The effect of azacitidine on interleukin-6 signaling and nuclear factor-κB activation and its *in vitro* and *in vivo* activity against multiple myeloma

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ABSTRACT

Background
Azacitidine is a DNA methyltransferase inhibitor and cytotoxic agent known to induce apoptosis of some cancer cells. This study evaluated the pre-clinical potential of azacitidine as a therapeutic agent for multiple myeloma.

Design and Methods
Dose responsiveness to azacitidine was determined utilizing a panel of genetically heterogenous human multiple myeloma cell lines. Azacitidine was also tested against primary multiple myeloma cells and in the 5T33MM murine model of systemic myelomatosis. Mechanistic studies included immunoblotting of key apoptosis signaling proteins, analysis of p16 gene methylation status, and characterization of both the interleukin-6 and nuclear factor-κB signaling pathways following azacitidine treatment.

Results
Human myeloma cell lines and primary multiple myeloma cells underwent apoptosis following exposure to clinically achievable concentrations of azacitidine (1 μM–20 μM). Similarly, azacitidine prolonged survival from 24.5 days to 32 days (p=0.001, log rank) in the 5T33MM model. At a mechanistic level azacitidine down-regulated two crucial cell survival pathways in multiple myeloma. First, it inhibited the elaboration of both interleukin-6 receptor-α and interleukin-6 resulting in the reduced expression of both phospho-STAT3 and Bcl-xl. Secondly, azacitidine inhibited both nuclear factor-κB nuclear translocation and DNA binding in a manner independent of IκB. The kinetics of these azacitidine-induced responses was more consistent with protein synthesis inhibition than with either hypomethylation or another DNA-mediated effect.

Conclusions
Azacitidine rapidly induces apoptosis of multiple myeloma cells, is effective *in vivo* against multiple myeloma and inhibits two crucial cell survival pathways in this disease. We conclude that azacitidine demonstrates novel and highly relevant anti-myeloma effects and warrants further evaluation in a clinical context.

Key words: multiple myeloma, Stat3, Bcl-XL, SOCS3, nuclear factor kB.

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Introduction

Multiple myeloma (MM) is a clonal malignancy of plasma cells characterized by the presence of a monoclonal protein in serum and/or urine, widespread osteolysis, renal failure and anemia.[1] It is a disorder principally of older patients whose median age at diagnosis is 70 years. The median survival of the majority of patients is only 2.5-3.0 years.[2] Over the past decade the outlook for younger patients has improved with the widespread introduction of high-dose chemotherapy conditioned autologous stem cell transplantation resulting in median survivals in the range of 5.0-6.0 years.[3,4] Furthermore, the introduction of bisphosphonates as a standard component of supportive care in the prevention of osteolysis has reduced the likelihood of skeletal-related morbidity and may have enhanced survival in subgroups of patients.[5,6] Unfortunately, despite these significant advances, disease recurrence following initial disease response is inevitable, with successive relapses manifesting increasing drug resistance, invariably culminating in uncontrollable and fatal disease. Within this context the demonstration that the immunomodulatory agent thalidomide was able to produce clinically meaningful responses in patients with advanced MM was highly significant.[7] Not only did the success of thalidomide provide an additional therapeutic option for patients, it also provided an impetus for further exploration of novel therapeutic approaches to MM. Recently published data describing significant anti-MM activity for the thalidomide analog lenalinomide and the proteasome inhibitor bortezomib attest to this.[8,9]

The underlying pathogenesis of MM and mechanisms of progression are complex and remain poorly understood.[10] What is, however, becoming evident is that certain non-random genetic and epigenetic abnormalities may be associated with particularly poor outcomes in patients managed with either conventional or high-dose chemotherapeutic strategies. Structural karyoptypic abnormalities of chromosome 13 and t(4;14) are well recognized examples of the former.[11-14] The role of epigenetic abnormalities in MM is less clear; however, several studies have demonstrated that methylation-mediated silencing of the cell cycle regulatory protein p16 is a common event in MM, occurring in from 19%-61% of cases.[15-18] The p16 gene is located at 9p21 and is responsible for the production of a 15.8 kD protein that plays a crucial role in the prevention of cell cycle progression at the G_1/S check-point.[19] Furthermore, preliminary data support the notion that the silencing of p16 may be not only associated with progression of monoclonal gammopathy of undetermined significance to MM, but in already established MM, is associated with a more proliferative tumor phenotype and shorter survival.[17-20] Based on these observations strategies aimed at re-activating expression of the p16 gene may provide a potential therapeutic approach for MM.

Azacitidine (AZA) is a ring analog of the naturally occurring pyrimidine nucleoside, cytidine and was first synthesized over 40 years ago.[2] *In vitro* studies have demonstrated that AZA is not only cytotoxic but that it is also capable of inducing cellular differentiation.[22,23] Kiziltepe *et al.*[24] demonstrated that 5-azacitidine induces apoptosis and has synergistic cytotoxicity with bortezomib and doxorubicin in MM. Furthermore, following incorporation into DNA, AZA is capable of covalently binding to and inhibiting DNA methyltransferase with resultant hypomethylation and transcriptional reactivation of previously silenced genes.[21] It is this latter characteristic that has led to revived interest in the therapeutic potential of AZA in the treatment of hematologic malignancies, particularly myelodysplastic syndromes.[25,26]

In this study we examined the effect of AZA on human myeloma cell lines, primary myeloma cells and in an *in vivo* model of MM. Significant anti-MM activity via previously undescribed modes of action was observed. These data coupled with the well characterized toxicity profile and track record of AZA in clinical practice provide a strong rationale for the further exploration of AZA as a potential therapeutic agent in MM.

Design and Methods

Human myeloma cells lines and azacitidine

The human myeloma cell lines U266, NCI H929 and RPMI 8226 were obtained from the American Type Culture Collection (Rockville, MD, USA). OPM-2 and LP 1 cell lines were from Deutshe SammLung von Mikroorgaanismen und Zellculturen (Braunshwieig, Germany). KMS 12PE and KMS 18 were a kind gift from Dr. Takemi Otsuki, Kawasaki Medical School, Japan. All cell lines were grown in RPMI-1640 medium supplemented with 10% iron fortified bovine calf serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin. AZA was kindly provided by Pharmion Pty Ltd. (Melbourne, Australia) and was resuspended in 0.9% NaCl solution to make a final stock solution of 10 mM.

