IKKα-deficient lung adenocarcinomas generate an immunosuppressive microenvironment by overproducing Treg-inducing cytokines

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The tumor microenvironment (TME) provides potential targets for cancer therapy. However, how signals originating in cancer cells affect tumor-directed immunity is largely unknown. Deletions in the CHUK locus, coding for IκB kinase α (IKKα), correlate with reduced lung adenocarcinoma (ADC) patient survival and promote KrasG12D-initiated ADC development in mice, but it is unknown how reduced IKKα expression affects the TME. Here, we report that low IKKα expression in human and mouse ADC cells correlates with increased monocyte-derived macrophage and regulatory T cell (Treg) scores and elevated transcription of genes coding for macrophage-recruiting and Treg-inducing cytokines (CSF1, CCL22, TNF, and IL-23A). By stimulating recruitment of monocyte-derived macrophages from the bone marrow and enforcing a TNF/TNFFR2/c-Rel signaling cascade that stimulates Treg generation, these cytokines promote lung ADC progression. Depletion of TNFR2, c-Rel, or TNF in CD4+ T cells or monocyte-derived macrophages dampens Treg generation and lung tumorigenesis. Treg depletion also attenuates carcinogenesis. In conclusion, reduced cancer cell IKKα activity enhances formation of a protumorigenic TME through a pathway whose constituents may serve as therapeutic targets for KRAS-initiated lung ADC.

Reduced IKKα activity increases expression of inflammatory cytokines and growth factors and enhances macrophage infiltration into sites of Ras-initiated pancreatic and skin carcinomas (10, 11). However, it remains to be determined how ADC cell-derived inflammatory responses influence cancer immunoeediting to impact tumor elimination or escape (12). An adoptive CD4+ T cell TDI has the potential to eliminate Ras-initiated tumors at an early stage. Indeed, CD4+ T cells antagonize Kras-initiated lung ADC development, while tolerance-related Treg cells dampen TDI (13). We hypothesized that impaired IKKα expression or activity unleashes KrasG12D ADC intrinsic signaling pathways that blunt TDI and allow ADC to escape immunosurveillance and undergo further progression. Because reduced IKKα expression or activity worsens survival of patients with lung ADC (4), we investigated how IKKα deficiency affects TDI in lung ADC with the hope of identifying new targets for therapeutic intervention.

Here we describe shared patterns of increased monocyte-derived macrophage and Treg-cell scores and regulators of macrophage and Treg differentiation in human and mouse lung ADC lacking IKKα. ADC-intrinsic Ikkα ablation up-regulated immunosuppressive response | Treg cells | lung cancer | NK-κB signaling | inflammation

Cancer-related genomic aberrations generate neoantigens, which drive tumor-directed immune responses that affect tumor progression. Identifying cancer-cell-intrinsic alterations that shape tumor-directed immunity (TDI) is important for choosing and improving treatments for lung cancer, which remains the leading cause of cancer-related mortality (1). Immune checkpoint blockade has improved 5-y survival rates for patients with nonsmall cell lung carcinoma (NSCLC), which includes lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) (2, 3). Previously, we found that the CHUK gene, coding for IKKα, is a suppressor of lung ADC (4). Oncogenic KRAS mutations and CHUK nonsense mutations or homozygous and hemizygous deletions are present in ~35% and 25% of human lung ADC patients, respectively (4–6). Patients with lung ADC whose tumors contain both CHUK hemizygous deletions and oncogenic KRAS mutations die earlier than patients with ADC with KRAS mutations alone (4, 6). IKKα inactivation in mouse lung epithelium results in spontaneous ADC formation and enhanced KrasG12D-initiated lung ADC development (4). Urethane-induced lung ADC incidence and ADC weights were also higher in mice lacking IKKα in type II lung epithelial cells compared to wild-type (WT) counterparts (7). Ikkα deletion in pancreatic epithelial cells enhances KrasG12D-induced pancreatic ductal ADC pathogenesis by interfering with the completion of autophagy, which results in chronic pancreatitis (8, 9).

