IRF8 Regulates Acid Ceramidase Expression to Mediate Apoptosis and Suppresses Myelogeneous Leukemia

Xiaolin Hu1, Dafeng Yang2, Mary Zimmerman1, Feiyan Liu1, Jine Yang1, Swati Kannan1, Andreas Burchert3, Zdzislaw Szulc4, Alicja Bielawska4, Keiko Ozato5, Kapil Bhalla2, and Kebin Liu1,2

Abstract

IFN regulatory factor 8 (IRF8) is a key transcription factor for myeloid cell differentiation and its expression is frequently lost in hematopoietic cells of human myeloid leukemia patients. IRF8-deficient mice exhibit uncontrolled clonal expansion of undifferentiated myeloid cells that can progress to a fatal blast crisis, thereby resembling human chronic myelogeneous leukemia (CML). Therefore, IRF8 is a myeloid leukemia suppressor. Whereas the understanding of IRF8 function in CML has recently improved, the molecular mechanisms underlying IRF8 function in CML are still largely unknown. In this study, we identified acid ceramidase (A-CDase) as a general transcription target of IRF8. We demonstrated that IRF8 expression is regulated by IRF8 promoter DNA methylation in myeloid leukemia cells. Restoration of IRF8 expression repressed A-CDase expression, resulting in C16 ceramide accumulation and increased sensitivity of CML cells to FasL-induced apoptosis. In myeloid cells derived from IRF8-deficient mice, A-CDase protein level was dramatically increased. Furthermore, we demonstrated that IRF8 directly binds to the A-CDase promoter. At the functional level, inhibition of A-CDase activity, silencing A-CDase expression, or application of exogenous C16 ceramide sensitized CML cells to FasL-induced apoptosis, whereas overexpression of A-CDase decreased CML cells’ sensitivity to FasL-induced apoptosis. Consequently, restoration of IRF8 expression suppressed CML development in vivo at least partially through a Fas-dependent mechanism. In summary, our findings determine the mechanism of IRF8 downregulation in CML cells and they determine a primary pathway of resistance to Fas-mediated apoptosis and disease progression. Cancer Res; 71(8); 2882–91. ©2011 AACR.
Mouse CML cell line 32Dcl was also obtained from ATCC. Peripheral blood was obtained from CML/AML patients in the Georgia Health Sciences University Medical Center. All studies with human specimens were carried out in accordance with approved NIH and Georgia Health Sciences University guidelines.

**Mice**

BALB/c mice were obtained from the National Cancer Institute Frederick mouse facility. IRF8 knock out mice were maintained as described (2). FasLgd mice were obtained from the Jackson Laboratory. All mice were housed, maintained, and studied in accordance with approved NIH and Georgia Health Sciences University guidelines for animal use and handling.

**Reverse transcriptase-PCR analysis**

Reverse transcriptase (RT) PCR analysis was carried out as previously described (24). The PCR primer sequences are as follows: human IRF8: forward: 5'-CCAGTTTTGAGGAGTGC-3', reverse: 5'-TGGGAAATGTGAAGCTGAC-3'; mouse IRF8: forward: 5'-GGGGAAAGAGGTTACAGCGT-3'; reverse: 5'-GCTGAATGTTGTTGATGCTACGC-3'; mouse A-CDase: forward: 5'-CTTTTGAGGAATGAGTGGG-3'; reverse: 5'-GTCTTGTAGTGTTGTGGTGC-3' and β-actin: forward: 5'-ATGTGTAAGAAGAAAGACGACTG-3', reverse: 5'-CTCTGTTAGTACCTGTTGAC-3'. PCR band intensity was quantified using NIH Image J program (NIH). Quantitative PCR reactions were done in a StepOnePlus Real-Time PCR system (Applied Biosystem).

**MS-PCR analysis**

Sodium bisulfite modification of genomic DNA was carried out using CpGenome Universal DNA Modification Kit (Chemicon). Mutagenically separated (MS)-PCR primers were carried out as previously described (25). The PCR sequences are as follows: the human IRF8 promoter: unmethylated forward primer: 5'-CCACACACACACCTACTACACCA-3', unmethylated reverse primer: 5'-GATGGTGTA-GTATTTGTCGCCGCGTTACT-3', methylated forward primer: 5'-TCCCGTTAAATACCCAGCGAGGAA-3', and methylated reverse primer: 5'-CGTGTTAGCTGGTGGTTTTCCG-3'.

