Cell-Selective Inhibition of NF-κB Signaling Improves Therapeutic Index in a Melanoma Chemotherapy Model

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ABSTRACT

The transcription factor NF-κB promotes the survival of cancer cells exposed to doxorubicin and other chemotherapeutic agents. IκB kinase is essential for chemotherapy-induced NF-κB activation and considered a prime target for anticancer treatment. An IκB kinase inhibitor sensitized human melanoma xenografts in mice to killing by doxorubicin yet also exacerbated treatment toxicity in the host animals. By using mouse models that simulate cell-selective targeting, we found that impaired NF-κB activation in melanoma and host myeloid cells accounts for therapeutic and adverse effects, respectively. Ablation of tumor-intrinsic NF-κB activity resulted in apoptosis-driven tumor regression after treatment with doxorubicin. By contrast, chemotherapy in mice with myeloid-specific loss of NF-κB activation led to a massive intratumoral recruitment of interleukin-1β-producing neutrophils and necrotic tumor lesions, a condition associated with increased host mortality but not accompanied by tumor regression. Therefore, a molecular target-based therapy may be steered toward different clinical outcomes depending on the drug's cell-specific effects.

SIGNIFICANCE: Our findings show that the IκB kinase–NF-κB signaling pathway is important for both promoting treatment resistance and preventing host toxicity in cancer chemotherapy; however, the two functions are exerted by distinct cell type–specific mechanisms and can therefore be selectively targeted to achieve an improved therapeutic outcome. Cancer Discovery; 1(6): 496–507. ©2011 AACR.

INTRODUCTION

Treatment resistance poses a major challenge in cancer chemotherapy. Tumors refractory to chemotherapeutic agents emerge as intrinsic resistance traits are selected for and stabilized in the tumor cell population (1, 2). Moreover, hematopoietic-derived host cells influence chemotherapy-induced tumor cell death (3, 4) and modulate tumor-specific immune responses triggered by primary treatment (5, 6). Tumor cells may also contribute to host cell–mediated resistance mechanisms; for example, tumor-derived factors can mobilize and recruit specific subsets of circulating and stromal cells and influence their function (7–9), thereby playing an active role in the construction of a microenvironment that defies cytotoxic treatment. Therefore, both tumor-intrinsic and host cell–mediated mechanisms of treatment resistance are likely dictated, albeit to varying extents, by the tumor's inherent genetic properties.

Another obstacle for successful cancer treatment is the toxicity of therapeutic agents that inevitably limits the dose and frequency of treatment. Treatment-induced toxicity, in most cases, manifests as a mixed condition of direct drug-inflicted adversity and secondary inflammation-mediated damage (10). Toxicity either arises from nonspecific "off-target" effects of the drug or occurs as the result of interference with an essential physiologic function of the intended drug target. In the latter circumstances, substantial clinical benefits can be derived from selectively interfering with the disease-related function of the target molecule while sparing other functions whose loss is responsible for adverse side actions. Cell-specific drug targeting may offer such selectivity if treatment efficacy and toxicity are mechanistically based in different cell types.

The transcription factor NF-κB is activated in response to diverse stress and inflammatory stimuli, and it mediates cellular interpretation of the triggering stimuli via regulation of gene expression. NF-κB activation requires the IκB kinase (IKK), which phosphorylates and induces degradation of the NF-κB–bound inhibitor IκB (11). DNA-damaging anticancer drugs such as doxorubicin are potent inducers of NF-κB activity. Doxorubicin is known to activate IKK via a DNA damage–induced signaling cascade involving phosphorylation, sumoylation, and ubiquitination of the inhibitor of IKK-γ/NF-κB essential modulator, the IKK regulatory subunit (12, 13). Among genes induced by chemotherapeutic agents in an NF-κB–dependent manner are several inhibitors of apoptosis that can prevent drug-induced cell death (14–16). Therefore, inhibition of NF-κB signaling has been considered a promising way to overcome tumor cell-intrinsic resistance to killing by cytotoxic agents. In fact, pharmacologic inhibitors of IKK were found to increase the apoptotic sensitivity of melanoma and other chemoresistant tumor cells to doxorubicin treatment (17–19).
of NF-κB pathway inhibitors on tumor–host interactions under conditions of chemotherapy as well as its contributions to treatment-induced toxicity.

