PML mediates IFNα induced apoptosis in myeloma by regulating TRAIL induction

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Abstract

Interferon (IFN) induces expression of pro-apoptotic genes and has been used in the clinical treatment of multiple myeloma. The promyelocytic leukaemia (PML) gene is an IFN induced target that encodes a tumor suppressor protein. PML protein is typically localized within discrete speckled nuclear structures termed PML nuclear bodies (NBs). Multiple myeloma cells demonstrate differential responses to IFN treatment, the mechanism of which is largely unknown. Herein, we show that growth inhibition effects of IFNα in myeloma cells correlate with PML NBs and TNF-related apoptosis inducing ligand (TRAIL) induction, whereas known IFN targets including STAT1, STAT3, p38 and Daxx cannot account for these differential responses. RNAi silencing of PML blocks IFNα induced apoptosis in myeloma cells and correspondingly down regulates TRAIL expression. Similarly, stable expression of a dominant negative TRAIL receptor DR5 partially blocks IFN induced cell death. These results demonstrate that PML and TRAIL play important roles in IFN induced apoptosis and identify TRAIL as a novel downstream transcriptional target of PML. Identification of PML and PML NBs as effectors of IFN responses provides insights into mechanisms by which tumor cells exhibit resistance to this class of agents and may prove useful in assessing treatment regimens.
**Introduction**

Multiple myeloma (MM) is an incurable form of cancer characterized by the accumulation and proliferation of malignant plasma cells in the bone marrow. Molecular mechanisms leading to the development of this disease are still poorly understood although a variety of lesions have been described in both patient samples and myeloma cell lines. Currently there is no clear picture as to the requirements for initiation or progression of this disease although multiple events such as chromosomal abnormalities and oncogene expression are likely involved\(^1,2\).

Standard treatment of MM involves combination chemotherapy with or without bone marrow transplantation. More recently, a series of new treatment regimens have been initiated including agents such as thalidomide, proteasome inhibitors and Interferons (IFNs), which have all been employed with varying success\(^1,3,4\). Since the mechanisms by which such agents inhibit tumor growth are poorly understood, it is probably not surprising that optimal regimens and combinations have not been defined. Among these compounds, IFNs have been used for the treatment of multiple forms of cancer including MM\(^3\) as well as other diseases.

IFNs are pleiotropic cytokines that were first identified as anti-viral agents secreted by infected mammalian cells, but have later been shown to also be important regulators of cell growth (reviewed in\(^5\)). Following binding to cell type specific receptors, IFNs regulate target genes through activation of Jak/STAT signaling pathways. It has been shown in MM that IFN\(^\alpha\) activates STAT3 as well as a transcription factor complex consisting of STAT1, STAT2, and IFN regulatory factors 1 and 2\(^6-8\). One of the consequences of IFN treatment is induction of apoptosis through up regulation of TNF-
Related Apoptosis Inducing Ligand (TRAIL) as demonstrated in the human multiple myeloma cell line U266\textsuperscript{9}. The death ligand TRAIL triggers apoptosis through activation of its cognate receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) which, in turn, recruit adapter proteins and activate caspases\textsuperscript{10}. TRAIL has been shown to effectively induce apoptosis and overcome drug resistance in several types of transformed cells including MM, but not most normal cells\textsuperscript{11-13}. The mechanism by which IFN induces TRAIL in MM cells is currently unknown.

Another target of IFNs that is specifically up-regulated at the transcriptional level is the promyelocytic leukaemia (PML) gene\textsuperscript{14,15}. Multiple isoforms of PML have been identified most of which are found in the nucleus where they form PML nuclear bodies (PML NBs)\textsuperscript{16}. Microscopy studies have shown that there are typically 10-20 PML NBs in most normal cells varying in size between 0.2-1.0 μm. More than 50 different proteins have been found to localize to PML NBs, but are conditionally present and include several different classes of proteins such as transcription factors and co-factors, oncoproteins, and ribosomal proteins\textsuperscript{17}. Post-translational modifications such as SUMOylation and phosphorylation of PML have been reported to be important for formation and maturation of PML NBs\textsuperscript{18}. The exact molecular function of PML and PML NBs is still largely unclear, but a biological role for PML in cell proliferation, apoptosis and regulation of transcription is well established\textsuperscript{19}.

