We have shown that thalidomide (Thal) and its immunomodulatory derivatives (IMiDs), proteasome inhibitors PS-341, and AS2-O3 act directly on multiple myeloma (MM) cells and in the bone marrow (BM) milieu to overcome drug resistance. Although Thal/IMiDs, PS-341, and AS2-O3 inhibit nuclear factor (NF)-κB activation, they also have multiple and varied other actions. In this study, we therefore specifically address the role of NF-κB blockade in mediating anti-MM activity. To characterize the effect of specific NF-κB blockade on MM cell growth and survival in vitro, we used an IκB kinase (IKK) inhibitor (PS-1145). Our studies demonstrate that PS-1145 and PS-341 block TNFα-induced NF-κB activation in a dose- and time-dependent fashion in MM cells through inhibition of IκBα phosphorylation and degradation of IκBα, respectively. Dexamethasone (Dex), which up-regulates IκBα protein, enhances blockade of NF-κB activation by PS-1145. Moreover, PS-1145 blocks the protective effect of IL-6 against Dex-induced apoptosis. TNFα-induced intracellular adhesion molecule (ICAM)-1 expression on both RPMI8226 and MM.15 cells is also inhibited by PS-1145. Moreover, PS-1145 inhibits both IL-6 secretion from BMSCs triggered by MM cell adhesion and proliferation of MM cells adherent to BMSCs. However, in contrast to PS-341, PS-1145 only partially (20–50%) inhibits MM cell proliferation, suggesting that NF-κB blockade cannot account for all of the anti-MM activity of PS-341. Importantly, however, TNFα induces MM cell toxicity in the presence of PS-1145. These studies demonstrate that specific targeting of NF-κB can overcome the growth and survival advantage conferred by tumor cell binding to BMSCs and cytokine secretion in the BM milieu. Furthermore, they provide the framework for clinical evaluation of novel MM therapies based upon targeting NF-κB.

NF-κB is a member of the Rel family of proteins and is typically a heterodimer composed of p50 and p65 subunits (1). NF-κB is constitutively present in the cytosol and inactivated by its association with IκB family inhibitors (2). IκBα is, therefore, a key molecular target regulating NF-κB activation. Various stimuli, including TNFα, lipopolysaccharide, phorbol esters, and viruses, trigger IκB protein phosphorylation on two serine residues located in its NH2 terminus by the multishunit IκB kinase (IKK) complex. Once phosphorylated, IκB is targeted for ubiquitination and degradation by the 26S proteasome (3), allowing translocation of NF-κB into the nucleus where it binds to specific DNA sequences in the promoters of target genes and stimulates their transcription. The protein products of these genes include cytokines, chemokines, cell adhesion molecules, and proteins mediating cellular growth control. Importantly, many of these proinflammatory proteins also act, either in an autocrine or paracrine fashion, to further stimulate NF-κB activation.

Many studies have reported growth and anti-apoptotic roles of NF-κB in normal and malignant cells. For example, NF-κB promotes cell growth by up-regulating cyclin D transcription, with associated hyperphosphorylation of Rb, G1 to S-phase transition, and inhibition of apoptosis (4). NF-κB also regulates transcription of the catalytic subunit of telomerase in mice (5). NF-κB is constitutively activated in Hodgkin’s tumor cells, whereas inhibition of NF-κB blocks their growth (6). Moreover, inhibition of NF-κB via expression of the super-repressor of IκBα induces apoptosis, even in the presence of an oncogenic allele of H-Ras (7). Although the precise role of NF-κB activation in pathogenesis of multiple myeloma (MM) has not been fully characterized, we have previously shown that MM cell adhesion to bone marrow stromal cells (BMSCs) induces NF-κB-dependent up-regulation of transcription of IL-6, a growth and anti-apoptotic factor in MM (8). In addition, TNFα secreted by MM cells: 1) activates NF-κB in BMSCs, thereby directly up-regulating IL-6 transcription and secretion in BMSCs; and 2) activates NF-κB in MM cells, thereby up-regulating intracellular adhesion molecule-1 (ICAM-1) (CD54) and vascular cell adhesion molecule-1 (VCAM-1) (CD106) expression on both MM cells and BMSCs and increasing MM cell to BMSC binding (9). Because IL-6 is the major growth and survival factor in MM cells (10–12) and adherence of MM cells to fibronectin confers resistance to drug-induced apoptosis (13, 14), specific blockade of NF-κB signaling may represent a novel therapeutic strategy
in MM. We and others have shown that novel agents with both preclinical and early clinical anti-MM activity, including thalidomide (Thal) (15) and its immunomodulatory derivatives (IMiDs),2 proteasome inhibitor PS-341 (17, 18), and arsenic trioxide As2O3 (19), all inhibit NF-κB activation and overcome conventional drug resistance in preclinical and early clinical trials, supporting this view. However, these novel drugs also have multiple other biologic actions, and the benefit of specifically targeting NF-κB in novel MM therapeutics is not yet defined.

