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Myeloid IKKβ Promotes Antitumor Immunity by Modulating CCL11 and the Innate Immune Response

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Abstract

Myeloid cells are capable of promoting or eradicating tumor cells and the nodal functions that contribute to their different roles are still obscure. Here, we show that mice with myeloid-specific genetic loss of the NF-κB pathway regulatory kinase IKKβ exhibit more rapid growth of cutaneous and lung melanoma tumors. In a $BRAF^{V600E/PTEN-/-}$ allograft model, IKKβ loss in macrophages reduced recruitment of myeloid cells into the tumor, lowered expression of MHC class II molecules, and enhanced production of the chemokine CCL11, thereby negatively regulating dendritic-cell maturation. Elevated serum and tissue levels of CCL11 mediated suppression of dendritic-cell differentiation/maturation within the tumor microenvironment, skewing it toward a Th2 immune response and impairing $CD8^+$ T cell–mediated tumor cell lysis. Depleting macrophages or $CD8^+$ T cells in mice with wild-type *IKKβ* myeloid cells enhanced

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No potential conflicts of interest were disclosed.

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tumor growth, where the myeloid cell response was used to mediate antitumor immunity against melanoma tumors (with less dependency on a $CD8^+$ T-cell response). In contrast, myeloid cells deficient in IKKβ were compromised in tumor cell lysis, based on their reduced ability to phagocytize and digest tumor cells. Thus, mice with continuous IKKβ signaling in myeloid-lineage cells ($IKK\beta^{CA}$) exhibited enhanced antitumor immunity and reduced melanoma outgrowth. Collectively, our results illuminate new mechanisms through which NF-κB signaling in myeloid cells promotes innate tumor surveillance.

Introduction

Malignant melanoma is a lethal disease due to its aggressive capacity for metastasis and resistance to therapy. For decades, considerable effort has gone toward development of immunotherapy for treatment of metastatic melanoma. Tumors can potentially be recognized as “altered self,” akin to allogeneic immunity, and leading to an antitumor immune response of potential value in the adjuvant setting. This motivated investigations of interactions between melanoma and immune cells and translation of this knowledge into effective clinical strategies. The majority of the early studies strove to increase T-cell responses to the tumor partly through manipulation of dendritic cells (DC), a key antigen-presenting cell (APC) type. However, macrophages and neutrophils were also found to be key mediators of inflammation and immunity in cancer. Their phenotypes depend on the physiologic or pathologic milieu in which they reside. Protumor macrophages (M2) and neutrophils (N2) can be contrasted with the classically activated macrophages (M1) and neutrophils (N1) that present antigen and/or produce reactive oxygen species (ROS) involved in the killing of foreign organisms and tumor cells (1, 2). Moreover, the cytokines and chemokines produced by myeloid cells can significantly affect DC and the Th1 (antitumor) versus Th2 (protumor) skew of the immune cells in the tumor microenvironment (TME).

Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor that regulates expression of proinflammatory genes, playing a crucial role in immune response (3). NF-κB activation is regulated by the IκB kinase complex (IKKα, IKKβ, NEMO) that has become a major target for anti-inflammation and cancer therapy (4–6). Considering the importance of IKK, particularly IKKβ, in tumor immunity, a myriad of efforts have focused on the molecular mechanism for IKKβ regulation of the myeloid-mediated immune response during tumor development. Deletion of the *Ikkβ* gene in myeloid cells led to inhibition of colitis-induced colon cancer (7) and expression of an IκB-super repressor in resident macrophages (Kupffer cells) inhibited progression of hepatocellular carcinoma (8). Furthermore, introduction of NF-κB–deficient macrophages into mice with early ovarian cancer lesions slowed cancer progression (9). Despite these indications of a protumorigenic role of NF-κB in macrophages, other reports indicate that NF-κB is needed for the antitumorigenic function of macrophages in breast cancer metastasis and angiosarcoma (10, 11). Thus, the role of IKKβ/NF-κB signaling in macrophage pro- or antitumor responses remains controversial.

To address the role of IKKβ function in myeloid cells during melanoma tumorigenesis, we generated a C57Bl/6 mouse model with Cre-recombinase–mediated *IKKβ* deletion in

myeloid cells (*IKKβ*$^{myeΔ/Δ}$) and evaluated how loss of myeloid IKKβ affects melanoma tumor growth in allogenic and syngeneic melanoma models. In the allogenic model, melanoma cells were derived from a melanoma in a *BRAF*^{V600E}/*Pten*$^{-/-}$ mouse that had been backcrossed from FVB to C57Bl/6 but retained FVB MHC. We evaluated the ability of these tumor cells to establish metastatic lesions in lung or liver in *IKKβ*^{WT} and *IKKβ*$^{myeΔ/Δ}$ C57Bl/6 mice. In the syngeneic model, growth of B16 melanoma tumors in mice with myeloid cells expressing IKKβ constitutively active (IKKβ^{CA}), IKKβ^{WT} or IKKβ$^{myeΔ/Δ}$ was evaluated. We now show that loss of *Ikkβ* in myeloid cells enhanced melanoma tumor growth in both the allograft and the syngeneic model, even though the mechanisms differed. In the allograft model, melanoma growth was enhanced in *IKKβ*$^{myeΔ/Δ}$ mice due to defects in myeloid cell MHCII expression and function, altered myeloid cytokine/chemokine expression, defects in DC maturation, and poor T-cell activation. In contrast, *IKKβ* and to a greater extent *IKKβCA* myeloid cells exhibited strong antitumor response to syngeneic B16 melanoma, compared with *IKKβ*$^{myeΔ/Δ}$ mice. These results indicate that IKKβ activity is important for the antitumorigenic function of myeloid cells, thus providing important therapeutic implications.

Materials and Methods

Myeloid *Ikk*β knockout models and melanoma metastatic models

All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee. To knockout Ikkβ in myeloid cells in either C57BL6 or FVB strain mice, LysM-Cre mice with Cre-recombinase expressed under the control of the murine lysozyme M gene regulatory region (1) were bred with *Ikk*β$^{f/f}$ mice (2). The C57Bl/6 mice *IKKβ*$^{f/f}$ mice were backcrossed from FVB to C57BL/6 five generations. These mice were then bred to mice harboring the *loxP*-flanked tdTomato (mT) following the EGFP (mG) cassette, which was inserted into the *Gt(ROSA)26Sor* locus. These mT/mG mice served as a Cre-reporter strain and after Cre-mediated recombination, myeloid cells that are Ikkβ-null are green (3, 4). The mice with *Ikkβ*-null myeloid cells are designated here as "*Ikkβ*$^{MyeΔ/Δ}$ mice." Littermates *LysMCre::mT/mG* mice without the *Ikkβ*$^{f/f}$ alleles were used as *Ikk*^{WT} controls. *Ikkβ*^{CA} mice with a genetic background of *cfms-rtTA::TetOn-cIkkβ* express a constitutively active form of Ikkβ in myeloid cells in response to doxycycline induction.

For generating metastasis models, melanoma cell lines expressing Gluc were derived from melanoma lesions (Braf^{V600E}/Pten$^{-/-}$) arising in the mixed strain of C57BL6/FVB (12), or B16F0 cells derived from C57Bl/6 mice were injected or implanted into *Ikkβ*$^{MyeΔ/Δ}$ or *Ikkβ*^{WT} mice. To evaluate whether deletion or constitutive activation of *Ikkβ* in myeloid cells affected tumor growth in a syngeneic model of melanoma, *Ikkβ*$^{MyeΔ/Δ}$ mice, *Ikkβ*^{CA} mice (10), or *Ikkβ*^{WT} littermates were intravenously (i.v.) injected with 5×10^4 Gluc–expressing B16F0 melanoma cells to obtain lung metastases in 4 weeks. For both models, after 20 days tumor burden was determined by tumor-expressing Gluc reporter activity in 20 μg protein from lung tissue lysate or 5 μL of peripheral blood. $N = 3$ independent experiments with 6 mice per group/experiment. Detailed descriptions of breeding procedures and characterization of the mice and tumors are found in the Supplementary Methods.