We suspected that, given the nodal position of NF-κB in the inflammatory signaling network, systemic inhibition of NF-κB signaling might not only sensitize tumor cells to chemotherapy but will also produce pleiotropic effects on inflammation-mediated processes associated with cancer treatment. We investigated this possibility by using mouse models of melanoma chemotherapy because melanoma is among the group of highly chemoresistant malignancies for which very few effective treatment options are available (20).

**RESULTS**

**Role of NF-κB Signaling in Chemoresistance of Human Melanoma Cells**

NF-κB is constitutively activated in many cancers, and aberrant NF-κB activation has been linked to the maintenance of the malignant state and resistance to cytotoxic chemotherapy (21). To investigate the link between NF-κB and melanoma chemoresistance, we first examined NF-κB expression and activation in 5 independently established human melanoma cell lines: A375, MM455, MM608, Hs944T, and Roth. The NF-κB proteins RelA and p50, which form a heterodimer, were abundantly expressed in all cell lines (Supplementary Fig. S1A). NF-κB activation entails IkB degradation and nuclear translocation of the liberated NF-κB dimer. The amount of nuclear RelA in untreated and doxorubicin-treated cells varied considerably among the melanoma lines, and the pattern of NF-κB activation ranged from constitutive to inducible to nearly undetectable (Supplementary Fig. S1B), Hs944T and Roth were competent and defective, respectively, in tumor-intrinsic NF-κB activation in response to doxorubicin (Fig. 1A) and were chosen as representing 2 contrasting subtypes of melanoma lines for further analysis.

We sought to set up an experimental model in which a poorly performing chemotherapeutic regimen for melanoma was converted to a highly effective one by the addition of a chemosensitizing agent. Doxorubicin seemed suitable for this purpose; melanoma cells are highly resistant to doxorubicin in vitro (22), and treatment of melanoma with doxorubicin has been largely ineffective in clinical trials (23, 24). We used BMS-345541, a small-molecule inhibitor of IKK, to determine the consequence of NF-κB blockade in melanoma cell response to doxorubicin. BMS-345541 efficiently blocked doxorubicin-induced NF-κB activation in Hs944T and other NF-κB–competent cells (Fig. 1B and Supplementary Fig. S1C) and sensitized them to killing by doxorubicin (Fig. 1C and Supplementary Fig. S1D and E). BMS-345541 treatment alone was cytotoxic to Hs944T cells, albeit to a lesser degree than in conjunction with doxorubicin (Supplementary Fig. S1D and E). These results show that NF-κB–dependent resistance to apoptosis is frequently co-opted by melanoma and can be effectively reduced by treatment with IKK inhibitor. With Roth cells, wherein neither constitutive nor doxorubicin-induced NF-κB activation was detected, BMS-345541 did not exhibit similar cytotoxic or sensitizing effects (Fig. 1C). Therefore, the IKK inhibitor does not appear to act on other NF-κB–independent mechanisms of apoptotic resistance.

**Effects of IKK Inhibitor and Doxorubicin Treatment on Melanoma Xenografts in Mice**

The drug sensitivity of cancer cells in vitro does not always mirror tumor response to treatment in vivo. To investigate the response of tumors derived from Hs944T and Roth cells

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**Figure 1.** Doxorubicin-induced NF-κB activation and NF-κB–mediated chemoresistance in Hs944T and Roth human melanoma cells. **A,** cytoplasmic (Cyto) and nuclear (Nuc) extracts from Hs944T and Roth cells treated with doxorubicin (Dxr; 2 µg/mL) were analyzed by immunoblotting with anti-RelA antibody. Values in the plot represent relative amounts of nuclear RelA. Hs944T and Roth cells were treated with doxorubicin and BMS-345541 (75 µM; added 1 hour before doxorubicin treatment) as indicated. Cyto and Nuc extracts were prepared 2 hours after doxorubicin treatment and analyzed as in panel A. **C,** Hs944T and Roth cells were treated with doxorubicin and BMS-345541 as in panel B. Cell viability was determined by MTT assay 24 hours after doxorubicin treatment.
to BMS-345541 and doxorubicin in the animal host, we established subcutaneous melanoma xenografts in Rag2−/−γc−/− mice, a strain devoid of T cells, B cells, and natural killer cells shown to support engraftment of human melanoma cells (25). Under our treatment regimen, the administration of doxorubicin alone did not induce remission in mice bearing Hs944T or Roth tumors or delay the growth of the xenografts. However, BMS-345541 alone was effective against Hs944T tumors in mice and dramatically sensitized Roth tumors to doxorubicin chemotherapy (Fig. 2A and B), suggesting that NF-κB blockade by BMS-345541 could subvert melanoma chemoresistance in vivo independently of whether the engrafted cells responded to the inhibitor in vitro. Almost complete tumor regression occurred in animals in whom BMS-345541 exhibited therapeutic efficacy, either alone or in conjunction with doxorubicin. However, combined treatment of tumor-bearing mice with BMS-345541 and doxorubicin caused severe side effects: mice carrying Hs944T and Roth tumors became lethargic, ataxic, and emaciated (Fig. 2C). These signs of illness were most visible and intense when the size of the tumor was rapidly decreasing.

