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REPORT

Hypoxia favors the generation of human plasma cells

Matthieu Schoenhals [a,b], Michel Jourdan[a], Angélique Bruyer[a], Alboukadel Kassambara[a,b], Bernard Klein[a,b,c,†], and Jérôme Moreaux [a,b,c,†]

[a]Institute of Human Genetics, CNRS-UM UMR9002, Montpellier, France; [b]Laboratory for Monitoring Innovative Therapies, Department of Biological Hematology, CHU Montpellier, Montpellier, France; [c]University of Montpellier, UFR de Médecine, Montpellier, France

ABSTRACT

Plasma cells (PCs) generation occurs in hypoxic conditions *in vivo*, whereas the relevance of O_2 pressure in PC differentiation remains unknown.

Using our *in vitro* PC differentiation model, we investigated the role of hypoxia in PC generation.

Hypoxia increases the generation of plasmablasts (PBs) starting from memory B cells, by increasing cell cycle and division number. Reactome analysis demonstrated a significant enrichment of genes involved in HIF1α and HIF2α transcription factor network, metabolism and MYC related pathways in hypoxic compared with normoxic PBs. Hypoxia-induced metabolism alteration and MYC pathway are involved in malignant PC pathophysiology. Therefore, the expression of 28 out of the 74 genes overexpressed in hypoxic PBs compared with normoxic ones was found to be associated with an adverse prognosis (event free survival and overall survival) in newly diagnosed multiple myeloma patients.

According to the role of hypoxia in supporting PBs generation through cell cycle induction, c-MYC activation and metabolism alteration, it could be involved in plasma cell tumorigenesis.

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KEYWORDS
differentiation; hypoxia; multiple myeloma; plasma cell; tumorigenesis

Introduction

Plasma cells (PCs) represent the end point of B cell differentiation. PCs produce large amounts of antibodies capable of neutralizing antigens. As terminally differentiated cells, PCs remain quiescent, unable to proliferate.

B-cell activation results in the formation of either short-lived or long-lived PCs. B cells activated in the presence of T cells providing help through ligation of CD40 generate prolonged responses characterized by the presence of germinal centers (GCs) where activated B cells endure somatic hypermutation and isotype switching.[1] Those that compete successfully and receive survival signals exit the GC and continue to mature as either plasmablasts (PBs) then early PCs, or memory B cells (MBCs). These PBs migrate to medullary cords, exit into the lymph through a sphingosine phosphate gradient and reach the peripheral blood.[2] Circulating PBs/early PCs must find a specific niche in the bone marrow (BM) or mucosa that will provide them with the factors to survive and fully differentiate. Migration to specific microenvironment niches is controlled by chemokines and their receptors. Coordinated expression of chemokines and their receptors orchestrates the movement of PBs to the BM where growth factors produced by the PC niche control PC survival.[2] Factors that promote the differentiation of PBs into bone marrow PCs (BMPCs) are poorly identified *in vivo*, due to the rarity of BMPCs and the difficulties to harvest them.

Using *in vitro* models of B to PCs differentiation, we and others have shown that *in vitro* generated PCs are early PCs with a phenotype close to that of circulating PBs and PCs in healthy individuals.[3,4] Recently, we and others also demonstrated that IL-6 in combination with APRIL, BAFF, or soluble stromal cell factors supports the generation and survival of long-lived PCs (LLPCs).[5,6]

In mammals, oxygen distribution is robustly regulated by the vascular system, which continually distributes oxygen-saturated red blood cells throughout the body. Despite the sturdiness of this system, oxygen distribution in the body is not uniform. Following air inspiration [partial pressure of oxygen (PO_2) of 159 mmHg], red blood cells absorb oxygen resulting in a maximal PO_2 in the pulmonary vasculature of 98 mmHg. As oxygen is delivered to tissue, PO_2 decrease to 50 mmHg in the BM, and 40 mmHg in venous blood.[7] Previous studies have shown that a large drop of pO_2 occurred across blood/tissue interface, reaching hypoxic levels from 30–40 μm from blood vessels.[8] In a recent paper, Abbott, *et al.*[9] showed that most of GC were located > 40 μm from the nearest blood vessel and that GC B cells from spleens, Peyer's patches and mesenteric lymph nodes were hypoxic. These unique microenvironments affect cell phenotype,

CONTACT Jérôme Moreaux jerome.moreaux@igh.cnrs.fr Laboratory for Monitoring Innovative Therapies, Department of Biological Hematology, Hôpital Saint-Eloi - CHRU de Montpellier, 80, av. Augustin Fliche, 34295 Montpellier Cedex 5, IGH - Institute of Human Genetics, CNRS-UM UMR 9002, http://www.igh.cnrs.fr/.
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[†]These authors share last authorship.

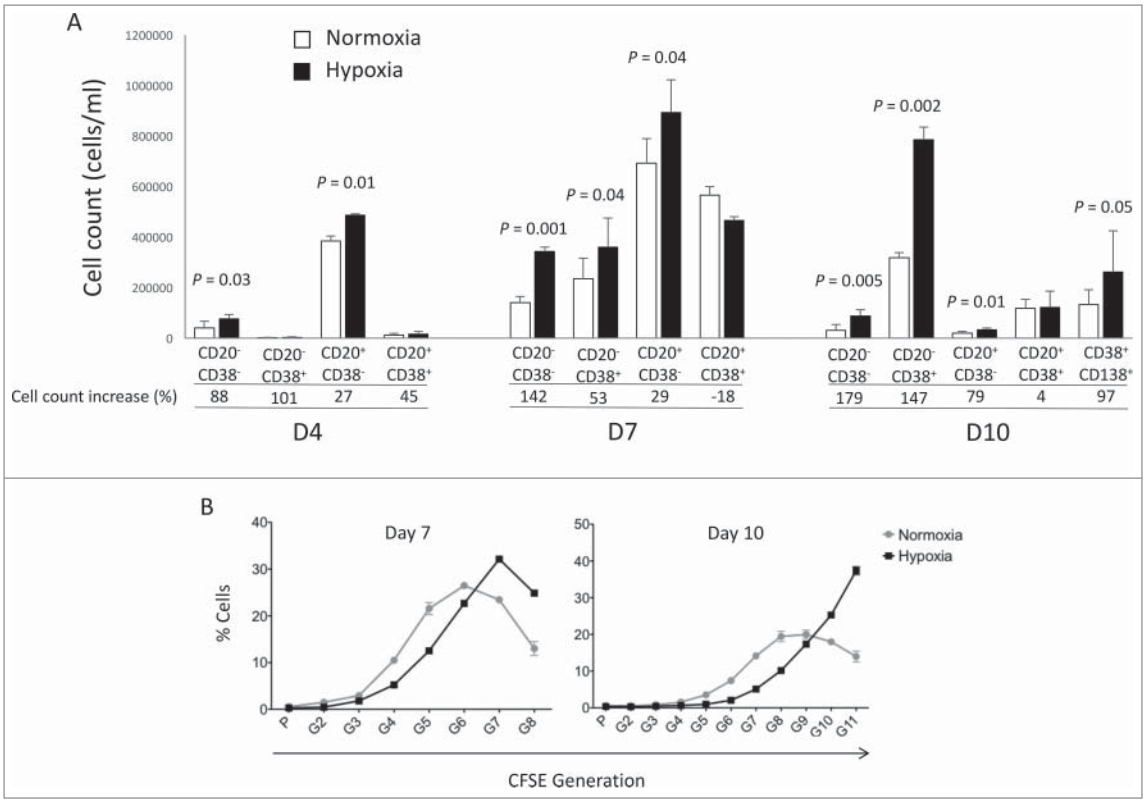

Figure 1. Hypoxia promotes PB generation *in vitro* and induces cell cycle progression. (A) Number of cells obtained at days 4, 7 and 10 *in vitro* either under hypoxic conditions (5% O_2) or normoxic ones (20% O_2). At D4, D7 and D10 of cell culture, the cells were counted and CD20, CD38 and CD138 expressions assessed by flow cytometry. The percentage of cell count increase in hypoxic conditions compared with normoxic conditions is indicated. Results are the means ± SD of 4 experiments. Statistical significance was tested using a Student's t-test for pairs. (B) Percentages of live cells according to their CFSE fluorescence obtained at days 7 and 10 *in vitro* under hypoxic conditions or normoxic ones following MBC CFSE incorporation at day 0. The statistical comparisons were done using a t-test.