**Genomic DNA sequencing**

The bisulfite-modified genomic DNA was used as template for PCR amplification of the mouse IRF8 promoter region. Bisulfite PCR primer pairs were designed using MethPrimer program (Chemicon). The Primer sequences are: forward: 5'-GGGTTAGAGTTTTTTAAAATTTGAA-3', reverse: 5'-AACAA-CCAAAACACACACTACTAC-3'. The 503 bp PCR-amplified DNA fragment was cloned to pCR2.1 plasmid using TA cloning kit (Invitrogen). The cloned DNA was then sequenced.

**Cell surface marker analysis**

Spleens were minced to make single cell suspension through a cell strainer (BD Biosciences). The cell suspension was stained with FITC-conjugated anti-mouse CD4, CD8, CD11b, and NK1.1 mAbs (BD Biosciences), respectively. The stained cells were analyzed by flow cytometry.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) assays were carried out according to protocols from Upstate Biotech. Immunoprecipitation was carried out using anti-IRF8 antibody (C-19; Santa Cruz) and agarose-protein A beads (Upstate). The DNA was purified from the eluted solution and used for PCR. The PCR primers used to amplify region 5 of the A-CDase promoter (Fig. 4B) are as follows: forward: 5'-TGTTCGTGAAAGCAGAAAATG-3', reverse: 5'-GTATTGTCGCCGCTTTACT-3'.

**Protein–DNA interaction assay**

Electrophoresis mobility shift assay (EMSA) was carried out as previously described (25). The DNA probes are as follows: wt forward: 5'-GCCAGAGGAAAAATCTGGAGTCTCCGCC-3', reverse: 5'-GGGGGCAGCTTTCCAGTTTCTCTCT-3'; mutant forward: 5'-GGCAAGCTGATACTCTTCTGTCGCG-3', reverse: 5'-GGGAGGACTGAGTAGTGAC-3'. Goat IgG and anti-IRF8 antibody (C-19) were obtained from Santa Cruz Biotech.

**Measurement of apoptotic cell death**

Cells were treated with either IFN-γ (R&D Systems) overnight, A-CDase inhibitor LCL85 overnight, or C16 ceramide (Santa Cruz) for 1 hour, followed by incubation with recombinant FasL (100 ng/mL, PeproTech) for approximately 24 hours. Cells were then collected and incubated with propidium iodide (PI) solution (R&D Systems) and analyzed by flow cytometry. For measurement of apoptosis in CD11b+ primary cells, total spleen cells were treated with LCL85 (2 μmol/L) for 4 hours, followed by incubation with FasL (25 ng/mL) for approximately 16 hours. The cells were then collected and stained with anti-CD11b mAb (BD Biosciences) and PI. The CD11b+ cells were gated out to determine the percentage of PI+ CD11b+ cells. The percentage of methylation status of cytosine was analyzed using Qumas program (26).

Western blotting analysis

Western blotting analysis was carried out as previously described (28). The blots were probed with the following antibodies: anti-IRF8 (C-19; Santa Cruz) at a 1:200 dilution; anti-mouse A-CDase (T-20; Santa Cruz) at 1:1,000; anti-Cytochrome C (BD Biosciences) at 1:500; and anti-β-actin (Sigma) at 1:8,000. Blots were detected using the ECL Plus (Amersham Pharmacia Biotech) Western detection kit.
cell death was calculated by the formula: % cell death = % PI+ cells after FasL treatment – % PI+ cells without FasL treatment.

**Gene silencing**

32D-BA cells were transduced with Scramble shRNA (catalogue no. sc-108080) and A-CDase-specific shRNA-expressing lentiviral particles (catalogue no. sc-140807-V; Santa Cruz) and selected for stable lines with puromycin. Cells were analyzed for A-CDase mRNA level by RT-PCR and sensitivity to Fas-mediated apoptosis by PI staining as described above.

**A-CDase overexpression**

Mouse A-CDase coding region was amplified by RT-PCR and cloned to pEGFP-N1 plasmid to generated pEGFP.mA-CDase. The insert was verified by DNA sequence. 32D-BA.IRF8 cells were transiently transfected with pEGFP-N1 and pEGFP.mA-CDase, respectively, and analyzed for A-CDase expression by RT-PCR and sensitivity to Fas-mediated apoptosis as described above.