We examined the histologic features of the tumors obtained immediately after treatment but before their complete destruction. Under the treatment conditions that induced tumor regression, areas of apoptotic nuclei emerged in Hs944T tumors (Fig. 2D, a). Apoptotic nuclei (green) and neutrophils (red) are shown together with the counter staining of DNA (blue). Scale bar, 100 µm.

**Figure 2.** The efficacy and toxicity of doxorubicin and IKK inhibitor treatment in mice harboring Hs944T and Roth tumors. Hs944T and Roth cells were infected with lentivirus expressing luciferase (Luc) and injected subcutaneously into Rag2−/−γc−/− mice (n=3). After tumors grew to 100 mm³ in volume, mice were administered doxorubicin intraperitoneally (Dxr; 2 mg/kg) and BMS-345541 (125 mg/kg) as indicated on day 0, 2, 4, and 6. A, tumors were visualized in pseudocolor by bioluminescence imaging on day 10. Representative images from each group are shown along with the reference scale of bioluminescence depicted on the right. Tumor sizes (B) and the severity of illness (C) were determined on day 10. Triangles and circles indicate values in individual mice. Severity of illness was expressed in scores representing decrease in liveliness (DIL) and grade of emaciation (GOE). D, tumor sections from the indicated groups were analyzed by TUNEL staining (top) and immunostaining with Ly6G-specific antibody (bottom). The number within each image indicates relative fluorescence intensity (TUNEL and Ly6G signal, respectively) and represents mean ± SD from 3 independent areas. Apoptotic nuclei (green) and neutrophils (red) are shown together with the counter staining of DNA (blue). Scale bar, 100 µm.
tumors exposed to a cytotoxic agent and likely create a cytokine-rich microenvironment at the tumor site. Both Hs944T and Roth cells were indeed able to activate NF-κB in response to interleukin (IL)-1β (Supplementary Fig. S2A), a cytokine whose production has been associated with chemotherapy-induced tumor cell death (8). We noted that inhibition of NF-κB signaling produced mixed effects on gene expression in melanoma cells. BMS-345541 suppressed basal and IL-1β-induced expression of CXCL8 (encoding the chemokine IL-8) but not CXCL5 (encoding another chemokine, ENA-78); rather, basal CXCL5 expression was markedly elevated by IKK inhibitor treatment (Supplementary Fig. S2B). Because both these genes encode neutrophil chemoattractants, we examined neutrophil recruitment in Hs944T and Roth tumors in vivo. Intriguingly, areas of Ly6G+ neutrophil infiltration were greatly increased in tumors from BMS-345541-treated mice regardless of doxorubicin treatment (Fig. 2D, bottom). Of note, BMS-345541-induced neutrophil recruitment in Roth tumors was dissociated from tumor regression. Therefore, we postulated that intratumoral neutrophil recruitment was not mechanistically linked to the antitumor efficacy of BMS-345541 but is indicative of inflammatory responses in tumor tissue that might contribute to or serve as a surrogate marker for BMS-345541 toxicity.

**Experimental Models Simulating Cell-Selective Inhibition of NF-κB Activation**

Our results from the melanoma xenograft experiments revealed mixed effects of NF-κB pathway inhibition on melanoma chemotherapy sensitization to treatment at the cost of severe host toxicity. In all likelihood, the chemosensitizing effect was attributable to inhibition of tumor-intrinsic NF-κB activation. The origin of toxicity was not as apparent but possibly hinted at by the observation that BMS-345541 treatment promoted a neutrophil inflammatory milieu within the tumor. Related to this finding were previous reports in which authors demonstrated that mice with myeloid cell-specific deficiency of IKKβ, a catalytic subunit of inhibitor of IKK, failed to limit inflammatory responses upon endotoxemia and bacterial infection and were prone to inflammation-mediated damage (26, 27). Of note, myeloid cells represent the major type of hematopoietic cells spared in the Rag2−/− mice we used as hosts for our melanoma xenografts. Therefore, under the conditions of doxorubicin-induced tumor injury and inflammation, BMS-345541 might have caused toxicity, at least in part, through interference with the anti-inflammatory mechanism mediated by myeloid NF-κB signaling.

