Class 1 HDAC-specific inhibition augments but HDAC6 inhibition mitigates CD38 upregulation in myeloma cells by interferon-α and all-trans retinoic acid.

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To the editor:

CD38 levels on the surface of myeloma (MM) cells appear to be a major determinant of MM cell sensitivity to daratumumab[1]. Therefore, augmentation of CD38 expression on MM cells is urgently needed to improve therapeutic efficacy of daratumumab especially for patients whose MM cells express CD38 at low levels. In this regard, Nijhof et al. nicely reported that all-trans retinoic acid (ATRA) increased CD38 expression on MM cells to enhance ADCC and CDC with daratumumab *in vitro*[2]. Consistent with the observation that the upstream sequence of the *CD38* gene contains an interferon regulatory factor1 (IRF1)-binding site, interferon (IFN)-γ is also accepted to upregulate CD38 expression through activation of the signal transducers and activators of transcription 1 (STAT1)-IRF1 pathway[3-5]. Interestingly, IFN-α was reported to more efficiently enhance CD38 expression on adult T cell leukemia cells than IFN-γ, and synergistically upregulate their CD38 expression in combination with ATRA[6,7]. Improvement of clinical efficacy of therapeutic anti-CD38 antibodies can therefore be expected for patients with MM in combination with IFN-α and/or ATRA. More recently, panobinostat has been reported to upregulate CD38 expression on both MM cell lines and primary MM samples to successfully improve the *in vitro* cytotoxic effects of daratumumab on MM cells[8]. We explored here the effects of HDAC inhibitors in combination with IFN-α as well as ATRA on the regulation of CD38 expression in MM cells. KMS-11, INA-6 and U266 cells expressed CD38 at relatively low levels, while MM.1S, RPMI8226 and OPM2 cells expressed it at high levels (Supplementary Figure 1A). IFN-α as well as ATRA dose-dependently (Supplementary Figure 1B) and cooperatively (Figure 1A) upregulated CD38 expression in KMS-11 and

MM.1S cells, low and high expressers of CD38, respectively. IFN-α much more markedly enhanced CD38 expression on the surface of KMS-11 and MM.1S cells than IFN-γ, while IFN-α and IFN-γ upregulated PD-L1 to the similar levels (Supplementary Figure 1C), indicating preferential induction of CD38 expression by IFN-α. Furthermore, IFN-α and ATRA cooperatively enhanced CD38 expression on the surface of all the MM cell lines tested (Figure 1B) and *CD38* mRNA expression in KMS-11 and MM.1S cells (Supplementary Figure 2). However, we unexpectedly found that panobinostat blunted or rather mitigated the CD38 upregulation in these MM cells most obviously under treatment with IFN-α and ATRA in combination (Figure 1B and Supplementary Figure 2). Because panobinostat is a pan-HDAC inhibitor, we next looked at the effects of HDAC inhibitors specific for class1 HDACs or HDAC6 on the CD38 upregulation by IFN-α and/or ATRA to further dissect the regulatory roles of class-specific HDAC inhibitors in CD38 expression in MM cells. The class1 HDAC-specific inhibitor MS-275 alone was able to increase CD38 expression on all the MM cell lines more than panobinostat (Figure 1B). MS-275 further increased CD38 expression upregulated by IFN-α or ATRA alone or both in combination on all the MM cell lines, suggesting restoration of CD38 expression repressed by class 1 HDACs in MM cells without interfering with its upregulation by IFN-α as well as ATRA. Intriguingly, in contrast to MS-275, the HDAC6 inhibitor ACY-1215 as well as panobinostat only marginally increased the CD38 upregulation by IFN-α or ATRA in INA-6 and U266 cells and rather reduced it in KMS11 and MM.1S cells (Figure 1B). Of note, ACY-1215 and panobinostat mitigated CD38 expression upregulated cooperatively by IFN-α and ATRA in combination on all the MM cell lines. These

results suggest that HDAC6 inhibition may antagonize the upregulation of CD38 expression in MM cells by IFN-α and ATRA.