Methyl tetrazolium salt (MTS) assays

MTS assays (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega) were used to quantify the percentage of metabolically active AZA-treated and untreated cells. Briefly, 20 000 cells per well were plated onto a 96-well plate and AZA, at a range of concentrations (1-10 μM), was added every 24 hours. At various time-points 20 μL of MTS reagent were added and the cells were incubated for a further 4 hours at 37°C. The plates were then read at 490 nm. To determine the impact of caspase inhibition on the activity of AZA, U266 and NCI H929 were treated with AZA 5 μM with or without 10, 50 and 100 μM of one of three different caspase inhibitors; caspase 3/Z-DEVD-FMK, caspase 8/Z-IETD-FMK, caspase 9/Z-LEHD-FMK) or a broad caspase inhibitor/Z-VAD-FMK (Merck Pty Ltd., Darmstadt, Germany) and incubated at 37°C for 24 hours. Cell proliferation was then determined using the MTS assay as described above.

The ability of exogenous interleukin-6 (IL-6) to prevent the killing of AZA-treated U266 was evaluated. U266 cells were treated or not with 5 μM AZA daily up to 96 hours with the addition of either 100 pg/mL or 3 ng/mL IL-6 (R&D Systems, Minneapolis, USA) every 24 hours. Cell proliferation at 24, 48, 72 and 96 hours was measured by the MTS assay and paired protein lysates

were prepared for western blot analysis of phosphorylated STAT3 as described below.

To measure the functional role of caspase-3 on cell apoptosis, we transfected NCI H929 and U266 utilizing the SignalSilence(R) Caspase-3 siRNA Kit from Cell SignalingTechnology, Inc., USA. Forty-eight hours post-transfection NCI H929 and U266 cells were treated, respectively, with 1 or 5 μM AZA daily for 72 hours and cell death was measured by propidium iodide (PI) staining on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) and analyzed with EXPO 32 software.

Methylation-specific polymerase chain reaction

Genomic DNA was isolated from RPMI 8226 and LP-1 cells that were treated or not with AZA 5 μM daily for 72 hours. The DNA was isolated using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. The resultant genomic DNA was then bisulphated using a CpGenome Universal DNA Modification Kit (Chemicon, Billerica, USA). The polymerase chain reaction (PCR) was performed under the following conditions: 1 x PCR Buffer, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 0.2 μM each of forward and reverse primers, 2.5 U of Platinum Taq DNA Polymerase and 40 ng DNA template. The primers for MSP and unmethylation specific PCR (UMSP) were as follows: forward MSP - $p16^{INK4a}$: TTATTAGAGGGTGGGGCGGATCGC, reverse MSP-$p16^{INK4a}$: GACCCCGAAC CG CGACCG-TAA, forward UMSP-$p16^{INK4a}$: TTATTAGAGGGTGG-GGTGGATTGT and reverse UMSP-$p16^{INK4a}$: CAACCC-CAAACCACAACCATAA. The MSP-$p16^{INK4a}$ primers generated 150 bp products while the UMSP-$p16^{INK4a}$ generated 151 bp products. Reactions were amplified in a Hybaid PCR machine (Eppendorf, Westbury, USA) and products were run on 2% agarose gel/TBE using a 1 Kb Plus DNA Ladder (Invitrogen Corporation, Carlsbad, USA) as the reference.

Primary myeloma cells

Bone marrow mononuclear cells from patients with advanced, multiply relapsed MM (n=13) were isolated by Ficoll Paque Plus (Amersham Biosciences, Piscataway, USA). Buffy layers containing the mononuclear cells were removed and the red blood cells were lysed using red blood cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl and 0.1 mM EDTA, pH 8.0) for 5 min at 37°C followed by a wash with sterile phosphate-buffered saline (PBS). Cells were then cultured overnight in RPMI 1640 medium supplemented with 10% iron fortified bovine calf serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin. The next day aliquots of 5×10^5 cells were treated with 10 or 20 μM AZA for 48 hours. The cells were then stained with CD45-FITC and CD38-PerCP for 15 min at room temperature, washed with FACS buffer (0.5% FCS/PBS) then fixed on ice with 2% PFA for 20 min. After incubation, cells were washed and then stained with Apo 2.7 PE in permeabilization buffer (0.3% saponin, 1% FCS in PBS) for 20 min on ice. After a final wash in FACS buffer the cells were resuspended in 300 μL of FACS buffer. Samples were studied on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) and analyzed with EXPO 32 software.

Evaluation of in vitro and in vivo activity using the 5T33 cell line and 5T33MM model

5T33 cells were cultured in McCoy's medium supplemented with 10% iron fortified bovine calf serum, 2 mM L-glutamine and 100 U/mL penicillin/streptomycin. Subsequently, 20,000 5T33 cells/100 μL complete medium were treated with 5-100 μM AZA for 72 hours and cell proliferation was measured by the MTS assay as described previously. C57BL/KaLwRij mice (8-10 weeks old) were obtained from the Animal Resources Center (Perth, Australia). Animals were housed and treated according to conditions approved by the Alfred Medical Research and Education Precinct (AMREP) animal ethics committee. The murine myeloma 5T33 cell line was a gift from Associate Professor Pamela Sykes, Flinders University, Australia. The 5T33 cells were maintained in McCoy's 5A modified medium (Sigma-Aldrich, Sydney, Australia) supplemented with 10% iron fortified fetal bovine serum (JRH Biosciences, Brooklyn, VIC, Australia), 50 U/mL penicillin, 50 μg/mL streptomycin and 2.92 mg/mL glutamine (Invitrogen) at 37°C in 5% carbon dioxide. Prior to inoculation 5T33 cells were washed three times in sterile 0.9% saline for irrigation then resuspended in sterile 0.9% saline for injection and quantification. Each mouse was administered 5×10^5 5T33 cells via the tail vein. Commencing on day 7 following 5T33 cell transfer, the mice were treated with 1 mg/kg, 3 mg/kg or 10 mg/kg AZA (n = 10 at each dose level) or vehicle (sterile saline, n = 10) on days 7, 9, 11, 13 and 15 by intraperitoneal injection. The mice were monitored daily for signs of hind limb paralysis or cachexia. When mice displayed paraparesis or other obvious signs of disease[26] they were immediately killed by carbon dioxide inhalation. Time to euthanasia was determined using the method of Kaplan and Meier.

