

ORIGINAL ARTICLE

CD93 is Selectively Expressed on Human Myeloma Cells but Not on B Lymphocytes

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ABSTRACT

Background: CD93 has originally been known as a C1q receptor, and many studies have demonstrated that CD93 is expressed on hematopoietic stem cells, B cell progenitors, myeloid and monocytic cells. Moreover, CD93 is shown to be expressed on long-lived plasma cells, and CD93 deficient-mice display an impairment in plasma cell development. **Objective:** To investigate the expression of CD93 on multiple myeloma (MM) cells. **Methods:** Human MM and B cell lines were cultured, and the expression of CD93 was examined on these cells by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and Fluorescence Activated Cell Sorting (FACS). In addition, CD19⁺ primary B cells and CD19⁻/CD138⁺ primary MM cells were isolated by MACS columns, and CD93 expression was further analyzed on these cells. **Results:** qRT-PCR data showed that CD93 expression at mRNA level was much higher in MM cell lines compared with B cell lines. In addition, MM cell lines expressed a higher amount of surface CD93 at protein level compared with B cell lines. More importantly, CD93 expression was significantly higher in CD19⁻/CD138⁺ primary MM cells than in CD19⁺ primary B cells isolated from the bone marrow of patients with MM. **Conclusion:** We demonstrated that CD93 is expressed on myeloma cells and, that CD93 could play a key role in the pathogenesis of MM. Further studies are necessary to explore this possible role.

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INTRODUCTION

CD93 is a Type I transmembrane glycoprotein that has been implicated in the regulation of cell-cell interaction and phagocytosis. It was initially identified as a receptor for complement component 1, subcomponent q phagocytosis (C1qRP) and was shown to be involved in C1q-enhancement phagocytosis (1-3). Although CD93 deficient-mice display an impairment in uptake apoptotic cells *in vivo*, they are viable and show no major developmental abnormalities (4). A previous study reported that CD93 was expressed on endothelial cells, platelets, circulating monocytes and granulocytes and stem cells, suggesting that this molecule may have certain important biological activities in the body (5). The expression of CD93 on granulocytes and monocytes has further been shown to be upregulated by inflammatory peptide FMLP (6,7). Interestingly, CD93 defines the earliest human bone marrow (BM) stem cells because it is expressed on both CD34-positive and -negative hematopoietic stem cells. CD93 is a common marker for early development (pre-B cells) of murine B cells lineages (8,9). In the line with these observations, we have previously shown that CD93 is expressed on CD34+ cells from human cord blood, BM and granulocyte-stimulating colony stimulating (G-CSF)-mobilized peripheral blood. In contrast to *ex-vivo* expanded granulocytes and megakaryocytes, which express CD93 at high levels, erythroid progenitors do not express CD93 (10). Furthermore, a recent study has demonstrated that CD93 is a functional marker for leukemic stem cells in mixed-lineage leukemia (MLL)-rearranged of AML subtype and it is required for MLL leukemogenesis and AML maintenance (11). Although CD93 is expressed at the early development of B cells in BM, it downregulates following maturation in the spleen. However, CD93 is re-induced during plasma differentiation in BM and is essential for the maintenance of long-lasting plasma cells. In addition, CD93 deficient-mice were unable to maintain the numbers of plasma cells and antigen-specific immunoglobulins levels following immunization with T-cell dependent antigens, indicating that CD93 may play a crucial role in differentiation and survival of plasma cells (12,13). However, the expression of this marker on multiple myeloma (MM) cells, as malignant plasma cells with a long-lasting life span, has not yet been elucidated. Herein, we show that CD93 is selectively expressed on MM cell lines and primary MM cells isolated from patients with MM.