We sought to verify the hypothesis that efficacy and toxicity of IKK inhibitor treatment arise from inhibition of
NF-κB signaling in different target cells—tumor cells and host myeloid cells, respectively. To this end, we used B16 murine melanoma cells and C57BL/6 hosts to construct syngeneic tumor models in which NF-κB activation was specifically blocked in only 1 of the 2 cell types. B16 cells expressed RelA and p50 (Supplementary Fig. S3) and were able to induce their nuclear translocation in response to doxorubicin (Fig. 3A). To ablate NF-κB activation in B16 melanoma, we generated a B16 derivative stably expressing the IκBα “super-repressor,” a mutant IκBα refractory to IKK phosphorylation and degradation (Fig. 3B, top). The resultant cell line, B16SR, exhibited impaired NF-κB activation after doxorubicin treatment (Fig. 3B, bottom) and defects in doxorubicin-induced expression of NF-κB-responsive antiapoptotic genes (Fig. 3C; Supplementary Fig. S4A and B).

Compared with control cells (B16GFP), B16SR cells were highly sensitive to killing by doxorubicin in vitro (Fig. 3D). Silencing of RelA expression in B16 cells with siRNA produced a similar chemosensitizing effect (Supplementary Fig. S5A and B). To selectively suppress myeloid NF-κB activation in host mice, we established B16 tumors in mice whose IκBκ gene was floxed and deleted by Cre recombinase expressed under the control of the lysozyme M promoter (IκBκΔM). Myeloid cells from these mutant mice have been shown to have defects in NF-κB activation and NF-κB–dependent gene transcription (28, 29).

**Chemosenzitization via Inhibition of Tumor-Intrinsic NF-κB Signaling**

The growth of B16GFP and B16SR tumors in C57BL/6 mice was comparable. However, the administration of doxorubicin resulted in rapid regression of B16SR tumors, whereas B16GFP tumor growth was largely refractory to the treatment (Fig. 4A). In neither group did the host animals exhibit signs of illness similar to those of BMS-345541–treated animals. Histologically, B16SR tumor sections from doxorubicin-treated mice exhibited karyopyknosis (Fig. 4B) and widespread areas of apoptotic nuclei (Fig. 4C, top). Apoptotic cells signal to promote an anti-inflammatory milieu in the surrounding tissue and recruit phagocytes for their engulfment and clearance. Accordingly, the apoptotic B16SR tissues were infiltrated with F4/80+ macrophages but not Ly6G+ neutrophils (Fig. 4C, middle and bottom). None of these histologic features was detected in B16GFP tumors under doxorubicin treatment. The findings from the analysis of B16SR tumors indicate that melanoma chemoresistance is mainly attributable to tumor-intrinsic NF-κB signaling, and its targeted inhibition may substantially increase therapeutic efficacy in melanoma treatment.

**Host Toxicity as the Result of Inhibition of NF-κB Signaling in Myeloid Cells**

We next examined how myeloid cell-specific deficiency of NF-κB signaling affects the response of B16 tumors and the tumor-bearing animals to chemotherapy. Growth of B16 tumors in both wild-type and IκBκΔM hosts was unabated during and after doxorubicin treatment (Fig. 5A, left), indicating that myeloid NF-κB signaling did not exert a critical influence on the treatment resistance of B16 tumors. Meanwhile, IκBκΔM host animals developed severe illness and eventually died, resulting in a drastically increased mortality (Fig. 5A, middle). This increase in lethality was accompanied by incidents of local tumor necrosis (Fig. 5A, right), a condition reminiscent of tumor lysis syndrome (30). Even before gross necrotic lesions occurred, tumor sections obtained from doxorubicin-treated IκBκΔM mice displayed hyperpigmented...
regions of micronecrosis and karyolysis but few apoptotic areas (Fig. 5B; and Fig. 5C, top). In marked contrast to apoptotic B16SR tumor tissues, these necrotic tumor areas were heavily infiltrated with Ly6G+ neutrophils but not F4/80+ macrophages (Fig. 5C, middle and bottom; and Fig. 5D). Therefore, perturbed NF-κB signaling in myeloid cells during cytotoxic cancer therapy may engender an intratumoral tissue environment prone to necrotic injury and neutrophilic inflammatory responses and ultimately result in adverse, potentially lethal toxic effects in the host.