Annexin-V/PI and cell cycle analyses of AZA-treated human myeloma cell lines

U266, NCI H929, LP-1 and RPMI8226 cells were treated daily with AZA 5 μM for 72 hours and the percentages of apoptotic cells at 24, 48 and 72 hours were determined by flow cytometry using PI and annexin-V staining and compared to those of untreated control cells. The cells were washed with 0.01M PBS (0.0027M KCl and 0.137M NaCl, pH 7.4, at 25°C), resuspended in binding buffer, and then stained with FITC-labeled annexin-V antibody. The cells were incubated for 15 min in the dark, washed with 0.01M PBS, resuspended in binding buffer with PI and then analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). To evaluate the impact of AZA on cell cycling, 10^6 cells of each of the four human myeloma cell lines treated or not with AZA 1-5 μM for 24, 48 or 72 hours were pelleted at 8,000 g for 5 min and then resuspended in ethanol-0.01M PBS (70/30 v/v). After 30 min, the cells were pelleted and resuspended in 100 μL lysis buffer (LPR, BD Biosciences) followed immediately by 0.5 μL RNase/PI. The samples were analyzed after 15 min on the FACScan (Becton Dickinson) and the percentages of cells in G_0-G_1, S and G_2+M phases of the cell cycle were analyzed using EXPO 32 software.

Western blotting

Nuclear and cytosolic extracts were isolated using a NE-PER kit (Pierce Biotechnology Rockford, USA). Briefly, 10^6 cells were pelleted then solubilized in Buffer CER I for 10 min on ice followed by addition of CER II for 1 min, then spun at 8,000g for 5 min at 4°C. The resultant supernatant containing the cytosolic fraction was collected and used or stored at -20°C. Buffer NER was added to the pellet, vortexed and kept on ice. The cells were vortexed a further three times at 10 min intervals then spun at 8,000g for 10 min at 4°C. Supernatant containing the nuclear extract was collected and used immediately or stored at -20°C. Proteins were resolved by SDS-PAGE on 1.5 mm gels and electrotransferred onto a nitrocellulose membrane (Pall). Blots were incubated with antibodies against caspases 3, 8, and 9, PARP, cytochrome c, STAT3, pSTAT3 (Tyr 705), IL6-Rα and α-tubulin for 2 hours at room temperature and then with appropriate secondary antibodies linked to horse-radish peroxidase.

All antibodies were purchased from Santa Cruz Biotechnology Inc. USA except caspase 3 and pSTAT3 antibody, which were from Cell Signalling Technology Inc. USA. The blots were subjected to SuperSignal West Pico (Pierce Biotechnology) for 5 min then exposed on CL-Xposure film and developed using a CP100 X-Ray Film Processor (Agfa, Mortsel, Belgium).

Evaluation of IL-6 and IL-6 receptor-α (IL6-Rα) elaboration

The concentrations of IL-6 and soluble IL6-Rα in U266 conditioned medium following treatment with AZA 5 μM daily for 72 hours were determined at various time-points using enzyme-linked immunosorbent assay (ELISA) kits from Quantikine (R&D Systems) according to the manufacturer's instructions. Likewise, soluble IL6-Rα levels following treatment with cyclohexamide 20 μg/mL were also measured.

In addition, protein lysates were prepared for western blot analysis to determine whole cell IL6-Rα expression as described above. Samples were immediately collected, snap frozen in liquid nitrogen and stored at –80°C until required. Total RNA from U266 cells treated with AZA for 2, 4, 8, 24, 48 and 72 hours was isolated using RNeasy columns (Qiagen) and cDNA was synthesized using oligo(dT)18 for priming and Sensiscript Reverse Transcriptase (Qiagen) according to manufacturer's instructions.

Primers used for real time-PCR were IL6-Rα forward primer: 5' AAA GGC TGT GCT CTT GGT GAG 3' and IL6-Rα reverse primer: 5' GAA TAC TGG CAC GGC TCC TG 3', β-actin forward primer: 5' GAC AGG ATG CAG AAG GAG ATT ACT 3' and β-actin reverse primer: 5' TGA TCC ACA TCT GCT GGA AGG T 3' as the house-keeping gene. A master mix consisting of Quantifast SYBR green PCR (Qiagen), 1 μM forward primer and 1 μM reverse primer was combined and added to 100 ng cDNA template in a 96-well plate. The PCR was initiated at 95°C for 2 min followed by 95°C for 30 seconds, 55°C for 30 seconds and 68°C for 15 seconds for 40 cycles in a Mastercycler ep realplex (Eppendorf).

Measurement of NFκB nuclear translocation and DNA binding

NCI H929 and U266 cells were treated daily with AZA 5 μM for 72 hours. Nuclear lysates were obtained pre-treatment and at 24, 48 and 72 hours as described previously. Subsequently the level of nuclear p65 was measured using the NoShift Transcription Factor assay kit (Merck) according to the manufacturer's instructions. NF-κB luciferase activity in lysates from remaining viable cells (prepared following the manufacturer's instructions) were measured and normalized by Renilla luciferase activity using the Dual Luciferase assay system (Promega) on a luminometer. NCI-H929 cells were co-transfected with pNF-κB-Luc (Stratagene, La Jolla, USA) and phRL-SV40 plasmid (Promega) to control for cell number and viability by measurement of the production of Renilla luciferase using Lipofectamine 2000 (Invitrogen). Eighteen hours post-transfection cells were treated with tumor necrosis factor-α 50 ng/mL (positive control) or AZA (5 μM or 10 μM) for 24 hours, at which time luciferase activity in cell lysates was measured.

Evaluation of SOCS3 and Bcl-$_{XL}$

NCI H929 and/or U266 cells were treated daily with AZA 5 μM for up to 72 hours. At various time-points cells were harvested, spun at 8000g for 5 min and then washed in FACS buffer (0.5% [fetal calf serum]FCS/PBS). Cell pellets were resuspended in Fix Buffer (2% paraformaldehyde in 0.01M PBS) and stored at 4°C for a minimum of 4 hours. Antibodies against SOCS3 and Bcl-$_{XL}$ were purchased from Santa Cruz Biotechnology Inc. (USA). Fixed cells were washed twice with FACS buffer before addition of primary antibody diluted in permeabilization buffer (0.1% saponin, 1% FCS in 0.01M PBS) for 30 min at room temperature then washed again in FACS buffer before secondary FITC-conjugated antibody was added for 15 min in the dark. Cells were washed as above and resuspended in 300 μL FACS buffer and studied on a FACS Calibur (Becton Dickinson) and analyzed with EXPO 32 software.