Bone marrow transplant and inducible/spontaneous melanoma models

Recipient C57BL6/FVB mixed background mice carrying *Braf*$^{+/-}$/*Pten*$^{-/-}$/*Tyr-Cre*$^{+}$ alleles were given 100 mg/L neomycin, 10 mg/L polymyxin B in pH2 water 1 week before transplant and continuously for 6 weeks after transplantation. Mice received one dose of 10-Gy irradiation (Cesium Gamma irradiator). Four hours later, the mice were injected via tail vein with bone marrow cells (1×10^6) from C57BL6 donor mice (myeloid *Ikk*^{WT} mice or myeloid *Ikk*$^{MyeΔ/Δ}$ mice). The reconstitution of bone marrow in recipient mice was validated 3 weeks after transplant (Supplementary Fig. S1J–S1L) and proper function of recipient myeloid cells in response to tumor cells was verified and compared with that of donor mice (Supplementary Fig. S1J).

FACS analysis and antibodies

For FACS analyses, tissues were minced on a programmable dissociator and digested with an enzyme solution of collagenase, Dispase and DNase. A detailed list of antibodies used, staining, and FACS analyses protocols is found in Supplemental Methods.

Characterization of macrophage killing of tumor cells in the peritoneum

To study the role of myeloid Ikkβ activity on the ability to migrate into the peritoneum in response to tumor, Braf^{V600E}/Pten$^{-/-}$ melanoma cells were injected into the peritoneum of *mT/mG Ikk*^{WT} mice. The infiltrating myeloid cells (GFP^{+}, Tomato RFP^{-}), lymphocytes (GFP^{-}, Tomato RFP^{+}), and melanoma cells were quantified by FACS analysis over an 8-hour time course (0, 2, 4, and 8 hours). To investigate the role of NF-κB in tumor cell phagocytosis, macrophages were isolated from *Ikkβ*$^{MyeΔ/Δ}$ mice or litter mate *Ikkβ*^{Wt} mice 18 hours after the mice had received a peritoneal injection of dead melanoma cells that had been fixed in 4% paraformaldehyde. The purity of isolated macrophages was more than 92% (Supplementary Fig. S3A).

Purification of mouse neutrophils and depletion of cellular subsets *in vivo*

Mouse blood was isolated as previously described (13). Cells were cultured in OptiMEM with 0.5% FBS. F4/80^{+} macrophages were depleted 90% or 97% *in vivo* by i.v. injections of 0.1 or 0.2 mL of clodronate (5 mg/mL), respectively, or liposome vehicle (without depletion of Gr1^{+} neutrophils or CD11c^{+} cells; Supplementary Fig. S2Bc and S2Bf). To evaluate the effect of macrophage depletion on tumor growth, clodronate or liposome vehicle were injected into mice 1 day before and every other day after implantation of 10^6 tumor cells, continuing throughout the experiment.

CD8^{+}T cells or neutrophils were depleted using injections of 250 μg of anti-CD8 monoclonal antibody (mAb) YTS, or the Ly6G neutrophil marker mAb 1A8, or IgG2a mAb 2A3 (BioX-cell) as isotype control Gr1 for 3 days before implanting with melanoma cells, with 100-μg mAb injections every other day thereafter. Systemic depletion of these leukocytes in bone marrow was evaluated at the study endpoint using flow cytometry.

Immunocytochemistry, immunohistochemistry, cytokine array and ELISA

Immunostaining was performed according to the previously described protocol (5), using antibodies against S-100 and MART1. Inflammatory Cytokine Arrays and ELISA were performed as described previously (5).

Phagocytic latex bead assay *ex vivo*

Peritoneal cells were collected from mice 3 days after injection with 2 mL of 4% thioglycollate, then cultured in DMEM with 20% FBS 1 hour. The nonadherent cells were removed and adherent cells were cultured in fresh medium overnight, then cultured with 5 μL of fluorescent blue–labeled latex beads (size 2 μm; Sigma; #L0280) for 2 hours. Cells that phagocytized latex beads were analyzed by FACS and GFP^+ macrophages that express blue fluorescence were counted.

Tetramer assay

A single-cell suspension was prepared from lung tumor tissues. H-2K(b) monomer loaded with SVYDFFVWL (TRP2) peptide was provided by the NIH Tetramer Core Facility and tetramerized using APC-labeled streptavidin. Cells were stained with PerCP-Cy5.5–conjugated anti-CD8 antibody and fluorescent TRP2 tetramer. The TRP2-tetramer–positive $CD8^+$ T cells were enumerated by flow cytometry.

Statistical analysis

Data are expressed as mean ±SEM; the unpaired, two-tailed Student *t* test was used to determine *P* values. $P < 0.05$ were considered significant.

Results

Myeloid IKKβ is essential for antitumorigenic immunity

C57/BL6 and FVB mice with targeted deletion of *Ikkβ* in myeloid cells ($Ikk\beta^{Mye\Delta/\Delta}$) were generated by efficient Cre-*loxP*–mediated recombination in macrophages and neutrophils, but not in T cells or in the majority of B cells and DCs (Fig. 1A; refs. 11, 14). To test whether IKKβ in myeloid cells influences immunity against melanoma, 10^6 melanoma cells derived from $Braf^{V600E}/Pten^{-/-}$ mice (on a mixed C57BL/6 × FVB background) after induction with 4-HT (Supplementary Fig. S1Aa; ref. 12), were i.v. injected into C57BL/6 $Ikk\beta^{Mye\Delta/\Delta}$ mice (11) or into control $Ikk\beta^{WT}$ C57BL/6 litter mates (20 mice/group). Three weeks later, all $Ikk\beta^{Mye\Delta/\Delta}$ mice had difficulty breathing and had large abnormal lungs (0.99 ± 0.116 g) in contrast to the $Ikk\beta^{WT}$ *recipients* (0.29 ± 0.013 g; Fig. 1B). To quantitate melanoma masses in the lung, melanoma cells were engineered to express Gaussian luciferase (Gluc). Four weeks after reporter animals (5 mice/group) received Gluc-expressing melanoma cells, Gluc activity was dramatically higher in lung tissue of $Ikk\beta^{Mye\Delta/\Delta}$ mice (319,763 ± 176,717) compared with the $Ikk\beta^{WT}$ mice (623 ± 182), or tumor-free controls (732 ± 117; Fig. 1C). Similar results were obtained when the $Braf^{V600E}/Pten^{-/-}$ melanoma cells were injected into FVB mice (Supplementary Fig. S1B and S1C). H&E staining detected melanoma lesions in $Ikk\beta^{Mye\Delta/\Delta}$ lungs, but not in $Ikk\beta^{WT}$ controls (Fig. 1D), and lesions were verified as melanocytic by immunohistochemical staining with

melanocyte and melanoma markers S-100 and MART-1, respectively (Supplementary Fig. S1D). When *Braf*^{V600E}*/Pten*$^{-/-}$ melanoma cells (1×10^6) were injected intrasplenically into C57BL6 or FVB mice, similar results were obtained (Supplementary Fig. S1E–S1H), indicating that the metastatic potential in IKKβ$^{MyeΔ/Δ}$ mice was not organ-specific. Moreover, when *Braf*^{V600E}*/Pten*$^{-/-}$ melanoma cells were implanted subcutaneously, xenografts grew steadily in *Ikkβ*$^{MyeΔ/Δ}$ mice, while cells implanted to *Ikkβ*^{WT} mice grew significantly slower for the first 2 weeks and subsequently regressed (Supplementary Fig. S1A and S1B; $P < 0.01$, $n = 7$).