MATERIALS AND METHODS

Cell Culture and Isolating Primary Cells. The MM cell lines (U266, Karpas-707) and B cell lines (NC37, Nalm-6, Raji) were purchased from Iran Pasteur Institute (Tehran, Iran) and were cultured in RPMI-1640 (Gibco, Manchester, UK) containing 10% FBS, at 37°C in a humid incubator with 5% CO₂. All cells were sub-cultured when they reached approximately 80% confluence. The BM aspirations were carried out from 6 patients with MM referred to Hemato-Oncology Ward of Tohid Hospital (Sanandaj, Iran), whose demographic characteristics are listed in Table 1. All samples were taken after an informed consent was obtained from each subject. In order to sort the primary B cells and MM cell, light-density mononuclear cells (MNCs) were separated from the BM, by density gradient centrifugation over Ficoll Paque Plus (Amersham Biosciences). MNCs were incubated with human CD19 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and kept on ice for 15 min. After that, MNCs were passed through MS

separation MACS columns as stated by manufacturer's instruction (Miltenyi Biotec). Next, the CD19-negative fractions were incubated with human CD138 microbeads (Miltenyi Biotec) as described above and CD19⁻/CD138⁺ primary MM cells were isolated by MS separation columns. As determined by fluorescence activated cell sorter (FACS) analysis, the purity of the isolated CD19⁺ and CD19⁻/CD138⁺ cells was greater than 95%.

Table 1. Demographic characteristic of MM patients.

	Number	Mean \pm SD
Number of case	6	-
Gender (Female/male)	1/5	-
Age		73.83 \pm 11.03
Hgb (g/dL)		10.82 \pm 2.19
WBC (10 ³ /mL)		6.22 \pm 2.96
RBC (10 ⁶ /mL)		3.59 \pm 0.63
ESR (mm/hr)		85 \pm 22.13
PLT (10 ³ /mL)		146.33 \pm 88.75
Ca (mg/dL)		8.7 \pm 0.5
T.Protein (g/dL)		8.25 \pm 1.69
Alb (g/dL)		3.45 \pm 0.73
β 2M (mg/dL)		4.16 \pm 1.17

FACS Analysis. In order to determine the surface CD93 expression, cell lines and primary cells were stained as previously reported (14). Cells were washed three times with cold FCM buffer (PBS containing 1% BSA) and incubated with 10 μ L of mouse anti-human CD93-PE (Biolegend, London, United Kingdom) or isotype antibody (Dako, Tehran, Iran) at 4° C for 45 min; subsequently, these cells were washed three times with FCM buffer, fixed in 1% paraformaldehyde, and subjected to flow cytometric analysis (FACSCalibur, Beckman Dickinson, San Jose, CA). FACS data were analyzed by FCM Express, Glendale, (CA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Total RNA from cell lines or primary cells were isolated using NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and mRNA was transcribed into cDNA by use of PrimeScript™ RT reagent Kit (Takara, Japan). qRT-PCR was performed by Corbett rotor gene 6000 Real-Time PCR system (Corbett Research, Australia) and carried out using SYBR Green dye detection protocol. Nucleotide sequences for human β -actin (housekeeping gene) and CD93 were obtained from Gen Bank (NCBI) and used to design the following primers; human CD93 sense: AACCAGTACAGTCCGACAC, antisense: CCAACACACAGCTTCCA and human β -actin sense: AGATCATTGCTCCTCCTGAG, antisense: AGTCATAGTCCGCCTAGAAG). Relative quantitation of CD93 mRNA expression was calculated using the comparative CT method (15). The relative quantitative value of mRNA expression for CD93 was normalized to endogenous housekeeping gene, β -actin, and calculated relative expression values.

Statistical Analysis. Data are presented as mean value \pm SD. Comparisons between the expression of CD93 in MM and B cells were made by Student *t test*, where values below 0.05 were considered statistically significant.

RESULTS

CD93 is selectively expressed on MM cell lines.