Results

AZA de-methylates $p16^{INK4a}$ and demonstrates in vitro and in vivo anti-MM activity

The dose responses of human myeloma cell lines treated with 1 – 10 μM AZA were determined by the MTS cell viability assay. Figure 1A shows that all seven genetically heterogenous cell lines were susceptible in a time- and dose-dependent manner to AZA with the median IC_{50} at 72 hours being, in all cases, ≤5 μM AZA. MSP demonstrated that the $p16^{INK4a}$ gene in both the RPMI 8226 and LP-1 cell lines was hypermethylated (Figure 1B) with evidence of $p16^{INK4a}$ gene demethylation in RPMI 8226 at 48 hours following AZA treatment, consistent with the necessity for AZA incorporation into the DNA of dividing cells prior to DMT inhibition. Interestingly, no evidence of demethylation was observed in the more AZA-sensitive LP-1 cell line, suggesting only a minor role, if any, for demethylation in the observed anti-MM effect; it must, however, be noted that we chose to use $p16^{INK4a}$ as a marker of overall

demethylation and more complete analyses are warranted. Clearly, evidence of inhibitory activity within the first 24 hours following AZA treatment confirmed non-methylation-dependent anti-MM activity. Primary MM cells from patients with advanced disease demonstrated approximately 25% apoptosis at 48 hours following AZA treatment (Figure 1C), an effect comparable to that seen with the 5T33 cells *in vitro* (Figure 1D). Subsequently, C57BL/KaLwRij mice treated with 1, 3 or 10 mg/kg AZA from 7 days following inoculation with syngeneic 5T33 cells demonstrated no evidence of significant drug toxicity but there was also no evidence of any relevant *in vivo* anti-MM activity at either the 1 or 3 mg/kg dose level (*data not shown*). In contrast, at 10 mg/kg AZA (Figure 1E) there was a statistically significant prolongation of time to hind limb paralysis- the median time to hind limb paralysis of mice treated with vehicle or AZA was 24.5 days and 32 days, respectively (p=0.001, log rank).

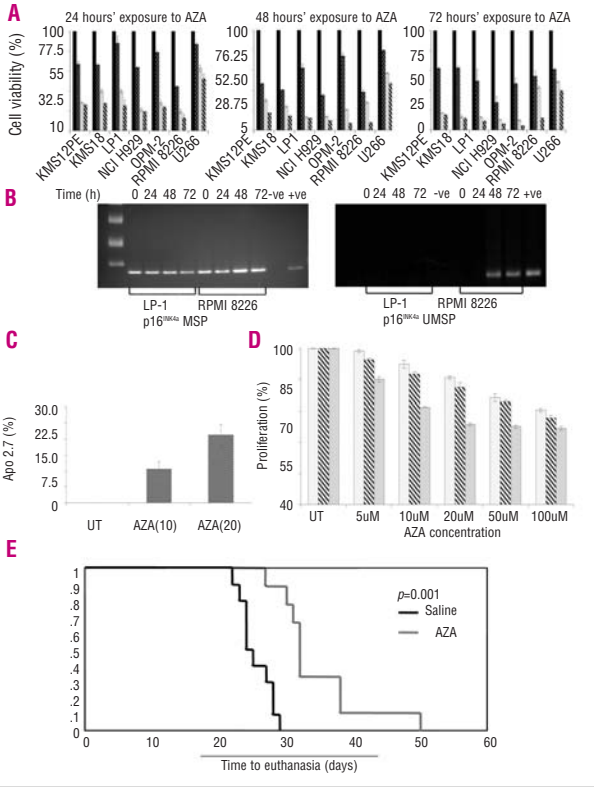

Figure 1. AZA de-methylates p16^{INK4a} and demonstrates *in vitro* and *in vivo* anti-MM activity. (A) Levels of inhibition induced by AZA (1 μM [▨], 5 μM [■] and 10 μM [▧]) in seven human myeloma cell lines, as measured by MTS assay over 24-72 hours. Data are the mean ± standard error of the mean from three individual experiments. (B) Genomic DNA from LP-1 and RPMI 8226 cells was isolated using the DNeasy Tissue Kit (Qiagen) and was bisulphated with the CpGenome Universal DNA Modification Kit (Chemicon). PCR using primers specific for methylated or unmethylated fragments were used to amplify p16^{INK4a} prior to and at 24, 48 and 72 hours following treatment with AZA 5 μM. By 48 hours after AZA treatment unmethylated p16^{INK4a} was observed in RPMI 8226 cells. (C) Primary myeloma cells from 13 patients with multiply relapsed MM were treated with AZA (10 and 20 μM) for 48 hours and apoptosis was measured by apo 2.7 expression. (D) 5T33 cells treated with AZA (0-100 μM) for 24 (■), 48(▧) and 72 (□) hours demonstrated modest *in vitro* sensitivity, as measured by the MTS assay. Data are the mean ± standard error of the mean from three individual experiments. (E) C57BL/KaLwRij syngeneic hosts were inoculated with 5×10^5 5T33 cells. Commencing on day +7 they were treated with five intra-peritoneal doses of AZA (10 mg/kg) on alternate days or similarly with a 0.9% (w/v) NaCl solution. AZA-treated mice had a significantly prolonged median time to hind limb paralysis, and thus euthanasia, compared to saline-treated controls (32 days versus 24.5 days, respectively, p=0.001 log rank).