IKKβ and NF-κB exert a negative control on the processing of pro-IL-1β into its mature functional form. Myeloid cells lacking IKKβ, hence deprived of this control, demonstrate excessive production of bioactive IL-1β upon lipopolysaccharide challenge (26). Similar dysregulation might have occurred in IKKβΔM mice under doxorubicin treatment. B16 tumor sections from IKKβΔM hosts indeed included intratumoral areas intensely stained for IL-1β (Fig. 6A). IL-1β was almost always detected at or near the necrotic tumor lesions. Immunoblot analysis showed that both unprocessed and mature IL-1β were present in the tumors (Fig. 6B). IL-1β was not detected in tumors from wild-type hosts by either immunostaining or immunoblotting. We identified Ly6G+ neutrophils as the principal source of intratumoral IL-1β expression in IKKβΔM mice (Fig. 6C and D). Consistent with an earlier report (26), IKKβ-deficient neutrophils exhibited enhanced production of fully processed IL-1β upon inflammatory challenge in vitro (Fig. 6E).

Therefore, tumor necrosis in doxorubicin-treated IKKβΔM mice is not only associated with a recruitment of IL-1β–producing neutrophils but likely also with a greater rate of IL-1β production by the recruited neutrophils. We found that B16 melanoma cells were directly responsive to IL-1β in vitro as were the human melanoma cells (Supplementary Fig. S2A and B), inducing NF-κB activity and the expression of neutrophil-specific...
Chemokine genes (Supplementary Fig. S6A and B). Thus, it appears that IL-1β production by intratumoral neutrophils establishes a self-sustaining or even self-amplifying state by maintaining neutrophil influx via tumor-derived chemoattractants.

We tested whether IL-1β production by intratumoral neutrophils is essential for the formation of necrotic lesions and inflammatory infiltration of the tumors. Administration of anakinra, an IL-1 antagonist, in doxorubicin-treated IKKβΔM hosts substantially prevented the occurrence of hyperpigmented necrotic lesions and neutrophil infiltration in the tumors (Fig. 6F; and Fig. 6G, top). Anakinra exerted similar effects on tumors in BMS-345541—treated wild-type mice (Supplementary Fig. S7). Under anakinra treatment, tumor cell apoptosis was preserved or moderately enhanced in IKKβΔM mice (Fig. 6G, lower). After doxorubicin treatment, IKKβΔM host animals had greater...
amounts of circulating IL-1β compared with wild-type animals. The serum concentration of IL-1β was greatly reduced in both wild-type and IKKβΔM mice by anakinra treatment (Fig. 6H). Therefore, IL-1β overproduction and resulting inflammation likely caused both local (intratumoral) and systemic responses in IKKβΔM mice during melanoma chemotherapy. Some reversal of the inflammatory events notwithstanding, anakinra-administered IKKβΔM animals experienced even greater rates of mortality under doxorubicin treatment than the anakinra-untreated group. This finding suggests that although IL-1 signaling potentially contributes to treatment-associated toxicity via inflammation-mediated mechanisms, it may play some indispensable protective role in IKKβΔM mice during cytotoxic cancer therapy.

**DISCUSSION**

Tumor cells exposed to cytotoxic agents incur injury and invoke damage-induced signaling cascades. Our findings illustrate that inhibition of NF-κB signaling in doxorubicin-treated mice can lead damaged tumor cells to 2 alternative fates: apoptotic demise or proinflammatory necrosis (Fig. 7). The mode of tumor cell death and the pattern of intratumoral infiltration in doxorubicin-treated mice differed markedly depending on the cell type in which NF-κB signaling is targeted: apoptosis and macrophage recruitment (tumor cell-restricted inhibition) versus necrosis and neutrophil recruitment (myeloid cell-restricted inhibition). These histologic parameters were associated with, and therefore predictive of, the rate of tumor regression and the severity of host toxicity. Necrotic lesions with massive neutrophilic infiltration characterized the histology of tumors in doxorubicin-treated IKKβΔM mice. Given the observed effect of anakinra, IL-1β appears to be instrumental for intratumoral neutrophil recruitment and other pathologic features in this setting. IL-1β was found to induce the expression of neutrophil-recruiting chemokines in the melanoma cell lines tested even in the presence of the IKK inhibitor. Importantly, the neutrophils themselves turned out to be the major source of IL-1β in the necrotic tumor areas.

