Myeloid IKKβ Promotes Anti-tumor Immunity by Modulating CCL11 and the Innate Immune Response

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Abstract

Myeloid cells are effectors of both anti-tumor and pro-tumor immune responses, but much needs to be determined as to signals that determine which function of the myeloid lineage dominates. Shown here, mice with myeloid-specific IKKβ loss exhibit more rapid growth of cutaneous and lung melanoma tumors. Specifically, in a BRAF<sup>V600E</sup>/PTEN<sup>−/−</sup> allograft model, IKKβ loss in macrophages resulted in reduced recruitment of myeloid cells into the tumor, reduced expression of MHCII, and enhanced production of the chemokine, CCL11, which negatively regulated dendritic cell maturation. The elevated serum and tissue levels of CCL11 mediated suppression of dendritic cell differentiation/maturation within the TME, resulted in a Th2 skew of the immune response, and impaired CD8<sup>+</sup>T cell-mediated tumor cell killing. Macrophage depletion or CD8<sup>+</sup>T cell depletion in mice with IKKβ<sup>WT</sup> myeloid cells enhanced tumor growth in the melanoma allograft. In contrast, mice with IKKβ<sup>WT</sup> myeloid cells used the myeloid cell response to mediate anti-tumor immunity against the syngeneic B16 melanoma, with less apparent dependency on a CD8<sup>+</sup>T cell response. Myeloid cells deficient in IKKβ were compromised in tumor cell killing based upon reduced ability to phagocytize and digest tumor cells. Conversely, mice with continuous IKKβ signaling in myeloid-lineage cells (IKKβ<sup>CA</sup>) exhibited enhanced anti-tumor immunity and reduced B16 melanoma tumor growth. Collectively, these data uncover new mechanisms by 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 anti-tumor 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 (DCs), 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 physiological or pathological milieu in which they reside. Pro-tumor 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 (anti-tumor) versus Th2 (pro-tumor) 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 pro-inflammatory genes, playing a crucial role in immune response(3). NF-κB activation is regulated by the IκB kinase complex (IKKα, IKKβ, NEMO) which 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 pro-tumorigenic role of NF-κB in macrophages, other reports indicate that NF-κB is needed for the anti-
tumorigenic function of macrophages in breast cancer metastasis and angiosarcoma.(10, 11). Thus, the role of IKKβ/NF-κB signaling in macrophage pro- or anti-tumor 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 (IKKβmyeΔ/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 BRAFV600E/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 IKKβWT and IKKβmyeΔ/Δ C57Bl/6 mice. In the syngeneic model, growth of B16 melanomas in mice with myeloid cells expressing IKKβ constitutively active (IKKβCA), IKKβWT or IKKβmyeΔ/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 IKKβmyeΔ/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 anti-tumor response to syngeneic B16 melanoma, compared to IKKβmyeΔ/Δ mice. These results indicate that IKKβ activity is important for the anti-tumorigenic function of myeloid cells, thus providing important therapeutic implications.

MATERIALS AND METHODS

Myeloid Ikkβ 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 IkkβΔ/Δ mice (2). The C57Bl/6 mice IKKβΔ/Δ mice were back-crossed 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 “IkkβMyeΔΔ mice”. Littermates LysMCre::mT/mG mice without the Ikkβft alleles were used as IkkWT controls. IkkβCA mice with a genetic background of cfms-rtTA::TetOn-clkkβ express a constitutively active form of Ikkβ in myeloid cells in response to doxycycline induction. Detailed descriptions of breeding procedures, characterization of the mice, bone marrow transplant and melanoma models used in this study are found in the Supplementary Methods.

To evaluate whether deletion of Ikkβ in myeloid cells also resulted in a pro-tumor effect in a syngeneic model of melanoma, IkkβMyeΔΔ mice, IkkβWT and IkkβCA littermates were intravenously injected with 5 X 10^4 Gluc expressing B16F0 melanoma cells derived from C57BL6 mice and after 20 days tumor burden was determined by tumor-expressing Gluc reporter activity in 20 µg protein from lung tissue lysate. N=3 independent experiments with 6 mice per group/experiment.