**Synthesis of LCL85**

(1R,2R)-2-N-[(1′-pyridinium)-hexadecanoylamino]-1-(4′-nitrophenyl)-1,3-propandiol bromide, was synthesized.
by Lipidomics Shared Resource at Medical University of South Carolina, as previously described (29).

**Measurement of endogenous ceramide level**

Cellular levels of endogenous ceramides were measured by high-performance liquid chromatography/mass spectrometry (LC/MS) as described (30, 31). Ceramide levels were normalized to the total cellular protein contents.

**Results**

**IRF8 expression is regulated by the IRF8 promoter DNA methylation in CML**

IRF8 expression is dramatically downregulated in the majority of human myeloid leukemia (11, 12). Because IRF8 expression is mediated by the IRF8 promoter DNA methylation in colon carcinoma cells (32) and multiple other types of tumors (33–36), we hypothesized that downregulation of IRF8 might be mediated by the IRF8 promoter DNA methylation in human myeloid leukemia. To test this hypothesis, we first compared IRF8 expression level in human myeloid leukemia cell lines and patient peripheral blood mononuclear cell (PBMC) to that in PBMC from normal donors. RT-PCR analysis indicated that IRF8 expression is indeed significantly lower in human myeloid leukemia cells than in normal PBMC cells as previously reported (ref. 11; Fig. 1A). MS-PCR analysis revealed that the IRF8 promoter is not methylated in normal PBMC but methylated in human myeloid leukemia cell lines and PBMC derived from human myeloid leukemia patients (Fig. 1B). Both myeloid leukemia cell lines K562 and LAMA84 exhibited a resistant phenotype to FasL-induced apoptosis (Fig. 1C and D). It is known that IFN-γ can sensitize tumor cells to Fas-mediated apoptosis (37). However, IFN-γ treatment also failed to sensitize the 2 myeloid leukemia cell lines to Fas-mediated apoptosis (Fig. 1C and D). Therefore, we concluded that human myeloid leukemia cells are resistant to Fas-mediated apoptosis.

To elucidate the molecular mechanisms underlying IRF8 promoter DNA methylation in myeloid leukemia pathogenesis, we used the myeloid 32D progenitor cell model for in vitro and in vivo studies. To induce a Bcr/Abl-dependent leukemia system, 32D cells were stably transfected with a vector containing the coding sequence of Bcr-Abl (32D-BA). The transfected cells thus mimic human myeloid leukemia. MS-PCR analysis revealed that the IRF8 promoter is methylated in 32D and 32D-BA cells (Fig. 2A). Consistent with the IRF8 promoter methylation status, IRF8 protein is undetectable in 32D and 32D-BA cells, and inhibition of DNA methylation restored IRF8 expression (Fig. 2B).

We next cloned a 503 bp long IRF8 promoter region (−350 to +153 relative to the IRF8 transcription initiation site) of 32D cells and sequenced the entire region. There are 51 CpG dinucleotides in this 503 bp region. Overall, an average of 73% of the cytosines of the 51 CpGs was methylated in human myeloid leukemia cells. The transcription initiation site is marked by +1. Each circle represents a CpG dinucleotide. Open circle, unmethylated CpG; closed circle, methylated CpG. Results from 5 independent clones are shown.