To further explore the impact of *Ikkβ*$^{MyeΔ/Δ}$ myeloid cells on tumorigenesis in the inducible *Braf*^{V600E}*/Pten*$^{-/-}$ mice, bone marrow cells from donor C57BL6 *Ikkβ*$^{MyeΔ/Δ}$ mice or littermate *Ikkβ*^{WT} mice were transplanted into recipient C57BL6/FVB (*Braf*$^{f/f}$*::Pten*$^{f/f}$*::Tyr-Cre*) mice (Supplementary Fig. S1J–S1L). Recipient animals (20 mice/group) were treated with topical 4-HT to induce Tyr-Cre–mediated expression of BRAF^{V600E} and deletion of PTEN in melanocytes, which then progress to melanoma (12). Five weeks after induction, typical pigmented melanomas occurred at the treatment site more frequently in irradiated mice transplanted with bone marrow from C57BL6 *Ikkβ*$^{MyeΔ/Δ}$ mice as compared mice transplanted with marrow from *Ikkβ*^{WT} mice (Supplementary Fig. S1M and S1N). Because of the leakiness of the inducible Tyr-Cre system (15), spontaneous tumors appeared more frequently on mice transplanted with *Ikkβ*$^{MyeΔ/Δ}$ than *Ikkβ*^{WT} marrow (Supplementary Fig. S1O and S1P).

When a syngeneic B16F0 melanoma model was examined using similar protocols, we observed that melanoma tumor burden was significantly enhanced in the lung of *Ikkβ*$^{MyeΔ/Δ}$ mice in comparison with that of *Ikkβ*^{WT} mice (Gluc activity respectively: 30,489 ± 2,759 vs. 6,549 ± 3,457; $P < 0.01$; n =6; Fig. 1E). Also using this B16F0 model, mice expressing a constitutively active IKKβ (*IKKβ*^{CA}) showed only a few lung lesions based on gross visual and H&E-stained histologic analyses, in contrast to the numerous melanoma lesions in the lungs of *IKKβ*^{WT} littermates (Fig. 1F and Supplementary Fig. SIQ). Tumor reporter-Gluc activity was significantly reduced in the lungs of *IKKβ*^{CA} mice compared with controls (4,304 ± 1,479 vs. 31,396 ± 6,493; $P < 0.01$; n =6) with two experimental repeats. It should be noted that the B16F0 tumors grow faster in the pure C57Bl/6 vector control mice (control for the Ikkβ^{CA}) as compared with the *Ikkβ*^{WT} (control for the *Ikkβ*$^{MyeΔ/Δ}$ mice) that have been bred from FVB/129 background onto the C57Bl/6 background. Altogether, these data show deletion of *IKKβ* in myeloid cells results in a dramatic reduction of antitumor immunity in both syngeneic and allogenic models of melanoma, while enhanced IKKβ activity (Ikkβ^{CA}) results in enhanced antitumor response.

The macrophage is a key mediator in antitumor immunity

We sought first to explore whether macrophages might influence antimelanoma immunity using a peritoneal tumor cell recruitment assay. These analyses revealed that there were progressive increases in leukocytes and decreases in tumor cells in the peritoneum (Fig. 2A). As this finding suggested that recruited leukocytes may be eliminating the melanoma, macrophages were depleted by clodronate treatment, leading to a 5.25-fold increase in tumor cells ($12.6 \pm 3.0 \times 10^5$) compared with the liposome control group ($2.4 \pm 1.2 \times 10^5$; $P <$

0.05; n =8; Fig. 2Ba–c). Clodronate treatment had no effect on $CD11c^+$ DCs within the time course of this assay (Supplementary Fig. S2Bf) and did not deplete $Gr\text{-}1^+$ neutrophils or DCs (Supplementary Fig. S2Bc). Moreover, *in vitro* experiments demonstrated that effects of clodronate were similar to that of liposome vehicle controls on survival and growth of melanoma cells (Supplementary Fig. S2E).

To extend this finding, Gluc $Braf^{V600E}/Pten^{-/-}$ melanoma cells were delivered i.v. to $IKK\beta^{WT}$ C57BL6 mice (5 per group). Melanoma lesions were identified in the lungs of clodronate-treated mice, but not in the liposome vehicle controls (Fig. 2C). Gluc activity was 439-fold higher in macrophage-depleted mice (788,198 ± 264,690) than in controls (1,793 ± 609; $P < 0.01$; $n = 5$). The lung Gluc activity was not different between control group and tumor-free mice (1,530 ± 191; $P = 0.44$; $n = 5$; Fig. 2D). In contrast, when neutrophils were depleted in $Ikk\beta^{WT}$ mice using anti-Ly6G antibody (Supplementary Fig. S2C) there was only a 6-fold increase in Gluc reporter activity in lung, indicating enhanced melanoma growth in the lung of neutrophil-depleted mice (Supplementary Fig. S2D; 6,769 ± 4,085 vs. 1,131 ± 344; $P < 0.05$; $n = 5$). We conclude that both macrophages and neutrophils lead to inhibition of melanoma tumor growth, but macrophages have a much more striking effect (439- vs. 6-fold increases in tumor size after macrophage depletion vs. neutrophil depletion).

NF-κB modulates macrophage-mediated tumor cell death

To determine whether defects in macrophage tumor cell killing and phagocytosis were responsible for the increased tumor growth in the $Ikk\beta^{Mye\Delta/\Delta}$ mice, we cocultured macrophages isolated from the peritoneum (Supplementary Fig. S3A) with RFP-tagged tumor cells. In contrast to $Ikk\beta^{WT}$ macrophages (Fig. 3A), $Ikk\beta^{Mye\Delta/\Delta}$ macrophages exhibited poor phagocytic activity toward RFP-tagged $Braf^{V600E}/Pten^{-/-}$ melanoma cells based on uptake of RFP (Fig. 3B). Moreover, the tumor cell kill after 5-hour coculture with macrophages was significantly lower with $Ikk\beta^{Mye\Delta/\Delta}$ macrophages (0.9% ± 0.8%) than $Ikk\beta^{WT}$ cells (8.2% ± 2.1%; $P < 0.02$; $n = 5$). Cell death in the macrophage-free cultured melanoma cells was comparable with that of cells incubated with $Ikk\beta^{Mye\Delta/\Delta}$ macrophage (0.8% ± 0.6%; Fig. 3C). This result was confirmed by latex bead assays that found the efficiency of macrophage phagocytosis was significantly reduced upon loss of IKKβ (Supplementary Fig. S3C). However, when cell–cell interaction was observed after prolonged culture (3 days), $IKK\beta^{\Delta/\Delta}$ macrophages did eventually engulf tumor cells, though engulfed tumor cells appeared not to undergo digestion (Supplementary Fig. S3B). Thus, loss of macrophage IKKβ resulted in less efficient phagocytosis of tumor cells.