In the present study, we demonstrate the specific biologic sequelae of NF-κB activation and blockade on MM cell growth and survival in the BM microenvironment. The novel specific IKK inhibitor PS-1145 inhibits phosphorylation of IκBα in MM cells and modestly directly inhibits their growth. Importantly, however, PS-1145 abrogates NF-κB activation related up-regulation of adhesion molecules on MM cells, MM cell to BMSC adherence, as well as the MM cell growth and survival advantage conferred both by adherence and cytokine secretion in the BM milieu. Furthermore, PS-1145 blocks the protective effect of IL-6 against apoptosis induced by both conventional (dexamethasone, Dex) and novel (immunomodulatory derivative of Thal 3, IMiD3) therapies. These studies therefore confirm a central role for NF-κB in regulating growth and survival of MM cells in the BM milieu and further suggest the potential utility of novel therapeutics targeting NF-κB in MM.

**EXPERIMENTAL PROCEDURES**

**MM-derived Cell Lines and Patient MM Cells**—The Dex-sensitive human MM cell line MM.1S was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). RPMI8226 and U266 human MM cells were obtained from American Type Culture Collection (Rockville, MD). All MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY). Patient MM cells were purified from patient

| Kinase | IC50 (μM) |
|-------|-----------|
| IKK   | <0.1 (88 nM) |
| IRAK  | >100p |
| NIK   | >100 |
| Protein kinase A | >100 |
| Protein kinase C | >100 |
| Casein kinase II | >100 |
| c-Src | >100 |
| Lck   | >100 |
| Calmodulin kinase | >100 |

**TABLE I**

Inhibitory activity of PS-1145 on various kinases

**FIG. 1.** PS-1145 blocks phosphorylation and degradation of IκBα triggered by TNFα. A, structure of PS-1145; B, relative IKK inhibitory activity of PS-1145; C, MM.1S cells were pre-treated with PS-1145 (10 μM for 90 min) and then stimulated by TNFα (5 ng/ml for 0–20 min); D, MM.1S cells were pre-treated with 0.125–40 μM PS-1145 for 90 min before stimulation by TNFα (5 ng/ml for 5 min); E, patient MM cells were pre-treated with PS-1145 (10 μM for 90 min) and then stimulated by TNFα (5 ng/ml for 0–10 min). The cells were lysed, electrophoresed, and then immunoblotted with phospho-IκBα and IκBα Abs; F, MM.1S cell were pre-treated with PS-1145 (10 μM for 90 min) and then stimulated by TNFα (5 ng/ml for 0–20 min). Nuclear extracts of the cells were subjected to EMSA. PS-341 served as a positive control for inhibition of NF-κB activation, as previously described (9, 18).
and immunoblotted with anti-phospho-STAT3, phospho-p42/44 MAPK, and p42/44 MAPK Abs.

and analyzed for cell cycle profile by flow cytometry. Percentages represent cells in G0/G1.