Significance

This study reveals that impaired IKKα expression or activity in lung cancer enhances differentiation of protumorigenic Treg cells through a TNF/TNFFR2/NF-κB signaling pathway in both human and mouse lung ADC. Depletion of one of the molecules that are required for Treg cell induction represses lung ADC development. Thus, the components that interfere with this particular Treg differentiation provide targets for the generation of TME-modifying therapies.

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expression of regulators of macrophage recruitment and Treg differentiation, thereby converting the tumor microenvironment (TME) from cancer restrictive to cancer supportive. A major effector of ADC-intrinsic IKKα deficiency is a Treg-promoting TNF/TNFFR2/NF-κB signaling cascade.

Results

IKKα Deficiency in Human Lung ADC Correlates with Increased FOXP3+ Treg and Monocyte-Derived Macrophage Scores. By exploring the relationship between reduced IKKα expression and the TME in human lung ADC, we found that low IKKα correlated with increased intratumoral macrophage (CD68+), a monocyte lineage marker) and elevated Treg (FOXP3+) numbers in a tissue array of 80 human lung ADC (Fig. 1A and B (SI Appendix, Fig. S1 A and B)). Immunohistochemical (IHC) staining revealed an identical pattern of CD68+ and CD163+ macrophages in human lung ADC and adjacent tissues (Fig. 1C and SI Appendix, Fig. S1C), suggesting that either CD68 or CD163 can be used to detect intratumoral macrophages. We did not observe a significantly correlated distribution between CD8+ T cell numbers and IKKα expression in the lung ADC array, although some high IKKα ADC showed increased CD8+ T cell infiltration (SI Appendix, Fig. S1 D and E). IKKα expression, which was grouped by median and quartiles, was inversely correlated with Treg and macrophage infiltration (including monocyte scores) in human lung ADC cohorts (Fig. 1D, ref. 14, and SI Appendix, Fig. S1 F and G). Furthermore, IKKB2 mRNA, which encodes IKKβ, interacts with IKKα and IKKγ to form the IKK complex (15), positively correlated with macrophage scores, though its expression did not show a significant correlation with Treg scores in the same ADC cohort (Fig. 1E) (14). Curiously, IKKα and IKKβ expressions were inversely correlated (SI Appendix, Fig. S1H).

By analyzing a human lung ADC cohort (6), we found that expression levels of gene pairs encoding CSF1R/CSF1, TNFRSF1B/FOXP3, FOXP3/CSF1R, FOXP3/CSF1, TNF/FOXP3, and CSF1R/CCL2 were significantly correlated (Fig. 1F), indicating an association between TNF/TNFRSF1B-linked Treg and macrophage numbers in human lung ADC. Of note, IKKα amounts were inversely correlated with expression of CSF1, CSF1R, FOXP3, TNFRSF1B, TNF, CCL2, and IKKβ in a dose-dependent manner (Fig. 1G and SI Appendix, Fig. S1I). These results suggest that reduced IKKα expression is associated with increased macrophage and Treg numbers and their regulators in human lung ADC.

IKKα Loss Enhances KrasGISD-Induced Lung ADC Development and Macrophage and Treg Infiltration. To investigate whether IKKα deficiency increases intratumoral macrophage and Treg numbers in mouse ADC, we expressed KrasGISD (16) and ablated Ikkα in lung epithelial cells (IkkαLU) by intratracheally injecting adenovirus-cyclization recombinase (Ad.Cre) (4) to generate KrasGISD and KrasGISD, IkkαLU mice on a C57BL/6 background. Ad.Cre is expressed only in the cytosolic compartment, disappearing after cell division, and KrasGISD, IkkαLU developed heavier lungs with significantly higher ADC burden compared to KrasGISD, IkkαLU mice (Fig. 2A and SI Appendix, Fig. S2A). Flow cytometry showed that ADC-associated F4/80+ cell numbers or F4/80+CD11b+ monocyte-derived macrophage numbers normalized to lung ADC weight were higher in Ikkα-deficient tumors (Fig. 2 B and C). F4/80+ and F4/80+CD11b+ cell numbers versus CD45+ cells were also higher in Ikkα-deficient tumors (SI Appendix, Fig. S2B). Treg cell numbers were elevated but CD8+ T cells were lower in KrasGISD, IkkαLU lung ADCs compared to KrasGISD lung ADCs (Fig. 2 B–E and SI Appendix, Fig. S2C).