Table 1. REACTOME analysis of genes significantly overexpressed in hypoxic PBs compared with normoxic PBs.

GeneSet	Ratios of genes in GenSet	Number of genes in GeneSet	Genes from Network	P-value	FDR	Nodes
HIF-1-α transcription factor network(N)	0.0068	66	9	0	2.54E-12	ALDOA,HK2,BNIP3,EGLN1,PKM, VEGFA,BHLHE40, PGK1, ENO1
Glycolysis / Gluconeogenesis (K)	0.0069	67	8	0	1.35E-10	ALDOA,PGAM1,HK2,PKM,GPI, TPI1,PGK1,ENO1
Carbon metabolism(K)	0.0109	106	8	0	3.36E-09	ALDOA,PGAM1,HK2,PKM,GPI, TPI1,PGK1,ENO1
HIF-2-α transcription factor network(N)	0.0034	33	5	0	4.74E-07	EPAS1,EGLN1,VEGFA,BHLHE40, PGK1
Biosynthesis of amino acids(K)	0.0076	74	6	0	4.74E-07	ALDOA,PGAM1,PKM,TPI1, PGK1,ENO1
Metabolism of carbohydrates (R)	0.0229	223	8	0	5.54E-07	ALDOA,PGAM1,HK2,PKM,GPI, TPI1,PGK1,ENO1
HIF-1 signaling pathway(K)	0.0109	106	6	0	2.75E-06	ALDOA,HK2,EGLN1,VEGFA, PGK1,ENO1
Central carbon metabolism in cancer(K)	0.0069	67	5	0	8.39E-06	PGAM1,HK2,PKM,PDGFRA,MYC
Regulation of Hypoxia-inducible Factor (HIF) by oxygen(R)	0.0026	25	3	0	5.00E-04	EPAS1,EGLN1,VEGFA
Glycolysis(P)	0.0005	5	2	0.0001	9.64E-04	PGK1,ENO1
Renal cell carcinoma(K)	0.0068	66	3	0.0001	0.006062074	EPAS1,EGLN1,VEGFA
Pathways in cancer(K)	0.0408	398	5	0.0004	0.021593392	EPAS1,EGLN1,VEGFA,PDGFRA, MYC
Bladder cancer(K)	0.0039	38	2	0.0017	0.036304882	VEGFA,MYC
Metabolic pathways(K)	0.1244	1214	8	0.0033	0.036304882	ALDOA,PGAM1,HK2,PKM,GPI, TPI1,PGK1,ENO1
Fructose and mannose metabolism(K)	0.0034	33	3	0.0036	9.64E-04	ALDOA,HK2,TPI1

Table 2A. High expression of genes induced by hypoxia in PBs could predict for shorter event free survival in myeloma patients. Patients of the LR-TT2 cohort (n = 345) were ranked according to increasing gene expression and a maximum difference in event free survival (EFS) was obtained using the Maxstat R function.

ID	Name	Maxstat Cutpoint	P value	Hazard Ratio	Prognostic Event Free Survival
204348_s_at	AK4	216	0.0041	1.7	Bad
200966_x_at	ALDOA	10163	3.70E-05	2	Bad
214687_x_at	ALDOA	9747	0.00027	1.8	Bad
210427_x_at	ANXA2	4194	3.50E-05	1.9	Bad
202207_at	ARL4C	519	3.00E-04	1.7	Bad
202206_at	ARL4C	790	0.00066	1.7	Bad
201849_at	BNIP3	1486	0.0061	1.7	Bad
208683_at	CAPN2	13644	0.026	1.4	Bad
209795_at	CD69	1646	0.024	1.6	Bad
223046_at	EGLN1	2423	0.0012	1.6	Bad
201231_s_at	ENO1	7048	0.00052	2.1	Bad
200859_x_at	FLNA	1659	0.00024	2.1	Bad
208308_s_at	GPI	5562	0.03	1.5	Bad
202934_at	HK2	1079	0.00036	1.7	Bad
203914_x_at	HPGD	117	0.0052	1.6	Bad
212192_at	KCTD12	543	0.0033	1.6	Bad
227021_at	KDM1B	399	0.0041	1.8	Bad
222572_at	PDP1	1641	0.0026	1.7	Bad
201037_at	PFKP	232	0.045	1.5	Bad
217356_s_at	PGK1	6648	0.00082	1.8	Bad
201251_at	PKM	2325	0.02	1.4	Bad
219014_at	PLAC8	8879	0.02	1.6	Bad
218984_at	PUS7	982	0.00085	1.7	Bad
223168_at	RHOU	588	0.00095	2	Bad
200872_at	S100A10	3788	2.20E-06	2.2	Bad
203186_s_at	S100A4	3389	0.0011	1.7	Bad
200822_x_at	TPI1	5484	0.0076	1.5	Bad
213011_s_at	TPI1	3598	0.039	1.5	Bad
201426_s_at	VIM	27572	0.0024	1.7	Bad
243764_at	VSIG1	87	0.016	1.6	Bad
228280_at	ZC3HAV1L	1384	0.012	1.5	Bad

Table 2B. High expression of genes induced by hypoxia in PBs could predict for shorter overall survival in myeloma patients. Patients of the LR-TT2 cohort (n = 345) were ranked according to increasing gene expression and a maximum difference in overall survival (OS) was obtained using the Maxstat R function.

ID	Name	Maxstat Cutpoint	P value	Hazard Ratio	Prognostic Overall Survival
204348_s_at	AK4	214	0.014	1.9	Bad
214687_x_at	ALDOA	9747	3.90E-05	2.2	Bad
200966_x_at	ALDOA	10126	0.00038	2	Bad
210427_x_at	ANXA2	7582	5.30E-06	2.8	Bad
202207_at	ARL4C	694	0.0085	1.6	Bad
202206_at	ARL4C	841	0.0099	1.6	Bad
201849_at	BNIP3	1487	0.018	1.8	Bad
208683_at	CAPN2	16155	0.039	1.5	Bad
209795_at	CD69	1646	0.00068	2.1	Bad
223046_at	EGLN1	3314	0.028	1.6	Bad
201231_s_at	ENO1	7048	0.00062	2.4	Bad
200859_x_at	FLNA	1414	0.0015	2	Bad
208308_s_at	GPI	5927	0.0071	1.8	Bad
202934_at	HK2	1079	0.00084	1.9	Bad
203914_x_at	HPGD	314	0.024	1.7	Bad
212192_at	KCTD12	563	0.0065	1.7	Bad
227021_at	KDM1B	404	0.012	1.8	Bad
222572_at	PDP1	1701	0.011	1.7	Bad
201037_at	PFKP	244	0.012	1.9	Bad
217356_s_at	PGK1	5760	0.0023	1.8	Bad
201251_at	PKM	2388	0.05	1.4	Bad
219014_at	PLAC8	9612	0.02	1.8	Bad
218984_at	PUS7	982	0.00025	2	Bad
223168_at	RHOU	529	0.015	1.8	Bad
200872_at	S100A10	4600	5.70E-05	2.3	Bad
203186_s_at	S100A4	3389	0.00089	2	Bad
200822_x_at	TPI1	5484	0.0021	1.8	Bad
213011_s_at	TPI1	5411	0.0092	1.6	Bad
201426_s_at	VIM	30262	0.00014	2.5	Bad
243764_at	VSIG1	87	0.04	1.7	Bad
228280_at	ZC3HAV1L	1915	0.029	1.7	Bad

Table 3. REACTOME analysis of genes significantly overexpressed in hypoxic PBs compared with normoxic PBs and associated with an adverse prognosis in MM.