We further examined the effects of HDAC inhibition on downstream signaling of IFN-α or ATRA in MM cells. Treatment with IFN-α promptly and sustainably phosphorylated STAT1 in KMS-11 cells, followed by upregulation of IRF1 protein levels at 1 hour and later (Supplementary Figure 3A). The upregulation of IRF1 protein levels in KMS-11 cells by IFN-α was reduced with ACY-1215, while not apparently affected with MS-275 (Figure 2A). Because *CD38* gene is known to be one of target genes of the transcription factor IRF1, the reduction of IRF1 protein levels is suggested to contribute to the downregulation of CD38 by ACY-1215. However, ACY-1215 did not reduced *IRF1* mRNA levels, which were upregulation by IFN-α and/or ATRA (Figure 2A), suggesting IRF1 protein degradation.

IRF1 and HDAC6 are known to be client proteins of the molecular chaperone heat shock protein 90 (Hsp90). Hsp90 chaperone activity is regulated by reversible acetylation and controlled by the deacetylase HDAC6 [9,10]. Treatment with the Hsp90 inhibitor 17-allylamino-demothoxy geldanamycin (17-AAG) inhibits chaperone function of Hsp90, thereby inducing polyubiquitylation and proteasomal degradation of HSP90 client proteins [9]. The synergism between inhibition of both HDAC6 and Hsp90 has also demonstrated [9,10]. In this way, Hsp90 and HDAC6 interact with each other to maintain their function and activity. Therefore, Hsp90 and HDAC6 are suggested to be critical modulators of the activity and stability of IRF-1. Consistent with these observations, CD38 upregulation in MM cells by IFN-α and/or ATRA was reduced with ACY-1215 or 17-AAG alone and cooperatively with both in combination (Figure 2B).

IFN-α activates multiple signaling pathways other than the JAK/STAT signaling pathway, including the MAPK-activated protein-1 (AP-1) pathway. NF-κB and AP-1 activation is known to transcriptionally increase CD38 expression[11,12]. The AP1 inhibitors SR11302 or T-5224 reduced the CD38 upregulation in KMS-11cells by IFN-α but not by ATRA (Supplementary Figure 3B). To further clarify the role of IRF-1, we examined the effects of *IRF1* gene silencing on CD38 upregulation by IFN-α in KMS-11cells (Supplementary Figure 3C). *IRF1* gene silencing mostly reduced CD38 expression in KMS-11 cells at baseline; however, IFN-α was able to induce CD38 expression in the KMS-11 cells with *IRF1* gene silencing, indicating IRF1-dependent and independent CD38 upregulation by IFN-α. The AP1 inhibitor SR11302 was able to further reduce the CD38 upregulation in the *IRF1* gene-deleting KMS-11 cells in the presence of IFN-α (Figure 2C), suggesting the role of AP1 activation in IRF1-independent CD38 upregulation by IFN-α.

These results collectively demonstrate that IFN-α and ATRA cooperatively enhance CD38 expression in MM cells, and suggest that IRF1 and HDAC6 critically involve in CD38 upregulation in MM cells by IFN-α and/or ATRA, although class1 HDACs repress their CD38 expression. Therefore, the present study can provide a rationale for possible combinatory treatment of therapeutic anti-CD38 antibodies for MM with IFN-α +/− ATRA and a caveat against further addition of HDAC inhibitors with anti-CD38 antibodies in combination with IFN-α or ATRA.

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Figure legends

Figure 1. Alteration of CD38 expression on MM cells by IFN-α and ATRA in the presence of HDAC inhibitors.

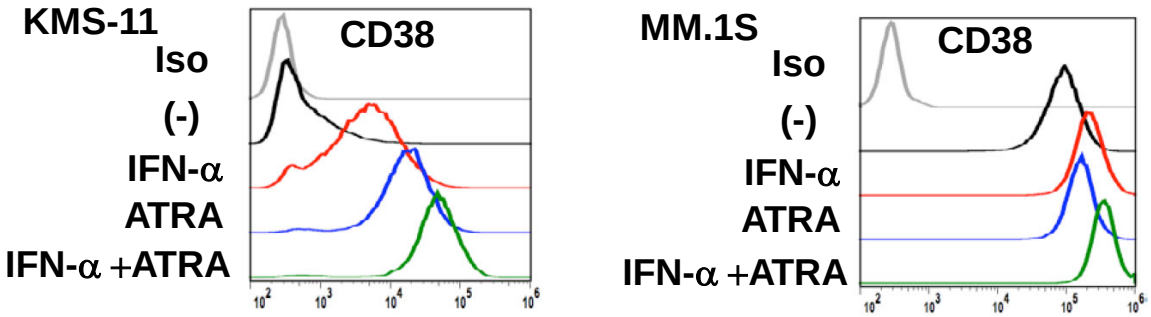
(A) Upregulation of CD38 expression on MM cells by IFN-α and ATRA. KMS11 and MM.1S cells were cultured for 24 hours without stimulation or in the presence of 100 U/ml IFN-α or 10 nM ATRA alone or both in combination. Surface levels of CD38 on the cells were analyzed by flow cytometry. Overlay histograms show CD38 expression on the cells upon the indicated treatments. CD38 expression at baseline is indicated as (-). Mouse IgG_1 was used as an isotype control (Iso). (B) 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. Surface levels of CD38 on the cells were then analyzed by flow cytometry.

