M. (2018), Class 1 HDAC and HDAC6 inhibition inversely regulates CD38 induction in myeloma cells via interferon-α and ATRA. Br J Haematol, 185: 969-974, which has been published in final form at https://doi.org/10.1111/bjh.15673. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

Supplemental information

Materials and Methods

Reagents

The following reagents purchased from the indicated manufacturers: rabbit polyclonal antibodies against human phosphorylated STAT1, STAT1 and CD38, rabbit monoclonal anti-human IRF1 antibody, horseradish peroxidase (HRP)-anti-rabbit IgG and anti-mouse IgG from Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-β-actin antibody and all trans retinoic acid from Sigma-Aldrich (St. Louis, MO); phycoerythrin-conjugated mouse anti-human CD38 antibody and mouse IgG_1 from Biolegend (San Diego, CA); 17-AAG from Calbiochem EMD Biosciences, Inc. (La Jolla, CA), recombinant human IFN-α and IFN-γ from R&D systems, Inc. (bio-techno, USA); and BAY11-7085, entinostat (MS-275) from Santa Cruz Biotechnology (Santa Cruz, CA); panobinostat, SR11302 and T-5224 from Cayman Chemical Company (Ann Arbor, MI).

Cells and cell culture

The use of human samples was approved by the Institutional Review Board at Tokushima University, and informed consent was obtained according to the Declaration of Helsinki. Peripheral blood mononuclear cells were isolated from fresh peripheral blood as previously described[1,2]. The human MM cell lines RPMI8226, U266-B1 and KMS-11 were obtained from the American Type Culture Collection (ATCC, Rockville, MD); OPM-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The human MM cell lines INA-6 and MM.1S were kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany) and Dr. Steven Rosen (Northwestern University, Chicago, IL, USA). Cells were cultured in RPMI1640 medium (Sigma, Aldrich, MO) supplemented with 10% FBS (Life Technologies, Grand Island, NY), penicillin G at 50 μg/mL and streptomycin at 50 μg/mL (Sigma).

Flow cytometry

Cell preparation and staining in flow cytometry were performed as described previously.[1,2] Briefly, approximately 10^6 cells were incubated in 100 μL FACS buffer (Japan Blood Products Organization, Tokyo, Japan) with phycoerythrin-conjugated monoclonal antibodies on ice for 45 minutes, and then washed. Samples were analyzed by flow cytometry using a Gallios flow cytometer (Beckman Coulter, Miami, FL). Deta were edited with FlowJo software (BD Biosciences, San Diego, CA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIZOL reagent (Gibco BRL, Rockville, MD). Two μg total RNA was reverse-transcribed with Superscript II (Gibco) in a 20 μL reaction solution. One tenth of the RT-PCR products were used for subsequent PCR analysis with 23–30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The primers used were listed in (Table 1).

Table 1. The list of primers

	Sense	Antisense
CD38	5'- AAACGGTTTCCCGCAGGTTTG -3'	5'- GTGTCTGAACCTTCTCTGGTTGC-3'
IRF1	5'- CAAATCCCGGGGCTCATCTG -3'	5'- CTGCTTTCTATCGGCCTGTGTG-3'
STAT1	5'- TGGTGAAATTGCAAGAGCTGA-3'	5'-GTGTGCGTGCCCAAAATG-3'
PPIE	5'- TGGACGTACAATTCGTGTCAA -3'	5'- GGCTCTGACCCTTCTTCCTC -3'
GAPDH	5'- TGTCTTCACCACCATGGAGAAGG -3	5'- GTGGATGCAGGGATGATGTTCTG -3'

Short hairpin RNA (shRNA) transfection

293T cells at a density of 3×10^5/mL were seeded in 6-wellculture plates in Dulbecco's Modified Eagle's medium supplemented with 10% FBS and incubated 24-30 hours. The shRNA encoding transfer vector (500 ng/well), dvpr (500 ng/well), and VSV-G (50 ng/well) were mixed in TransIT-LT1 transfection reagents containing OPTI-MEM media. After 30 minutes incubation at room temperature, 293T cells were transfected with 3 kinds of plasmids for 18 hours at 37℃ in 5% CO_2. After transfection, 293T cells were re-fed with fresh media. 24 hours later, virus particles were harvested. KMS-11 cells were transfected with Mission Lentiviral transduction particles, containing *IRF1* shRNA (NM_002198 clone TRCN0000218571, TRCN0000229658, and TRCN0000229659, Sigma-Aldrich) in the presence of polybrene (8 mg/mL). After incubating for 3-5 hours in 6-well culture plates, cells were washed with PBS and resuspended in fresh growth media. Transfected cells were selected by incubating with puromycin (1□g/mL) for 48 hours. *Luciferase* shRNA was used as a control. Efficacies of gene silencing by shRNA were evaluated by RT-PCR.