PML has previously attracted intense interest in cancer biology due to its role in the pathogenesis of acute promyelocytic leukaemia (APL)\textsuperscript{20-22}. In APL the PML gene is fused to the retinoic acid receptor \(\alpha\) gene (RAR\(\alpha\)) as a result of a \(t(15; 17)\) chromosomal translocation. This translocation results in expression of a PML-RAR\(\alpha\) fusion protein.
leading to disruption of the PML NBs and de-localization of PML NB components. The disruption of PML NBs leads to a growth advantage for leukemic blast cells. Degradation of PML-RARα and induction of the remaining wild type PML allele by treatment with As2O3, retinoic acid, or IFN results in reformation of PML NBs and complete remission of the disease. Furthermore, a role for PML as a tumor suppressor has been suggested by studies on mice with impaired PML function. Investigations of PML-RARα transgenic and PML-/- mice have demonstrated that PML inactivation results in impaired induction of apoptosis by multiple apoptotic pathways and PML-/- mice are highly susceptible to developing tumors when challenged by carcinogens. Recently, comprehensive studies have shown that PML protein is frequently lost in human cancers of various histologic origins. These observations demonstrate that PML is crucial for critical tumor-suppressive pathways although its mechanism of action is largely unknown.

To date, a functional role for PML in MM has not been addressed. Herein, we describe the IFNα mediated induction of PML NBs in MM cells and the impaired regulation of the PML gene in a subset of lines that correlates with unresponsiveness to IFN induced apoptosis. TRAIL induction is directly involved in the cytotoxic effect of IFN against MM. Furthermore, induction of PML appears important for enhanced expression of TRAIL and subsequent activation of death receptors and apoptosis.
Materials and Methods

Cell lines

Human myeloma cell lines were kindly provided by Dr. W. Michael Kuehl (NCI, Bethesda, MD). All lines were maintained in RPMI1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA). In addition, human IL-6 was added at 10 ng/ml for IL-6-dependent myeloma lines ANBL-6, XG1 and XG2.

Western blot and immunofluorescence analysis

For western blot analysis, lysates were prepared as previously described 27. Thirty-five μg of total protein per sample was fractionated on 4-12% NuPAGE gels (Invitrogen) and transferred to PVDF membranes (Millipore Corporation, Bedford, MA). Antibodies used for western blot analysis were: rabbit polyclonal anti-PML, polyclonal anti-Stat3 (K15), polyclonal anti-Daxx (M112), goat anti-p38 (Santa Cruz, Santa Cruz, CA); anti-p-84/91STAT1 (E23), rabbit anti-p-Tyr701-Stat1, anti-p-Tyr705-Stat3, anti-p-Thr180/Tyr182-p38 (Cell Signaling, Beverly, MA); anti-GAPDH (Biodesign International, Saco, Maine); and goat anti-human TRAIL (R&D System, Minneapolis, MN).

Cells were prepared for confocal immunofluorescence studies as previously described 28. Confocal fluorescent images were collected with a Bio-Rad MRC 1024 confocal scan head mounted on a Nikon Optiphot microscope with a 60X planapochromat lens in the Confocal Core Facility, Center for Cancer Research, NCI. A krypton-argon gas laser provided excitation at 488 nm. Emission filters of 522/532 were used for sequentially collecting green fluorescence. Z-sections were collected at 0.5 μm.
intervals for each cell. Image files were collected and analyzed using Bio-Rad LaserSharp software.

**Cell staining and analysis by Laser Scanning Cytometry (LSC)**

Cells were washed twice with PBS and re-suspended in PBS at approximately $2 \times 10^5$ cells/ml. Cells were then added to poly-L-lysine coated chambers (Nalge Nunc, Naperville, IL) for 15 minutes to allow attachment, fixed with 70% ethanol at $-20^0 \text{C}$ for 20 minutes, followed by 15 minutes incubation at room temperature. Chambers were then rinsed twice with PBS and subjected to blocking using 5% BSA in PBS. Fixed cells were incubated for 1h at room with monoclonal anti-PML antibody (Santa Cruz), washed three times with PBS containing 0.1% Tween 20 then incubated in Alexa 488 conjugated goat-anti-mouse secondary antibody (Molecular Probe) for 30 minutes at room temperature. After washing three times with PBS containing 0.1% Tween 20, cells were treated with PBS containing 5 μg/ml propidium iodine (PI) and 100 μg/ml RNase for 30 minutes at $37^0 \text{C}$. Slides were washed with PBS and mounting medium (5 μg/ml PI, 75% Glycerol in PBS) added before placement of coverslips. Slides were stored at $4^0 \text{C}$ prior to scanning. PML NBs fluorescence was measured by LSC (CompuCyte, Cambridge, MA) in the FACS core facility of the Center for Cancer Research, NCI. Slides were scanned using an Argon laser at 5mW and 40X objectives. Green and red channels were used for Alexa 488 and PI respectively, cells were contoured on PI fluorescence and scored for PML NBs in the green channel. All samples were scanned using the same PMT settings. At least 3,000-5,000 cells were measured per sample.
**siRNA transfection**

