFSE-labeled apoptotic BMMCs (ratio 1:4) at 37°C and then stained with CD14-FITC and DAPI. Macrophages incubated with CFSE-labeled freshly isolated BMMCs were also examined as control samples. Green fluorescent macrophages which have not phagocytosed apoptotic cells are shown in the left panel (images A-C). Macrophages engulfing the intense green apoptotic cells (white arrows) are shown in the right panel (images D-F). The nuclei of the macrophages are demonstrated by blue DAPI staining. The efferocytosis index was calculated after examining 200 macrophages randomly under a Zeiss Axioskop microscope (Zeiss, Jena, Germany). Images were documented with the ISIS analyzing system (MetaSystems, Altlussheim, Germany) using a charge coupled device (CCD)-camera (ISIS-Metasystems, Altlussheim). Original magnifications are x100 for all images. Abbreviations: BM, bone marrow; CFSE, carboxyfluorescein diacetate succinimidyl ester; DAPI, 4',6-diamidino-2-phenylindole; BMMC, bone marrow mononuclear cells.



Online Supplementary Figure S2. Clonogenic potential of normal CD34+ cells in the presence of apoptotic cells or HMGB1. Irradiated macrophage layers from MDS patients (n=6) or healthy subjects (n=6) were recharged with $2x10^4$ allogeneic normal CD34+ BM cells in the presence or absence of $2x10^6$ apoptotic or live allogeneic normal peripheral blood mononuclear cells (PBMCs) in the presence or not of a TLR4 blocking monoclonal antiboby. The clonogenic potential of the non-adherent cell fraction containing the CD34+ cells was assessed in a week's time by scoring the total colonies, characterized as total colony-forming cells (CFC). (A) Columns represent the mean (plus one standard deviation) of CFC numbers produced by the non-adherent cells of recharged MDS-derived macrophage culture cells in the presence or absence of either apoptotic or live allogeneic PBMC with or without the addition of a specific TLR4-inhibitor. (B) The graph depicts the mean CFC numbers (plus one standard deviation) produced by the non-adherent cells of recharged macrophage cultures derived from healthy subjects following treatment with apoptotic cells in the presence or absence of a specific TLR4-inhibitor. The fourth bar depicts CFC produced by the non-adherent cells of normal macrophage cultures assessed in the presence of a higher concentration (HC) of apoptotic PBMC ($4x10^6$). (C) The bars represent the mean CFC numbers (plus one standard deviation) in the non-adherent cell fraction of normal macrophage cultures recharged with allogeneic normal CD34+ BM cells in the presence or absence of recombinant human (rh) HMGB1 at a concentration of 300 ng/mL corresponding to the mean cytokine levels measured in the BM plasma of MDS patients. In all cases comparisons were performed using the non-parametric Wilcoxon signed rank test for paired samples and the *P* values are indicated. N.S. denotes a non-statistically significant difference.





Online Supplementary Table S1. Clinical and laboratory data of the patients studied.

UPN	Age	Sex	WHO	IPSS	Karyotype
1	87	M	RCMD	Intermediate-1	46, XY
2	75	M	RA	Low	45,X, -Y, 16qht
3	83	M	RAEB-1	Intermediate-1	46, XY
4	75	M	RA	Intermediate-1	47, XY, +8
5	62	M	RAEB-2	Intermediate-2	46, XY
6	60	M	RAEB-2	Intermediate-2	46, XY
7	82	F	RCMD	Intermediate-1	46, XX
8	79	M	RA	Low	46, XY
9	72	M	RAEB-1	Intermediate-1	46, XY
10	71	F	RA	Low	46, XX
11	81	M	RA	Low	45, X, -Y
12	83	M	RAEB-2	Intermediate-2	46, XY
13	76	F	RA	Low	46, XX
14	84	F	RAEB-2	Intermediate-2	46, XX
15	82	F	RAEB-1	Intermediate-1	46, XX
16	81	M	RCMD	Intermediate-1	46, XY
17	80	F	MDS-associated with isolated del (5q)	Intermediate-1	46, XX, del(5)(q13q33)
18	81	F	MDS-associated with isolated del (5q)	Low	46, XX, del(5)(q13q33)
19	70	M	RCMD	Intermediate-1	46, XY, 22pst
20	62	M	RAEB-1	Intermediate-1	46, XY
21	65	M	RA	Low	46, XY
22	89	M	RA	Low	46, XY
23	67	M	RCMD	Intermediate-1	46, XY
24	82	M	RA	Low	45, X, -Y
25	72	M	RCMD	Intermediate-1	46, XY
26	74	M	RA	Low	46, XY
27	86	F	RAEB-1	Intermediate-1	46, XX

UPN: unique patient number; WHO: World Health Organization; IPSS: International Prognostic Scoring System; RCMD: refractory cytopenia with multilineage dysplasia; RA: refractory anemia; RAEB: RA with excess blasts; MDS: myelodysplastic syndrome.