GeneSet	Ratio of genes in GenSet	Number of genes in GeneSet	Genes from Network	P-value	FDR	Nodes
Glycolysis / Gluconeogenesis(K)	0,0069	67	8	0,0000	3.94E-11	ALDOA,HK2,PKM,TPI1,ENO1, PFKP,GPI,PGK1
Carbon metabolism(K)	0,0109	106	8	0,0000	7.40E-10	ALDOA,HK2,PKM,TPI1,ENO1, PFKP,GPI,PGK1
HIF-1-α transcription factor network(N)	0,0068	66	7	0,0000	1.02E-09	ALDOA,HK2,BNIP3,EGLN1,PKM, ENO1,PGK1
Biosynthesis of amino acids(K)	0,0076	74	6	0,0000	9.54E-08	ALDOA,PKM,TPI1,ENO1,PFKP, PGK1
Metabolism of carbohydrates(R)	0,0229	223	8	0,0000	9.54E-08	ALDOA,HK2,PKM,TPI1,ENO1, PFKP,GPI,PGK1
Fructose and mannose metabolism(K)	0,0034	33	4	0,0000	6.68E-06	ALDOA,HK2,TPI1,PFKP
HIF-1 signaling pathway(K)	0,0109	106	5	0,0000	1.84E-05	ALDOA,HK2,EGLN1,ENO1,PGK1
Pentose phosphate pathway(K)	0,0029	28	3	0,0000	2.08E-04	ALDOA,PFKP,GPI

gene expression profiles, and functional behavior.[10] In mice, hypoxic parts of germinal centers are associated with accelerated class switch recombination and antibody secretion.[9]

Thus, the generation of PCs from B cells occurs in hypoxic conditions *in vivo* and the relevance of O_2 pressure in PC generation remains unknown. We therefore sought to evaluate the impact of decreased oxygen partial pressures in plasma cell generation.[3,6,11]

Results

Bone marrow microenvironment is physiologically hypoxic. This hypoxic microenvironment is important for normal bone marrow hematopoiesis. We investigated the impact of decreased oxygen partial pressures in plasma cell generation using our *in vitro* model[3,6,11,12] at each step (day 4, day 7 and day 10) of B cell differentiation (Fig. 1A). Five percent oxygen (hypoxia) culture compared with 20% oxygen (normoxia) led to an increased generation of $CD20^{-}CD38^{-}$ prePBs and $CD20^{-}CD38^{+}$ PBs at day 7 starting from MBCs by triggering CD40 and Toll-like receptor 9 and adding IL2, IL10 and IL15 ($P = 0.04$). A 2-fold increase in $CD20^{-}CD38^{+}$ PB generation was maintained at day 10 ($P = 0.002$) (Fig. 1A). Of note, the increase in PBs count under hypoxic conditions resulted in a significant increase in the generation of $CD138^{+}$ PCs at day 10 ($P = 0.05$) (Fig. 1A). Reduced PO_2 pressure (1% or 3%) did not increase further PB generation, but on the contrary led to decreased cell survival (Fig. S1). Using CFSE incorporation at day 0 and monitoring its level throughout the differentiation process, cells growing in hypoxia were roughly one division ahead of the normoxic cells at day 7 (Fig. 1B). Moreover, these cells continued cycling at day 10, unlike the normoxic ones, explaining the increase in PB cell count until day 10.

Analyzing gene expression profiles of sorted $CD20^{-}CD38^{+}$ PBs at day 7, 175 genes were differentially expressed between normoxic and hypoxic PBs (fold change ≥ 1.5, $P \leq 0.0001$, Supplementary Tables S1 and S2) including 74 genes with increased expression and 101 with a significant downregulation in hypoxic condition. Reactome analysis demonstrated a significant enrichment of genes involved in HIF1α and HIF2α transcription factor network, metabolism and cancer related pathways in hypoxic PB compared with normoxic ones (Table 1).

HIF1α and HIF2α are the main regulators of oxygen homeostasis. When complexed with ARNT, they activate target gene transcription involved in glycolysis, iron transport, cell proliferation and survival and angiogenesis such as vascular endothelial growth factor (VEGF).[13,14] Among the genes significantly upregulated in hypoxic PBs, *HIF2A (EPAS1)* (fold change = 5.3) and *VEGFA* (fold change = 1.5) were identified (Supplementary Table S1 and Fig. S2A) and validated by quantitative RT-PCR (Supplementary Fig. S2B). No significant changes in *HIF1A* and *ARNT* gene expression was identified between normoxic and hypoxic PBs (Supplementary Fig. S2A). Cancer related pathways were also enriched in hypoxic PB (Table 1). Interestingly, HIF2α was shown to promote cell-cycle progression of hypoxic VHL-deficient renal cell carcinoma cells through c-Myc activation.[15] Therefore, a significant induction of *c-MYC* gene expression was identified in hypoxic PBs (fold change = 1.9) compared with normoxic PBs (Fig. S3). *c-MYC* upregulation in hypoxic conditions was validated by quantitative RT-PCR (Supplementary Fig. S3).

Hypoxic PBs are characterized by *CD19* (2.1-fold), CD69 (1.9-fold), CD52 (1.6-fold) and *HLA-DQB1* B cell markers overexpression (11.5-fold) suggesting hypoxic PBs are less differentiated than normoxic ones.

Normoxic PB overexpressed genes are significantly enriched in mitotic genes and nucleosome assembly (Supplementary Table 3).

A score equal to the sum of standardized expressions of these 175 genes differentially expressed between hypoxic and normoxic PBs was high in germinal center or bone marrow sorted primary samples (centroblasts, centrocytes and BMPCs) compared with blood circulating cells and cells that have undergone normoxic differentiation *in vitro* (naive B cells, MBCs and in vitro PCs, Supplementary Fig. S4).

Discussion

Altogether, our data demonstrated that hypoxia increases the generation of PBs starting from MBCs by activating cell cycle and division number. Hypoxic PBs are less differentiated than normoxic PBs and continue cycling at day 10, favoring the