siRNA duplexes were obtained from Dharmaco (Lafayette, CO). siPML corresponds in nucleotide sequence to positions 398-418 of the PML coding region relative to the first nucleotide of the start codon and is present in all PML isoforms. Luciferase GL2 duplex (siluc) was used as a negative control. Transfection was performed using an Amaxa electroporation apparatus (Amaxa, Cologne, Germany). Transfection efficiency was monitored using cy3-conjugated siluc and FACS analysis. A series of optimization transfections were first performed according to the manufacturer’s instructions. The program (C16) and solution (T) that gave the best efficiency and least cell death was chosen for later studies. One day before transfection, cells were diluted in fresh growth medium and 5X10^6 cells were mixed with 2 mM siRNA in 100 µl of solution T and transfected using program C16. All analyses were performed 36h after transfection. Using the above conditions transfection efficiencies above 70% were consistently obtained.

**Proliferation assay**

Cell proliferation assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following the manufacturer’s instructions as previously described 27. Proliferation (% of untreated cells) was calculated using the following equation:

\[100 + 100 \times (\text{abs. 490nm of treated cells} - \text{abs. 490nm of untreated cells})/\text{abs. 490nm of untreated cells}\]. This calculation assigns a value of 100% proliferation to untreated cells.
Trypan blue viability assay

Viability of cells was assessed by trypan blue (TB) staining followed by counting unstained cells. Viability (% of untreated) was calculated similarly as described above for proliferation.

Caspase-3 assay

Caspase-3 activity was detected using ApoAlert™ Colorimetric Assay Kits (BD Biosciences, Palo Alto, CA) as described in the User Manuals. Protein content was determined by BCA Protein Assay kit (Pierce, Redfod, IL). 400 μg of total protein per sample in duplicate was used. All experiments were repeated at least 3 times.

Terminal Deoxynucleotide Transferase (TdT) Assay.

Cells were washed with cold PBS and fixed in -20°C methanol. After removal of methanol, cells were incubated in TdT TUNEL Label solution (Roche, Mannheim, Germany) and TUNEL Enzyme (Roche, Mannheim, Germany) for 30 min. at 37°C. Cells were then washed and resuspended in PBS containing Hoechst. TUNEL positive cells were quantitated using flow cytometry (FACSCalibur; Becton Dickinson).

Preparation of IFNα-stimulated culture supernatants

Supernatants were prepared as previously described 29. Briefly, cells (2x10^5 cells/mL) treated with or without IFNα for 60 h were resuspended in complete RPMI-1640 medium (5x10^6 cells/mL), incubated for an additional 6 h and harvested by centrifugation. Supernatant was passed through 0.22 μm filters (Millipore Corporation, Bedford, MA) and assayed for cytotoxicity. Blocking of secreted TRAIL was performed by adding 10 mg/mL anti-TRAIL antibodies (R&D Systems, Minneapolis, MN).
**RT-PCR**

Total RNA was isolated using TRIzol Reagent (Invitrogen). RNA was then treated with DNA-free™ kit (Ambion, Austin, TX) to remove contaminating genomic DNA. Purity of RNA samples was confirmed by agarose gel analysis. RT-PCR of TRAIL expression was conducted using TITANIUM™ ONE-Step RT-PCR kit (BD Biosciences) according to the User Manual. TRAIL primer set was as follows: sense; 5’-GGCTATGATGGAGGTCCAGG-3’; anti-sense; 5’-GGTCCATGTCTATCAAGTGCTC-3’. PML primer set: sense; 5’-TTCTGGTGCTTTGAGTGCGAG-3’; anti-sense; 5’-TCACTGTGGCTGCTGCAAG-3’. Samples were subject to 25 cycles of amplification under the following conditions: 94°C for 30s, 55°C (TRAIL) or 57°C (PML and GAPDH) for 30s, and 68°C for 1 min, with a final extension at 68°C for 7 min. Reaction mixtures were separated on 1% agarose gels. For control reactions, human GAPDH amplimer set (BD Biosciences) was used.