To verify whether IKKα-deficient ADC cells cause similar TME alterations when growing in WT mice, we intratracheally injected mouse Kras-CL and KrasIKKα-deficient cell lines, derived from KrasGISD-initiated ADC without or with IKKα deficiency, respectively (4) into lungs of WT mice. KrasIKKα-deficient ADC cells express low amounts of IKKα and have increased tumorigenic activity compared to Kras-CL cells (4). KrasIKKα-deficient tumor burden and KrasIKKα-deficient ADC-associated F4/80+ and Treg numbers were higher, but CD8+ cells were lower than in Kras-CL ADC (Fig. 2F and G and SI Appendix, Fig. S2D). Most intratumoral F4/80+ macrophages also expressed CD11b, a monocyte marker (Fig. 2H and SI Appendix, Fig. S2E).

Monocyte-derived macrophages isolated from KrasIKKα-deficient ADC showed elevated reactive oxygen species (ROS) levels and expressed reduced antioxidant genes but more ROS-, inflammation-, and mitogenesis-related genes compared to macrophages isolated from Kras-CL ADC (Fig. 2 I and J and SI Appendix, Fig. S2 F and G). These results show that ADC-intrinsic IKKα deficiency alters the TME to become more immunosuppressive (Fig. 2K).

Bone Marrow–Derived Macrophages and Treg Cells Support Growth of Ikkα-Deficient ADC. To determine the effect of macrophage-derived ROS on Ikkα-deficient ADC development, we transferred IkkαLU or Nox2KO bone marrow (BM) into irradiated KrasGISLD and KrasGISLD, IkkαLU mice. Lung weights and ADC burden were decreased in chimeric KrasGISLD, IkkαLU mice with Nox2KO BM compared to KrasGISLD, IkkαLU mice with WT BM (Fig. 3 A and B). Interestingly, lung ADC with Nox2KO BM decreased macrophage and Treg numbers and elevated CD8+ T cell numbers compared to lung ADC with WT BM, in addition to the expected decrease in ROS (Fig. 3 C and D and SI Appendix, Fig. S3 A and B). By contrast, lung ADC burden and infiltrating BM-derived macrophages, Treg cells, and CD8+ T cell numbers in chimeric KrasGISLD mice were not affected by the Nox2 status of the transplanted BM (Fig. 3 E and F and SI Appendix, Fig. S3C). Lung tumor burden and tumoral F4/80+ and Treg cell numbers were significantly reduced in Nox2−/− mice receiving KrasIKKα-deficient cell injections compared to WT hosts transplanted with the same cells (SI Appendix, Fig. S3D). Thus, ROS produced by BM-derived macrophages may stimulate Treg differentiation to support the development and progression of Ikkα-deficient lung ADC.