Ki curve for quantification. For PS-1145 phosphoantibodies in an ELISA format with appropriate standard

associated kinase, p38 kinases, PKA (protein kinase A), protein kinase C, casein kinase II, PKC, (MAPK kinases) 1/2, MAPK-activated protein kinase 2, ERK2 (extracellular signal regulated kinases), JNK2 (c-Jun NH2-terminal kinase 2), and epidermal growth factor receptor tyrosine kinase. Percentage inhibition of these kinases was negligible at PS-1145 (100 μM) (Table I). Target selectivity of PS-1145 was confirmed in the receptor binding and formation assays by NovaScreen (NovaScreen Bioscience, Hanover, MD) and Cerep (Cerep Inc., Redmond, WA). PS-341 and PS-1145 were diluted in culture medium immediately before use; PS-341 and PS-1145 control media contained <0.1% Me2SO. MAPK kinase (MEK) inhibitor PD98059 was purchased from Cell Signaling (Beverly, MA).

DNA Synthesis—Proliferation was measured as previously described (21). MM cells 3 × 105/well were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media, PS-341, PS-1145 and/or Dex or recombinant human IL-6 (Genetics Institute, Cambridge, MA) for 48 h at 37 °C. DNA synthesis was measured by [3H]thymidine ([3H]TdR, PerkinElmer Life Sciences, Boston, MA) uptake. Cells were pulsed with [3H]TdR (0.5 μCi/well) during the last 8 h of 48-h cultures. All experiments were performed in duplicate.

Growth Inhibition Assay—The inhibitory effect of PS-341 and PS-1145 on MM growth was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye absorbance of the cells. Cells from 48-h cultures were pulsed with 10 μl of 5 mg/ml MTT to each well for the last 4 h of 48-h cultures, followed by 100 μl of isopropanol containing 0.04 M HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Immunoblotting—MM cells were cultured with PS-341 or PS-1145, harvested, washed, and lysed using lysis buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM NaF, 2 mM Na3VO4, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. For detection of phospho-IκBα, IκBα, phospho-MAPK, phospho-STAT3, ERK2, or α-tubulin, cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), and immunoblotted with anti-phospho-IκBα, IκBα,

BM aspirates using RosetteSep separation system (StemCell Technologies, Vancouver, Canada). The purity of MM cells was confirmed by flow cytometry using PE-conjugated anti-CD138 Ab (BD PharMingen, San Diego, CA).

BMSC Cultures—BM specimens were obtained from patients with MM. Mononuclear cells separated by Ficoll-Hypaque density sedimentation were used to establish long term BM cultures, as previously described (20). When an adherent cell monolayer had developed, cells were harvested in Hanks’ buffered saline solution containing 0.25% trypsin and 0.02% EDTA, washed, and collected by centrifugation.

Inhibitors—A proteasome inhibitor PS-341 and an IKK inhibitor PS-1145 (Millennium Pharmaceuticals, Cambridge, MA) were dissolved in Me2SO and stored at −20 °C until use. Ki value of PS-1145 against the IKK complex was determined by measuring K_m,ATP against varying fixed concentration of the inhibitor. Briefly, partially purified IKK complex obtained from unstimulated HeLa S3 cells were pre-activated in a fixed concentration of the inhibitor. Briefly, partially purified IKK complex was first preincubated in varying fixed concentration of the inhibitor (0.1–1 μM) for 1 h. Then apparent Km isopropanol containing 0.04 N HCl. Absorbance was measured at 570 nm using a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

FIG. 2. Effect of PS-1145 on DNA synthesis, cell cycle profile, and signaling cascades triggered by IL-6. MM.1S ( ), U266 ( ), and RPMI8226 ( ) cells were cultured in the presence of (A) PS-1145 (1.5–50 μM) or (B) PS-341 (0.00001–10 μM) for 48 h. DNA synthesis was assessed by [3H]TdR uptake. C, U266 cells were cultured in the presence of PS-1145 (10 μM for 24, 36, and 48 h), harvested, stained with propidium iodide, and analyzed for cell cycle profile by flow cytometry. Percentages represent cells in G0/G1.

36h: 66.7% 48h: 77.2%
NF-κB in Multiple Myeloma

- phospho-MAPK, or -phospho-STAT3 Abs (Cell Signaling, Beverly, MA), and anti-α-tubulin (Sigma) Abs.