IL-1β production by newly recruited neutrophils presumably requires activation by biochemical stimuli released from doxorubicin-injured tumor cells because unstimulated IKKβ-deficient neutrophils do not produce significant amounts of IL-1β in vitro. A role for such “danger” signals in neutrophil recruitment under inflammatory conditions was recently demonstrated (31, 32). Taken together, we postulate that neutrophil-derived IL-1β and tumor-derived chemokines constitute a self-sustaining cycle of local signaling that maintains or amplifies intratumoral necrosis and inflammatory responses. Different enzymatic mechanisms of pro-IL-1β cleavage appear to operate in distinct subsets of myeloid cells: caspase-1-dependent and -independent (33). Therefore, there is a possibility that pro-IL-1β processing in neutrophils can be specifically inhibited, thus preventing its adverse clinical effects, without interfering with any protective function that IL-1β from other subtypes of myeloid cells may serve. Notably, it was demonstrated that dendritic cells link chemotherapy-induced tumor cell death to tumor-specific CD8⁺ T-cell-mediated immune responses through IL-1β production (8). Tumor-derived chemokines responsible for intratumoral neutrophil recruitment may be considered as another group of therapeutic targets for the prevention of neutrophil-mediated treatment toxicity.

The protumor function of NF-κB is not merely confined to promoting chemoresistance and other mechanisms of tumor persistence. NF-κB activity in premalignant and malignant cells is critically required for the development and growth of many different forms of cancer (34–37). Some melanoma cells tested in this and other studies, as well as many other tumor cells of different origin, are “addicted” to NF-κB signaling to such an extent that treatment with an IKK inhibitor or other means of NF-κB blockade was sufficient to induce their apoptosis and regression (17, 38–40).

Although these findings offer new opportunities in cancer treatment, it is becoming increasingly clear that the
IKK–NF-κB signaling axis also serves important physiologic functions whose loss could result in undesired pathologic conditions. For example, systemic ablation of inhibitor of IKKβ expression in mice perturbs myeloid homeostasis; increased proliferation of IKKβ-deficient myeloid progenitors leads to neutrophilia and neutrophil-mediated tissue inflammation (41, 42). Importantly, we demonstrate these adverse events can be suppressed by eliminating IL-1 receptor 1-mediated signaling in IKKβ-mutant mice. Therefore, long-term treatment with an NF-κB–blocking agent either alone or in conjunction with other cytotoxic agents may give rise to clinical adversities resulting from excessive production and activity of IL-1β.

On the basis of the target cell-specific effects of NF-κB pathway inhibition identified in the current study, we thus suggest that antitumor therapeutic modalities that are built upon their ability to block NF-κB activity be specifically delivered to or activated in tumor cells. Such selectivity will prevent or alleviate adverse clinical events that result from dysregulation of NF-κB signaling in host myeloid cells.

**Methods**

**Melanoma and Primary Cells**

A375, MM455, MM608, Hs944T, and Roth cells are independently established cell lines from human melanoma obtained from the sources described previously (43, 44). These cell lines were authenticated by DNA fingerprinting on the basis of PCR amplification and DNA sequencing of specific loci (44). B16 melanoma cells are of C57BL/6 mouse origin and were obtained from the American Type Culture Collection; no validation was performed. Neutrophils were isolated from mouse bone marrow as described (45).

**Animals**

Rag2−/− and C57BL/6 mice were obtained from Taconic and The Jackson Laboratory, respectively. Inhibitor of IKKβ&κB (IκB)−/−,LysMCre mice were described previously (28). All animal studies were conducted under Institutional Animal Care and Use Committee-approved protocols.

**Preparation of BMS-345541**

BMS-345541, N(2,3-dimethylimidazo[1,2-α]quinolin-4-yl)-ethane-1,2-diamine, was prepared according to the procedure described in the Patent Cooperation Treaty patent application (WO 02/060386 A2) beginning with commercially available starting materials, 3-fluoro-4-nitrotoluene and ethyl 4-methyl-5-imidazolecarboxylate. The purity of the BMS-345541 preparation used was greater than 96%, as assessed by high-performance liquid chromatography.