**Bone Marrow transplant (BMT)**

Recipient C57BL6/FVB mixed background mice carrying Braff/+Ptenf/+Tyr-Cre+ alleles were given 100mg/L neomycin, 10mg/L polymyxin B in pH2 water one week before transplant and continuously for 6 weeks post transplantation. Mice received one dose of 10Gy irradiation (Cesium Gamma irradiator). Four hours later the mice were injected via tail vein with bone marrow cells (1 x 10^6) from C57BL6 donor mice (myeloid IkkWT mice or myeloid IkkMyeΔΔ mice). The reconstitution of bone marrow in recipient mice was validated 3 weeks post-transplant as detailed in Supplemental Data.

**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.

**Purification of mouse neutrophils and depletion of cellular subsets in vivo**
Mouse blood was isolated as previously described (12). Cells were cultured in OptiMEM with 0.5% FBS. Macrophages were depleted in vivo by intravenous injections of clodronate or liposome vehicle followed by intraperitoneal injection of $1 \times 10^6$ melanoma cells, depletion was validated, and effects on tumor growth were scored as described in Supplemental Methods. To evaluate the effect of macrophage depletion on tumor growth, clodronate or liposome vehicle were injected into mice 1 day prior to and every other day after implantation of $10^6$ tumor cells, continuing throughout the experiment.

As detailed in Supplemental Methods, CD8+ T cells or neutrophils were depleted using injections of mAb or isotype control for 3 days prior to implanting with melanoma cells, with 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).

**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( see Supplemental Methods for additional details).

**Statistics**

Data are expressed as mean ± SEM; the unpaired, 2-tailed Student’s $t$ test was used to determine $P$ values as detailed in Supplemental Methods. $P < 0.05$ were considered significant.
RESULTS

Myeloid IKKβ is essential for anti-tumorigenic immunity

C57/BL6 and FVB mice with targeted deletion of Ikκβ in myeloid cells (IkκβMyeΔΔ) 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)(11, 13). To test whether IKKβ in myeloid cells influences immunity against melanoma, 10⁶ melanoma cells derived from BrafV600E/Pten−/− mice (on a mixed C57BL/6 x FVB background) after induction with 4-HT (Fig.S1Aa), were intravenously injected into C57BL/6 IkκβMyeΔΔ mice or into control IkκβWT C57BL/6 litter mates (20 mice/group). Three weeks later, all IkκβMyeΔΔ mice had difficulty breathing and large abnormal lungs (0.99±0.116g) in contrast to the IkκβWT recipients (0.29±0.013g)(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 IkκβMyeΔΔ mice (319,763±176,717) compared to the IkκβWT mice (623±182), or tumor free controls(732±117)(Fig.1C). Similar results were obtained when the BrafV600E/Pten−/− melanoma cells were injected into FVB mice(Fig.S1B-C). H&E staining detected melanoma lesions in IkκβMyeΔΔ lungs, but not in IkκβWT controls(Fig.1D), and lesions were verified as melanocytic by immunohistochemical staining with melanocyte and melanoma markersS-100 and MART-1, respectively (Fig. S1D). When BrafV600E/Pten−/− melanoma cells were injected intra-splenically into C57BL6 or FVB mice, similar results were obtained (Fig.S1E-H), indicating that the metastatic potential in IKKβMyeΔΔ mice was not organ-specific. Moreover, when BrafV600E/Pten−/− melanoma cells were implanted subcutaneously, xenografts grew steadily in IkκβMyeΔΔ mice, while cells implanted to IkκβWT mice grew significantly slower for the first two weeks and subsequently regressed(Fig.1A-B, p<0.01, n=7)

To further explore the impact of IkκβMyeΔΔ myeloid cells on tumorigenesis in the inducible BrafV600E/Pten−/− mice, bone marrow cells from donor C57BL6 IkκβMyeΔΔ mice or littermate IkκβWT mice were transplanted into recipient C57BL6/FVB (Brafft::Ptenft::Tyr-Cre) mice (Fig.S1J-L). Recipient animals (20 mice/group) were treated with topical 4-HT to induce Tyr-Cre mediated expression of BRAFV600E and deletion of PTEN in
melanocytes which then progress to melanoma(14). Five weeks post-induction, typical pigmented melanomas occurred at the treatment site more frequently in irradiated mice transplanted with bone marrow from C57BL6 Ilkβ^{MeyΔ/Δ} mice as compared mice transplanted with marrow from Ilkβ^{WT} mice (Fig. S1M-N). Due to the leakiness of the inducible Tyr-Cre system (15), spontaneous tumors appeared more frequently on mice transplanted with Ilkβ^{MeyΔ/Δ} than Ilkβ^{WT} marrow (Fig. S1O-P).