Electrophoretic Mobility Shift Analysis—Electrophoretic mobility shift analyses (EMSA) were carried out as in our previous studies (9, 18). Briefly, MM.1S cells were preincubated with PS-341 (5 μM for 1 h) and PS-1145 (10 μM for 90 min) before stimulation with TNFα (5 ng/ml) for 10 or 20 min. Cells were then pelleted, resuspended in 400 μl of hypotonic lysis buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM Na3VO4, 5 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin), and kept on ice for 20 min. After centrifugation (14,000 × g for 5 min) at 4 °C, the nuclear pellet was extracted with 100 μl of hypotonic lysis buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 5 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin) on ice for 20 min. After centrifugation (14,000 × g for 5 min) at 4 °C, the supernatant was collected as nuclear extract. Double-stranded NF-κB consensus oligonucleotide probe (5'-GGGGACTTTCCC-3', Santa Cruz Biotechnology) was end-labeled with [γ-32P]ATP (50 μCi at 222 TBq/mM, PerkinElmer Life Sciences, Boston, MA). Binding reactions containing 1 ng of oligonucleotide and 5 μg of nuclear protein were conducted at room temperature for 20 min in a total volume of 10 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol (v/v)), and 0.5 μg of poly(dI-dC) (Amersham Biosciences, Inc., Peapack, NJ). The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, UK), and visualized by autoradiography.

Flow Cytometric Analysis—For cell cycle analysis, MM cells cultured for 24–48 h in PS-1145 (10 μM), or control media were harvested, washed with phosphate-buffered saline, fixed with 70% ethanol, and treated with 10 μg/ml of RNase (Roche Diagnostics Corp., Indianapolis, IN). Cells were then stained with propidium iodide (Sigma) (5 μg/ml) and visualized by autoradiography. The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, UK). The samples were loaded onto a 4% polyacrylamide gel, transferred to Whatman paper (Whatman International, Maidstone, UK), and visualized by autoradiography.

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Effect of PS-1145 on Paracrine MM Cell Growth in the BM—To evaluate growth stimulation and signaling in MM cells adherent to BMSCs, 3 × 105 MM.1S cells were cultured in BMSC coated 96-well plates for 48 h, in the presence or absence of PS-1145. DNA synthesis was measured as described above. The Duoset ELISA (R&D System) was used to measure IL-6 in supernatants of 48-h cultures of BMSCs with or without MM.1S cells, in the presence or absence of PS-1145.

Statistical Analysis—Statistical significance of differences observed in drug-treated versus control cultures was determined using the Student’s t test. The minimal level of significance was p < 0.05.

RESULTS

PS-1145 Inhibits IκBα Phosphorylation and NF-κB Activation in MM.1S Cells and Patient MM Cells—The effect of PS-1145, a novel specific IKK inhibitor (Fig. 1, A and B) on IκBα phosphorylation and NF-κB activation in MM cells was first examined. Inhibition of IκBα phosphorylation by PS-1145 was assayed in MM.1S and patient MM cells triggered by TNFα. Serine phosphorylation and degradation of IκBα were significantly induced by TNFα at 5 and 10 min in cells cultured in MeSO control media, whereas phosphorylation and degradation of IκBα were completely blocked by PS-1145 pre-treatment of MM.1S cells (Fig. 1C). To study the dose-dependent effect of PS-1145, MM.1S cells were pre-treated with 1.25–40 μM PS-1145 for 90 min, and then stimulated by TNFα (5 ng/ml). Phosphorylation of IκBα was completely inhibited by ≥5 μM PS-1145 (Fig. 1D). As in MM.1S cells, PS-1145 also inhibited phosphorylation and degradation of IκBα triggered by TNFα in patient MM cells (Fig. 1E). These results demonstrate a time- and dose-dependent inhibitory effect of PS-1145 on phosphorylation and degradation of IκBα.

Because NF-κB activation requires phosphorylation, ubiquitination, and degradation of IκBα, we next examined whether PS-1145 could inhibit NF-κB activation, assessed by EMSA. MM.1S cells pre-treated with either MeSO control media or PS-1145 (10 μM for 90 min) were stimulated by TNFα (5 ng/ml for 0–20 min). NF-κB activation was completely inhibited by PS-1145 pre-treatment (Fig. 1F). PS-341 served as a positive control for inhibition of NF-κB activation, as previously reported (9, 18).