Western blot analysis

Cells were collected and lysed in lysis buffer (Cell Signaling, Beverly, MA) supplemented with 1 mmoL/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma-Aldrich). The cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millpore, Billerica, MA). Membranes were blocked with 3% non-fat dry milk in Tris-buffered saline with 0.01% Tween 20 for 1 hour at room temperature and incubated for 16 hours at 4^{o}C with primary antibodies. After washing, secondary horseradish peroxidase-conjugated antibody was added, and membranes were then developed, using the enhanced chemiluminescence plus Western blotting detection system (American Biosciences, Piscataway, NJ).

References

1. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood* 2004 Oct; **104**(8)**:**2484-2491.
2. Abe M, Hiura K, Wilde J, Moriyama K, Hashimoto T, Ozaki S et al. Role for macrophage inflammatory protein (MIP)-1alpha and MIP-1beta in the development of osteolytic lesions in multiple myeloma. *Blood* 2002 Sep; **100**(6)**:**2195-2202.

Legends to Supplementary Figures

Supplementary Figure 1. (A) Surface expression of CD38 on MM cells. Surface levels of CD38 on MM cell lines as indicated were analyzed by flow cytometry. Shaded histograms show staining with mouse IgG1 isotype control. (B) CD38 upregulation on MM cells by IFN-α and ATRA. KMS-11 and MM.1S cells were cultured for 24 hours in the presence or absence of IFN-α or ATRA at the indicated concentrations. Surface levels of CD38 on the cells were analyzed by flow cytometry. Overlay histograms show CD38 expression on the cells upon the indicated treatments. CD38 expression at baseline is indicated as (-). Mouse IgG1 was used as an isotype control (Iso). (C) Upregulation of CD38 and PD-L1 on MM cells by IFN-α and IFN-γ. KMS-11 and MM.1S cells were cultured for 24 hours without stimulation or in the presence of 100 U/ml IFN-α or 100 U/ml IFN-γ alone or both in combination. Surface levels of CD38 and PD-L1on the cells were analyzed by flow cytometry.

Supplementary Figure 2. Effects of HDAC inhibitors on CD38 expression on MM cells. MM cell lines as indicated were cultured for 24 hours in the absence or presence of 100 U/ml

IFN-α or 10 nM ATRA alone or both in combination. The pan-HDAC inhibitor panobinostat (Pano), the class I HDAC-selective inhibitor MS-275 and the HDAC6-selective inhibitor ACY-1215 were further added at 25 nM, 1 μM and 3 μM, respectively, as indicated. *CD38* mRNA expression was analyzed in the MM cells by RT-PCR. *GAPDH* was used as an internal control.

Supplementary Figure 3. (A) Activation of the STAT1-IRF-1 pathway in MM cells by IFN-α. After overnight starvation by culture media containing 1% FBS, KMS-11 and MM.1S cells were incubated in the presence of IFN-γ (100 U/ml) for the indicated time periods. The cells were then harvested, and STAT1, tyrosine-phosphorylated STAT1 (p-STAT1) and IRF1 protein levels were examined by Western blot analysis. β-actin was blotted as a loading control. (B) Effects of AP-1 inhibition on CD38 upregulation on MM cells by IFN-α and ATRA. KMS-11 cells were cultured for 24 hours in the presence or absence of 100 U/ml IFN-α or 10 nM ATRA. AP-1 inhibitors, SR-11303 or T-5224 were further added at 10 μM as indicated. Surface levels of CD38 on the cells were analyzed by flow cytometry. Overlay histograms show CD38 expression on the cells upon the indicated treatments. CD38 expression at baseline is indicated as (-). Mouse IgG1 was used as an isotype control (Iso). (C) *IRF1* gene knockdown efficacy. KMS11 cells were transfected with *IRF1* shRNA (IRF1 KD) or control *Luciferase* shRNA. The cells were then cultured for 24 hours in the presence or absence of 100 U/ml IFN-α, 10 nM ATRA or 25 nM panobinostat (Pano) alone, or upon treatment with various combination of these reagents. *IGF1* mRNA expression was analyzed in the MM cells by RT-PCR. *PPIE* (protein coding gene of peptidylprolyl isomerase E) was used as an internal control.