In order to determine the expression levels of CD93 on MM cell lines, we examined its expression on two MM cell lines (U266, Karpas-707) and three B cell lines (NC37, Nalm-6, Raji) by qRT-PCR. Our data showed that CD93 expression was significantly (50 time) higher in myeloma cells (Figure 1). The expression of CD93 on these cells was further examined at the protein levels by employing FACS analysis. Our FACS data demonstrated that CD93 was expressed at a higher level on MM in comparison with B cell lines (Figure 2A). The mean fluorescent intensity (MFI) of CD93 expression in MM cells was 9 times higher than that in B cell lines (Figure 2B), corroborating our qRT-PCR data.

CD93 expression in CD19⁺ primary B cells and CD19⁻/CD138⁺ primary MM cells.

So as to determine the expression of CD93 in primary MM cells, the CD19⁻/CD138⁺ primary MM cells and CD19⁺ primary B cells were isolated, via MACS, from the BM of the patients with MM.

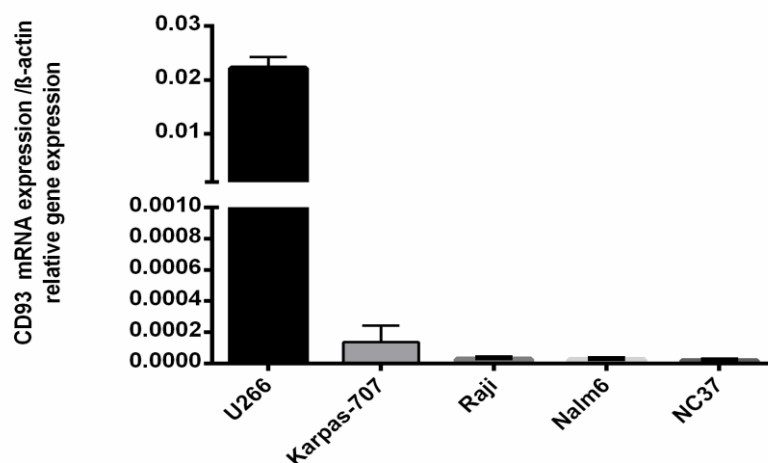


Figure 1. CD93 expression at mRNA level in MM cells lines. Human MM cell lines (U266, Karpas-707) and B cell lines (NC37, Nalm-6, Raji) were cultured as described in “materials and methods”. The expression of CD93 was determined by qRT-PCR and normalized to endogenous β -actin. Data are expressed as mean \pm SD; *, $p < 0.05$.

As shown in Figure 3, the expression level of CD93 was significantly higher (2 times) in primary CD19⁻/CD138⁺ MM cells compared with CD19⁺ B cells. The expression of CD93 on CD19⁻/CD138⁺ MM cells was further analyzed by flow cytometry; as is shown in Figure 4, CD93 was expressed on the surface of at least 26% of the primary MM cells, yet not the normal lymphocytes (Figure 4B, F). Moreover, CD93 was strongly expressed on monocytes and granulocytes (Figure 4C, D), confirming the previous data (1).