Myeloid IKKβ is important for macrophage and CD8 T cell–mediated cytotoxicity

To determine the impact of IKKβ loss on the infiltrating lung macrophages and their expression of MHC II^+, we performed FACS analysis of lungs from $Ikk\beta^{Mye\Delta/\Delta}$ mice or $Ikk\beta^{Wt}$ littermates that had received $BRAF^{V600E}/PTEN^{-/-}$cells (1×10^6) i.v. The number of $F4/80^+$ macrophages infiltrating into lungs of $Ikk\beta^{Wt}$ mice was nearly twice that of $Ikk\beta^{Mye\Delta/\Delta}$ 24 hours after tumor cell injection (12,060 ± 1,660 vs. 6,943 ± 1,294, respectively). The $Ikk\beta^{Mye\Delta/\Delta}$ mice exhibited greatly diminished numbers of $F4/80^+$/MHC II^+ double-positive cells in comparison with the $Ikk\beta^{WT}$ mice (2.9% ± 1.0% vs. 50% ± 4.3%;

$P < 0.01$; $n = 6$; Fig. 4A). These data suggest that loss of IKKβ reduces the number of F4/80$^+$ cells in the lung in response to tumor by about 42%, but the number of F4/80$^+$ macrophages expressing MHCII is reduced 16-fold (or >7-fold taking into consideration the reduction in total F4/80$^+$macrophages in the lung).

To learn the impact of IKKβ expression in macrophages on CD4$^+$ T-cell phenotype, *Ikkβ*$^{MyeΔ/Δ}$ mice or *Ikkβ*^{WT} littermates received 10^6 *Braf*^{V600E}/*Pten*$^{-/-}$ cells i.v.. Three days later, immune cells expressing Tomato-RFP were isolated from lung, F4/80$^+$ macrophages were excluded, and expression of Th2 and Treg markers (CD25 and Foxp3) was analyzed by FACS. The number of CD25$^+$/Foxp3$^+$ CD4$^+$ T cells (Tregs) from *IKKβ*$^{MyeΔ/Δ}$ mice was significantly increased over *Ikkβ*^{WT} mice (69% ± 7.8% vs. 23% ± 5.9%; $P < 0.01$; $n = 4$; Fig. 4B). Thus, an anti-inflammatory skewing occurs as a consequence of IKKβ loss in myeloid cells.

Activated CD8$^+$T cells release perforin and granzymes from their lytic granules to kill targets by exocytic merging of the CD107a/b-containing granule membrane with the plasma membrane (16–19). To investigate the activation status of CD8$^+$ T cells in lung of mice with melanoma tumors, lymphocytes expressing Tomato-RFP were sorted from lung tissues and CD8$^+$T/CD107b$^+$double-positive cells identified by FACS. The percentage of CD107b$^+$ CD8$^+$ cells from tumor-bearing *Ikkβ*$^{MyeΔ/Δ}$ mice declined by 40% in comparison with tumor-bearing *Ikkβ*^{WT} mice (19% ± 6.0% vs. 60% ± 7.8%; $P < 0.01$; $n = 4$; Fig. 4C). Thus, loss of IKKβ activity in myeloid cells results in poor activation of CD8$^+$ T cells in lung, additionally supported by Supplementary Fig. S4A. A similar immune response was observed in the cutaneous melanoma model. Together, the increase in Treg and decrease in activated CTL suggest that myeloid IKKβ activity is pivotal for driving tumor cytotoxicity of CD8$^+$ T cells.

To test the role of CTL directly, CD8$^+$ T cells were depleted using CD8-YTS antibody to achieve 98.4% CD8$^+$ T-cell depletion (Supplementary Fig. S4D). In contrast to control mice that completely rejected melanoma formation after input of Gluc-*Braf*^{V600E}/*Pten*$^{-/-}$ melanoma cells, mice with depleted CD8$^+$ T cells exhibited 1,152-fold increased Gluc activity, indicating significant outgrowth of metastatic melanoma lesions (Fig. 4D; 1,303,308 ± 187,269 vs. 1,131 ± 344; $P < 0.01$). Thus, data suggest that CD8$^+$ T cells are required for antitumor cytotoxicity and myeloid IKKβ is essential for activation of CD8$^+$ T cells in response to melanoma cells in the tumor allograft model.

In the syngeneic model in which less immunogenic B16F0 melanoma cells were implanted into C57/BL6 mice, there was no influence on tumor progression compared with IgG-treated mice in either of *Ikkβ*$^{MyeΔ/Δ}$ mice or *Ikkβ*^{WT} mice when CD8$^+$T cells were depleted (Fig. 4E and Supplementary Fig. S5A–S5C). A similar result was observed in the *Ikkβ*^{CA} mice (Supplementary Fig. S5D). Thus, immune cells other than CD8$^+$ T cells play the major antitumor role in the syngeneic model. Also, TRP2 (SVYDFFVWL) tetramer staining did not reveal B16 melanoma-specific CD8$^+$ T cells in the lung tumor milieu (Fig. 4F and Supplementary Fig. S5E), indicating that for B16 melanoma in the syngeneic melanoma tumor model, CD8$^+$ T cells contributed little to the antitumor response. B16 cells are

reported to be poor activators of an anti-melanoma CTL response due to a significant population of Treg cells (20, 21); our data are in agreement with those prior observations.

NF-κB is required for cytokine-mediated immunity

To learn whether myeloid *IKKβ* deletion leads to any alteration in cytokine profiles *in vivo*, which might affect the Th1- versus Th2-skew of immune cells, sera from non–tumor-bearing $Ikk\beta^{WT}$ and $Ikk\beta^{MyeΔ/Δ}$ mice were analyzed. CCL11 was elevated 19-fold in serum of $Ikk\beta^{MyeΔ/Δ}$ mice compared with $Ikk\beta^{WT}$ mice (Fig. 5A) and the CCL11 was expressed mainly in $IKKβ^{MyeΔ/Δ}$ macrophages and to a lesser extent in the $IKKβ^{MyeΔ/Δ}$ neutrophils (Fig. 5B). Because CCL11 can hinder DC differentiation (22) and affect a Th2 response (23), we hypothesized that CCL11 may link myeloid IKKβ loss with the TME. To examine the *in vivo* impact of CCL11 on the DC population, splenocytes isolated from $Ikk\beta^{WT}$ and $Ikk\beta^{MyeΔ/Δ}$ mice and stained for DCs ($CD11c^+$, $CD80^+$) were analyzed by FACS. $Ikk\beta^{WT}$ mice had over 4-fold more DCs than mice whose myeloid lineage lacked IKKβ (9.5% ± 1.3% of DCs vs. 2.2% ± 0.12%, respectively; $P < 0.01$; $n = 5$; Fig. 5C). To further study DC maturation, the cells were stained for CD83 (a marker for DC maturation) and subjected to FACS analysis. CD83 expression on the DCs of $Ikk\beta^{MyeΔ/Δ}$ mice was very low (6.8% ± 3.8%), in contrast to the CD83 expression on the DCs of $Ikk\beta^{WT}$ mice (82% ± 4.8%; $P < 0.01$; $n = 4$; Fig. 5D). To gain insight into the effect of CCL11 on DC generation, murine bone marrow cells from $Ikk\beta^{WT}$ mice were cultured 7 days in medium with 20 ng/mL of GM-CSF and 20 ng/mL of IL4 ± 100 ng/mL of CCL11 or control PBS, collected on day 7, stained with CD11c-Alexa Fluor 700, and analyzed by FACS. CCL11 significantly reduced generation of DCs (7.7% ± 0.78% vs. 43.6% ± 1.72%; $P < 0.01$; $n = 4$; Fig. 5E). To test DC function, $CD11c^+$ DCs were incubated with FITC-dextran 15 minutes and dextran endocytosis was analyzed by flow cytometry. CCL11-treated IKKβ-deficient DCs exhibited a 65% reduction in endocytosis compared with controls (Fig. 5F; 15.8% ± 0.84% vs. 44.7% ± 0.83%, respectively; $P < 0.01$; $n = 4$). Thus, IKKβ deletion in myeloid cells resulted in overexpression of CCL11, which contributed to reduced generation of DCs and reduced DC maturation. The loss of DC maturation in the $Ikk\beta^{MyeΔ/Δ}$ mice could have significant consequences on the T-cell activation in the allograft tumor model.