PS-1145 Inhibits Proliferation of MM Cell Lines—To study the direct effect of PS-1145 on MM cells, we measured [3H]TdR uptake by MM.1S, U266, and RPMI8226 cell lines cultured for 48 h in the presence of PS-1145 (1.5–50 μM). 20–50% inhibition in proliferation was observed at doses > 12.5 μM PS-1145 (Fig. 2A). In contrast, PS-341 completely inhibited [3H]TdR uptake in all cell lines tested at IC50 of 0.02–0.005 (Fig. 2B). These

![Fig. 3.](image-url)
results indicate that complete blockade of NF-κB activation cannot achieve >50% inhibition of DNA synthesis in MM cell lines and that complete inhibition by PS-341 is mediated through inhibition of another signaling pathway, such as p42/44 MAPK (22). Cell cycle profile, assessed by propidium iodine staining, was also examined in these MM cell lines. Interestingly, PS-1145 induced G1 growth arrest, but not apoptosis, in U266 cells (Fig. 2C). Similar results were observed in RPMI8226 cells (data not shown). These data show that NF-κB blockade induces G1 growth arrest in MM cells.

We have previously shown that IL-6 stimulates p42/44 MAPK, JAK2/STAT3, and PI3K/Akt signaling pathways in MM.1S cells (23) and that PS-341 inhibits p42/44 MAPK, but not JAK2/STAT3 or PI3K/Akt, cascades triggered by IL-6 (18). IL-6 induces phosphorylation of both p42/44 MAPK and STAT3 in MM.1S cells, and the MEK1 inhibitor PD98059 selectively inhibits p42/44 MAPK phosphorylation (Fig. 2D). As in our prior report (18), PS-341 also inhibits p42/44 MAPK phosphorylation; however, PS-1145 does not inhibit either p42/44 MAPK or STAT3 phosphorylation. Phosphorylation of Akt triggered by IL-6 was also unaffected by PS-1145 pre-treatment (data not shown). These results indicate that PS-1145 specifically blocks NF-κB, without affecting other known signaling pathways in MM.1S cells triggered by IL-6.

Dex Up-regulates IκBα Protein and Enhances the Inhibitory Effect of PS-1145 on NF-κB Activation in MM.1S Cells—We next determined whether PS-1145 enhances the inhibitory effect of Dex on NF-κB activation in MM.1S cells. Because Dex has been shown to up-regulate IκBα expression in monocytic cell lines and T cells (24, 25), we first examined whether Dex could induce IκBα protein in MM cells. MM.1S cells were cultured in the presence of Dex (1 μM for 0–36 h), and expression of IκBα protein was assessed by Western blotting. Dex significantly induces IκBα expression, and IL-6 partially blocks Dex-induced up-regulation of IκBα (Fig. 3A). NF-κB activation was next directly assayed in the presence of Dex, with or without IL-6. TNFα-induced activation of NF-κB in MM.1S cells is abrogated by pretreatment with Dex (Fig. 3B). Moreover, PS-1145 also inhibits NF-κB activation triggered by TNFα in MM.1S cells in a dose-dependent fashion, and pre-treatment with Dex enhances the inhibitory effect of PS-1145 (Fig. 3C). These results suggest that Dex inhibits NF-κB activation via up-regulation of IκBα, with related G1 growth arrest and apoptosis.

The effect of Dex, PS-1145, PS-341, and/or IL-6 on TNFα-induced phosphorylation of IκBα in MM.1S cells was next examined. Dex, PS-341, and/or IL-6 do not inhibit TNFα-induced phosphorylation of IκBα; moreover, PS-341 enhances phosphorylation of IκBα, due to inhibition of proteasome activity and accumulation of IκBα (Fig. 3D). In contrast, PS-1145, in the presence or absence of IL-6, inhibits phosphorylation of IκBα. Dex inhibits TNFα-induced degradation of IκBα, whereas IL-6 blocks this effect. Both PS-341 and PS-1145 inhibit degradation of IκBα, in the presence or absence of IL-6.