AZA induces apoptosis of MM cells via the intrinsic apoptotic pathway

FACS demonstrated a dose-dependent accumulation of annexin V positive cells in all four human myeloma cells analyzed (NCI H929, LP-1, RPMI 8226 and U266) following treatment with AZA 5 μM, confirming the induction of apoptosis and suggesting that apoptosis was the predominant mode of AZA-induced cell death. Representative results from U266 cells are shown in Figure 2A. Figure 2B shows different stages of cell cycling in LP-1 cells treated with 1 and 5 μM AZA for 24 hours. LP-1 and RPMI 8226 cells (*data not shown*) both showed a reduction in the proportion G_o/G_1 populations at 72 hours after treatment with 5 μM AZA, compared to the proportion of the same populations in untreated controls: 13% *vs.* 39% and 18% *vs.* 43%, respectively. While there was an increased number of cells in G_o/G_1 among AZA-treated NCI H929 cells, this almost certainly represented overwhelming induction of apoptosis rather than cell cycle arrest. Both U266 (Figure 2C) and NCI H929 (*data not shown*) treated with AZA 5 μM demonstrated PARP cleavage, confirming the induction of apoptosis. Furthermore, this was associated with cleavage of caspase 9 and cytoplasmic accumulation of cytochrome c consistent with apoptosis occurring via the intrinsic (mitochondrial-dependent) apoptotic pathway (Figure 2C). Evaluation of both U266 and NCI H929 utilizing AZA 5 μM and caspase inhibition confirmed this. Specifically, the caspase 9 inhibitor (Z-LEHD-FMK) inhibited apoptosis of both cell lines, whereas caspase 8 (Z-IETD-FMK) inhibition demonstrated no protective effect (for U266, Z-LEHD-FMK + AZA *vs.* AZA alone and Z-IETD-FMK + AZA *vs.* AZA alone, p<0.001 and p=1.0, respectively, Student's t-test; and for NCI H929 - Z-LEHD-FMK + AZA *vs.* AZA alone and Z-IETD-FMK + AZA *vs.* AZA alone, p=0.004 and p=0.9, respectively, Student's t-test) (Figure 2D). The almost total abrogation of apoptosis in the U266 cell line with Z-LEHD-FMK, compared to that of NCI H929, is consistent with the lower sensitivity to AZA of the former. The involvement of caspase 3 in AZA-induced apoptosis was validated by quantitation of cell death, assessed by PI staining, of U266 and NCI H929 cells that were untreated or transfected with caspase-3 siRNA, at 72 hours after treatment with 5 and 1 μM AZA, respectively. The percentage of PI cells was reduced in caspase 3-silenced cells in both groups: for U266 and NCI H929 – caspase 3 siRNA + AZA versus AZA alone, p=0.002 and p=0.003, respectively, Student's t-test (Figure 2E). The level of pro-caspase 3 was also validated by western blot analysis following AZA treatment and to confirm the silencing of caspase-3 expression (Figure 2E).

AZA inhibits the IL-6 signaling pathway

AZA-induced apoptosis prior to any evidence of gene demethylation mandated further mechanistic evaluation. The multi-functional cytokine IL-6 is a critical

mediator of MM cell survival.[28-29] The U266 cell line, which exhibits both autocrine IL-6 elaboration and constitutive phosphorylation of STAT3,[30] provides a useful model to examine the impact of AZA on activation of the IL-6 signaling pathway. Whole cell lysates from U266 cells treated with AZA 5 μM showed a marked and progressive reduction in the IL-6-specific receptor (gp80/IL-6Rα) from 4 hours after treatment (Figure 3A). Consistent with this, there was a dramatic reduction in shedding of IL-6Rα into conditioned medium from as early as 2 hours after-AZA treatment and throughout the 72-hour observation period ($p<0.05$ at all time points for AZA-treated versus untreated controls, Student's t-test) (Figure 3B). Similarly, surface expression of IL-6Rα in response to AZA fell over 72 hours to approximately 50% of pre-treatment levels (*data not shown*). Treatment of U266 with cyclohexamide recapitulated the effect of AZA, suggesting that the observed reduction in IL-6Rα may be secondary to the inhibition of protein synthesis (Figure 3C). In support of this hypothesis was the observation that pre-treatment with caspase inhibitors had no impact on the reduction in IL-6Rα protein expression (*data not shown*), confirming that the reduction was not secondary to caspase-mediated cleavage. Finally, quantitative reverse transcriptase-PCR, while demonstrating a modest initial reduction in the levels of IL-6Rα transcripts subsequently showed a sustained rise compared to pre-AZA levels despite a persisting reduction in IL-6Rα protein expression (Figure 3D). Importantly, the down-regulation of IL-6Rα levels was associated with a reduction in pSTAT3 to undetectable levels within 8 hours with no associated reduction in overall STAT3 protein expression (Figure 3E). Furthermore, in the context of inhibition of STAT3 phosphorylation, there was a progressive decline in Bcl-xL protein expression over the 72-hour observation period (Figure 3F). Finally, consistent with the reduction in IL6 signaling activity, and excluding SOCS3 upregulation as a possible mechanism for the inhibition of IL-6 signaling, there was reduced SOCS3 expression from as early as 4 hours after AZA treatment (Figure 3G).

Exogenous IL-6 partially restores STAT3 phosphorylation but does not restore viability of IL-6 dependent U266 cells following AZA treatment

We next examined whether we could overcome the AZA-induced down-regulation of IL-6 signaling with exogenous IL-6 and whether this would preserve the viability of AZA-treated U266 cells. U266 cells were treated over a 96-hour period with AZA 5 μM alone or in combination with exogenous IL-6 at a concentration of either 100 pg/mL (physiological – see Figure 3B) or 3 ng/mL (supra-physiological). AZA in combination with IL-6 100 pg/mL resulted in persisting inhibition of STAT3 phosphorylation (*data not shown*). In contrast, the addition of IL-6 at 3 ng/mL was able to partially restore STAT3 phosphorylation within 24 hours and this effect was sustained over the 96-hour observation period (Figure 4A). These findings confirm that AZA-induced inhibition of STAT3 phosphorylation is secondary to interruption of autocrine IL-6 signaling. Interestingly, however, despite this partial restoration of STAT3 phos-