What might be clinical meanings of these findings in patients with melanoma? To explore the potential relevance of these findings in human melanoma, we evaluated CCL11 expression in macrophages ($CD163^+$) of biopsy specimens from 6 patients with melanoma before and after treatment with the proteasome inhibitor, bortezomib (VELCADE), and temozolomide in a phase I/II clinical trial (24). VELCADE, an FDA-approved agent in some cancers, inhibits degradation of phosphorylated-IκB, thus reducing NF-κB activity by retaining RelA/p65 in the cytoplasm, but also affects a number of additional pathways (25–28). Tissues were stained for macrophages (CD163; ref. 29), phospho-RelA(p65), activated $CD8^+$ T cells (CD107a), and CCL11, visualized by confocal microscopy, and quantitated using Metamorph (Supplementary Fig. S6A and S6B). Treatment resulted in a small but significant reduction in nuclear phospho-REL-A/p65 (29,586 ± 2,741 vs. 33,462 ± 3,460; $P < 0.01$; Fig. 6A), a 17% upregulation of CCL11 (36,772 ± 1,860 vs. 31,452 ± 2,224; $P < 0.01$; Fig. 6B), and a significant reduction in $CD107a^+$ $CD8^+$ T cells (36,924 ± 2,009 vs. 48,146 ± 4,641; $P < 0.01$; Fig. 6C) compared with pretreatment controls. The specificity of

antibodies was verified on human peripheral lymphocytes (Supplementary Fig. S6C and S6D) and expression of CD107a on individual tumor-associated $CD8^+$ T cells from VELCADE-treated patient tumors (Supplementary Fig. S6 E and S6F) was significantly reduced. Thus, our findings suggest that systemic therapy with VELCADE may act via NF-κB to change chemokine expression and CTL effector activity in patients with cancer, and that targeting NF-κB signaling in human melanoma risks negative effects on anti-tumor immunity.

Discussion

Tumor-associated macrophages (TAM) exhibit both antitumor M1 and protumor M2 innate immunity phenotypes, indicating the highly complex milieu within the tumor (30–32). Loss-of-function studies indicate that various members of the NF-κB/Rel family of transcription factors regulate macrophage polarization (5, 10, 32, 33). Some work suggests that NF-κB activation confers an M2 phenotype to TAMs, based on observations that inhibition of NF-κB in myeloid cells elicits a switch from an M2 to M1 phenotype (33). However, Connelly and colleagues (10) showed that constitutive activation of NF-κB maintained the antitumor phenotype of macrophages, whereas NF-κB inhibition by expression of an IκBα “super-repressor” resulted in enhanced breast tumor promotion. Under certain chronic inflammatory conditions, lipopolysaccharide-tolerant macrophages accumulated p50 NF-κB homodimers that could act as negative regulators of the NF-κB signal pathway (34). Moreover, in an angiosarcoma model, loss of IKKβ in myeloid cells resulted in enhanced tumor growth (11), though in colon cancer and hepatocellular carcinoma, this myeloid loss of IKKβ had the opposite effect (7, 8). The controversy over the role of NF-κB in cells of the myeloid lineage in mediation of tumor immunity raises the intriguing question of whether one common NF-κB signal creates a diversity of transcriptional responses that are tailored to particular tissues and organs.

In this study, we advance understanding of how NF-κB affects innate immunity through the demonstration that deletion of IKKβ in myeloid cells results in macrophages with an M2-phenotype. Here, in allo- and syngeneic studies of melanoma as well as models in which melanoma is inducible *in situ*, loss of IKKβ in myeloid cells is associated with enhanced melanoma growth. In contrast, expression of a constitutively activated form of NF-κB in myeloid cells markedly inhibits tumor growth. Although neutrophils have been reported to have either tumor-inhibitory (N1) or tumor-enhancing (N2) properties (2), we observed that macrophages play a more dominant role in the innate immune response to melanoma.

Cytokines such as IL1 IL4, IL6, IL10, IL12, TNFα, IFNγ, and TGFβ, as well as chemokines, play an important role in the modulation of the pro- or antitumor properties of innate immune response (33). The chemokine CCL11 exhibits an inhibitory role on the differentiation of DCs and enhances subsequent Th2- polarization (22). CCL11 is upregulated by Th2 cytokines IL4 and IL13, whereas its expression is down-regulated by the Th1 cytokine IFNγ (35, 36). Here CCL11 was highly expressed in macrophages with IKKβ knockout and this blocked DC differentiation and enhanced the implied Th2 milieu, resulting in poor activation of $CD8^+$ T cells. Although the number of clinical samples analyzed was small and not large enough to predict prognosis, our clinical data

demonstrating that CCL11 was expressed by myeloid cells in melanoma tumors from a human trial using VELCADE (22) are of interest because they stress the importance of careful consideration of the immunologic effects of drugs that affect the NF-κB pathway.

A key advance of our studies is the definitive demonstration that the antitumor activity of TAMs requires NF-κB, because myeloid-targeted deletion of IKKβ resulted in macrophages with decreased ability to kill tumor cells *in vitro*. Moreover, we show that macrophages from $IKK\beta^{mye\Delta/\Delta}$ mice exhibit marked reduction in expression of the MHC class II molecules needed to present antigens to $CD4^+$ T cells to prime $CD8^+$ T cells to become CTLs (37, 38). Moreover, our data suggest that in the absence of activation of the $CD8^+$ T-cell response, the innate immune response is the major guardian in the host response to tumor.