**Human and Mouse Melanoma Models and Treatment**

Hs944T and Roth cells were transduced with a lentivirus expressing luciferase under the control of the cytomegalovirus promoter according to established methods (46). A total of $5 \times 10^7$ transduced cells were injected subcutaneously into the right hind legs of 8-week-old Rag2−/− mice. B16 and their derivative cells were similarly injected into 8-week-old C57BL/6 mice. After tumors grew to 100 mm³ in volume, mice were administered doxorubicin intraperitoneally (2 mg/kg; Sigma-Aldrich) alone or together with BMS-345541 (125 mg/kg) on days 0, 2, 4, 6, and 8. Complete tumor regression occurred with 125 mg/kg of BMS-345541, either as such or in conjunction with doxorubicin; lower BMS-345541 doses tested were far less effective and produced inconsistent results.

**Monitoring and Measurement of Tumor Growth and Host Toxicity**

Luciferase-expressing tumors were visualized by bioluminescence-based imaging (IVIS 200; Caliper Life Sciences) on day 10. Tumor size was manually measured with a caliper. The toxicity of treatment was monitored and expressed in scores ranging from 0 to 3 representing a decrease in liveliness (0, normal; 1, mildly lethargic; 2, lethargic and/or ataxic; 3, severely lethargic and/or ataxic) and grade of emaciation, which was based on the extent of body weight loss: 0, 1%, 2, ≥10% and <20%; 3, ≥20%. These criteria were adapted from a previously described guideline (47).

**Histology and Immunofluorescence**

Tumor tissue sections were prepared and analyzed as described (48). Antibodies against the following markers were used: F4/80 (MCA497B, AbD Serotec), Ly6G/Gr-1 (553125; BD Biosciences), and IL-1β (AF-401-NA; R&D Systems). TUNEL staining was performed via use of the In Situ Cell Death Detection kit (Roche). Relative fluorescence intensity was determined using the ImageJ software (National Institutes of Health).

**Protein and RNA Analysis**

Whole-cell lysates were prepared and analyzed as described (48). To prepare cytoplasmic and nuclear extracts, $4 \times 10^6$ cells were resuspended in 0.5 mL of buffer L1 (50 mM Tris-chloride, pH 8.0, 2 mM EDTA; 0.1% Nonidet P-40; 10% glycerol; 25 mM β-glycerophosphate; and protease inhibitors), incubated for 5 minutes at 4°C, and centrifuged at 4,500g. Cytoplasmic supernatants were stored, and pelleted nuclei were rinsed by resuspending and centrifuging in 0.5 mL of buffer L1, and extracted further in buffer L2 (20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid-potassium, pH 7.6, 150 mM sodium chloride; 2 mM EDTA acid; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 10% glycerol; 25 mM β-glycerophosphate; and protease inhibitors). Lysed nuclei were centrifuged at 16,000g, and the supernatant was collected for use as the nuclear fraction. The purity of cytoplasmic and nuclear extracts was verified by immunoblotting with antibodies against various cytoplasmic and nuclear marker proteins (Supplementary Fig. S7). Antibodies against the following proteins were used in immunoblot analysis: IkBα (sc-371), RelA (sc-372), RelB (sc-226), c-Rel (sc-71), p105/50 (sc-1409), p100/p52 (sc-298), and p53 (sc-6243; all from Santa Cruz Biotechnology); IL-1β (AF-401-NA; R&D Systems); and actin (A4700; Sigma-Aldrich). Total RNA was isolated by use of the Trizol (Invitrogen) and analyzed by the use of quantitative real-time PCR as described previously (29). Serum IL-1β was assayed by ELISA (R&D Systems).

**Flow Cytometry**

Myeloid marker and IL-1β expression in tumor-associated cells were analyzed by flow cytometry by the use of FACSCanto (BD) and the FlowJo software (Tristar). Antibodies against the following markers were used: CD11b (12-0112; eBioscience), IL-1β (17-7114; eBioscience), and Ly6G (clone 1A8; 551460; BD Biosciences).

**Statistical Analysis**

Data values are expressed as mean ± SEM. P values were obtained with the unpaired, 2-tailed Student t-test, and the Log-rank test. P < 0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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