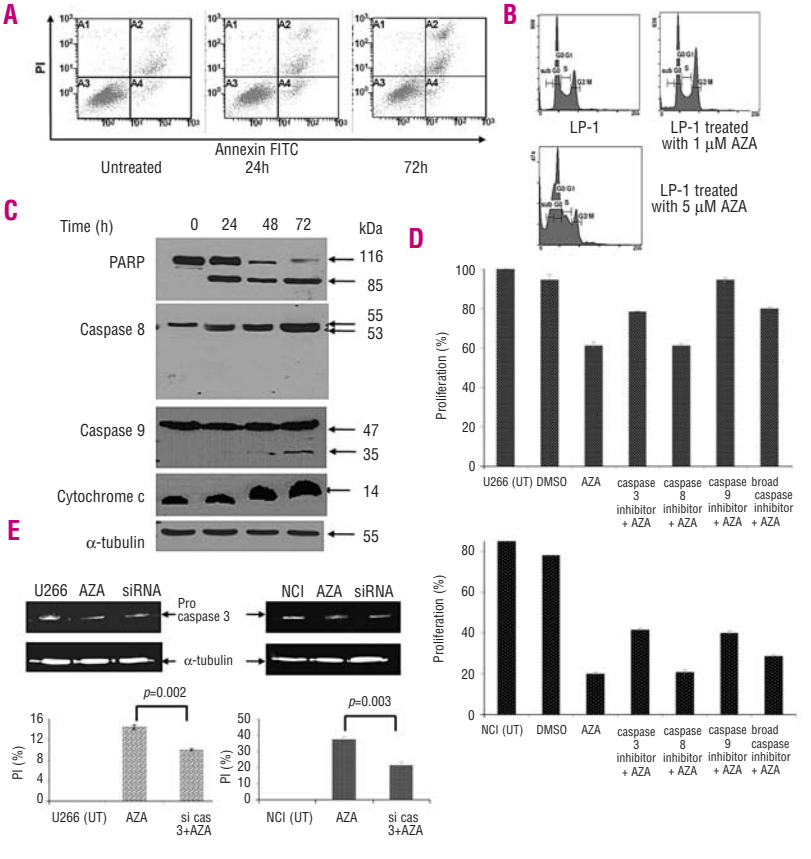

Figure 2. AZA induces apoptosis of MM cells predominantly via the intrinsic apoptotic pathway. (A) AZA-induced apoptosis of U266 with 5 μM AZA was confirmed by annexin-V FITC and PI staining at 24 and 72 hours post-treatment. (B) The different stages of cell cycling in LP-1 cells treated with AZA (1 and 5 μM) at 24 hours. (C) Western blot analyses of U266 following treatment with AZA 5 μM demonstrated PARP cleavage, reduction of caspase 9, accumulation of caspase 8 and addition of cytochrome c release, consistent with predominantly intrinsic apoptotic pathway activation. α-tubulin was used as the loading control. (D) MTS analyses of U266 and NCI H929 cells treated with AZA 5 μM incorporating pre-treatment with inhibitors of caspases 3 (C3), 8 (C8), and 9 (C9), or a broad range caspase inhibitor (BC) confirmed that apoptosis occurred primarily via the intrinsic apoptotic pathway. For U266, C8 + AZA versus AZA alone and C9 + AZA versus AZA alone, p=NS and $p<0.01$, respectively (Student's t-test). For NCI H929, C8 + AZA versus AZA alone and C9 + AZA versus AZA alone, p=NS and $p<0.01$, respectively (Student's t-test). Data are the mean ± standard error of the mean from three individual experiments. (E) The involvement of caspase 3 in AZA-induced apoptosis was validated by PI staining at 72 hours post-treatment in U266 and NCI H929 cells that were untreated or in which caspase 3 was silenced. Reduced expression of pro-caspase 3 in AZA-treated and caspase 3-silenced cells was also confirmed by western blotting.

phorylation, no increment in the viability of U266 cells (Figure 4B) was evident at any time-point, strongly suggesting that the inhibitory effect of AZA on U266 cells was, at least in part, mediated by other mechanisms, as would have been the situation with the non-IL-6-dependent human myeloma cell lines studied.

AZA inhibits NFκB activity and autocrine IL-6 secretion

NFκB represents a family of proteins involved in the transcriptional activation of a wide variety of genes critical to the control of immune function, cellular survival and apoptosis.[30] Recent insights into the role of NFκB in a variety of cancers, including MM,[32-33] prompted us to hypothesize that AZA may act via modulation of NFκB activation. This was clearly demonstrated in both U266 and NCI H929 cells, as evidenced by marked reductions in p65 nuclear translocation following treatment with AZA 5 μM (Figure 5A), and then confirmed by the use of a reporter gene assay showing AZA-induced inhibition of NFκB DNA binding (Figure 5B). Since growth factors and oncoproteins have been demonstrated, in the majority of cases, to enhance NFκB activation via regulation of IκB kinases (IKK)[32] we next sought evidence for modulation of IκB following AZA treatment. Surprisingly we saw no consistent changes in either phosphorylated IκB (pIκB) or total IκB (*data not shown*) to explain the observed inhibition of NFκB. Indeed, U266 cells showed a modest decrease in the level of pIκB following treatment with AZA 5 μM (median reduction of 17% compared to that of untreated control cells) (*data not shown*). Finally, evaluation of IL-6, a transcriptional target of NFκB, showed a concomitant reduction in autocrine secretion (Figure 5C) from 24 hours following AZA treatment, consistent with the observed inhibition of NFκB.