Experiments described herein have important implications for therapeutic use of inhibitors of NF-κB in melanoma therapy. Although targeted deletion of *Ikkβ* in *Ink4A/Arf*-null melanocytes blocks mutant RAS-induced melanoma (5), systemic targeting of NF-κB with an IKKβ inhibitor is less effective in inhibiting the growth of RAS-transformed murine melanoma in immunocompetent mice, indicating a potential negative impact of the inhibitor on antitumor immunity (Hawkins, in preparation). On the basis of our data, IKKβ inhibitors will be most effective when delivered directly to tumor cells. Moreover, developing ways to heighten or retain IKKβ activity in myeloid cells, while blocking IKKβ in melanoma tumor cells, may prove to be effective for inhibition of melanoma tumor growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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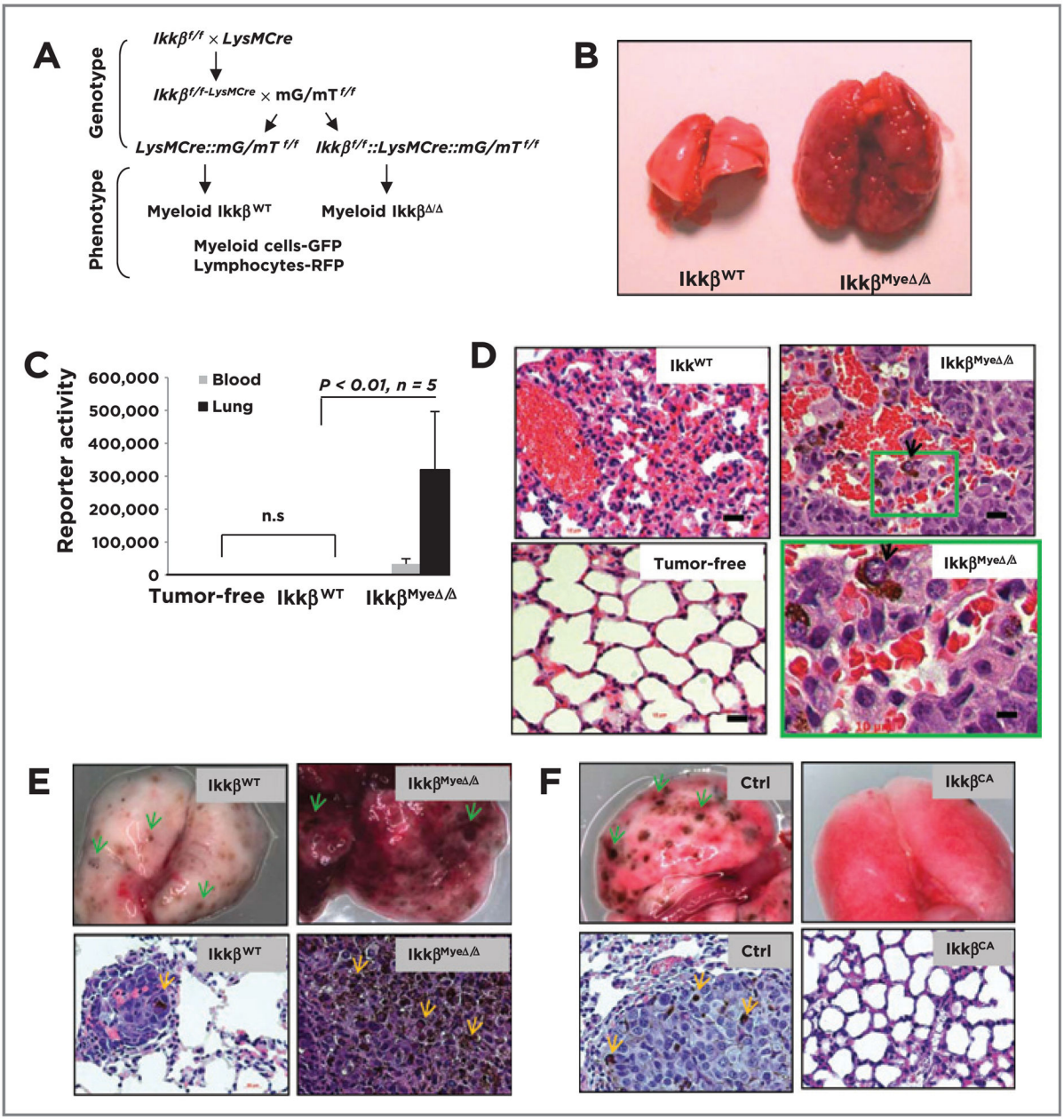

Figure 1.

Deletion of *Ikkβ* in myeloid cells leads to protumorigenic immunity. A, generation of mice with myeloid *Ikkβ* deletion. $Ikk\beta^{f/f}$ mice (C57BL6) crossed with myeloid specific LysMCre mice (C57BL6) and mG/mT mice (C57BL6). Cre^{+} myeloid cells express GFP, whereas other Cre^{+} cells (e.g., lymphocytes) express Tomato red protein. B, $Braf^{V600E}/Pten^{-/-}$ melanoma cells form melanoma lung lesions in $Ikk\beta^{MyeΔ/Δ}$ mice, but not in $Ikk\beta^{WT}$ mice. C, quantitation of lung melanoma lesions based on Gluc activity (mean ± SEM) in the blood or lung 4 weeks after G-luc-$Braf^{V600E}/Pten^{-/-}$ cells were i.v. injected into $Ikk\beta^{Δ/Δ}$ or $Ikk\beta^{WT}$ C57B/6 mice or tumor-free $Ikk\beta^{WT}$ mice. D, histology of melanoma lesions in lungs from C based on H&E staining. Arrows, melanoma cells in lungs of $Ikk\beta^{myeΔ/Δ}$ mice. E, myeloid

IKKβ deletion enhances B16F0-Gluc melanoma growth in lung in syngeneic *Ikkβ*$^{MyeΔ/Δ}$ mice versus littermate *Ikkβ*^{WT} mice. Twenty days after i.v. injection of tumor cells, lungs were perfused and photographed (top), or fixed and stained with H&E (bottom). F, B16F0 melanoma growth in C57BL6 myeloid *IKKβ*^{CA} mice and littermate control mice (single transgene cfms-rtTA or TetOn c*IKKβ*) received B16F0-Gluc melanoma cells i.v. and transgene was induced with doxycycline. After 20 days, lungs were collected for photography (top) and H&E staining (bottom). Arrows, the pigmented melanoma lesion. Scale bar, 50 μm.

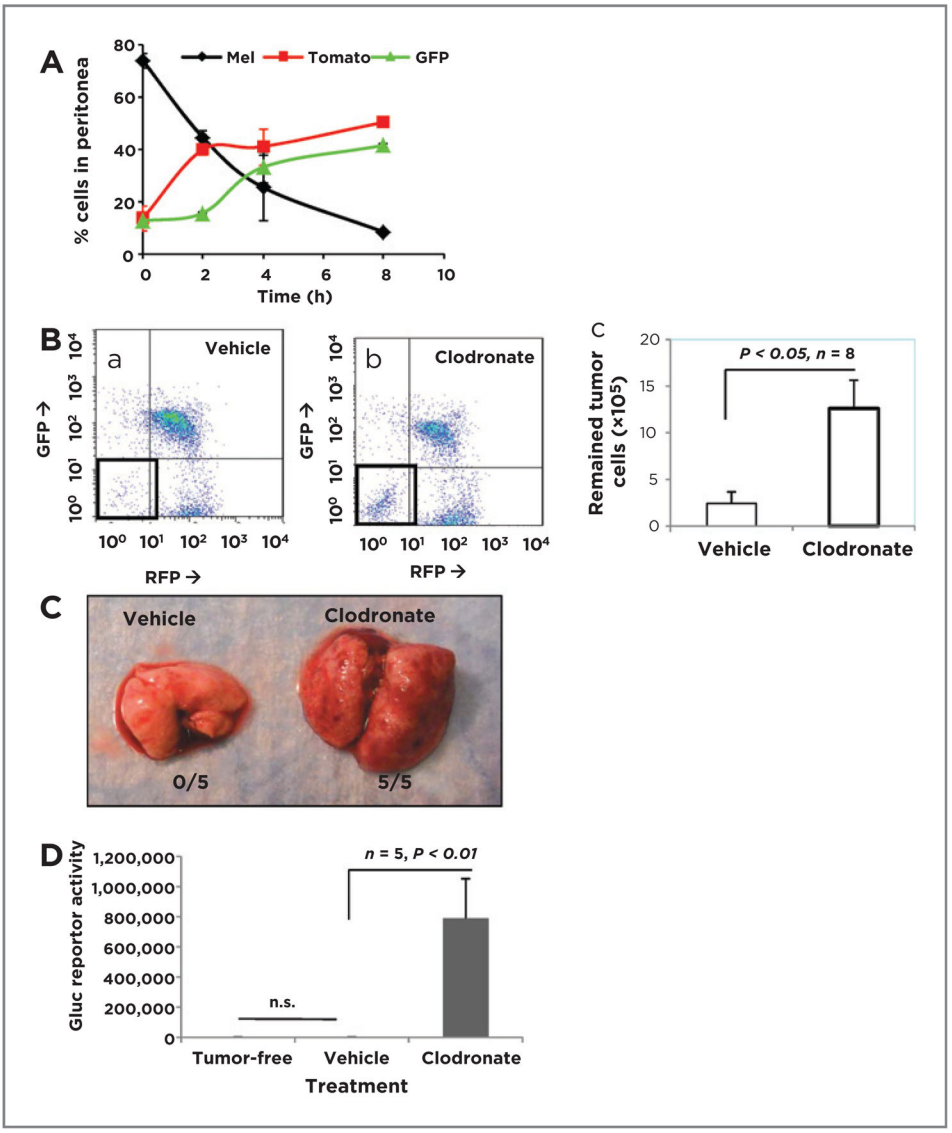

Figure 2.