**IRF8 is a transcription repressor of A-CDase in myeloid cells**

We next restored IRF8 expression by ectopic expression of IRF8 in 32D-BA cells and analyzed the expression levels of key apoptosis mediators in the Fas-mediated apoptosis signaling pathway. Among the multiple apoptosis mediators examined, A-CDase was found to be repressed by ectopically expressed IRF8. A-CDase is present in the organelle-enriched mitochondrial fraction of 32D-BA and 32D-BA-vector cells, but dramatically decreased in 32D-BA-IRF8 cells (Fig. 3A). The A-CDase transcript level is also significantly lower in 32D-BA-IRF8 cells as compared to the control cells (Fig. 3A). Consistent with the observation that inhibition of DNA methylation restores IRF8 expression (Fig. 2C), inhibition of DNA methylation repressed A-CDase expression (Fig. 2C).
To determine whether IRF8 directly regulates A-CDase transcription, we carried out ChIP assay to determine whether IRF8 directly binds to the A-CDase promoter region in vivo. Analysis of interactions between IRF8 protein and an approximately 6,000 bp region of the A-CDase promoter (−5,000 to +1,000 relative to the A-CDase transcription initiation site) revealed that IRF8 binds to the A-CDase promoter near the transcription start site in 32D-BA.IRF8 cells (Fig. 3B). To locate the IRF8 binding site at the A-CDase promoter region, we analyzed the A-CDase promoter and identified a potential IRF8 binding site [IRF-Ets composite sequence (IECS)] (ref. 38; Fig. 3C). Analysis of IRF8 and this IECS sequence interaction revealed that IRF8 specifically binds to this DNA sequence (Fig. 3C). Therefore, we concluded that IRF8 is a transcriptional repressor of A-CDase and IRF8 binds directly to the A-CDase promoter region to regulate A-CDase transcription in CML cells.

Restoration of IRF8 expression resulted in C16 ceramide accumulation in myeloid cells

A-CDase converts ceramide to sphingosine and sphingosine-1-phosphate and thus is a key mediator of the sphingosine signaling pathway (39, 40). We then measured C14-, C16-, C18-, C20-, C22-, C24-, and dhC16-ceramide contents in the 3 cell lines and found that these ceramide levels are very low to undetectable in 32D-BA and 32D-BA.Vector cells. Restoration of IRF8 significantly increased C16- and dhC16-ceramide levels (Fig. 4). Because accumulation of ceramide often increases cell sensitivity to apoptosis (30, 41, 42), we reasoned that 32D-BA.IRF8 cells should exhibit increased sensitivity to Fas-mediated apoptosis. Indeed, consistent with the increased ceramide level, 32D-BA.IRF8 cells became more sensitive to Fas-mediated apoptosis (Fig. 5A).

Figure 3. IRF8 binds to the A-CDase promoter region to repress IRF8 expression in CML cells. A, cytosol and mitochondrion fractions were prepared from 32D-BA, 32D-BA.Vector, and 32D.BA-IRF8 cells and analyzed for IRF8 and A-CDase protein level by Western blotting analysis. CytC was used as normalization control. Right, analysis of A-CDase mRNA level by real-time PCR. B, ChIP analysis of IRF8 and A-CDase promoter interaction. Top, mouse A-CDase promoter structure. The ChIP PCR regions are indicated at the bottom. Bottom, ChIP PCR results. 1, input genomic DNA; 2, IgG control; 3, anti-IRF8 antibody. Representative image of 1 of 2 independent experiments is shown. C, IRF8 binds to the A-CDase promoter DNA in vitro. Top, the A-CDase promoter structure. Bottom, EMSA of IRF8 association with the IECS sequence-containing DNA probe. Nuclear extracts (NE) prepared from 32D-BA and 32D-BA.IRF8 cells were incubated with mutant probe (lanes 4 and 5), wt probe (lanes 1, 2, 3, 6, 7, 8, and 9), in the presence of isotype control IgG (lane 6) or anti-IRF8 Ab (lanes 7), and analyzed for the DNA–protein interactions. Lanes 1 to 7, 5 μg nuclear extract/reaction. Lanes 8 and 9, 20 μg nuclear extract/reaction. The potential probe–IRF8 complex is indicated at the right.

Figure 4. C16-ceramide and dhC16-ceramide contents in CML cells. Ceramide contents were determined by LC/MS. Representative results of 2 independent measurements are shown.
A-CDase mediates myeloid cell sensitivity to Fas-mediated apoptosis

To further define the role of A-CDase in Fas-mediated apoptosis, we treated 32D-BA cells with A-CDase inhibitor LCL85. LCL85 represents a cationic analog of B13, the first discovered inhibitor of A-CDase, designed to act as mitochondriotropic inhibitor of this enzyme (29, 43). Pretreatment with LCL85 significantly increased 32D-BA cell sensitivity to Fas-mediated apoptosis (Fig. 5B). We also incubated 32D-BA cells with exogenous C16 ceramide and analyzed their sensitivity to Fas-mediated apoptosis. C16 ceramide pretreatment significantly increased 32D-BA cell sensitivity to Fas-mediated apoptosis (Fig. 5C). In addition, silencing A-CDase also significantly increased 32D-BA cell sensitivity to Fas-mediated apoptosis (Fig. 5D). Conversely, overexpression of A-CDase significantly decreased 32D-BA cells to Fas-mediated apoptosis (Fig. 5D). Taken together, we demonstrated, by 4 complementary approaches, that A-CDase plays an important role in mediating 32D-BA cell sensitivity to Fas-mediated apoptosis.