PS-1145 Overcomes the Protective Effect of IL-6 against Dex and IMiD3 in MM.1S Cells—To define the functional sequelae of PS-1145-related NF-κB blockade in MM.1S cells, we first examined its inhibitory effect on growth of MM cells, in the presence of Dex and/or IL-6. Dex inhibits IL-6-induced proliferation of MM.1S cells, but PS-1145 does not enhance its effect (Fig. 4A). Importantly, both constitutive and IL-6-induced DNA synthesis in MM.1S cells is abrogated in the presence of PS-1145 in a dose-dependent fashion (Fig. 4B). Furthermore, the protective effect of IL-6 on Dex-induced growth inhibition is
also abrogated by PS-1145 (Fig. 4C). These data suggest that promotion of cell growth and survival by IL-6 is mediated, at least in part, via NF-κB signaling.

Because we have previously reported that the immunomodulatory derivative of Thal 3 (IMiD3) inhibits MM.1S cell growth and that IL-6 abrogates this IMiD effect (21), we next examined the effect of PS-1145 on DNA synthesis of MM.1S cells treated with IMiD3, in the presence or absence of IL-6. IMiD3 significantly (p < 0.001) inhibits DNA synthesis in MM.1S cells in a dose-dependent fashion (0.25–1 μM, Fig. 4D). IL-6 (20 ng/ml) overcomes the effect of IMiD3; importantly, however, PS-1145 neutralizes the inhibitory effect of IL-6 against IMiD3. These studies suggest that NF-κB blockade can overcome resistance to IMiDs.

**PS-1145 Inhibits TNFα-induced ICAM-1 Expression and Enhances TNFα-induced Apoptosis in MM.1S Cells**—We have previously shown that TNFα induces ICAM-1 and VCAM-1 expression on both BMSCs and MM cells and that proteasome inhibitor PS-341 blocks induction of these molecules (9). Because PS-341 is not a specific NF-κB inhibitor, we cannot conclude from these earlier studies that TNFα-induced up-regulation of ICAM-1 and VCAM-1 is mediated through NF-κB. However, PS-1145 also inhibits TNFα-induced up-regulation of ICAM-1 expression on MM.1S and RPMI8226 cells (Fig. 5A). This result strongly suggests that up-regulation of ICAM-1 by TNFα is mediated via activation of NF-κB.

We have previously shown that TNFα induces modest proliferation, but not apoptosis, in MM.1S cells (9). In this study, we hypothesized that TNFα would induce apoptosis in MM.1S cells when NF-κB activation is blocked by PS-1145. To test this hypothesis, MM.1S cells were cultured with TNFα, in the presence or absence of PS-1145. TNFα treatment does not affect viability of MM.1S cells, assessed by MTT assay; however, in the presence of PS-1145, viability of the cells is significantly (p < 0.001) decreased (Fig. 5B). For example, TNFα (1 ng/ml) induces 60% growth inhibition of MM.1S cells in the presence of 10 μM PS-1145. This effect on MM.1S viability is also confirmed by trypan blue exclusion (data not shown). These studies suggest that NF-κB mediates protection of MM.1S cells against TNFα-induced apoptosis.

**Effect of PS-1145 on Paracrine MM Cell Growth and IL-6 Secretion**—To study the role of NF-κB activation in regulating IL-6 transcription and secretion, as well as related paracrine MM cell growth in the BM milieu, MM.1S cells were cultured with or without BMSCs, in the presence or absence of PS-1145. PS-1145 blocks constitutive secretion of IL-6 in BMSCs in a dose-dependent fashion (Fig. 6A). Importantly, adhesion of MM.1S cells to BMSCs triggers increased IL-6 secretion (1.6-fold, p < 0.01), and PS-1145 also blocks this response in a dose-dependent fashion (p < 0.01). Adherence of MM.1S cells to BMSCs also triggers increased MM.1S cell growth (1.9-fold, p < 0.01), and PS-1145 similarly inhibits this augmentation in a dose-dependent fashion (p < 0.01, Fig. 6B). These data confirm our earlier studies that induction of IL-6 secretion from BMSCs triggered by MM cell adhesion is mediated through NF-κB (8), and importantly, show that PS-1145 can abrogate this effect.