When a syngeneic B16F0 melanoma model was examined using similar protocols, we observed that melanoma tumor burden was significantly enhanced in the lung of Ilkβ^{MeyΔ/Δ} mice in comparison to that of Ilkβ^{WT} mice (Gluc activity respectively: 30,489 ± 2,759 vs. 6,549 ± 3,457, p<0.01, n=6) (Fig. 1 E). 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 histological analyses, in contrast to the numerous melanoma lesions in the lungs of IKKβ^{WT} littermates (Fig.1F and Fig.S1Q). Tumor reporter-Gluc activity was significantly reduced in the lungs of IKKβ^{CA} mice compared to controls (4,304 ± 1,479 vs. 31,396 ± 6,493, p<0.01, n=6) with 2 experimental repeats. It should be noted that the B16 tumors grow faster in the pure C57Bl/6 vector control mice (control for the Ilkβ^{CA}) as compared to the Ilkβ^{WT} (control for the Ilkβ^{MeyΔ/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 anti-tumor immunity in both syngeneic and allogenic models of melanoma, while enhanced IKKβ activity (IKKβ^{CA}) results in enhanced anti-tumor response.

The macrophage is a key mediator in anti-tumor immunity

We sought first to explore whether macrophages might influence anti-melanoma 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±3.0×10^5) compared to the liposome control group (2.4±1.2×10^5, p<0.05, n=8, Fig.2Ba-c). Clodronate treatment had no effect on CD11c^+ DCs within the time course of this
assay (Fig.S2Bf) and did not deplete Gr-1⁺ neutrophils or DCs(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(Fig.S2E).

To extend this finding, Gluc Bravl⁶⁰⁰E/Pten⁻⁺ melanoma cells were delivered intravenously to IKKβWT 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 mouse (1,530±191, p=0.44,n=5) (Fig.2D). In contrast, when neutrophils were depleted in IkkbWT mice using anti-Ly6G antibody(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 (Fig.S2D, 6,769±4085 vs. 1131±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 IkkbΔ/Δ mice, we co-cultured macrophages with RFP-tagged tumor cells. In contrast to IkkbWT macrophages(Fig.3A), IkkbΔ/Δ macrophages exhibited poor phagocytic activity toward RFP-tagged BrafV600E/Pten⁻⁺ melanoma cells based on uptake of RFP(Fig.3B). Moreover, the tumor cell kill after 5h co-culture with macrophages was significantly lower with IkkbΔ/Δ macrophages (0.9±0.8%) than IkkbWT cells(8.2±2.1%, p<0.02,n=5). Cell death in the macrophage-free cultured melanoma cells was comparable to that of cells incubated with IkkbΔ/Δ 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(Fig.S3C). However, when cell-cell interaction was observed after prolonged culture(3 days), IKKβΔ/Δ macrophages did eventually engulf tumor cells, though engulfed tumor cells
appeared not to undergo digestion (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 MHCII, we performed FACS analysis of lungs from Ikkβ<sup>MyeΔ/Δ</sup> mice or Ikkβ<sup>WT</sup> litter-mates that had received BRAF<sup>V600E/PTEN<sup>−/−</sup></sup> cells intravenously. The number of F4/80<sup>+</sup> macrophages infiltrating into lungs of Ikkβ<sup>WT</sup> mice was almost halved 24 h post tumor cell injection (12,060±1660 cells versus 6943±1294 cells in Ikkβ<sup>MyeΔ/Δ</sup> controls). The Ikkβ<sup>MyeΔ/Δ</sup> mice exhibited even more greatly diminished numbers of F4/80<sup>+</sup>/MHC II<sup>+</sup> double positive cells in comparison to the Ikkβ<sup>WT</sup> 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<sup>+</sup> cells in the lung in response to tumor by about 42%, but the number of F4/80<sup>+</sup> macrophages expressing MHC II is reduced 16-fold (or >7-fold taking into consideration the reduction in total F4/80<sup>+</sup> macrophages in the lung).