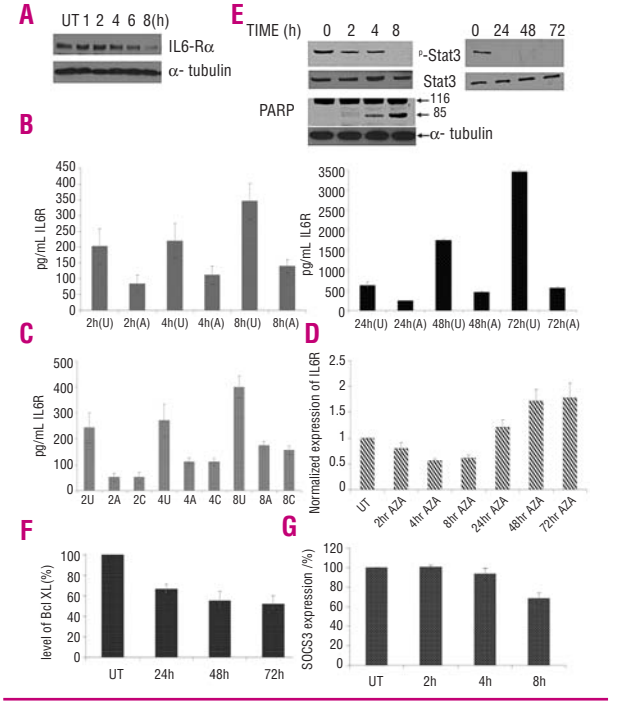

Figure 3 (left). AZA inhibits the IL-6 signaling pathway. (A) Western blot analysis of U266 cells showed a reduction in total cellular IL6-Rα protein level from 4 hours onward following treatment with AZA 5 μM. (B) Soluble IL6-Rα in conditioned medium (CM) from U266 cells after 0 to 72 hours of culture was quantified with an ELISA and compared to levels in condizioned medium over the same time period following treatment with AZA 5 μM. A marked reduction in soluble IL6-Rα was seen with AZA treatment at all time points. (C) Soluble IL6-Rα in CM from U266 cells from 0 to 8 hours following treatment with either AZA 5 μM [A] or cyclohexamide 20 μg/mL [C] compared to that of untreated controls [U]. Treatment with cyclohexamide reproduced identical kinetics of soluble IL6-Rα modulation to that seen with AZA. Data are the mean ± standard error of the mean from three individual experiments. (D) Quantitave real-time-PCR demonstrated a transient reduction in IL6-Rα transcripts with a subsequent sustained increase to greater than baseline levels. Data are the mean ± standard error of the mean from five individual experiments. (E) Western blot analysis demonstrated undetectable levels of p-STAT3 in U266 at 8 hours following treatment with AZA 5 μM but no associated change in total STAT3 protein levels. PARP cleavage was observed from 2 hours post-AZA treatment; α-tubulin was used as a loading control. (F) Phosflow measurement of βcl-xl expression from 0 to 72 hours following treatment with AZA 5 μM. Data are the mean ± standard error of the mean from three individual experiments. (G) Expression of SOCS3 at early time points following exposure to azacitidine, as measured by FACS.

Discussion

The continuing challenges in the management of MM are the inevitable treatment-resistant relapse following initial response and the less common scenario of primary refractory disease. In both instances a variety of mechanisms are recognized as potentially contributing to the drug-resistant phenotype; this heterogeneity mandates the ongoing search for novel therapeutic approaches exploiting potential therapeutic synergisms.[34,35] Recently published data describing high response rates to both bortezomib and lenalinomide have provided a rationale for the further exploration of both these agents in earlier phases of the disease and in combination with other therapeutic agents.[9,36] Furthermore, while quite dissimilar chemically, these two drugs share the common characteristic that they are both promiscuous from a mechanistic point of view, exerting pleiotropic effects against not only the MM cells but probably also the MM-supportive bone marrow micro-environment.[8] It is likely that this relative lack of specificity provides a therapeutic advantage in treating a genetically heterogenous disorder such as MM.

Our *in vitro* data on the action of AZA revealed a range of anti-MM effects. Inhibitory activity was seen within 24 hours of treatment in all human myeloma cell lines tested and was achieved at AZA concentrations well below those safely achievable *in vivo* with either intravenous or subcutaneous administration schedules.[37] These *in vitro* observations were consistent with the anti-MM activity that AZA demonstrated *in vivo* despite only modest inhibition of the 5T33 cell line *in vitro*. The 5T33 model that we utilized is a systemic model of aggressive MM with the unambiguous end-point of hind limb paralysis.[38] Using escalating doses of AZA we clearly demonstrated a significant survival advantage from treatment with AZA at a dose of 10 mg/kg daily for 5 days, which produced a 33% prolongation in time to hind limb paralysis when compared to that in vehicle-only treated animals. Importantly, animals were not treated until day 7 after inoculation, at which time estab-

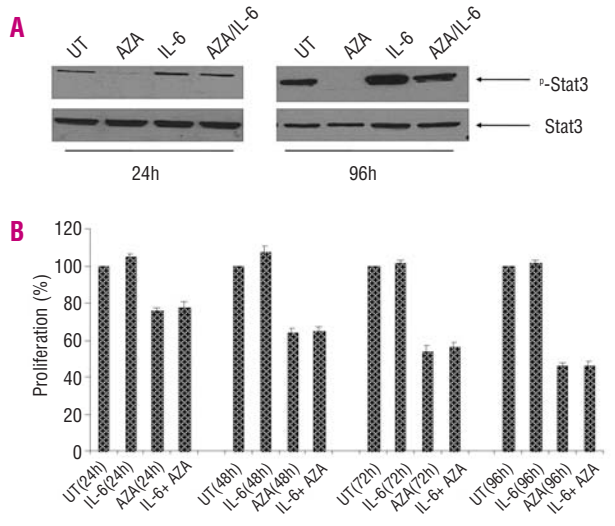

Figure 4. Exogenous IL-6 partially restores STAT3 phosphorylation but does not restore viability of IL-6-dependent U266 cells following AZA treatment. (A) Western blot analysis of U266 cells at 24 and 96 hours after treatment with AZA 5 mM showed a reduction in p-STAT3 to undetectable levels while total STAT3 remained unchanged. AZA (5 mM)-treated U266 cells in the presence of 3 ng/mL IL-6 showed partial restoration of STAT3 phosphorylation. (B) MTS assays following treatment with AZA 5 mM with or without exogenous IL6 (3 ng/mL) demonstrated no evidence of preservation of cell viability compared to that of untreated controls despite a partial restoration of STAT3 phosphorylation (see above). Data are the mean ± standard error of the mean from three individual experiments.