**Cloning and expression of dominant negative DR-5 in myeloma cells**

Dominant negative DR5 (DR5-DN) was constructed by introducing a SmaI fragment (residues 1-275 of wild type DR5) of pCMV-SPORT6/DR5 (Open Biosystems, Huntsville, AL) into retroviral construct pMSCVhyg vector (BD Biosciences, Palo Alto, CA). Myeloma cell line 8226 was first infected with murine ecotropic retroviral receptor (gift from Dr. Weguo Zhang, Department of Immunology, Duke University) as previously described. Following DR5-DN infection stable clones were generated by limited dilution. DR5-DN positive clones were selected using DR5 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA).
Results

PML is induced in IFNγ sensitive human MM lines

Although IFNγ has been shown to induce apoptosis in hematopoietic cells including MM, the mechanism of action by which it facilitates cell death in MM is currently unclear. As the PML gene has been associated with IFN mediated apoptotic pathways in other cell types, the current studies were undertaken to evaluate the role of PML in IFNγ induced apoptosis of human MM cells. Among nine MM lines tested, five demonstrated growth inhibition that correlated with PML induction upon IFNγ treatment. The four non-responsive lines failed to evidence PML induction (Table 1). From this set, two IFNγ responsive lines, H929 and 8226, and two non-responsive lines, Delta 47 and OPM-2, were selected for further studies. As shown in Figure 1a, IFNγ inhibited proliferation starting at concentrations as low as 500 U/ml in H929 and 8226 cells, but did not inhibit growth of Delta 47 and OPM-2 even at the highest concentration of 2,000U/ml. The growth inhibitory activity correlated with strong PML mRNA and protein induction (Figure 1b and c). Multiple forms of PML with varying molecular weight were induced which likely correspond to different isoforms and post-translational modifications of the PML protein.

Immunofluorescence analysis of the IFNγ treated cells showed a marked increase in both the number and size of PML NBs appearing as typical discrete speckled structures within the nuclei of H929 and 8226 cells, but not in Delta 47 and OPM-2 (Figure 2a). It can be noted that H929 and 8226 also have higher basal levels of PML NBs than OPM2 and Delta 47. Induction of PML was quantitated using LSC to analyze 3,000-5,000 cells. Figure 2b shows scattergrams representing bivariate distributions of integrated green
fluorescence vs maximal pixel of green fluorescence. In untreated cultures, the majority of cells were characterized by low values of integrated as well as maximal pixel fluorescence. Upon IFN expression in nearly 100% of cells. In contrast, no significant increase in fluorescence intensity was observed in OPM-2 cells after IFN treatment. These results indicate that the inhibition of proliferation induced by IFN in myeloma cell lines correlates with PML protein induction and NB formation.

**Downstream targets of IFN**

In an attempt to biochemically define the link between IFN treatment and PML induction, a series of known downstream targets of IFN were analyzed. Both STAT1 and STAT3 have been shown to be activated by IFN in other cell types. IFN treatment resulted in an increase in STAT1 protein and phosphorylated STAT1 in both responsive and non-responsive lines (Figure 3). Similarly, in both responsive and non-responsive lines STAT3 was readily phosphorylated although protein levels were unchanged. Moreover, co-immunostaining of either STAT1 or STAT3 with PML revealed no co-localization with PML NBs (data not shown). Therefore, activation of STAT1 and STAT3 is not likely to be sufficient for PML induction or the differential growth inhibition exhibited by IFN responsive and non-responsive lines.

Other downstream IFN targets include members of the MAP kinase family, particularly p38, which has been reported to play an important role in IFN signaling and to be involved in activation of the PML promoter. However, p38 was found to be
constitutively phosphorylated in all of the MM cell lines tested and IFN treatment did not enhance phosphorylation.

IFN induced apoptosis in B cells has been reported to occur through a STAT1 independent pathway involving a downstream element designated Daxx. Daxx is of particular interest in that association with PML has been demonstrated and a role in apoptosis, although controversial, has been proposed. In myeloma lines, Daxx protein was not induced by IFN and no difference was seen between responsive and non-responsive cells (Figure 3). Additionally, RT-PCR of H929 cells treated with IFN for various times (4h, 8h, 16h, 24h and 48h) showed no induction of Daxx mRNA and cellular protein localization was also unchanged (data not shown). Thus, IFN induced apoptosis in multiple myeloma is Daxx independent. Taken together, STAT1, STAT3, p38 pathway and Daxx induction are not sufficient for IFN induced apoptosis in MM cells suggesting an additional pathway associated with this phenotype. PML is the only IFN induced gene detected in this study that is restricted to responsive cell lines.