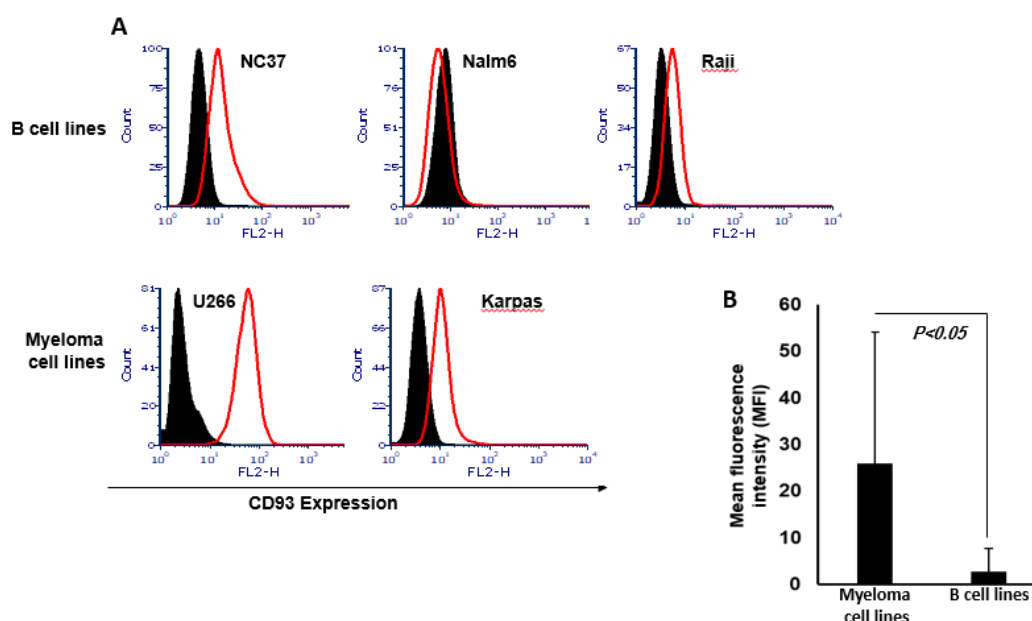


Figure 2. CD93 expression at protein level on MM cells. (A) The expression of CD93 on MM and B cell lines were analyzed by FACS analysis. Cells were stained with mouse anti-human CD93-PE or isotype-PE antibody and CD93 expression analyzed by FACSCalibur. Filled black and red histogram represent isotype antibody and CD93, respectively. (B) Shows mean fluorescent intensity (MFI) of FACS analysis of CD93 expression in MM and B cell lines.

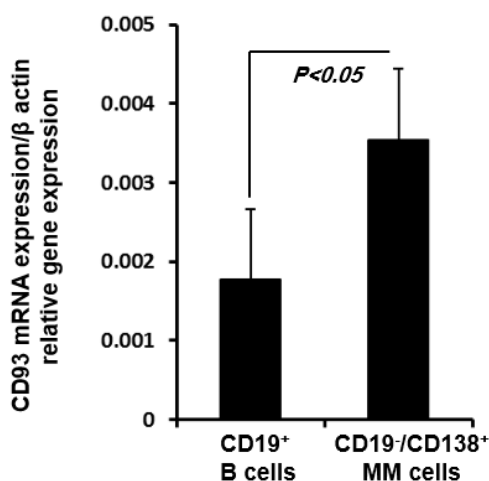


Figure 3. CD93 expression is higher in primary MM cells than B lymphocytes. CD19⁺ primary B cells and CD19⁻/CD138⁺ primary MM cells were isolated from BM aspiration of six patients with MM. The expression of CD93 was determined by qRT-PCR and normalized to endogenous β-actin. Data are expressed as mean ± SD; *, $p < 0.05$.

DISCUSSION

The expression of CD93 on MM cells has not been reported yet. In this research, on the other hand, it was shown that CD93 is expressed both on MM cells and primary MM cells.