To learn the impact of IKKβ expression in macrophages on CD4<sup>+</sup> T cell phenotype, Ikkβ<sup>MyeΔ/Δ</sup> mice or Ikkβ<sup>WT</sup> littermates received 10<sup>6</sup> Brafo<sup>V600E/PTen<sup>−/−</sup></sup> cells intravenously. Three days later, immune cells expressing Tomato-RFP were isolated from lung, F4/80<sup>+</sup> macrophages were excluded, and expression of Th2 and Treg markers (CD25 and Foxp3) was analyzed by FACS. The number of CD25<sup>+</sup>/Foxp3<sup>+</sup> CD4<sup>+</sup> T cells (Tregs) from Ikkβ<sup>MyeΔ/Δ</sup> mice was significantly increased over Ikkβ<sup>WT</sup> 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<sup>+</sup>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<sup>+</sup> T cells in lung of mice with melanoma tumors, lymphocytes expressing Tomato-RFP were sorted from lung tissues and CD8<sup>+</sup>T/CD107b<sup>+</sup> double positive cells identified by FACS. The percentage of CD107b<sup>+</sup> CD8<sup>+</sup> cells from tumor-bearing Ikkβ<sup>MyeΔ/Δ</sup> mice declined by 40% in comparison to tumor-bearing Ikkβ<sup>WT</sup> 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 Fig.S4A. A similar immune response was observed in the cutaneous melanoma model. Together, the increased Treg and decrease in activated CTL suggest that myeloid IKK\(\beta\) 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(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 anti-tumor cytotoxicity and myeloid IKK\(\beta\) is essential for activation of CD8\(^+\) T cells in response to melanoma cells in the tumor allograft model.

In the syngeneic model where 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 \(Ikk^{\beta\text{Mye}\Delta/\Delta}\) mice or \(Ikk^{\beta\text{WT}}\) mice when CD8\(^+\) T cells were depleted(Fig. 4E;Fig.S5A-C). A similar result was observed in the \(Ikk^{\beta\text{CA}}\) mice(Fig. S5D). Thus, immune cells other than CD8+T cells play the major anti-tumor 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 Fig.S5E), indicating that for B16 melanoma in the syngeneic melanoma tumor model, CD8\(^+\) T cells contributed little to the anti-tumor 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-\(\kappa\)B is required for cytokine-mediated immunity**

To learn whether myeloid IKK\(\beta\) deletion leads to any alteration in cytokine profiles \textit{in vivo}, which might affect the Th1- versus Th2-skew of immune cells, sera from non-tumor bearing \(Ikk^{\beta\text{WT}}\) and \(Ikk^{\beta\text{Mye}\Delta/\Delta}\) mice were analyzed. CCL11 was elevated 19-fold in serum of \(Ikk^{\beta\text{Mye}\Delta/\Delta}\) mice compared to \(Ikk^{\beta\text{WT}}\) mice (Fig. 5A) and the CCL11 was expressed mainly in IKK\(p^{\text{Mye}\Delta/\Delta}\) macrophages and to a lesser extent in the IKK\(p^{\text{Mye}\Delta/\Delta}\) neutrophils(Fig 5B). Since 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 \( \text{Ikk}^{\text{WT}} \) and \( \text{Ikk}^{\text{Mye}^{-/-}} \) mice and stained for DCs (CD11c\(^+\), CD80\(^+\)) were analyzed by FACS. \( \text{Ikk}^{\text{WT}} \) mice had over four-fold more DCs than mice whose myeloid lineage lacked IKKβ (9.5±1.3% of DCs versus 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 \( \text{Ikk}^{\text{Mye}^{-/-}} \) mice was very low (6.8±3.8%), in contrast to the CD83 expression on the DCs of \( \text{Ikk}^{\text{WT}} \) 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 \( \text{Ikk}^{\text{WT}} \) mice were cultured 7 days in medium with 20 ng/ml of GM-CSF and 20 ng/ml of IL-4 ± 100 ng/ml of CCL11 or control PBS, collected on day 7, stained with CD11c-Alexa Fluor700, 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. E). To test DC function, CD11c\(^+\) DCs were incubated with FITC-dextran 15 min and dextran endocytosis was analyzed by flow cytometry. CCL11-treated IKKβ-deficient DCs exhibited a 65% reduction in endocytosis compared to controls (15.8±0.84% vs. 44.7±0.83%, respectively; \( p<0.01, n=4 \)). Thus, IKKβ deletion in myeloid cells resulted in over-expression of CCL11, which contributed to reduced generation of DCs and reduced DC maturation. The loss of DC maturation in the \( \text{Ikk}^{\text{Mye}^{-/-}} \) mice could have significant consequences on the T cell activation in the allograft tumor model.