**DISCUSSION**

Five NF-κB family members have been identified and cloned, including RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). All have highly conserved Rel homology do-

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**Fig. 5. PS-1145 inhibits TNFα-induced ICAM-1 expression and triggers TNFα-induced apoptosis in MM cells.** A, MM.1S and RPMI8226 cells were cultured for 24 h with MeSO control (heavy solid line), TNFα (5 ng/ml) alone (light solid line), or TNFα + PS-1145 (•). The cells were harvested, stained with isotype control (dotted line) or fluorescein isothiocyanate-conjugated anti-ICAM-1 Ab, and analyzed using flow cytometry. B, TNFα induces apoptosis in MM.1S cells with PS-1145-related NF-κB blockade. MM.1S cells were cultured for 48 h with 0.2 and 1 ng/ml TNFα, in the absence (□) or presence of 2.5 μM (■), 5 μM (□), and 10 μM (■) PS-1145. Cell viability was assessed by MTT assay.
mains mediating DNA binding and interaction with IkBα. The NF-κB family regulates growth, cell differentiation, and apoptosis in cell lines and tissues, including embryonic limb, lymphocytes, lung and liver, skin, and bone (26–29). It has also been shown that NF-κB activation inhibits apoptosis of Kaposi’s sarcoma-associated herpes virus infected primary effusion lymphoma cells (30) and induces Bcl-xL expression in Hodgkin/Reed-Sternberg cells (31) and CD4⁺ T lymphocytes (32). In this study, we characterize the biologic sequelae of NF-κB activation in MM pathogenesis. Specific NF-κB blockade using the IKK inhibitor PS-1145 confirms the role of NF-κB in growth, survival, and drug-resistance in MM cells within the BM milieu and suggests the therapeutic benefit of targeting NF-κB in MM.

We first demonstrate the inhibitory effect of PS-1145 on serine phosphorylation of IkBα in MM.1S cells and patient MM cells in a dose-dependent fashion. Phosphorylation of IkBα triggered by TNFα is completely abrogated by pre-treatment with 5 μM PS-1145, and inhibition of DNA binding of NF-κB by PS-1145 is confirmed by EMSA. Even with NF-κB blockade, however, PS-1145 decreases cell proliferation of MM cells by only 50%, assessed by [³H]TdR uptake. In contrast, as we have previously shown (18), proteasome inhibitor PS-341 completely blocks cell proliferation at an IC₅₀ of 0.002–0.005 μM in these same MM cell lines. Moreover, PS-1145 induces G₀ growth arrest, but not apoptosis or necrosis, as does PS-341 in MM cells. Our result is consistent with previous reports demonstrating that NF-κB activity is increased during G₀ to G₁ cell cycle transition in mouse fibroblasts (33) and that inhibition of NF-κB causes impairment of cell cycle progression in human glioma cells (34). The identification of an NF-κB binding site in the cyclin D1 promoter may account, at least in part, for cell cycle regulation by NF-κB (35). Our results further indicate that a complete growth inhibitory effect cannot be induced in MM cells by NF-κB blockade and that the complete block in MM cell growth by PS-341 is not solely due to NF-κB blockade but also to targeting other signaling pathways. Indeed, PS-341 inhibits IL-6-triggered p42/44 MAPK activation, which mediates proliferation in MM cell lines as well as patient MM cells (22).

We have shown that Dex-induced apoptosis in MM cells is mediated via related adhesion focal tyrosine kinase activation;
second mitochondria-derived activator of caspases, but not cytochrome c release from mitochondria; and caspase-9 activation (23, 36, 37). Conversely, IL-6 protects against Dex-induced apoptosis via PI3K/Akt signaling (23) and activation of SH2 domain containing protein tyrosine phosphatase SHP2, thereby blocking related adhesion focal tyrosine kinase activation (37). Although Dex up-regulates IκBα in monocytic cell lines and T cells (24, 25), it inhibits NF-κB activation without affecting IκBα expression in epithelial cells (38). We therefore examined whether Dex induces IκBα protein and thereby inhibits NF-κB activation in MM cells. As expected, Dex markedly increases IκBα protein expression in MM cells in a time-dependent fashion and inhibits NF-κB activation triggered by TNFα in MM.1S cells. Inhibition of NF-κB activation by Dex is also augmented by pre-treatment with PS-1145; conversely, Dex-induced up-regulation of IκBα protein expression is inhibited by IL-6. Therefore, Dex inhibits cell proliferation and IL-6 overcomes this effect, at least in part, by altering expression of IκBα protein. These results suggest that NF-κB plays a critical role in DNA synthesis and protection against Dex-induced apoptosis in MM.1S cells and that PS-1145 may be useful to overcome clinical Dex resistance.