Macrophages play an essential role in melanoma innate immunity. A, $Braf^{V600E}/Pten^{-/-}$ melanoma cells (1×10^7) were i.p. injected into *mG/mT::lysM-Cre* C57BL6 mice ($n = 3$). Infiltrating GFP myeloid cells, Tomato red lymphocytes, and tumor cells in the peritoneum were subsequently collected over 0 to 8 hours, analyzed by FACS, and graphed as percentage cells in peritoneum. B, $Braf^{V600E}/Pten^{-/-}$ melanoma cells (10^7) were injected i.p. into each of 8 mice pretreated with clodronate or liposome vehicle to deplete macrophages. The next day, peritoneal cells were analyzed by FACS. C, Gluc-$Braf^{V600E}/Pten^{-/-}$ *cells* delivered i.v. colonize lungs of mice with macrophages depleted 3 weeks of clodronate treatment. D, quantitation of Gluc activity in lungs of *Gluc-*$Braf^{V600E}/Pten^{-/-}$ tumor-injected mice treated as in C ($n = 5$, $P < 0.01$).

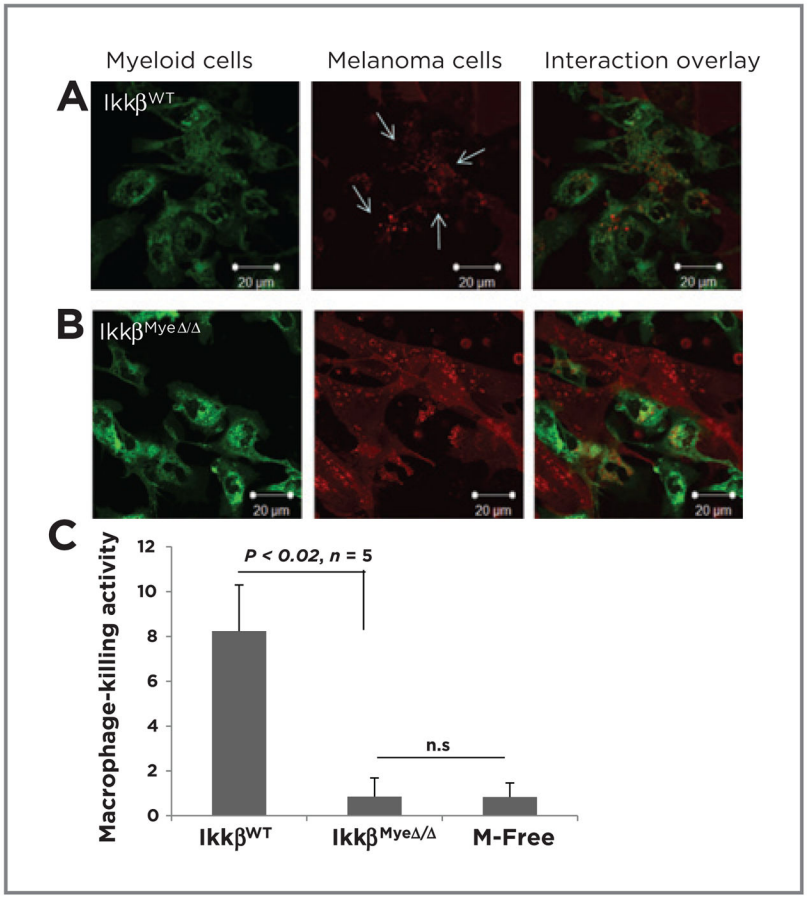

Figure 3.

IKKβ regulates macrophage phagocytosis. GFP-expressing macrophages isolated from *Ikkβ*^{WT} (A) or *Ikkβ*$^{MyeΔ/Δ}$ (B) were cocultured with melanoma cells expressing RFP (3:1) for 5 hours and examined by confocal microscopy. Scale bars, magnification. C, macrophage killing activity of cells recovered after coculture in A and B were stained with 7-AAD and subjected to FACS analysis.

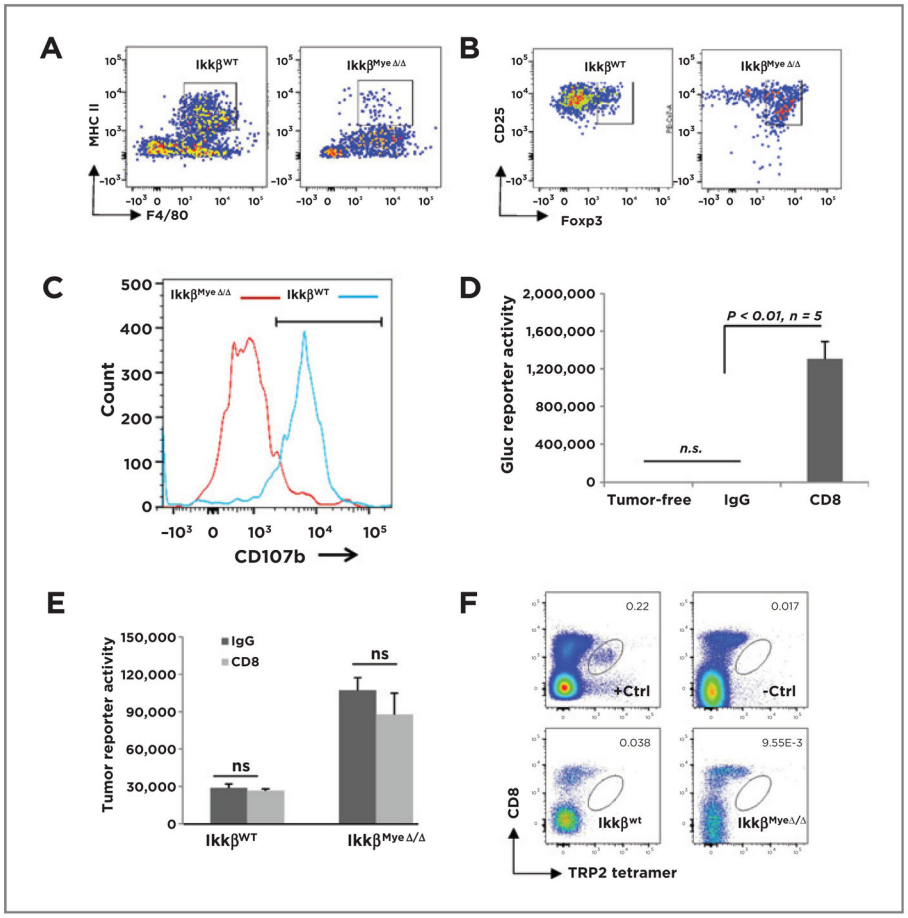

Figure 4.

Myeloid IKKβ mediates macrophage and CD8$^+$cytotoxicity. A, C57Bl/6 *Ikkβ*^{WT} or *Ikkβ*$^{MyeΔ/Δ}$ mice were i.v. injected with Braf^{V600E}/Pten$^{-/-}$ cells. After 24 hours, GFP$^+$ lung macrophages were stained for F4/80 and MHCII and analyzed by FACS. B, *Braf*^{V600E}/*Pten*$^{-/-}$ cells were injected i.v. into mice carrying *Ikkβ*^{Wt} or *Ikkβ*$^{MyeΔ/Δ}$ myeloid cells. Pulmonary Tomato-RFP CD4$^+$ T cells double-positive for CD25 and Foxp3 were analyzed by FACS 3 days after injection. C, using the protocol described in B, lung Tomato-RFP lymphocytes positive for both CD8 and CD107b were evaluated by FACS. D, CD8$^+$ cells were depleted and after 3 weeks, lung tumor burden was analyzed by Gluc activity. E, CD8$^+$ T cells were depleted 3 days before subcutaneous implantation of 5 × 10^4 Gluc-B16F0 melanoma cells. CD8 or control antibody injections continued 16 days before tumor burden was assessed by Gluc activity assay. F, tetramer analysis of CD8$^+$T cells infiltrating syngeneic melanoma tumor. *Ikkβ*^{Wt} or *Ikkβ*$^{MyeΔ/Δ}$ mice received B16F0 melanoma cells (5 × 10^4) i.v. After 16 days, cells from the lungs of these mice were stained with PerCP-Cy5.5–conjugated CD8 antibody and APC-labeled tetramer with monocyte-derived TRP2 (SVYDFFVWL) peptide and analyzed FACS. +Ctrl, positive control cells from splenocytes of TRP2-immunized mouse; −Ctrl, negative control cells from splenocytes of nonimmunized mouse.