Figure 2. Roles of IRF1 in regulation of CD38 expression on MM cells.

(A) IRF1 expression in MM cells. KMS11 and MM.1S cells were cultured for 24 hours without stimulation or in the presence of 100 U/ml IFN-α or 10 nM ATRA alone or both in combination. The class I HDAC-selective inhibitor MS-275 and the HDAC6-selective inhibitor ACY-1215 were further added at 1 and 3 μM, respectively, as indicated. The cells were then harvested, and IRF1 protein levels were examined by Western blot analysis (upper). β-actin was blotted as a loading control. *IRF1* mRNA expression was analyzed in the cells by RT-PCR (lower). *GAPDH* was used as an internal control. (B) Effects of HDAC6 and HSP90 inhibition on CD38 expression on MM cells. KMS-11 cells were cultured for 24 hours without stimulation or in the presence of 100 U/ml IFN-α or 10 nM ATRA alone or both in combination. The HDAC6-selective inhibitor ACY-1215 and the HSP90 inhibitor 17AAG were further added at 3 μM, as indicated. (C) Roles of IRF1 and AP1 in CD38 upregulation by IFN-α. KMS11 cells were transfected with *IRF1* shRNA (IRF1 KD) or control *Luciferase* shRNA (LUC). Knockdown efficacy was examined by RT-PCR (Supplementary Figure 3C). The cells were cultured for 24 hours in the presence or absence of 100 U/ml IFN-α. The AP1 inhibitor SR-11303 (AP1 inh) was 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 (-).