IRF8 functions as an A-CDase transcription repressor to regulate primary myeloid cell lineage differentiation in vivo

To determine whether IRF8 regulates A-CDase expression under physiological conditions, we examined A-CDase protein level in IRF8 knock out mice. Spleen cells from IRF8 knock out and age-matched wt control littermate mice were first analyzed for 4 major subsets of immune cells: CD4\(^+\) T cells, CD8\(^+\) T cells, CD11b\(^+\) macrophage, and NK cells. As compared to the wt mice, the percentage of CD4\(^+\) T cells,
CD8+ T cells and NK cells were decreased in spleens from IRF8 knock out mice. However, the total number of T and NK cells per spleen is similar between IRF8 knockout and wild type mice. As noted before, the size of spleen of the IRF8 knock out mice was larger than that of wt mice (Fig. 6B). CD11b+ cells that likely include macrophages and granulocytes were increased in the spleen of the IRF8 knock out mice (Fig. 6A), thus confirming the role of IRF8 as a key transcription factor in lineage-specific differentiation of myeloid cells (1, 2). Analysis of A-CDase level revealed that A-CDase protein level is weak to undetectable in CD4+ T cells, CD8+ T cells, CD11b+ myeloid cells, and NK cells. Knocking out IRF8 did not alter AC-Dase protein level in these 3 subsets of primary cells. However, A-CDase protein level is dramatically higher in CD11b+ cells in IRF8 knock out mice than that in control mice (Fig. 6C).

Next, we sought to determine whether inhibiting A-CDase increases the sensitivity of the IRF8 KO CD11b+ cells to Fas-mediated apoptosis. Spleen cells were pretreated with LCL85 and then incubated with FasL. The primary IRF8 KO CD11b+ cells are sensitive to Fas-mediated apoptosis (Fig. 6D). However, LCL85 significantly increased the IRF8 KO CD11b+ cell sensitivity to Fas-mediated apoptosis (Fig. 6D). Taken together, our data suggest that IRF8
regulates myeloid cell lineage differentiation at least partially through regulating apoptosis sensitivity in an A-CDase-dependent manner.

IRF8 suppresses CML development in vivo

Because IRF8 represses A-CDase expression to mediate apoptosis in myeloid leukemia cells (Fig. 5), we next tested whether restoration of IRF8 expression would suppress myeloid leukemia development in vivo. 32D-BA.Vector and 32D-BA.IRF8 cells were injected into naïve mice and mouse survival was recorded. Mice that received 32D-BA.Vector cells started to die on day 24 after tumor transplant and all mice were dead 56 days after tumor transplant. In contrast, only 1 of the 11 mice that received 32D-BA.IRF8 cells was dead 52 days after tumor transplant and 9 of the 11 mice were still alive 65 days after tumor transplant (Fig. 7A).

Fas-mediated apoptosis pathway is a key effector mechanism for the host immune system to eliminate unwanted or diseased cells to maintain normal homeostasis (44) and to control tumor development (45, 46). It has been shown that the host T lymphocytes play a key role in suppressing myeloid leukemia (47). T lymphocytes primarily use perforin and FasL to induce target tumor cell apoptosis (46, 48). Therefore, acquisition of resistance to Fas-mediated apoptosis may confer the tumor cell an advantage to avoid the T lymphocyte-mediated elimination. Our above observations that restoration of IRF8 expression confers the tumor cell sensitivity to Fas-mediated apoptosis (Fig. 5A) and immunocompetent naïve mice can survive 32D-BA.IRF8 tumor cell challenge (Fig. 7A) suggest that the host immune cells might have played a significant role in suppressing apoptosis-sensitive myeloid leukemia development through the Fas-mediated effector mechanism. In that case, we expected that 32D-BA.IRF8 cells should cause increased mortality in the FasL-deficient mice. Indeed, FasL-deficient mice exhibited significantly lower survival rate than the wt control mice after 32D-BA.IRF8 tumor cell challenge (Fig. 7B).