Online Supplementary Table S2. TLR expression in the BM and LTBMC adherent cell subsets of MDS patients.

	MDS patients	Healthy controls	P
BM CD14+ cell fraction			
% TLR1+ cells	5.46%±3.86%	4.14%±3.27%	0.1843
MRFI	1.21±0.22	1.18±0.50	0.2965
% TLR2+ cells	47.20%±22.55%	44.34%±22.48%	0.6081
MRFI	3.61±1.73	3.84±1.83	0.8691
% TLR3+ cells	16.36%±19.38%	12.72%±16.64%	0.1999
MRFI	2.29±1.16	2.15±1.53	0.0852
% TLR4+ cells	6.10%±5.27%	2.01%±1.38%	< 0.0001*
MRFI	1.29±0.31	0.96±0.25	0.0002*
% TLR9+ cells	35.40%±21.14%	28.85%±16.99%	0.1904
MRFI	3.24±1.54	2.98±1.12	0.7142
LTBMC CD45+/CD14+ cell fraction			
% TLR1+ cells	3.18%±2.98%	2.18%±0.98%	0.6842
MRFI	1.16±0.30	1.14±0.17	0.8534
% TLR2+ cells	3.44%±1.98%	3.25%±2.38%	0.6842
MRFI	1.28±0.26	1.22±0.27	0.7959
% TLR3+ cells	23.99%±23.41%	6.04%±2.73%	0.0524
MRFI	2.60±1.57	1.33±0.24	0.0524
% TLR4+ cells	9.57%±7.79%	3.54%±3.48%	0.0288*
MRFI	1.82±0.57	1.34±0.32	0.0232*
% TLR9+ cells	21.06%±22.60%	11.74%±7.11%	0.6305
MRFI	3.01±2.07	2.31±1.27	0.3930
LTBMC CD45- cell fraction			
% TLR1+ cells	3.30%±4.35%	2.65%±0.17%	0.4813
MRFI	1.05±0.21	1.24±0.49	0.6305
% TLR2+ cells	1.83%±1.08%	1.61%±1.06%	0.5787
MRFI	1.07±0.15	1.14±0.15	0.2475
% TLR3+ cells	12.73%±19.72%	3.07%±1.98%	0.6305
MRFI	1.70±1.31	1.35±0.72	0.2475
% TLR4+ cells	7.61%±6.26%	4.85%±5.47%	0.0524
MRFI	1.64±0.69	1.64±1.34	0.2176
% TLR9+ cells	22.99%±16.99%	11.86%±5.65%	0.0753
MRFI	2.56±0.72	2.23±0.75	0.1655

BM: bone marrow; LTBMC: long-term bone marrow culture; MRFI: mean relative fluorescence intensity. The values represent the means ± 1 standard deviation. BM data were obtained from 27 MDS patients and 25 healthy controls whereas LTBMC data came from 10 MDS patients and 10 healthy controls. Comparisons were performed by means of the non-parametric Mann-Whitney test and the P values are indicated. Asterisk (*) denotes statistically significant differences.

Online Supplementary Table S3. TLR-signaling related genes showing at least a 4-fold up-regulation in MDS CD14+ BM cells.