Figure 1

A


KMS-11
CD38
Iso
(-)
IFN-α
ATRA
IFN-α +ATRA
MM.1S
CD38
Iso
(-)
IFN-α
ATRA
IFN-α +ATRA

B

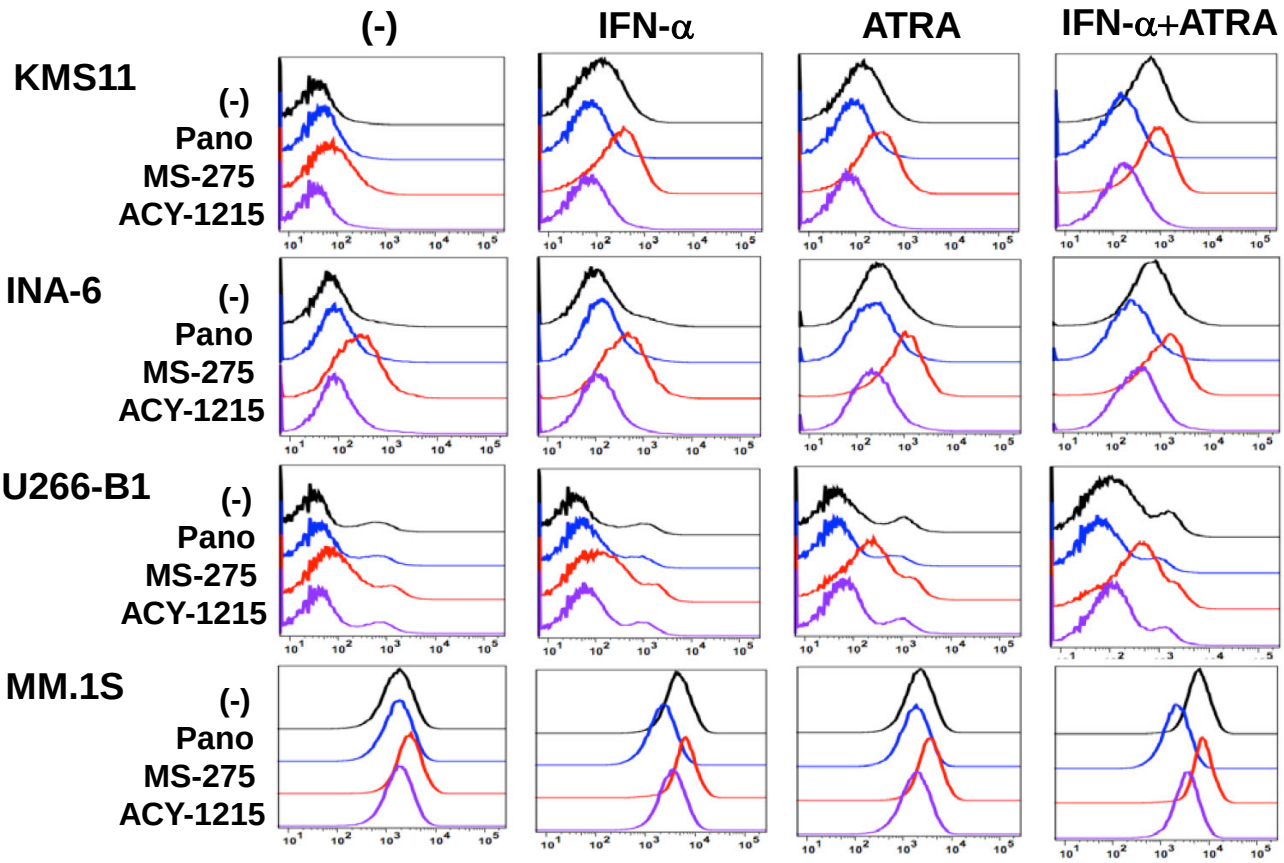
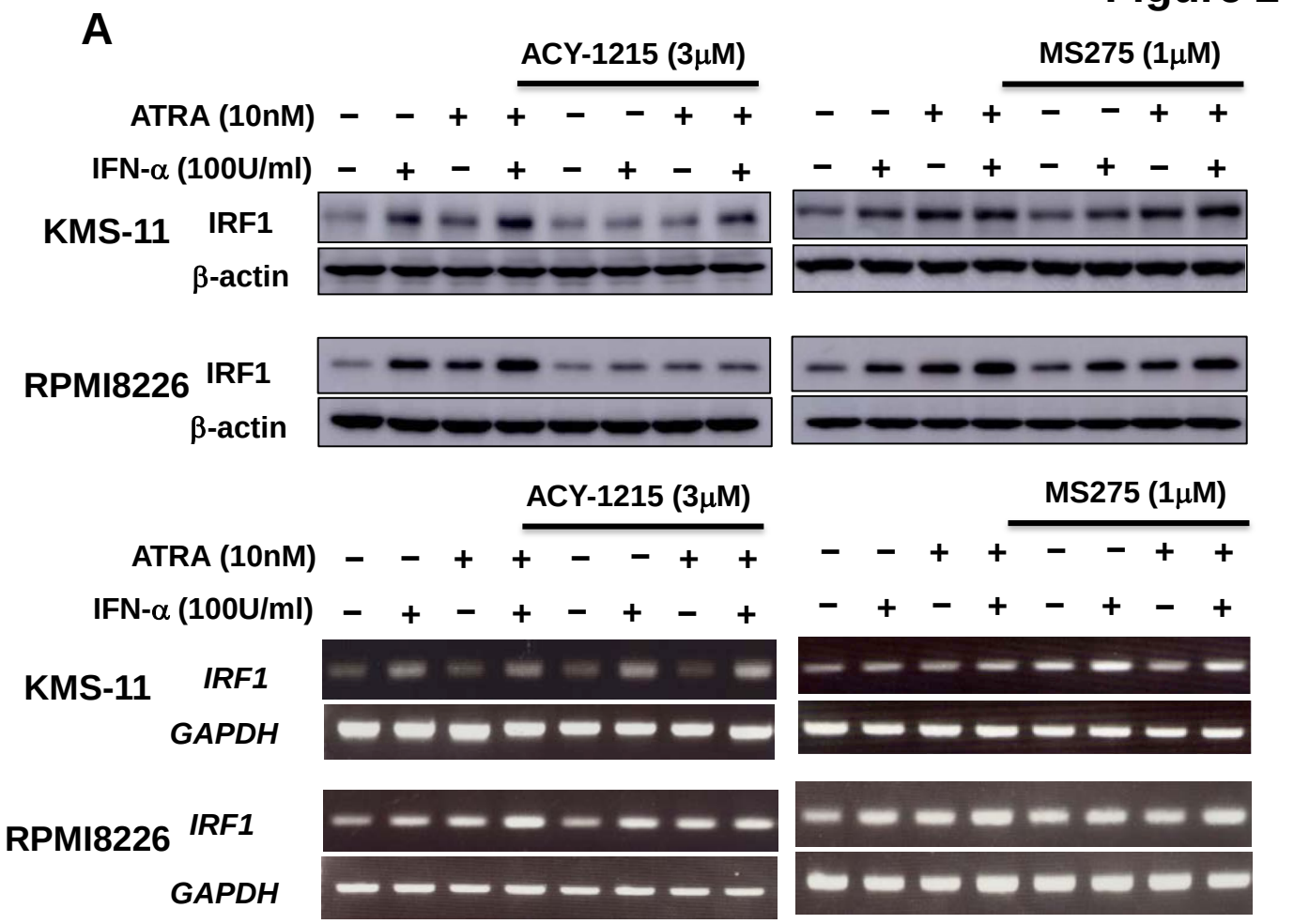