Immunocompetent mice can survive 32D-BA.IRF8 cell challenge (Fig. 7A). To determine whether the surviving mice would develop immunological memory against myeloid leukemia, we rechallenged these surviving mice with 32D-BA cells 90 days after the first tumor challenge. Naïve mice were used as control. While naïve mice receiving this first 32D-BA cell injection died all up to 43 days after transplantation, all of the 32D-BA.IRF8 cell prechallenged mice remained alive at the end of the experiment (56 days after the tumor challenge; Fig. 7C). Taken together, our data suggest that IRF8 functions as a tumor suppressor at least partially through regulating A-CDase expression to mediate CML sensitivity to Fas-mediated effector mechanism of the host immune system in vivo.

Discussion

Human myeloid leukemia patients exhibited significant decreased level of IRF8 protein in their hematopoietic cells (11, 12). A major phenotype of IRF8 knock out mice is the uncontrolled clonal expansion of granulocytes and macrophages that can progress to a fatal blast crisis (2). These observations suggest that IRF8 is a tumor suppressor. It has been proposed that acquisition of apoptosis resistance is responsible for the CML-like pathogenesis, and several apoptosis regulator, including Bcl-xL, Bcl-2, and FAP-1, have been shown to play a role in regulating apoptosis in myeloid leukemia cells in vitro (15, 18, 19). The function of IRF8 in apoptosis and tumor suppression has also been demonstrated in other nonhematopoietic cells (27, 32, 37, 49). However, it has been shown that the correlation between IRF8 and the Bcl-2 family members observed in vitro is not observed in vivo (21). Our data suggest that IRF8 is a transcription repressor of...
A-CDase in myeloid leukemia cells in vitro and in primary myeloid cells in vivo.

A-CDase converts ceramide to sphingosine and sphingosine-1-phosphate and thus is a key mediator of the sphingosine signaling pathway, which plays a key role in Fas-mediated apoptosis (39, 40). We demonstrated here, by 4 complementary approaches, that A-CDase directly mediates Fas-mediated apoptosis in CML cells (Fig. 5). Our data also revealed that IRF8 directly binds to the A-CDase promoter to repress IRF8 expression (Fig. 3B and C). Furthermore, restoration of IRF8 expression in myeloid leukemia cells decreased A-CDase protein level (Fig. 3A) and consequently led to accumulation of C16 ceramide (Fig. 4). C16 ceramide has been shown to protect HNSCC cells from ER stress and apoptosis (50). However, C16 ceramide has also been shown to increase the sensitivity of Jurkat T cells and hepatocytes to Fas-mediated apoptosis (41). Our data suggest that C16 ceramide enhances CML sensitivity to Fas-mediated apoptosis (Fig. 5).

More importantly, we demonstrated that IRF8 also represses A-CDase expression in primary myeloid cells in vivo. Thus, our data strongly suggest that IRF8 functions as an apoptosis mediator at least partially through repressing A-CDase transcription in myeloid cells.

The lymphocyte-executed and Fas-mediated apoptosis is a key effector mechanism for the host immune system to eliminate unwanted and/or diseased cells during lineage differentiation and homeostasis (44). The Fas-mediated apoptosis is also a critical component of the host immune-surveillance system in suppression of tumor development (45, 46). It has been shown that the host T lymphocytes play a key role in suppressing myeloid leukemia (47). We observed that restoration of IRF8 expression suppressed myeloid leukemia development in vivo (Fig. 7A). We further observed that IRF8-mediated tumor suppression function is significantly impaired in FasL-deficient mice. Based on these observations, we propose that the Fas-mediated effector mechanism of the host T lymphocytes plays an important role in the elimination of unwanted cells during myeloid cell lineage differentiation to maintain normal homeostasis. IRF8 regulates A-CDase and potentially other apoptosis-related genes (15, 18–21) to maintain myeloid cell sensitivity to Fas-mediated apoptosis for normal homeostasis. Loss of IRF8 expression (i.e., via the IRF8 promoter DNA methylation) leads to increased A-CDase, decreased C16 ceramide, and subsequently an apoptosis resistant phenotype, resulting in uncontrolled clonal expansion of undifferentiated myeloid cells that can progress to CML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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