**RNAi silencing of PML establishes an essential role in IFN induced apoptosis in MM**

To clarify the role of PML in IFN induced apoptosis of MM cells, an oligonucleotide corresponding to the published RNAi sequence of PML (siPML) was used to inhibit protein expression. PML duplex had no effect on cell survival in the absence of IFN (data not shown). RNAi treatment of 8226 cells markedly reduced the IFN induced levels of PML protein (Figure 4a). PML NB formation was also correspondingly decreased as readily visualized in representative microscopy of cells immuno-stained for PML (Figure 4b). LSC quantitation revealed that IFN treated
control cells transfected with siRNA corresponding to the luciferase gene (siluc) exhibited an induction of 37% which was reduced to 17% in the siPML treated cells (Table 2). The biological consequences of the reduction in PML expression are reflected in a decrease in the ability of IFNα to inhibit cell growth as measured by MTX assay (Figure 4c). IFNα stimulation reduced proliferation to 72% in 8226/siluc treated cells, but to a much lesser extent (91%) in 8226/siPML group when compared to untreated controls. Similar results are also seen in a trypan blue viability assay (Figure 4d).

**PML mediates IFNα induced apoptosis through TRAIL**

The molecular mechanisms of PML’s cellular function(s) remain unclear. However, there is increasing evidence suggesting a role for PML in transcriptional regulation (reviewed in 19,35). To address the issue of the molecular mechanism of PML action in IFNα induced apoptosis of MM cells, we next examined the status of TRAIL, a major IFNα induced apoptotic factor known to be transcriptionally regulated. TRAIL protein was induced only in MM lines sensitive to IFNa mediated apoptosis (Table 1, Figure 5a). Interestingly, exogenous TRAIL was effective at inhibiting the growth of both IFNα responsive and non-responsive cell types indicating that the TRAIL death receptor pathway is intact in all MM lines tested (Figure 5b). To further assess the role of TRAIL in the IFNα response, we analyzed TRAIL secretion from IFNα treated responsive human myeloma cells. Treatment of the IFNα non-responsive cell line Delta47 with supernatant from IFNα treated H929 cell resulted in growth inhibition of the Delta47 cells. This inhibition was reversed by the addition of anti-TRAIL antibody (Figure 5c). Supernatant from IFNα treated responsive myeloma cells therefore has a growth inhibitory effect on non-responsive cells that is mainly mediated by secreted TRAIL.
role of TRAIL in mediating the growth inhibitory response of IFNα was further confirmed by stably expressing a dominant negative death receptor 5 (DR5-DN) construct containing a deletion of the cytoplasmic death domain in 8226 cell line. Expression of DR5-DN not only blocks TRAIL induced cell death in 8226 cells (data not shown) but also rescues 8226 from IFNα induced growth inhibition (Figure 5d). These studies demonstrate that TRAIL mediates the majority, if not all, of the IFNα response exhibited by myeloma cells.

To address the question of whether TRAIL is transcriptionally regulated by, and downstream of PML, TRAIL protein and mRNA levels were analyzed in cells treated with control and siPML duplexes. As seen in Figure 6, both IFNα induced TRAIL protein and mRNA expression were reduced in cells treated with siPML whereas control treated cells were unaffected. These results strongly indicate that TRAIL is a downstream target of PML in IFNα induced apoptosis of MM cells.

As the biological consequences of TRAIL activation are mediated by the activation of caspases leading to apoptosis, we performed experiments to verify that IFN stimulation induces apoptosis as opposed to stasis in the MM cell lines used in this study. As expected, IFNα induced caspase-3 activity in the responsive MM cell lines H929 and 8226 and this activity could be inhibited by a caspase-3 specific inhibitor (Figure 7a). Furthermore, internucleosomal fragmentation was detected in about 60% of the H929 cells and 40% of the 8226 cells by TdT assay (Figure 7b). Thus, IFNα induces apoptosis in responsive MM cell lines, and this process is mediated by PML which is necessary for subsequent induction of TRAIL.
Discussion