(-)
IFN-α
ATRA
IFN-α+ATRA
KMS11
INA-6
U266-B1
MM.1S
(-)
Pano
MS-275
ACY-1215

Figure 2


A
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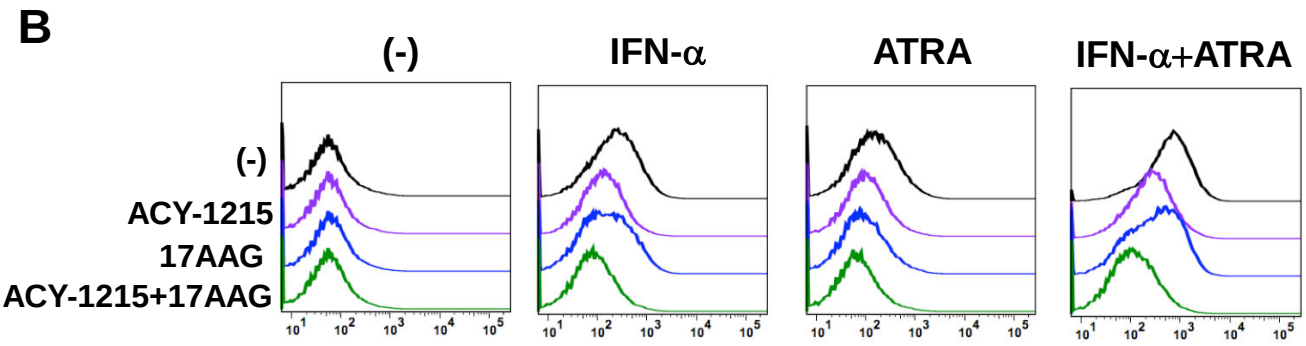
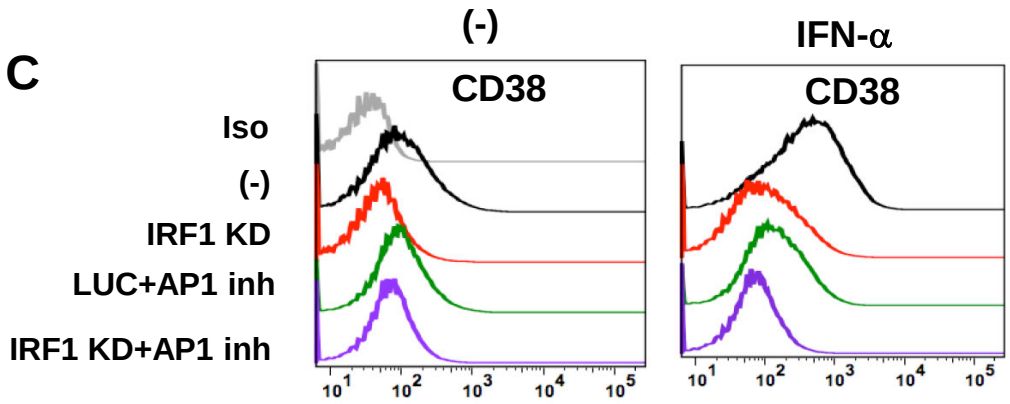
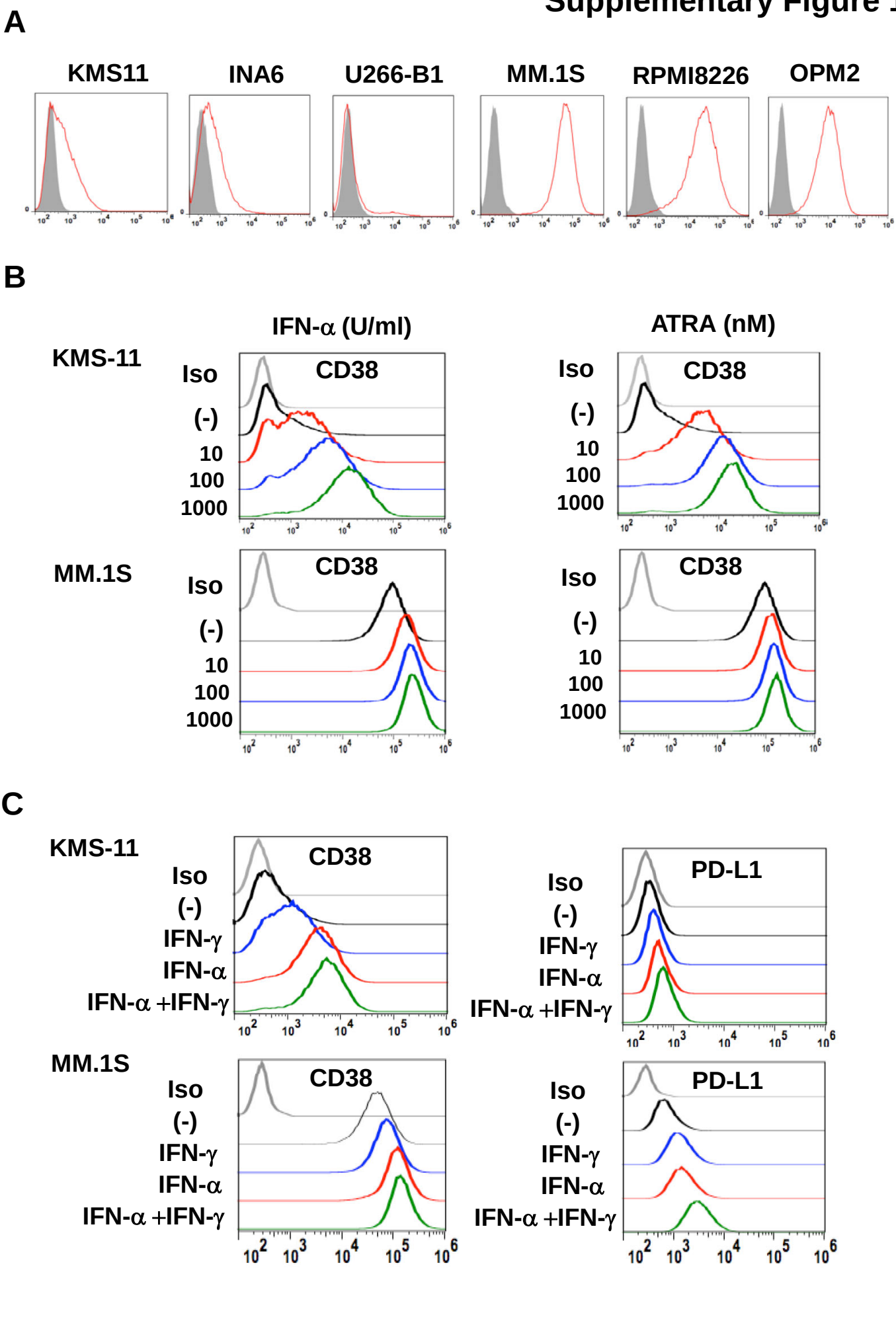
MS275 (1μM)
ATRA (10nM)
IFN-α (100U/ml)
KMS-11
IRF1
β-actin
RPMI8226
IRF1
β-actin
ACY-1215 (3μM)
MS275 (1μM)
ATRA (10nM)
IFN-α (100U/ml)
KMS-11
IRF1
GAPDH
RPMI8226
IRF1
GAPDH