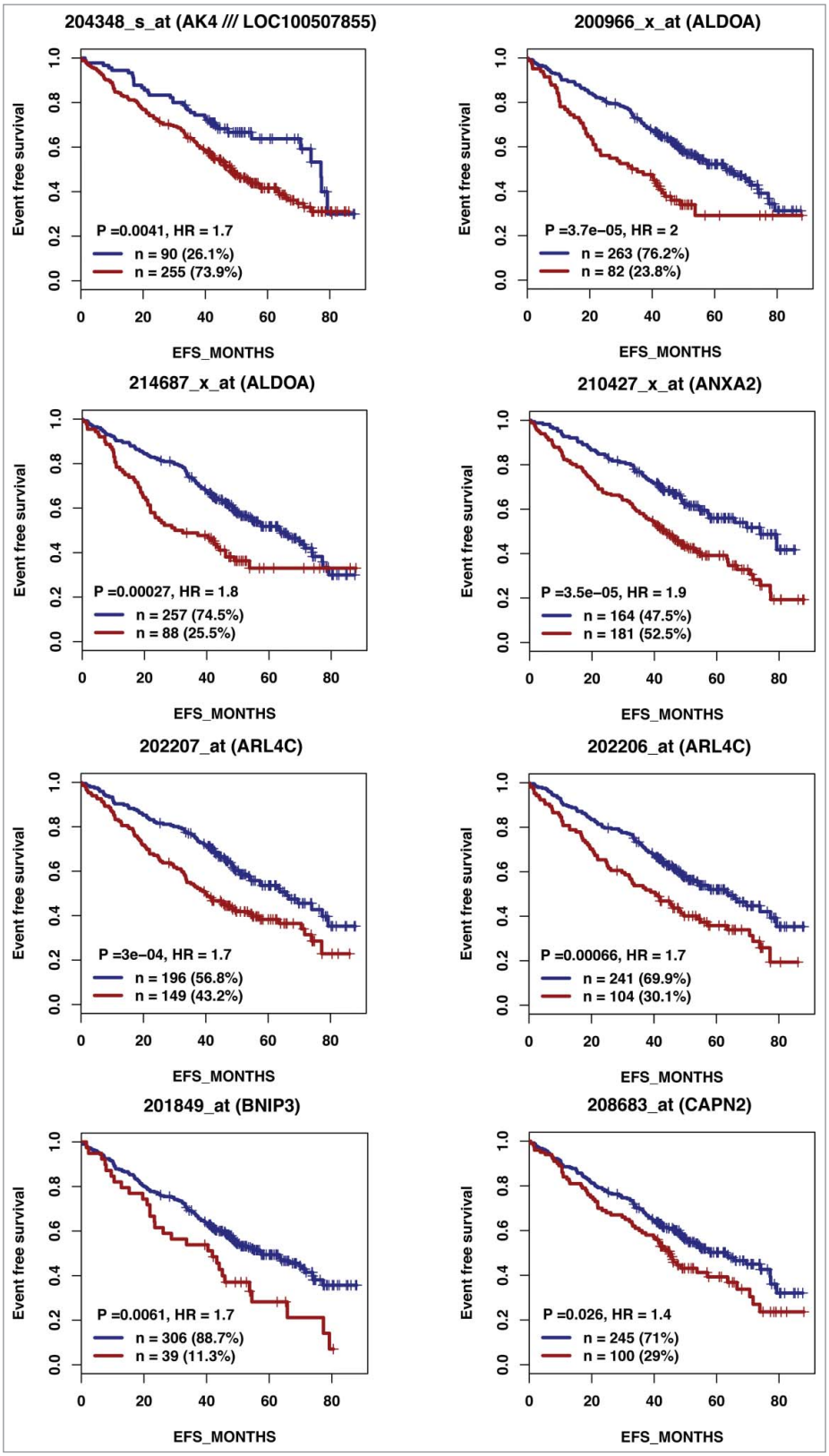

Figure 2A. The expression of 28 out of the 74 genes overexpressed in hypoxic PBs compared with normoxic ones is associated with a poor prognosis in MM. High expression of 28 out of the 74 genes induced by hypoxia in PBs could predict for shorter overall and event free survival in myeloma patients. Patients of the LR-TT2 cohort (n = 345) were ranked according to increasing gene expression and a maximum difference in event free survival (EFS) was obtained using the Maxstat R function.

generation of PCs *in vitro*. Our results are in agreement with recent data revealing a critical role of hypoxia in germinal center B cell differentiation.[9] We also confirmed that the genes upregulated in hypoxic PBs are also significantly overexpressed in germinal center B cells compared with circulating naive B cells (Supplementary Fig. S5). Furthermore, hyperoxia induced a significant inhibition of germinal center response and antibody production after immunization in mice.[9]

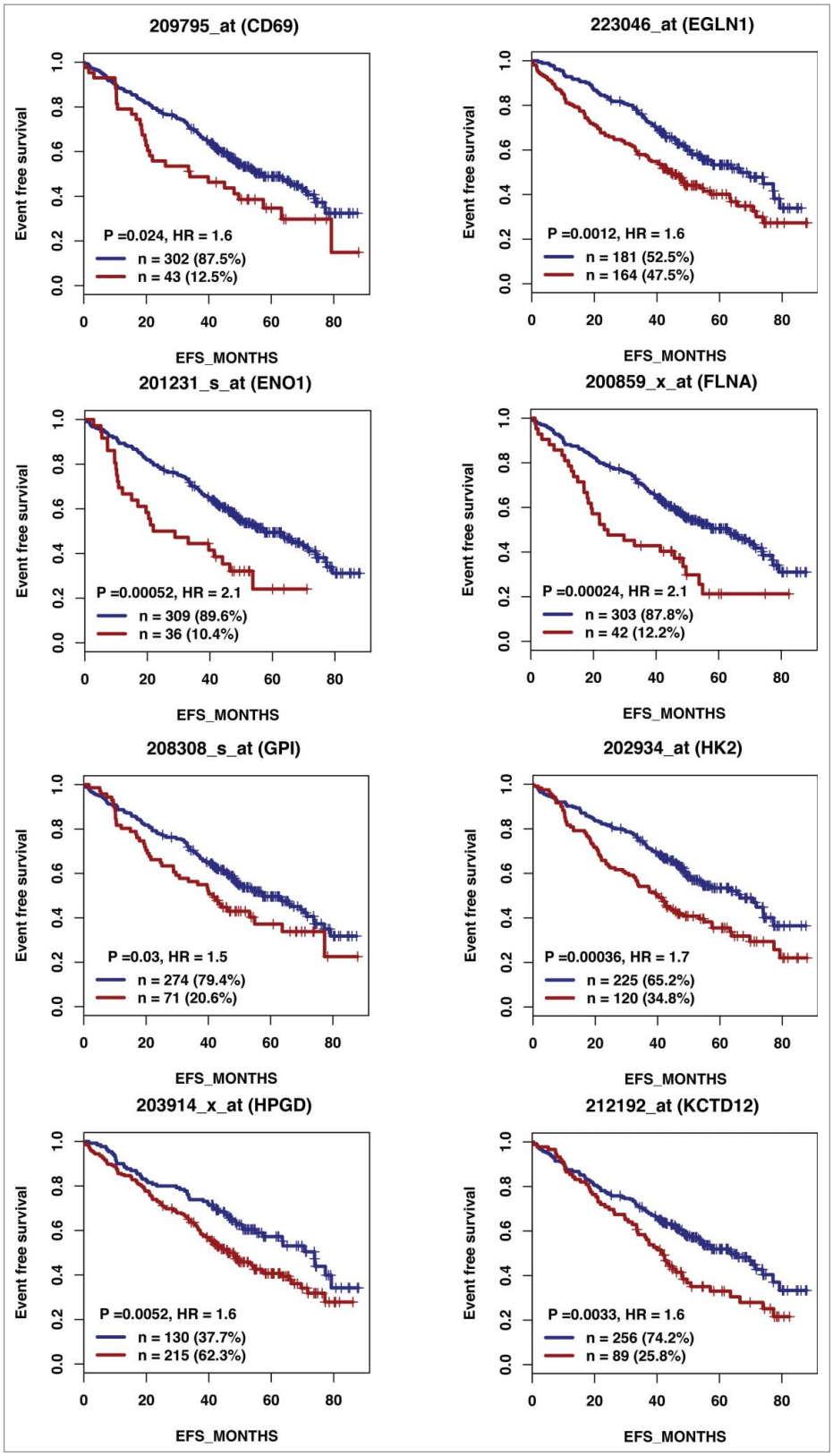

Figure 2A. (Continued).