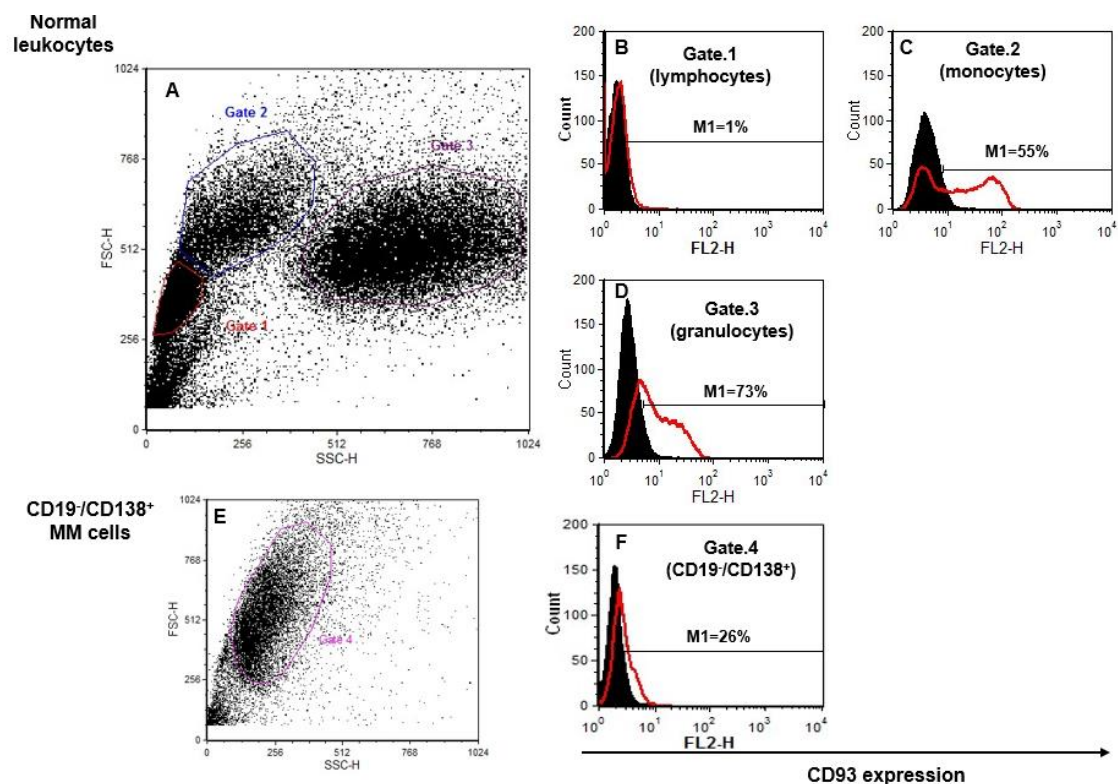


Figure 4. CD93 expression on normal peripheral blood and CD19⁻/CD138⁺ primary MM cells. Peripheral blood samples were taken from three health normal donors. RBC were lysed and then cells stained with either mouse anti-human CD93-PE or isotype-PE antibody and CD93 expression analyzed by FACSCalibur. **(A)** Demonstrate a representative dot plots depicts forward scatter (FSC) and side scatter (SSC) of normal leukocytes and the corresponding gates. The expression of CD93 is shown on lymphocytes **(B)**, monocytes **(C)** and granulocyte **(D)**. **(E)** Shows a representative sample of isolated CD19⁻/CD138⁺ primary MM cells and **(F)** demonstrates CD93 expression on these cells. Filled black and red histogram represent isotype antibody and CD93, respectively.

Chevrier *S et al.* have recently shown that although CD93 is a marker for early B cell development, its expression is re-induced during plasma cell differentiation. CD93 is strongly expressed on long-lived plasma cells, and CD93-deficient mice are impaired in antibody secretion following antigen stimulation, as the number of BM plasma cells decreases (12), indicating that CD93 plays a substantial role in maintaining plasma cells in BM. As MM cells are long-lived malignant plasma cells, we examined the expression of CD93 on MM cell lines and observed that CD93 was remarkably expressed in MM cells comparisons with B cell lines, suggesting that CD93 might play a role in the pathogenesis or biology of MM. This is in agreement with a previous observation where