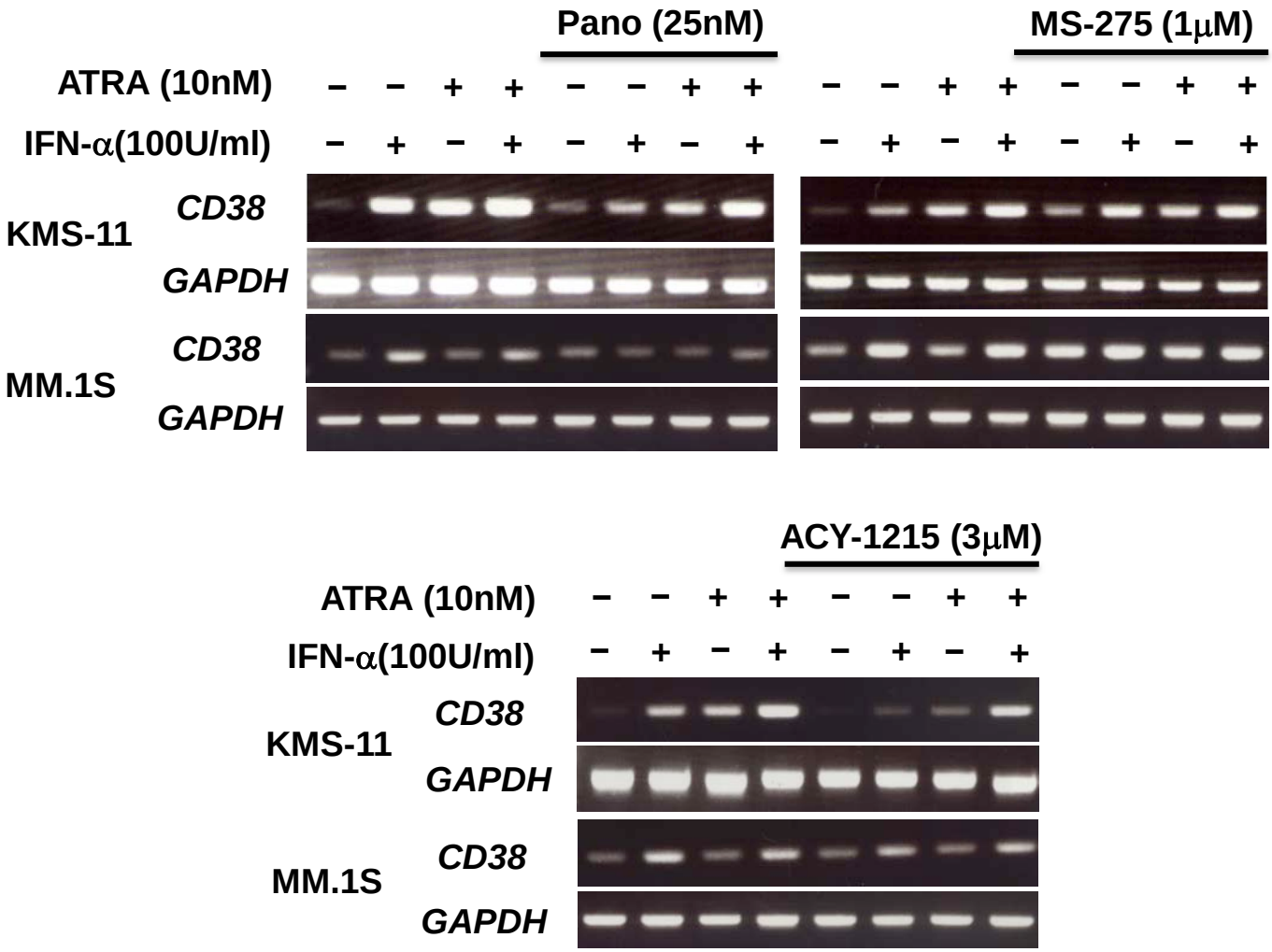

B
(-)
IFN-α
ATRA
IFN-α+ATRA
(-)
ACY-1215
17AAG
ACY-1215+17AAG


C
(-)
IFN-α
CD38
CD38
Iso
(-)
IRF1 KD
LUC+AP1 inh
IRF1 KD+AP1 inh

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3