IFNs have been used with varying effectiveness as pro-apoptotic agents in the treatment of multiple myeloma. Although only a small subset of patients appear to derive clinical benefit from IFN treatment, these results do not preclude the possibility that IFNs, in combination with newer agents such as proteasome inhibitors or thalidomide, may prove to be clinically effective. The precise mechanism by which IFNs exert anti-tumor activity remains unclear, but recent studies have indicated that IFN induces up-regulation of TRAIL as a critical downstream target in this apoptotic pathway. Moreover, it has been shown that IFN stimulated neutrophils and monocytes release a soluble form of TRAIL that has an apoptotic effect on leukemic cells suggesting a novel mechanism by which IFN might exert anti-tumor activity. The mechanism(s) of TRAIL regulation and the identity of other elements in this pathway remain to be elucidated. Herein, we present evidence that PML plays a major role in IFN induced apoptosis and contributes to the regulation of TRAIL in MM cell lines.

Interest in investigation of the possible involvement of PML in the IFN response in human myeloma was stimulated by the important role suggested for PML as a tumor suppressor in human cancers and as an essential mediator of various pro-apoptotic stimuli. Initial studies demonstrated that PML protein and mRNA are induced by IFN only in MM cell lines responsive to IFN treatment wherein both the number and size of PML NBs increase (Fig. 1, Table 1). Cell lines that are not responsive to IFN treatment do not show PML induction. Multiple isoforms of PML are induced, varying in size from about 50 to 150kD. These isoforms may be generated by alternative splicing and/or post-translational modification. Furthermore, IFN responsiveness and PML induction appear...
not to be dependent on elements associated with these processes in other systems (Fig. 3) including STATs 1 and 3\textsuperscript{14,15}, p38 MAPK\textsuperscript{30,40} and Daxx\textsuperscript{31,32,41} nor with the p44/42 MAPK or PI-3K pathways as determined by use of corresponding inhibitors (not shown). Co-localization of PML with previously reported binding partners such as STAT 3 and Daxx was also not observed although it should be noted that the present studies were limited to endogenously rather than over expressed proteins\textsuperscript{31,32,41}.

Although PML appears to act as a tumor suppressor, the mechanisms by which it achieves this function remain unclear. PML NBs have been suggested to be sites of transcriptional regulation\textsuperscript{35} and IFN\textsuperscript{a} stimulation has been reported to induce >300 genes\textsuperscript{42,43}. Among these is the TRAIL gene which has also been described as an important mediator of IFN\textsuperscript{a} induced apoptosis in human myeloma\textsuperscript{9,42,44,45}. Herein, it was observed that, similar to PML induction, TRAIL is induced by IFN\textsuperscript{a} only in responsive MM cell lines. That recombinant TRAIL kills both IFN responsive and unresponsive cells with equal efficiency indicates that the failure of IFN\textsuperscript{a} to induce apoptosis in certain lines is not due to TRAIL receptor defects. Rather, these observations suggest a failure in the induction of PML and TRAIL in response to IFN\textsuperscript{a} with TRAIL downstream of PML. TRAIL has also been reported to be secreted from cells stimulated with IFN\textsuperscript{29}, and the current studies also provide evidence that TRAIL is secreted upon IFN stimulation of responsive MM cell lines. The ability of secreted TRAIL to kill IFN non-responsive MM lines indicates that TRAIL may be important for paracrine and autocrine effects of IFN\textsuperscript{a} treatment in human myeloma. The functional significance of TRAIL as the biological mediator of IFN\textsuperscript{a} induced growth inhibition was further confirmed by expressing a dominant negative DR5 construct in 8226 cells (IFN\textsuperscript{a}-
responsive) to prevent TRAIL/TRAIL receptor signaling. Dominant negative expression resulted in a rescue of IFN\(\alpha\) induced growth inhibition. These findings are consistent with studies indicating that apoptosis induced by IFN\(\alpha\) in the myeloma cell line U266 was partially blocked by dominant negative DR5\(^9\).

To confirm a role for PML in IFN\(\alpha\) induced apoptosis in myeloma, RNAi was used to specifically down regulate PML expression\(^{34}\). Treatment of an IFN\(\alpha\)-responsive MM cell line with siPML led to a partial rescue of the growth inhibitory effect of IFN\(\alpha\) and correlated with the reduction in level of PML protein levels and formation of PML NBs. Importantly, reduced PML expression resulted in down regulation of both TRAIL mRNA and protein levels. Therefore, differential PML and subsequent TRAIL induction in MM lines appears to determine the response versus non-response phenotype to IFN\(\alpha\).