CD93 expression was reduced on bortezomib-resistant mouse MM cell lines (16). This intriguing observation prompted us to further explore CD93 expression in primary MM cells (CD19⁻/CD138⁺) belonging to the BM of patients with MM. In accordance with the results obtained in cell lines, we found that CD19⁻/CD138⁺ primary MM cells, but not CD19⁺ primary B cells, expressed CD93 both at protein and mRNA levels. However, a previous report demonstrated that the patients with higher expression of CD93 at mRNA levels in BM cells exhibited a better clinical outcome (16). Noteworthy, a certain study showed that long-lived normal plasma cells reside in the BM and are within the population of CD19⁻/CD138⁺/CD38^{high} of plasma cells (17). Similarly, we have recently observed that malignant plasma cells (CD38^{high}/CD45^{low}) express a higher level of CD93 in comparison with normal plasma cells (CD38⁺/CD45^{high}) (Fakhari S, *et al.* unpublished data). However, we have to consider that further experiments are required to explore the expression of CD93 in long-lived normal plasma and memory B cells in BM and germinal centers of lymphoid organs. Furthermore, a comprehensive study carried out by Iwasaki *et al.* has demonstrated that CD93 is a functional marker for leukemic stem cells in mixed lineage leukemia (MLL)-rearranged of AML subtype and that CD93 is required for MLL leukemogenesis and AML maintenance (11). Nevertheless, the expression and function of this stem cell biomarker for MM has not been explored yet. In addition, it has been previously shown that CD93 is expressed on certain myeloid cell lines (THP-1 and U937) and absent on the promyelocytic cell line (HL60) (5). This is in agreement with our previous observation where CD93 was expressed on hematopoietic stem cells, *ex vivo*-expanded myeloid and megakaryocytic. However, CD93 expression is downregulated upon the maturation of *ex-vivo*-expanded erythroid progenitors (10). In the current study, we demonstrated that CD93 is expressed on normal monocytes and granulocytes, which is only logical as CD93 is involved in the clearance of apoptotic cells and enhancement of bacterial phagocytosis (4,18). Although in the current study we observed that CD93 is not expressed on normal lymphocytes, Ikewaki N *et al.* reported that CD93 was expressed mostly on native T helper but not on memory T helper cells (19), suggesting that CD93 expression depends on the differentiation stage and lineage of blood cells. It is well known that MM cells survival is dependent on BM stromal cells in particular mesenchymal and endothelial cells (20). The interaction between MM cells and endothelial cells and the eventual formation of new vessels play crucial roles in the transition from monoclonal gammopathy unassociated/unattributable (MGUS) to MM, or from remission MM to relapse and plasma cell leukemia (21,22). Because CD93 extracellular domain FC fusion protein binds to activated endothelial cells, it is posited that CD93 is involved in leukocyte trafficking (23). However, its role in trafficking and adhesion of MM cells to endothelial cells needs to be further explored. In conclusion, we demonstrated that CD93 is expressed on MM cell lines and primary MM cells and it may play a major role in the pathophysiology of MM.