According to the role of hypoxia in supporting PB generation through cell cycle activation, it could be involved in plasma cell tumorigenesis or autoimmune diseases. HIF1α and HIF2α immunostaining in bone biopsies from multiple myeloma (MM) patients demonstrated a high level of HIF1α in 33% and of HIF2α in 13% of the cases.[16] Furthermore, overexpression of HIF1α and HIF2α in MM cells, resulted in significant induction of MM-induced angiogenesis in xenograft model.[17] Hypoxic PBs are characterized by c-*MYC* and *HIF2A* gene activation in association with an induction of genes

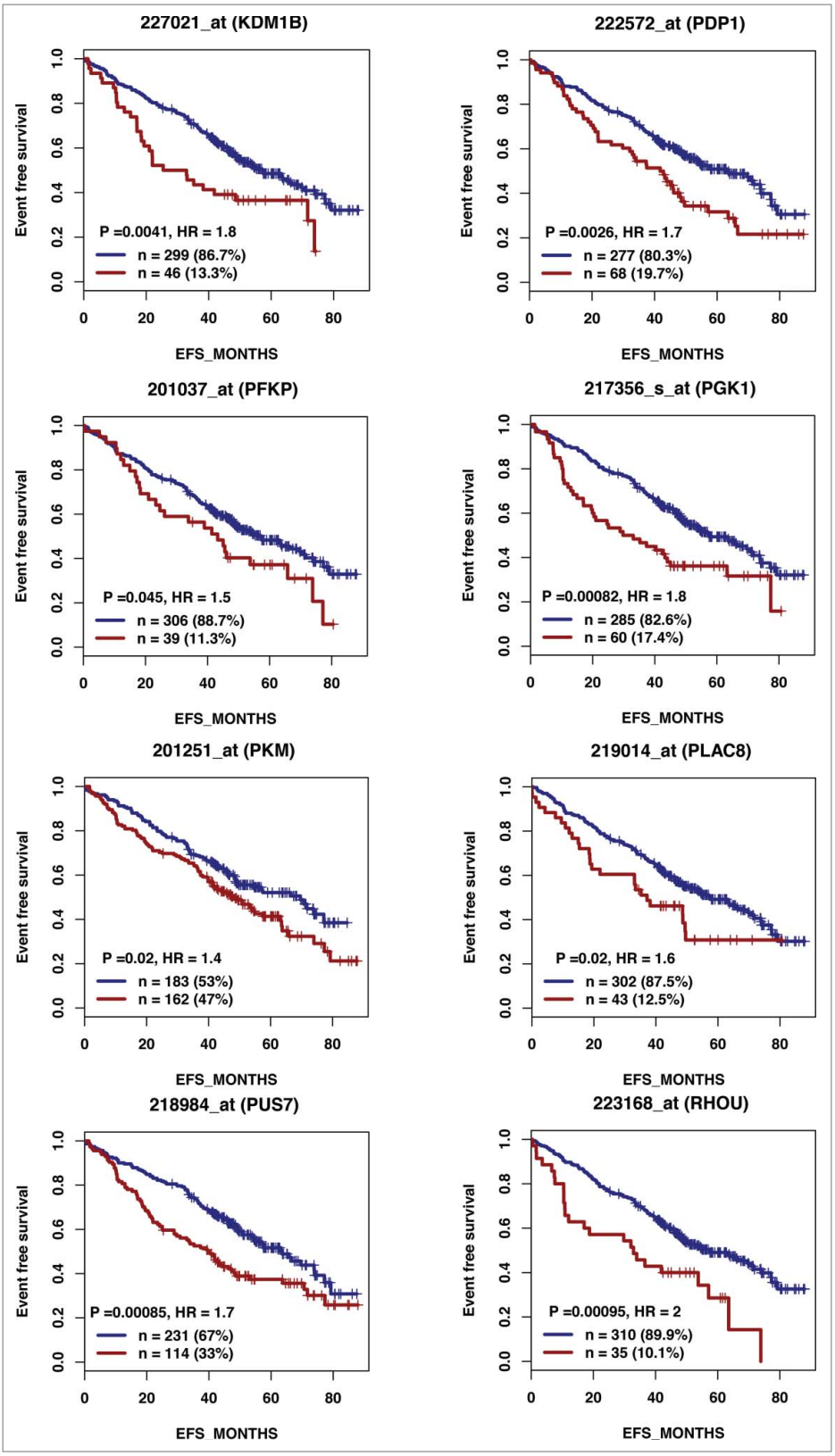

Figure 2A. (Continued).

enriched in metabolism and cancer pathways. Abnormal activity of several transcription factors including c-MYC have been implicated in MM development.[18,19] HIF2α can promote c-MYC-induced transcriptional modifications through binding and stabilization of MYC-MAX heterodimer.[15] These support the role of HIF2α in driving hypoxia-dependent proliferation.[15] Interestingly, a significant enrichment ($P < 0.0001$) of genes associated with a poor prognosis (event free survival and overall survival), in newly diagnosed MM patients (LR-TT2 cohort, n = 345), was identified among genes overexpressed in

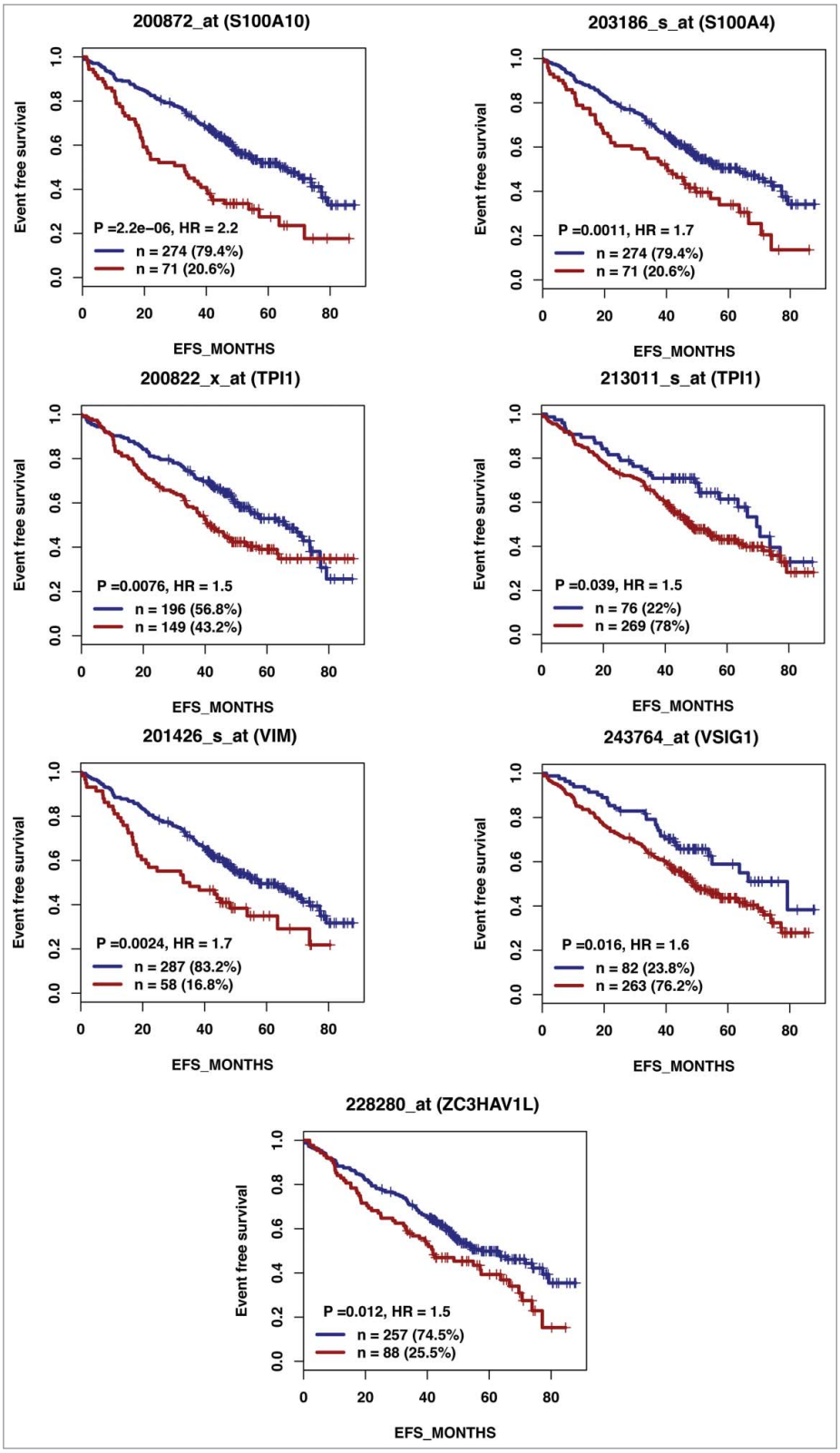

Figure 2A. (Continued).