PML expression is not lost in IFN non-responsive MM cell lines, but rather the amount of PML is much higher in IFN responsive cell lines particularly after IFN treatment. This observation indicates that PML's function in IFN signaling may be concentration dependent, and that certain minimal levels of PML protein are required for transcriptional induction of the TRAIL gene although other mechanisms contributing to IFN non-responsiveness can not be ruled out. Consistent with this interpretation is the suggestion that PML is haplo-insufficient for some of its tumor suppressive properties\(^{26,39}\) as has been reported for other tumor suppressors\(^{46}\). The present observations, in conjunction with other similar studies \((\text{as reviewed in}^{35})\), point toward a mechanism whereby PML protein levels regulate the availability and localization of transcription factors and can through this scenario affect downstream gene activation. This model for the role of PML in regulating transcription is consistent with the current understanding of
the role of the PML NB as a nuclear depot wherein a variety of transcription factors and other proteins are assembled in complexes the stoichiometry of which is likely to be highly dependent on PML levels.

As mentioned previously, the binding partners of PML appear to be quite diverse in different cell types. In order to understand its role in transcriptional control a major focus in the future will, therefore, be to identify transcription factors PML interacts with upon IFN stimulation and define which of these subsequently regulate activation of the TRAIL gene. The availability of both IFN responsive and non-responsive lines may provide an opportunity by using techniques such as array analysis to identify PML regulated target genes and subsequently additional tumor suppressive pathways that are regulated by PML upon IFN treatment. Finally, this study provides new insights in the understanding of IFN responsiveness that may be useful in evaluating the potential use of this agent in patient treatment.
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Figure legends

Figure 1. IFNα induced growth inhibition in MM correlates with enhanced PML expression.
(a) Proliferation of MM cell lines H929, 8226, Delta 47, and OPM-2 following treatment with indicated amounts of IFNα for 48h was determined by MTX assay as described in Materials and Methods. (b) RT-PCR analysis of transcriptional induction of PML mRNA in the indicated MM cell lines with (+) or without (-) IFNα (2,000 U/ml) treatment for 48h. The amplified fragment is located at 5’-end and appears in all PML isoforms. The level of GAPDH serves as loading control. (c) Western blot analysis of PML protein isoforms using rabbit polyclonal anti-PML with (+) or without (-) IFNα (2,000 U/ml) treatment for 48h. The level of GAPDH serves as loading control.

Figure 2. Confocal image and LSC analysis of PML NBs. (a) PML nuclear bodies induced by IFNα (2,000 U/ml) were visualized by immunostaining using monoclonal anti-PML. Pictures are a projection of confocal Z-sections. (b) IFNα treated (2,000 U/ml) and untreated H929 and OPM-2 cells were immuno-stained with mouse monoclonal anti-PML followed by incubation with Alexa 488 conjugated secondary antibody. LSC analysis was performed as described in Material and Methods. 3,000 cells per sample were scanned. PML NBs are represented by scattergrams of two parameters of LSC measurement. Green fluorescence integral represents overall fluorescent intensity within the nucleus and green max pixels represents maximal fluorescent signal (brightest cells).

Figure 3. STAT1, STAT 3, Daxx, and p38 do not correlate with IFNα effects on responsive and non-responsive cell lines
Western blot analysis of Stat1, Stat3, Daxx and p38 MAP kinase determined after 48 h in the presence (+) or absence (-) of IFNα (2,000U/ml).

**Figure 4. RNAi leads to specific down-regulation of PML and a corresponding loss of IFNα mediated growth inhibition.**

(a) Immunoblot analysis of PML induction by IFN (500 U/ml) in parental 8226 cells (left), 8226 cells transfected with control siRNA targeting luciferase (siluc, middle) and 8226 cells transfected with siRNA targeting PML (siPML, right). Protein loading control was measured by GAPDH blotting. (b) Fluorescence microscopy image of PML NB induction. Cells in left panels were untreated and in right panels treated with IFNα (c) MTX proliferation assay comparing the effect of IFNα on 8226 cells transfected with siluc or siPML. Data are expressed as percentage of OD value of IFN treated versus untreated cells as described in Material and Methods with untreated cells representing 100% proliferation and are averaged values of five independent experiments performed in triplicate (± SD). (d) Trypan blue viability assay of the same experiment as in (c). The data are average of three independent experiments performed in triplicate (±SD).