What might be clinical meanings of these findings in melanoma patients? To explore the potential relevance of these findings in human melanoma, we evaluated CCL-11 expression in macrophages (CD163\(^+\)) of biopsy specimens from 6 melanoma patients 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 impacts a number of additional pathways (25-28). Tissues were stained for macrophages (CD163\(^+\)), phospho-RelA(p65), activated CD8\(^+\)T cells (CD107a), and CCL-11, visualized by confocal microscopy, and quantitated using Metamorph (Fig. S6A&B). 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% up-regulation 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 to pre-treatment controls. The specificity of antibodies was verified on human peripheral lymphocytes (Fig.S6C&D) and expression of CD107a on individual tumor associated CD8+T cells from VELCADE treated patient tumors(Fig.S6 E&F) was significantly reduced. Thus, our findings both suggest that systemic therapy with VELCADE may act via NF-κB to change chemokine expression and CTL effector activity in cancer patients, and that targeting NF-κB signaling in human melanoma risks negatively impacting anti-tumor immunity.

**DISCUSSION**

Tumor associated macrophages (TAMs) exhibit both anti-tumor M1 and pro-tumor 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 upon observations that inhibition of NF-κB in myeloid cells elicits a switch from an M2 to M1 phenotype(33). However, Connelly et al. showed that constitutive activation of NF-κB maintained the anti-tumor phenotype of macrophages, whereas NF-κB inhibition by expression of an IκBα “super-repressor” resulted in enhanced breast tumor promotion(10). Under certain chronic inflammatory conditions, LPS-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 where 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. Though 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 IL-1, IL-4, IL-6, IL-10, IL-12, TNFα, IFNγ and TGFβ, as well as chemokines, play an important role in the modulation of the pro- or anti-tumor 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 up-regulated by Th2 cytokines IL-4 and IL-13, whereas its expression is down-regulated by the Th1 cytokine IFN-γ (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. Though 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 since they stress the importance of careful consideration of the immunological effects of drugs that impact the NF-κB pathway.

A key advance of our studies is the definitive demonstration that the anti-tumor activity of tumor associated macrophages requires NF-κB, since myeloid targeted deletion of IKKβ resulted in macrophages with decreased ability to kill tumor cells *in vitro*. Moreover, we show that macrophages from IKKβ<sup>myeΔ/Δ</sup> 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 *Ikκβ* 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 anti-tumor immunity (Hawkins, in preparation). Based on our data, IKK β inhibitors will be most effective when delivered directly to tumor cells. Moreover, developing ways to heighten or retain IKK β activity in myeloid cells, while blocking IKK β in melanoma tumor cells, may prove to be effective for inhibition of melanoma tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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LEGENDS

Figure 1. Deletion of Ikkβ in myeloid cells leads to pro-tumorigenic immunity. A, Generation of mice with myeloid Ikkβ deletion. Ikkβf/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, BrafV600E/Pten-/- melanoma cells form melanoma lung lesions in IkkβMyeΔΔ mice, but not in IkkβWT mice. C, quantitation of lung melanoma lesions based on Gluc activity (mean ± SEM) in the blood or lung 4 weeks after G-luc-BrafV600E/Pten-/- cells were intravenously (i.v.) injected into IkkβΔΔ or IkkβWT C57B/6 mice or tumor free IkkβWT mice. D, Histology of melanoma lesions in lungs from C based on H&E staining. Arrows indicate melanoma cells in lungs of IkkβMyeΔΔ mice. E, Myeloid IKKβ deletion enhances B16F0-Gluc melanoma growth in lung in syngeneic IkkβMyeΔΔ mice vs littermate IkkβWT mice. 20 days after i.v. injection of tumor cells, lungs were perfused and photographed (upper panels), or fixed and stained with
H&E (lower panels). F, B16F0 melanoma growth in C57BL6 myeloid IKKβCA mice and littermate control mice (single transgene cfms-rTA or TetOn cIKKβ) received B16F0-Gluc melanoma cells i.v. and transgene was induced with doxycycline. After 20 days, lungs were collected for photography (upper panels) and H&E staining (lower panels). Arrows indicate the pigmented melanoma lesion. Scale bar=50 µm.