hypoxic PBs (Table 2A and B and Fig. 2 A and B). These genes are significantly enriched in genes involved in HIF signaling pathway and glycolysis (*ALDOA, HK2, PKM, TPI1, ENO1, PFKP, GPI* and *PGK1*) (Table 3). Hypoxia-induced metabolic re-writing is known to be involved in tumorigenesis and cancer progression.[20] Furthermore, altered tumor cell metabolism is essential for MM cell drug resistance.[21] Annexin A2 (ANXA2) and KDM1B were also identified among the genes induced by hypoxia in PBs and associated with an adverse prognosis in MM (Fig. 2). ANXA2 is known to promote MM cell growth and survival, stimulate angiogenesis and increase osteoclast formation.[22-25] In MM, high *ANXA2* expression is associated

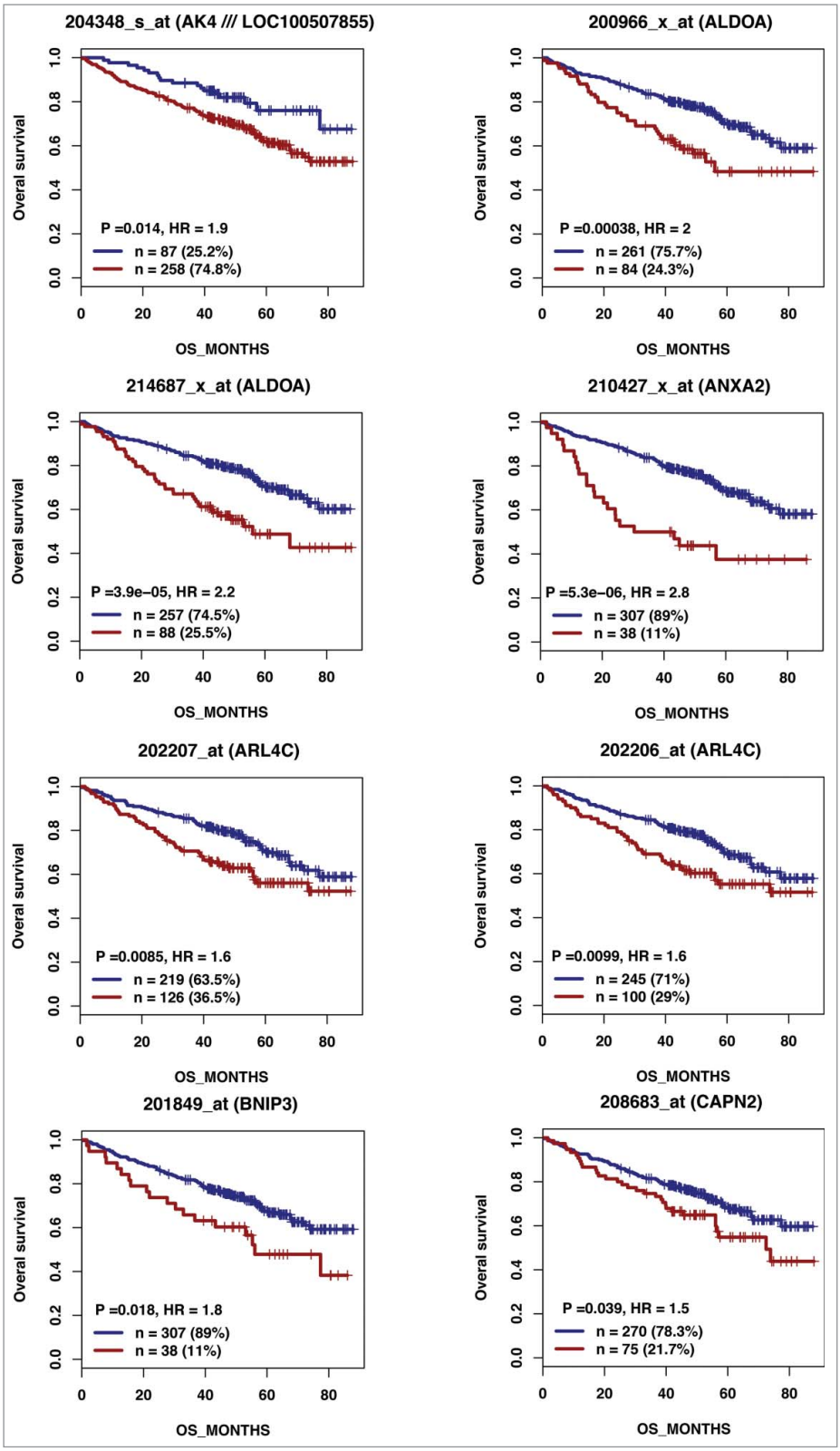

Figure 2B. The expression of 28 out of the 74 genes overexpressed in hypoxic PBs compared with normoxic ones is associated with a poor prognosis in MM. High expression of 28 out of the 74 genes induced by hypoxia in PBs could predict for shorter overall survival in myeloma patients. Patients of the LR-TT2 cohort (n = 345) were ranked according to increasing gene expression and a maximum difference in overall survival (OS) was obtained using the Maxstat R function.

with an adverse prognosis, high-risk proliferation and high-risk gene expression-based signatures.[26] KDM1B is a lysine-specific protein demethylase[27] that functions as an enhancer of gene transcription by modulating H3K4 methylation.[28] Interestingly, KDM1B was shown to control NFκB target gene activation,[29] a pathway playing a critical role in MM survival and proliferation.[30,31] Our data underline a potential role of hypoxia in plasma cell tumorigenesis through c-MYC activation, metabolic modifications and cancer related pathways induction.