Functional characterization of genes	Genes
Toll-like receptors	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, CD180 (LY64), SIGIRR
Adaptor and interacting proteins	CD14, HSPD1, LY86 (MD-1), MAPK8IP3, MYD88, , RIPK2, TICAM2, TOLLIP
Effectors	CASP8, EIF2AK2, FADD, IRAK1, IRAK2, PPARA, TRAF6, UBE2V1
TLR signaling	
Negative regulation	SIGIRR
MYD88-independent pathway	NR2C2, PELI1, TBK1, TICAM2, TICAM1, TRAF6, TLR4, TLR3
MYD88-dependent pathway	IRAK1, IRAK2, MYD88, NR2C2, TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TRAF6
Downstream pathways and target genes	
NF-κB Pathway	CASP8, FADD, IKBKB, IL10, IL1B, IRAK1, IRAK2, MAP3K1 (MEKK), MAP4K4, NFKB1, NFKB2, NFKBIA (IKBA/MAD3), PPARA, REL, RELA, TNFRSF1A, UBE2V1
JNK/p38 pathway	ELK1, IL1B, JUN, MAP2K3 (MEK3), MA2K4(JNKK1), MAP3K1 (MEKK), MAPK8IP3
JAK/STAT pathway	CCL2 (MCP-1), IL6
IRF pathway	CXCL10 (INP10), TBK1
Cytokine mediated pathway	CCL2 (MCP-1), IL1B, IL6, IRAK1, IRAK2, RELA, SIGIRR, TNFRSF1A
Regulators of adaptive immunity	CD86, HSPD1, IL10, IL1B, TRAF6

CCL2: chemokine (C-C motif) ligand 2, HSP: heat shock protein, IL: interleukin, IRF: interferon-regulatory factor , JNK: JUN N-terminal kinase, LY: lymphoid antigen, MAPK : mitogen-activated protein kinase, MAPK8IP3: mitogen-activated protein kinase 8 interacting protein 3, MCP-1: monocyte chemotactic protein-1, MyD88: myeloid differentiation factor 88 , NF-κB: nuclear factor-κB, TBK1: tank binding kinase 1, TLR: Toll-like receptor, TNF-α: tumor necrosis factor-α, TNFRSF1A: TNF receptor superfamily member 1A, UBE2N: ubiquitin-conjugating enzyme E2 N.

Online Supplementary Table S4. Gene set enrichment analysis of the genes showing at least 4-fold upregulation in the PCR array analysis.*

GO term	Description	Genes	Enrichment (N, B, n, b)	FDR q-value	*P*
0005125	Cytokine activity	CCL2 - chemokine (c-c motif) ligand 2 IL8 - interleukin 8 CXCL10 - chemokine (c-x-c motif) ligand 10 IL1B - interleukin 1, beta IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 LTA - lymphotoxin alpha (TNF superfamily, member 1)	2.68 (51,7,19,7)	0.195	0.000931
0008285	Negative regulation of cell proliferation	IL8 - interleukin 8 JUN - jun proto-oncogene IL1B - interleukin 1, beta IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 EIF2AK2 - eukaryotic translation initiation factor 2-alpha kinase 2 LTA - lymphotoxin alpha (TNF superfamily, member 1)	2.68 (51,7,19,7)	0.781	0.000931
0051336	Regulation of hydrolase activity	CCL2 - chemokine (c-c motif) ligand 2 IL8 - interleukin 8 HSPD1 - heat shock 60 kDa protein 1 (chaperonin) CASP8 - caspase 8, apoptosis-related cysteine peptidase JUN - jun proto-oncogene IL6 - interleukin 6 (interferon, beta 2) FADD - fas (tnfrsf6)-associated via death domain	2.83 (51,7,18,7)	1	0.000655
0005615	Extracellular space	IL8 - interleukin 8 CCL2 - chemokine (c-c motif) ligand 2 HSPD1 - heat shock 60 kDa protein 1 (chaperonin) CXCL10 - chemokine (c-x-c motif) ligand 10 IL1B - interleukin 1, beta CD14 - cd14 molecule IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 LTA - lymphotoxin alpha (TNF superfamily, member 1)	2.42 (51,10,19,9)	0.0984	0.000665
0044421	Extracellular region part	CCL2 - chemokine (c-c motif) ligand 2 IL8 - interleukin 8 HSPD1 - heat shock 60 kDa protein 1 (chaperonin) CXCL10 - chemokine (c-x-c motif) ligand 10 IL1B - interleukin 1, beta IL6 - interleukin 6 (interferon, beta 2) CD14 - CD14 molecule IL10 - interleukin 10 LTA - lymphotoxin alpha (TNF superfamily, member 1)	2.42 (51,10,19,9)	0.0492	0.000665

**For each GO term, the associated genes appear in the optimal top of the list. Each gene name is specified by its gene symbol followed by a short description of the gene GO: Gene Ontology; FDR: false discovery rate; Enrichment = (b/n) / (B/N); N: total number of genes; B: total number of genes associated with a specific GO term; n: number of genes in the top of the user's input list or in the target set when appropriate, b: number of genes in the intersection.*