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REFERENCES

1. Bohlson SS, Zhang M, Ortiz CE, Tenner AJ. CD93 interacts with the PDZ domain-containing adaptor protein GIPC: implications in the modulation of phagocytosis. *J Leukoc Biol.* 2005; 77:80-9.
2. Steinberger P, Szekeres A, Wille S, Stockl J, Selenko N, Prager E, et al. Identification of human CD93 as the phagocytic C1q receptor (C1qRp) by expression cloning. *J Leukoc Biol.* 2002; 71:133-40.
3. Bohlson SS, Silva R, Fonseca MI, Tenner AJ. CD93 is rapidly shed from the surface of human myeloid cells and the soluble form is detected in human plasma. *J Immunol.* 2005; 175:1239-47.
4. Norsworthy PJ, Fossati-Jimack L, Cortes-Hernandez J, Taylor PR, Bygrave AE, Thompson RD, et al. Murine CD93 (C1qRp) contributes to the removal of apoptotic cells in vivo but is not required for C1q-mediated enhancement of phagocytosis. *J Immunol.* 2004; 172:3406-14.
5. Nepomuceno RR, Tenner AJ. C1qRP, the C1q receptor that enhances phagocytosis, is detected specifically in human cells of myeloid lineage, endothelial cells, and platelets. *J Immunol.* 1998; 160:1929-35.
6. Guo WX, Ghebrehiwet B, Weksler B, Schweitzer K, Peerschke EI. Up-regulation of endothelial cell binding proteins/receptors for complement component C1q by inflammatory cytokines. *J Lab Clin Med.* 1999; 133:541-50.
7. Ishizaki J, Takemori A, Suemori K, Matsumoto T, Akita Y, Sada KE, et al. Targeted proteomics reveals promising biomarkers of disease activity and organ involvement in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Res Ther.* 2017; 19:218.
8. Danet GH, Luongo JL, Butler G, Lu MM, Tenner AJ, Simon MC, et al. C1qRp defines a new human stem cell population with hematopoietic and hepatic potential. *Proc Natl Acad Sci U S A.* 2002; 99:10441-5.
9. Haralambieva IH, Zimmermann MT, Ovsyannikova IG, Grill DE, Oberg AL, Kennedy RB, et al. Whole Transcriptome Profiling Identifies CD93 and Other Plasma Cell Survival Factor Genes Associated with Measles-Specific Antibody Response after Vaccination. *PloS one.* 2016; 11:e0160970.
10. Jalili A, Marquez-Curtis L, Shirvaikar N, Wyszczynski M, Ratajczak M, Janowska-Wieczorek A. Complement C1q enhances homing-related responses of hematopoietic stem/progenitor cells. *Transfusion.* 2010; 50:2002-10.
11. Iwasaki M, Liedtke M, Gentles AJ, Cleary ML. CD93 Marks a Non-Quiescent Human Leukemia Stem Cell Population and Is Required for Development of MLL-Rearranged Acute Myeloid Leukemia. *Cell Stem Cell.* 2015; 17:412-21.
12. Chevrier S, Genton C, Kallies A, Karnowski A, Otten LA, Malissen B, et al. CD93 is required for maintenance of antibody secretion and persistence of plasma cells in the bone marrow niche. *Proc Natl Acad Sci U S A.* 2009; 106:3895-900.
13. Matesanz-Isabel J, Sintes J, Llinas L, de Salort J, Lazaro A, Engel P. New B-cell CD molecules. *Immunol Lett.* 2011; 134:104-12.
14. Jalili A, Ozaki S, Hara T, Shibata H, Hashimoto T, Abe M, et al. Induction of HM1.24 peptide-specific cytotoxic T lymphocytes by using peripheral-blood stem-cell harvests in patients with multiple myeloma. *Blood.* 2005; 106:3538-45.
15. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008; 3:1101-8.
16. Stessman HA, Mansoor A, Zhan F, Linden MA, Van Ness B, Baughn LB. Bortezomib resistance can be reversed by induced expression of plasma cell maturation markers in a mouse in vitro model of multiple myeloma. *PloS One.* 2013; 8:e77608.
17. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoret IV, et al. Long-Lived Plasma Cells Are Contained within the CD19(-)CD38(hi)CD138(+) Subset in Human Bone Marrow. *Immunity.* 2015; 43:132-45.
18. Ikewaki N, Tamauchi H, Inoko H. Decrease in CD93 (C1qRp) expression in a human monocyte-like cell line (U937) treated with various apoptosis-inducing chemical substances. *Microbiol Immunol.* 2007; 51:1189-200.
19. Ikewaki N, Yamao H, Kulski JK, Inoko H. Flow cytometric identification of CD93 expression on naive T lymphocytes (CD4(+)CD45RA (+) cells) in human neonatal umbilical cord blood. *J Clin Immunol.* 2010; 30:723-33.

20. Terpos E, Ntanasis-Stathopoulos I, Gavriatopoulou M, Dimopoulos MA. Pathogenesis of bone disease in multiple myeloma: from bench to bedside. *Blood Cancer J.* 2018; 8:7.
21. Pellegrino A, Ria R, Di Pietro G, Cirulli T, Surico G, Pennisi A, et al. Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *Br J Haematol.* 2005; 129:248-56.
22. De Bruyne E, Menu E, Van Valckenborgh E, De Raeve H, Van Camp B, Van Riet I, et al. Myeloma cells and their interactions with the bone marrow endothelial cells. *Current Immunology Reviews.* 2007; 3:41-55.
23. McGreal EP, Ikewaki N, Akatsu H, Morgan BP, Gasque P. Human C1qRp is identical with CD93 and the mNI-11 antigen but does not bind C1q. *J Immunol.* 2002; 168:5222-32.