Figure 2. Macrophages play an essential role in melanoma innate immunity. A, BrafV600E/Pten−/− melanoma cells (1×10⁷) were intraperitoneally (ip) 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 0-8 h, analyzed by FACS, and graphed as % cells in peritoneum. B, BrafV600E/Pten−/− melanoma cells (10⁷) 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-BrafV600E/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-BrafV600E/Pten−/− tumor-injected mice treated as in C (n=5, p<0.01).

Figure 3. IKKβ regulates macrophage phagocytosis. GFP-expressing macrophages isolated from IkkβWT (A) or IkkβMyeΔ/Δ (B) were co-cultured with melanoma cells expressing RFP (3:1) for 5 h, and examined by confocal microscopy. Scale bars=magnification. C, Macrophage killing activity of cells recovered after co-culture in A,B were stained with 7-AAD and subjected to FACS analysis.

Figure 4. Myeloid IKKβ mediates macrophage and CD8+ cytotoxicity. A, C57Bl/6 IkkβWT or IkkβMyeΔ/Δ mice were intravenously injected with BrafV600E/Pten−/− cells. After 24h GFP+ lung macrophages were stained for F4/80 and MHC II and analyzed by FACS. B, BrafV600E/Pten−/− cells were injected i.v. into mice carrying IkkβWT or IkkβMyeΔ/Δ myeloid cells. Pulmonary Tomato-RFP CD4+ T cells double positive for CD25 and Foxp3 were analyzed by FACS 3 days post 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 prior to 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**MyeΔΔ** 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 non-immunized mouse.

**Figure 5.** A, Myeloid IKKβ restrains CCL11 expression. A. Results of cytokine arrays of sera isolated from Ikkβ**WT** and Ikkβ**MyeΔΔ** mice; green rectangle (right panel) shows position of CCL11 signal of sera from Ikkβ**MyeΔΔ** 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**MyeΔΔ** 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 IL-4 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 min then cells were stained for CD11c and double positive cells analyzed by FACS.

**Figure 6.** Paraffin-embedded patient melanoma tumor samples, pre- or post-treatment with VELCADE and TMZ, were stained for CD163, phosphor-p65 and CCL11 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).

**References**


Contributions of Respective Authors:

Conception and design: A. Richmond: Development of methodology: J. Yang, O.E. Hawkins, W. Barham, P. Gilchuk, F.E. Yull, A. Richmond; Acquisition of data: J. Yang, O.E. Hawkins, W. Barham, P. Gilchuk, F.E. Yull, A. Richmond; Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): G.D. Ayers, J. Yang, . M. Boothby, S. Joyce, F.E. Yull, A. Richmond; Writing, review, and/or revision of the manuscript: J. Yang, M. Boothby, S. Joyce, M. Karin, F.E. Yull, A Richmond; Administrative, technical, or material support: F.E. Yull, M. Karin, A Richmond; Study supervision: A. Richmond
Figure 1

A) Genotype

B) (Image of two organs labeled as ikkβWT and ikkβMyelD/Δ)

C) Reporter Activity

D) (Images of tissue sections labeled as ikkβwr, ikkβMyelD/Δ, and Tumor-free)

E) (Images of organ sections labeled as ikkβwr, ikkβMyelD/Δ, and Tumor-free)

F) (Images of organ sections labeled as Ctrl, ikkβCA, and ikkβWT)
Figure 2

A

% Cells in Peritoneal

Time (h)