Recently, we (21, 39, 40) and others (41, 42) have reported that Thal and IMiDs overcome drug resistance in MM cell lines and MM patient cells in vitro and achieve clinical responses even in refractory relapsed MM (21, 41, 42). A recent report (15) shows that Thal at high concentration inhibits NF-κB activity via indirect suppression of IKK activity. In this study, we further examine the effect of PS-1145 in Thal/IMiD-treated MM.1S cells and demonstrate that PS-1145 enhances inhibition of MM cell growth by IMiDs. Importantly, our prior study shows that IL-6 protects against Thal/IMiDs (21), and the current experiments demonstrate that PS-1145 blocks this protective effect of IL-6 against IMiD-induced apoptosis in MM.1S cells. These results indicate that the protective effect of IL-6 against Thal/IMiDs-induced apoptosis is also mediated via NF-κB activation. Importantly, they suggest the combined use of Thal/IMiDs with PS-1145 as a potential clinical strategy to overcome drug resistance.

Induction of NF-κB with related up-regulation of adhesion molecules, including CD54 (ICAM-1) and CD106 (VCAM-1), has been demonstrated in TNFα-stimulated MM cell lines and BMSCs, human umbilical vein endothelial cells, and fibroblast-like synoviocytes (9, 17, 43). Moreover, MM cell adhesion to fibronectin mediates an anti-apoptotic effect against chemotherapeutic agents (14). In our prior study, proteasome inhibitor PS-341 blocked TNFα-induced up-regulation of adhesion molecules and related increased MM cell to BMSC binding by inhibiting NF-κB activation (9, 18). In this study, IKK inhibitor PS-1145 also inhibits TNFα-induced ICAM-1 expression in MM.1S and RPMI8226 cells, suggesting that NF-κB directly targets ICAM-1 expression in MM. We further demonstrate that MM cell adherence to BMSCs induces up-regulation of IL-6 transcription and secretion in BMSCs, as well as proliferation of adherent MM cells, as we (18) and others (44) have previously reported. Importantly, PS-1145 abrogates this induction of IL-6 secretion in BMSCs and proliferation of adherent MM cells in a dose-dependent fashion, consistent with our previous report that adhesion-induced IL-6 transcription and secretion in BMSCs is conferred, at least in part, by NF-κB activation (8). This PS-1145-mediated inhibition of adhesion molecule expression, abrogation of protection against apoptosis conferred by MM cell to binding to BMSCs adhesion, and blockade of cytokine secretion in the BM milieu, provides further rationale for targeting NF-κB in novel MM therapies.

We have previously shown that TNFα mediates moderate cell proliferation associated with p24/44 MAPK activation in MM.1S cells (9). TNFα has also been reported to induce NF-κB activation and apoptosis via caspase cleavage (45). When NF-κB signaling was blocked by PS-1145 in this study, TNFα significantly inhibited MM.1S cell growth in a dose-dependent fashion. This result strongly suggests that NF-κB mediates protection against TNFα-induced apoptosis in MM cells and is consistent with a recent report that activation of IKKβ mediates protection against TNFα-induced apoptosis in T cells (16). It further suggests that TNFα, which is secreted by MM cells (9), may promote tumor cell apoptosis in patients treated with PS-1145.

In summary, our data demonstrate that NF-κB activation promotes growth, survival, and drug resistance of MM cells in the BM microenvironment and provide the framework for clinical trials of novel agents, such as PS-1145, specifically targeting NF-κB in MM.

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