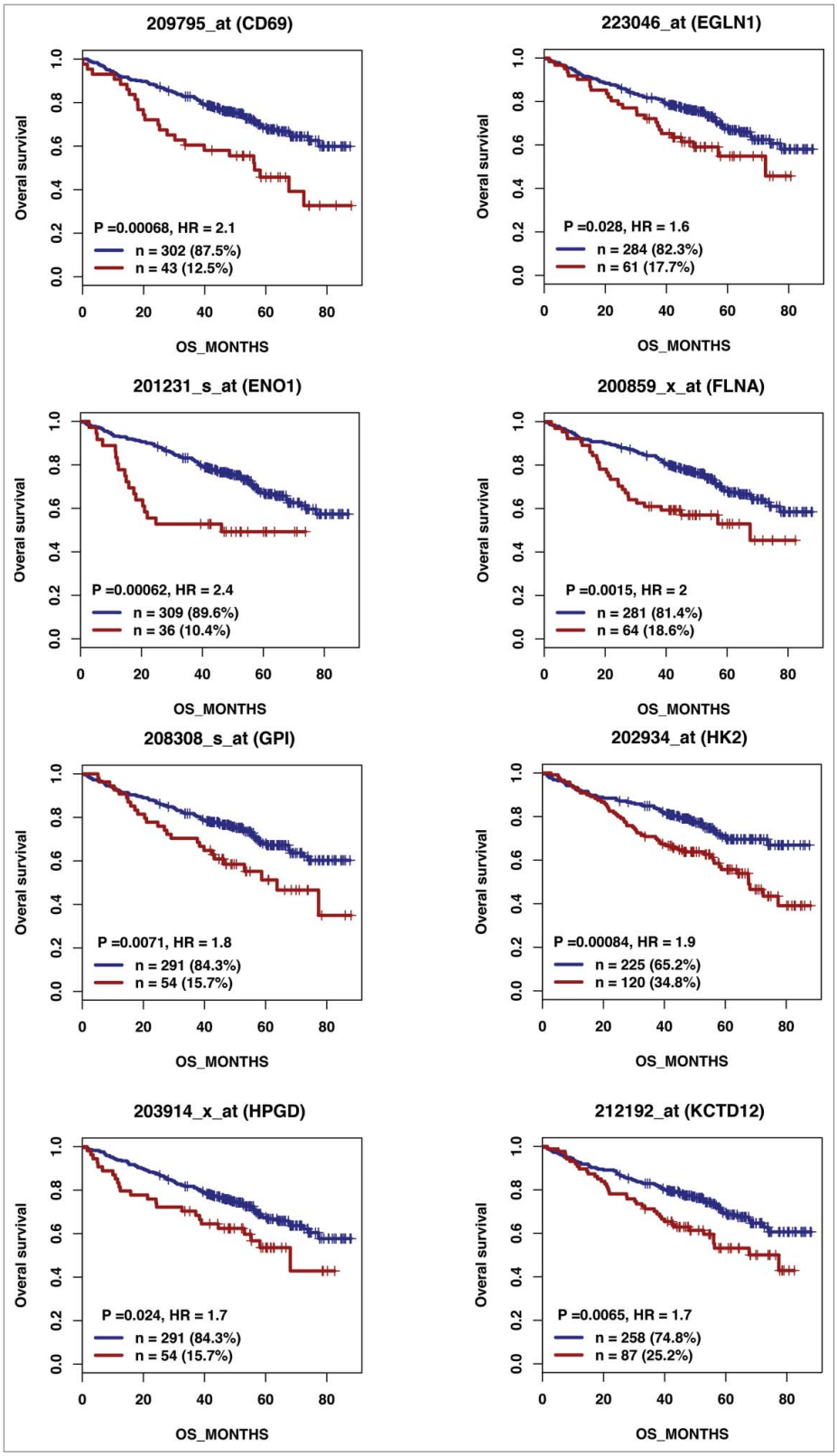

Figure 2B. (Continued).

Materials and methods

Cell cultures

Peripheral blood cells from healthy volunteers were purchased from the French Blood Center (Toulouse, France) and $CD19^+$ $CD27^+$ MBCs purified (> 95% purity) as described.[11] Standard normoxic culture conditions comprised 21% O2, 5% CO_2 and 37°C. Hypoxic culture conditions (5% O_2, 5% CO_2; 37°C) were obtained by oxygen displacement by nitrogen using the muti-Gas MCO-5M-PE Panasonic Biomedical incubator (Avons, France). PCs were generated as reported.[3,6] All cultures were performed in Iscove's modified Dulbecco medium (IMDM,

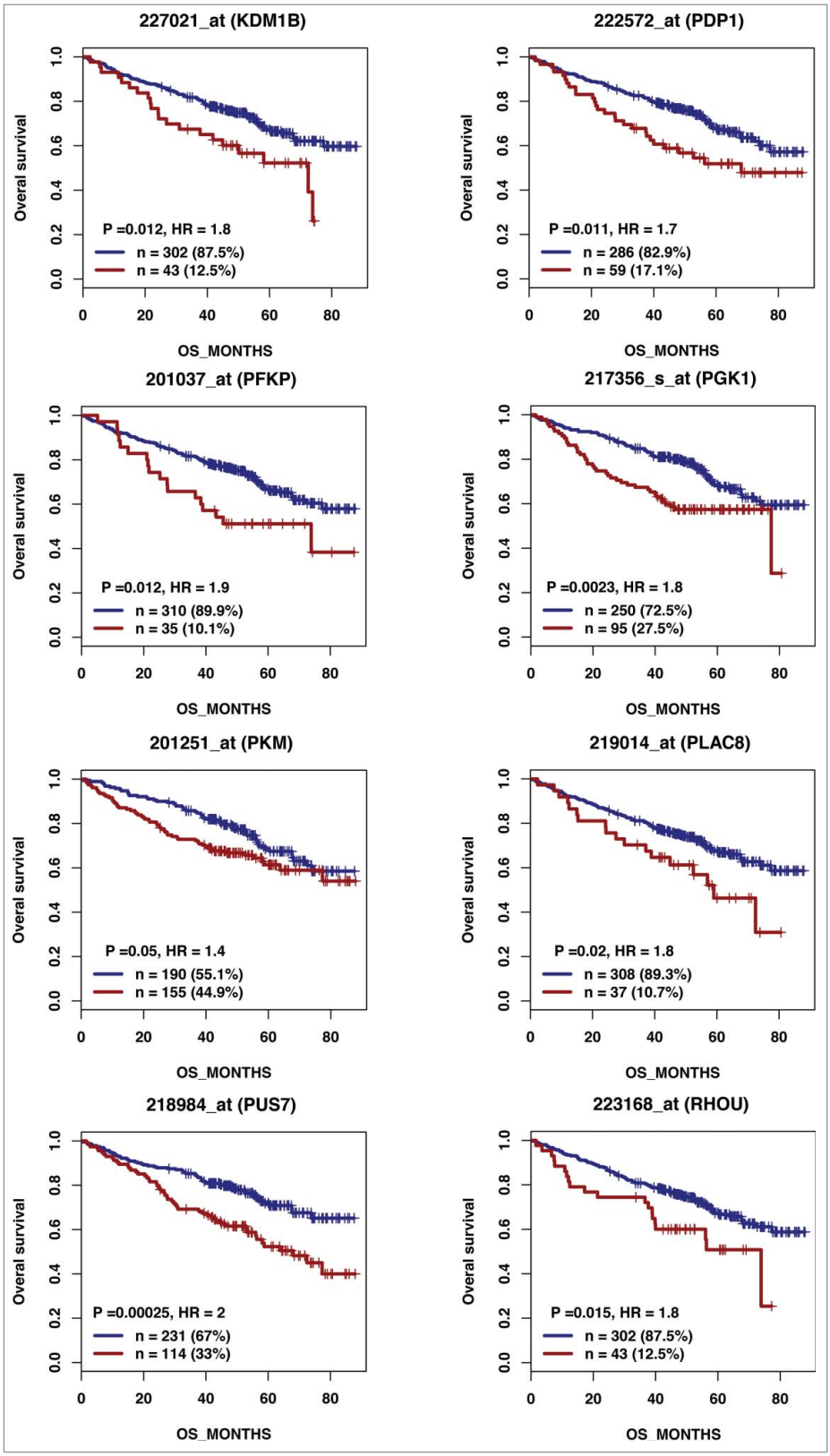

Figure 2B. (Continued).