B

Vehicle

Clodronate

C

Remained Tumor Cells (×10^4)

p<0.05, n=8

D

Glae Reporter Activity

n=5, p<0.01

Treatment

Tumor-free Vehicle Clodronate
Figure 3

A

Myeloid cells | Melanoma cells | Interaction overlay

B

Ikβ\textsuperscript{WT} | Ikβ\textsuperscript{MyeΔΔ} | Ikβ\textsuperscript{MyeΔΔ}

C

Macrophage-killing Activity

\[ p < 0.02, n = 5 \]

Ikβ\textsuperscript{WT} | Ikβ\textsuperscript{MyeΔΔ} | M-Free

n.s
Figure 4

A. MHC II vs F4/80

B. CD25 vs Foxp3

C. CD107b count for IkB WT vs IkB ΔΔ

D. Gluc Reporter Activity: Tumor-Free, IgG, CD8

E. Tumor Reporter Activity: IgG, CD8

F. TRPZ tetramer for IkB WT, IkB ΔΔ, Ctrl, and CD8
Figure 6

A) Phospho-p65

B) CCL11

C) CD107a

Fluorescent Intensity

Treatment Time

$P < 0.01$
Figure 1

A

\[
\begin{align*}
\text{Genotype} & : \\
Ikk^{\beta f/f} \times \text{LysMCre} & \rightarrow Ikk^{\beta f/f-}\text{LysMCre} \times mG/mT^{\#} \\
\text{LysMCre::mG/mT}^{\#} & \rightarrow Ikk^{\beta f/f-}\text{LysMCre::mG/mT}^{\#} \\
\rightarrow \text{Myeloid } Ikk^{\beta \text{WT}} \quad \text{Myeloid } Ikk^{\beta \Delta/\Delta} & \\
\rightarrow \text{Myeloid cells-GFP} \quad \text{Lymphocytes-RFP} &
\end{align*}
\]

B

C

D

E

F

\[\text{Blood} \quad \text{Lung} \quad p<0.01, n=5 \]

\[\text{Blood} \quad \text{Lung} \quad \text{n.s} \]

\[\text{Tumor-Free} \quad Ikk^{\beta \text{WT}} \quad Ikk^{\beta \text{Mye}\Delta/\Delta} \]

\[\text{Tumor-free} \quad \text{Tumor-free} \]

\[\text{Ctrl} \quad \text{Ctrl} \quad \text{Ikk^{\beta CA}} \]

\[\text{Ikk^{\beta CA}} \quad \text{Ikk^{\beta CA}} \]

\[\text{Ikk^{\beta CA}} \quad \text{Ctrl} \quad \text{Ikk^{\beta CA}} \]

\[\text{Ikk^{\beta CA}} \quad \text{Ctrl} \quad \text{Ikk^{\beta CA}} \]

\[\text{Ikk^{\beta CA}} \quad \text{Ctrl} \quad \text{Ikk^{\beta CA}} \]
Figure 2

A

% Cells in Peritonea vs. Time (h)

B

GFP vs. RFP fluorescence in Vehicle (a) and Clodronate (b) treated samples.

C

Remained Tumor Cells (×10⁵) in Vehicle and Clodronate treatments.

D

Gluc Reporter Activity in Tumor-free, Vehicle, and Clodronate treatments.

p<0.05, n=8

Tumor-free
Vehicle
Clodronate

GFP → RFP → RFP → GFP

Clodronate

n=5, p<0.01

n.s.
Figure 3

A

Myeloid cells

Melanoma cells

Interaction overlay

B

Ikβ\textsuperscript{WT}

Ikβ\textsuperscript{MyeΔΔ}

C

Macrophage-killing Activity

Ikβ\textsuperscript{WT}  Ikβ\textsuperscript{MyeΔΔ}  M-Free

p<0.02, n=5

n.s
**Figure 4**

**A**

- MHC II
- F4/80

**B**

- IkkβWT
- IkkβMyeΔ/Δ

**C**

- IkkβMyeΔ/Δ
- IkkβWT

**D**

- Gluc Reporter Activity

- Tumor-Free
- IgG
- CD8

**E**

- IgG
- CD8

**F**

- CD8
- TRP2 tetramer

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 5

A

B

C

D

E

F
Myeloid IKKβ Promotes Anti-tumor Immunity by Modulating CCL11 and the Innate Immune Response

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