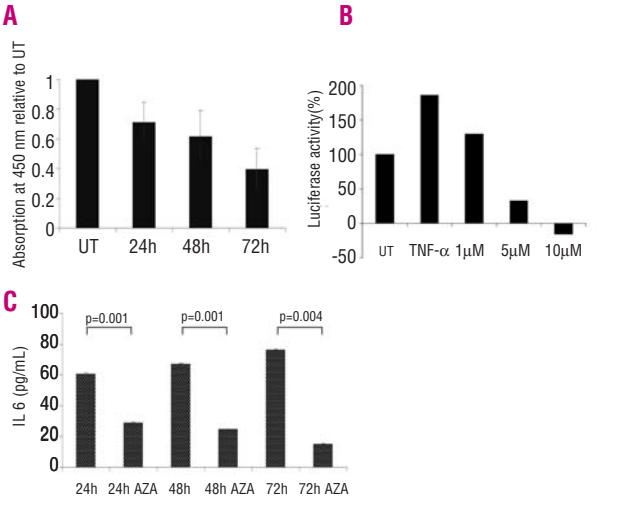

Figure 5. AZA inhibits NFκB activity and autocrine IL-6 elaboration. (A) A super shift assay demonstrated a time-dependent decrease in nuclear translocation of p65 in both U266 and NCI H929 *(data not shown)* cells following treatment with AZA 5 μM. (B) NFκB transcriptional activity in viable NCI H929 cells, as measured by the luciferase assay, confirmed inhibition by 24 hours following treatment with AZA (5 μM or 10 μM) as compared to that in untreated controls. Tumor necrosis factor-α (TNF-α) 50 ng/mL was used as a positive control. (C) Conditioned medium (CM) from U266 cells co-cultured with and without AZA 5 μM was assayed for IL-6 at 24, 48 and 72 hours using the IL-6 ELISA kit from R&D Systems. AZA induced a progressive decrease in IL-6 over time. In contrast, untreated U266 CM contained increased levels of IL-6 over the same time period ($p<0.05$, Student's *t*-test, for treated versus untreated cells at 24, 48 and 72 hours).

lished systemic disease is readily demonstrable (*data not shown*). Furthermore, unlike in clinical practice, in which repeated cycles of therapy are used, we demonstrated a significant anti-MM effect with only a single brief cycle of treatment.

In vitro induction of apoptosis by AZA was clearly evident and occurred predominantly via the intrinsic apoptotic pathway. This is consistent with the recognized cytotoxic activity of AZA and preceded the expected hypomethylating activity of AZA, which was first observed – using the hypermethylated *p16* gene as a marker of global demethylation – 48 hours after exposure to AZA. The mechanisms by which AZA induces cytotoxicity are not fully elucidated and may well be cell-type dependent. Studies undertaken in the 1970s led to the hypothesis that the principle mechanism of cytotoxicity was incorporation of AZA into RNA.[39] This, in turn, was thought to lead to inhibition of protein synthesis and cell death. Data also suggested that later incorporation into DNA could not only inhibit DNA synthesis but also sensitize cells to the effects of other DNA-damaging drugs.[40] More recently it has been shown that 5-aza-2'-deoxycytidine (decitabine) cell killing is dependent on the p53 DNA-damage response pathway and is related to enzyme-DNA adduct formation.[41] Our data, showing a rapid onset of cell killing and activity against both p53-wild type and p53-null human myeloma cell lines (*data not shown*) excludes the latter pathway as a dominant mechanism in AZA-induced MM cell killing.

IL-6 plays a critical role as an anti-apoptosis inducing agent in MM.[42,43] Its effects are mediated by both autocrine and paracrine pathways[30,44,45] and a cell surface receptor with two components, the IL-6-specific ligand-binding IL-6Rα (gp80) subunit and the signal transducing gp130 subunit.[46] STAT3 is a member of the signal transducers and activators of transcription (STAT) family of proteins, is a crucial component of the JAK-STAT signaling cascade and mediates the expression of IL-6 responsive genes following recruitment and phosphorylation by gp130.[47-49] Importantly, STAT3 is constitutively over-expressed in about one third of patients with MM, is associated with over-expression of the anti-apoptotic Bcl-2 family member, Bcl-x_L, and is thus thought to play a crucial role in mediating drug resistance.[30,50] Consistent with this, a variety of strategies that down-regulate STAT3 activity have been shown to enhance the sensitivity of MM cells to drug therapy.[51-53] Here we have demonstrated for the first time that AZA inhibits autocrine IL-6 signaling via the down-regulation of both IL-6Rα expression and autocrine IL-6 secretion. It is also possible that the reduced shedding of IL-6Rα may contribute to inhibition of the IL-6 pathway. Published data from studies on the role of soluble IL-6Rα suggest that this receptor not only enhances both the magnitude and duration of IL-6 signaling but also the formation of IL-6/IL-6Rα complexes, which markedly prolonged the half-life of IL-6,[46] a factor that assumes potentially greater significance in the context of reduced IL-6 secretion. Importantly, the combination of these effects

results in sustained inhibition of STAT3 phosphorylation and subsequently the expression of Bcl-xL. The mechanisms underlying the reduced expression of IL-6Rα are unclear; however, our data demonstrate that the effect is not secondary to either caspase-induced cleavage of IL-6Rα or a DNA-dependent reduction in IL-6Rα transcription, as shown by quantitative reverse transcriptase-PCR. In contrast, the rapidity of the effect on IL-6Rα protein levels (within 2 hours) and the recapitulation of the impact of AZA on IL-6Rα with cyclohexamide treatment supports the hypothesis that the early anti-MM effects seen with AZA may be secondary to incorporation into RNA species with subsequent inhibition of protein synthesis, although this clearly requires further evaluation.

A variety of conventional anti-cancer agents have been demonstrated to up-regulate NFκB activity in solid tumors; this, in turn, potentially abrogates the induction of apoptosis and may contribute to resistance to chemotherapy.[54] Furthermore, we recently demonstrated that the tyrosine kinase inhibitor PKC412 induces an up to 6-fold increase in NFκB activity in MM cells.[55] In contrast, AZA rapidly induces inhibition of NFκB. Clearly, in view of the latter and the proven efficacy of bortezomib in advanced MM, AZA warrants clinical evaluation for the treatment of MM. Furthermore, its ability to inhibit NFκB suggests that AZA may also have a role in sensitizing MM to other available therapeutic agents. The latter may be achieved via down-regulation of important pro-survival and anti-apoptotic (IL-6, IL-6Rα, Bcl-xL) factors but also by abrogating drug-induced NFκB stress responses as we, and others, have recently demonstrated.[55-57] Our data suggest an IKK-independent process; while such processes have been previously postulated, the underlying mechanisms remain to be elucidated.[58,59] In conclusion, we report that AZA at clinically relevant concentrations has significant anti-MM activity both *in vitro* and *in vivo*. AZA induces pleiotropic effects including the down-regulation of JAK-STAT signaling and inhibition of NFκB. Further investigation of these novel effects is clearly warranted; however, our data already provide sufficient rationale for the clinical evaluation of AZA in MM.

Authorship and Disclosures

TK designed, performed and analyzed the experiments and drafted the manuscript; JS performed the murine experimental procedures; AS conceived the study, analyzed and interpreted the data and prepared the manuscript. The authors reported no potential conflicts of interest.

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