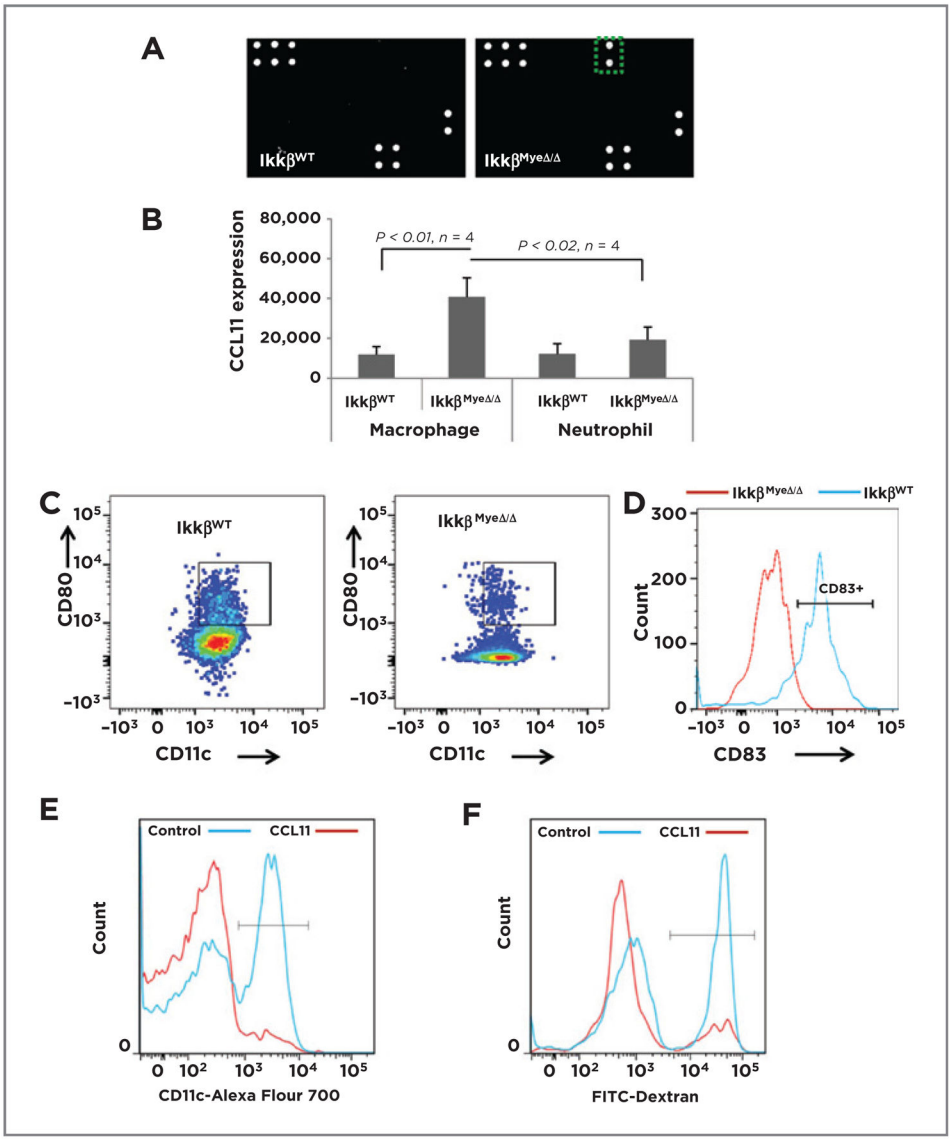

Figure 5.

A, myeloid IKKβ restrains CCL11 expression. Results of cytokine arrays of sera isolated from *Ikkβ*^{WT} and *Ikkβ*$^{Δ/Δ}$ mice; green rectangle (right) shows position of CCL11 signal of sera from *Ikkβ*$^{nyeΔ/Δ}$ mice. B, FACS analysis of intracellular CCL11 expression (mean ± SEM; $n = 4$) in macrophages or neutrophils isolated from spleen of the *Ikkβ*^{WT} and *Ikkβ*$^{Δ/Δ}$ mice. C, splenic cells were prepared as in B and CD45^{+} cells were stained for CD11c and CD80 and analyzed by FACS (mean ± SEM). D, splenocytes isolated as in B were stained with CD11c and CD83 and analyzed by FACS. E, bone marrow cells from *Ikkβ*^{WT} mice were cultured in medium containing GM-CSF and IL4 with or without 100 ng/mL of CCL11 for 7 days, then stained for CD11c by FACS. F, DCs generated from bone marrow as in E were incubated in 50 μL of FITC-dextran for 15 minutes, then cells were stained for CD11c and double-positive cells analyzed by FACS.

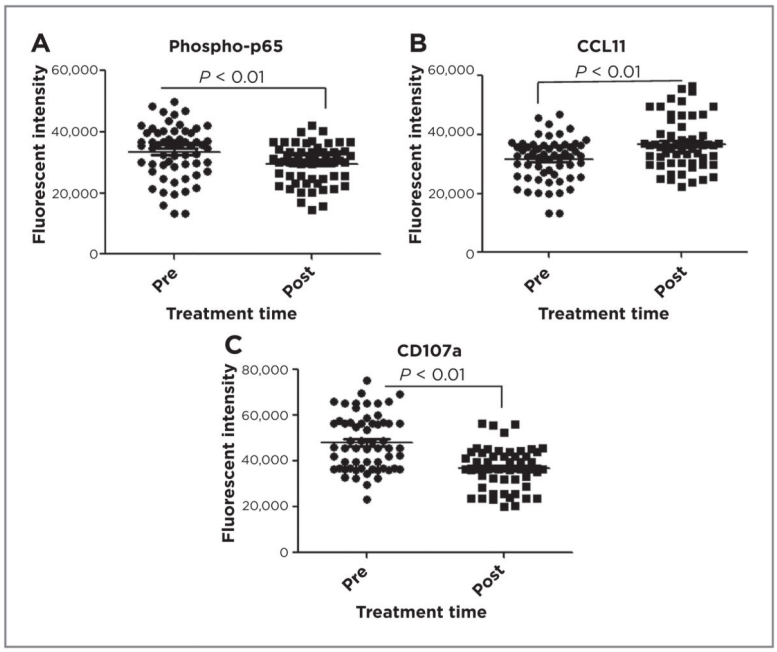

Figure 6.

Association of NF-κB with myeloid CCL11 expression and T cell activity in human melanoma. Paraffin-embedded patient melanoma tumor samples, pre- or posttreatment with VELCADE and temozolomide, were stained for CD163, phosphor-p65 (A) and CCL11 (B) and fluorescent intensity of each was quantified. C, human melanoma samples were stained for CD8 and CD107a, expression was quantified and graphed (GraphPad Prism, mean ± SEM, *t* test, $P < 0.01$).