Invitrogen) and 10% FCS.[3,6,11] Purified peripheral blood MBCs (1.5×10^5/ml) were activated for 4 d by CpG oligodeoxynucleotide and CD40 ligand (sCD40L) −10 μg/ml of phosphorothioate CpG oligodeoxynucleotide 2006 (Sigma), 50 ng/ml histidine tagged sCD40L, and anti-poly-histidine mAb (5 μg/ml), (R&D Systems) - with IL-2 (20 U/ml), IL-10 (50 ng/ml) and IL-15 (10 ng/ml) in 6-well culture plates. PBs were generated by removing CpG oligonucleotides and sCD40L and changing the cytokine cocktail (IL-2, 20 U/ml, IL-6, 50 ng/ml, IL-10, 50 ng/ml and IL-15, 10 ng/ml). PBs were differentiated into PCs adding IL-6 (50 ng/ml), IL-15 (10 ng/ml) and IFN-α (500 U/ml) for 3 d.

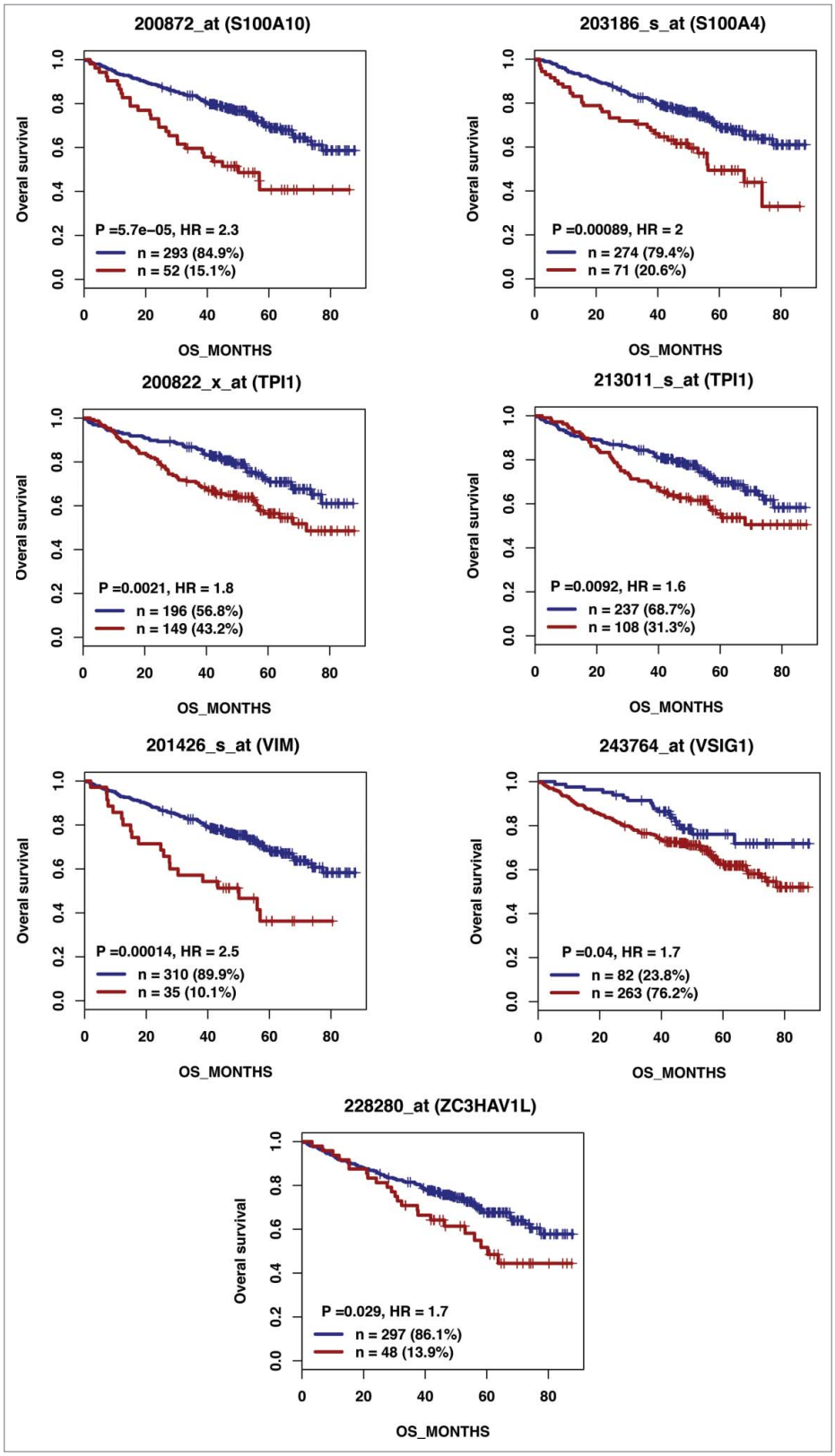

Figure 2B. (Continued).

Reagents

Human recombinant IL-2 was purchased from R&D Systems (Minneapolis, MN), IFN-α (IntronA) from Merck Canada Inc. (Kirckland, Canada), IL-6 and IL-15 from AbCys SA (Paris, France), IL-10, from Peprotech (Rocky Hill, NJ, USA). Mouse (or rat when indicated) mAbs conjugated to allophycocyanin (APC), fluorescein isothiocyanate (FITC), peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5), phycoerythrin (PE), specific for human CD19 (clone

HIB19), CD27 (clone M-T271), CD38 (clone HIT2) and CD138 (clone RF8B2) were purchased from BD Biosciences (Le Pont De Claix, France); CD20 (clone B9E9) from Beckman Coulter (Fullerton, CA).

Real-time RT-PCR

RNA was converted to cDNA using the Qiagen QuantiTect Rev. Transcription Kit (Qiagen, Hilden, Germany). The assays-on-demand primers and probes and the TaqMan Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). The measurement of gene expression was performed using the Roche LC480 Sequence Detection System. For each primer, serial dilutions of a standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve to assess PCR efficiency. Ct values were obtained for *GAPDH* and the respective genes of interest during log phase of the cycle. Gene expression was normalized to that of *GAPDH* (dCt = Ct gene of interest – Ct GAPDH) and compared with the values obtained for a known positive control using the following formula: 100/2ddCt where ddCt = dCt unknown – dCt positive control.

Microarray hybridization and bioinformatics analysis

RNA was extracted and hybridized to human genome U133 Plus 2.0 GeneChip microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Gene expression data are available on GenomicScape (http://www.genomicscape.com).[32] Gene expression data were normalized using MAS5 algorithm and analyzed with GenomicScape (http://www.genomicscape.com).[32] Gene expression profiles (GEP) of hypoxic and normoxic plasmablasts were analyzed with Affymetrix GCOS (GeneChip Operating Software) software using Wilcoxon test and Benjamini-Hochberg multiple testing correction and a fold change of 1.5.[33] The molecular pathways, encoded by the genes differentially expressed between hypoxic and normoxic PBs, were pointed out using reactome FI (Functional Interaction) cytoscape plugin (http://wiki.reactome.org/index.php/ Reactome_FI_Cytoscape_Plugin). Pathways significantly enriched in a given cell subpopulation with a FDR < 0.001 are selected.

GEP of MMCs were obtained from large patients' cohort from the University of Arkansas for Medical Sciences (UAMS, Little Rock, USA, cohort treated with total therapy 2, N = 345) (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE2658). Centroblast and centrocytes GEP are publicly available from GEO database (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE15271) and naive B cell GEP from ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/; accession number E-MTAB-1771).

Statistical analysis

Statistical comparisons were made using a student's t-test on Prism software. *P* values ≤ .05 were considered as significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

MS performed the experiments and wrote the paper. MJ developed the in vitro model for plasma cell generation. AB participated in research. AK provided help for microarray analysis and wrote the paper.

BK and JM are the senior investigators who designed research and wrote the paper.

ORCID

Matthieu Schoenhals http://orcid.org/0000-0001-8928-8669
Jérôme Moreaux http://orcid.org/0000-0002-5717-3207

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