**Figure 5. PML up-regulation correlates with TRAIL induction in MM lines.**

(a) TRAIL is induced only in IFN responsive cell lines H929 and 8226. Western blot analysis of TRAIL protein following treatment of indicated lines with or without IFNα for 48h (2,000 U/ml). GAPDH expression is included as protein loading control. (b) TRAIL inhibits proliferation of both IFN responsive and non-responsive cell lines. MM cell lines were treated with indicated amount of TRAIL and proliferation measured by MTX assay. (c) Biologically active TRAIL is secreted from IFNα responsive cell lines. Supernatant from IFNα treated and untreated H929 cells was added to IFNα non-
responsive Delta47 cells with or without anti-TRAIL antibodies (10 mg/mL) and proliferation measured by MTX assay. Data is presented as percent proliferation of cells treated with supernatant from untreated H929 cells as described in materials and methods. Data are average of three independent experiments done in triplicate (±SD). (d) Dominant negative TRAIL receptor blocks IFN-α growth inhibition. 8226 cells transfected with either vector control (8226/ctr) or DR5-DN construct (8226/DR5-DN) were stimulated with IFN-α 500 U/mL and proliferation was measured by MTX assay. Proliferation was calculated as described in material and methods. A representative experiment (one of three independent experiments) performed in triplicate (±SD) is shown.

**Figure 6.** siPML down regulates TRAIL.

(a) RNAi silencing of PML inhibits induction of TRAIL protein by IFN-α. 8226 cells transfected with siluc or siPML were treated with IFN-α 500U/ml for 48h and analyzed for TRAIL protein expression by immuno-blotting. GAPDH protein level serves as loading control. (b) RNAi silencing of PML inhibits induction of TRAIL mRNA by IFN-α. RT-PCR of cells treated as in (a) using primers specific for TRAIL and GAPDH as a control.

**Figure 7.** IFN-α induces cell death in MM lines by activation of caspases.

(a) IFN-α stimulation induces apoptosis in MM cell lines. IFN-α (2,000U/ml) activates caspase-3 in the MM cell lines H929 and 8226 and inhibitor of caspase-3 blocks IFN-α induced caspase-3 activity and cell death. (b) IFN-α (2,000U/ml) induces apoptosis in the MM cell lines H929 and 8226 as assayed by internucleosomal fragmentation using a TdT assay. Data are presented as average of three independent experiments (±SD).
Figure 1

A

B
Figure 3

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<tr>
<th></th>
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<td>+</td>
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Figure 4

A

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IB: PML

IB: GAPDH

B

untreated                             IFN (500 U/ml)

8226/siluc

8226/siPML
Figure 5

A

<table>
<thead>
<tr>
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<th>H929</th>
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<th>Delta 47</th>
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<tr>
<td>IB: TRAIL</td>
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<td>IB: GAPDH</td>
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B

![Graph showing absorbance at 490 nm for different conditions and cell lines.](image-url)
Figure 6

A

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IB: TRAIL

IB: GAPDH

B

<table>
<thead>
<tr>
<th></th>
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<th>8226/siPML</th>
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RT-PCR TRAIL

RT-PCR GAPDH
Table 1. PML and TRAIL induction following IFN-α treatment of MM lines

<table>
<thead>
<tr>
<th>MM Cell Line</th>
<th>Growth inhibition response</th>
<th>Induction of PML</th>
<th>TRAIL protein expression</th>
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<tr>
<td>OPM2</td>
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</tr>
<tr>
<td>XG1</td>
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</tr>
<tr>
<td>8226</td>
<td>Yes</td>
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<tr>
<td>XG2</td>
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MM cell lines were treated with or without IFN-α (2,000U/ml) for 48h. Growth inhibition was measured by MTS assay as described in Material and Methods. PML induction was assessed by western blot and confocal image analysis. TRAIL was detected by western blotting.
Table 2. LSC quantitation of PML NBs in 8226 cells transfected with siPML and siluc

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<th>IFN-α (500 U/ml)</th>
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<tbody>
<tr>
<td>8226/siluc</td>
<td>6.0%</td>
<td>37.1%</td>
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<tr>
<td>8226/siPML</td>
<td>5.3%</td>
<td>17.0%</td>
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</tbody>
</table>

5,000 cells per sample were scanned by LSC as described in Material and Methods. Percentage represents max green pixel value.
PML mediates IFNα induced apoptosis in myeloma by regulating TRAIL induction

Chun Crowder, Oyvind Dahle, R E Davis, Odd S Gabrielsen and Stuart Rudikoff