Because the BM contains multiple haemopoietic cell types, we verified that the effects of NOX2-dependent ROS on lung ADC development were exerted by macrophages. We intratracheally injected KrasIKKα-deficient cells into WT mice and then depleted host macrophages with clodronate-loaded liposomes, which kills monocyte-derived macrophages (17). In this setting, we could not use Kras-CL cells because they generated very small ADCs with a low number of infiltrating macrophages and Treg cells and were poorly responsive to macrophage-generated ROS. Clodronate-loaded liposome treatment significantly reduced lung ADC burden, ADC-associated F4/80+CD11b+ macrophages, and Treg cell numbers, as well as c-Rel amounts in CD4+Foxp3+ Treg cells, but increased CD8+ T cell numbers compared to vehicle control (Fig. 3 G and H and SI Appendix, Fig. S3E). To determine the role of CD4+ T cells, we used Rag1−/− and Cd4−/− mice as recipients, which developed heavier lungs with increased ADC burden compared to WT mice after intratracheal KrasIKKα-deficient cell injection (Fig. 3I and SI Appendix, Fig. S3 F and G). These results suggest that CD4+ T cells have antitumor activity. Intratumoral F4/80+ cell numbers were elevated in Rag1−/− and Cd4−/− hosts compared to WT hosts (SI Appendix, Fig. S3 H and I), suggesting that CD4+ T cells may inhibit macrophage infiltration.

To examine the effect of Treg cells on tumorigenesis, we intratracheally injected KrasIKKα-deficient cells into WT mice and treated the mice with an anti-CD25 antibody to deplete Treg cells (18). Treg reduction attenuated ADC development and...
reduced intratumoral monocyte-derived macrophages (Fig. 3 J and K and SI Appendix, Fig. S3J). We also depleted Foxp3\(^+\) cells using diphtheria toxin (DT) in Foxp3-DTR-GFP (DEREG) mice (19) that were intratracheally injected with Kras\(^{IKK\alpha}L\) cells, resulting in dampened lung ADC development (Fig. 3 L and M). These data indicate that Treg cells support lung tumorigenesis in this setting.

**TGFβ** as a strong Treg-cell inducer, was not highly expressed in Kras\(^{G12D}L\) ADC. Because c-Rel promotes Treg cell generation (20) and macrophage depletion reduced its expression.
IKKα loss promotes lung carcinogenesis associated with increased Foxp3 Treg-cell and macrophage numbers. (A) An approach (Top) of KrasG12D activation and Ikkα ablation in lungs of KrasG12D and KrasG12D;Ikkαf/f mice by adenovirus-Cre (Ad.Cre, or Cre) and the lung appearances (Bottom) of KrasG12D and KrasG12D;Ikkαf/f mice at 3 mo after Ad.Cre treatment. (B and C) Flow cytometric analyses of F4/80+CD11b+ (B) or F4/80+ (C) cells in KrasG12D (n = 5) and KrasG12D;Ikkαf/f (n = 6) lung ADCs. Representative images for flow cytometry on the Left and statistical analyses on the Right are shown. Data represent mean ± SEM (multiple experiments). This result is representative. **P < 0.01; ****P < 0.0001; Student's t test. (D) Flow cytometric analyses of Treg-cell numbers in KrasG12D and KrasG12D;Ikkαf/f lung ADCs. Data represent mean ± SEM (three experiments). **P < 0.01; Student's t test. (E) IHC staining for % CD8 T cells in KrasG12D and KrasG12D;Ikkαf/f lung ADCs, analyzed by n = 3 mice/group; three sections per mouse. Data represent mean ± SD (three repeats). ***P < 0.001; Student's t test. (F) Lung appearances with ADC (Left) and ADC burden (Right) derived from Kras-CL and KrasIKKαL ADC cells in WT mice (n = 3/group). This is representative. Data represent mean ± SEM (three experiments). **P < 0.01; Student's t test. (G) IHC analysis of % F4/80 cells in KrasG12D and KrasG12D;Ikkαf/f lung ADCs. Data represent mean ± SEM (three experiments). **P < 0.01; Student's t test. (H) Representative images of flow cytometric analyses for % macrophages (F4/80+CD11b+) in CD45+ cells isolated from KrasG12D ADCs in WT mice. (I) Relative DCF levels in macrophages isolated from Kras-CL and KrasIKKαL ADCs in WT mice (n = 3/group). Data represent mean ± SD (three repeats). *P < 0.05; **P < 0.001; Student's t test. (J) A heat map analyzing the gene expression profiles in macrophages isolated from Kras-CL and KrasIKKαL lung ADC in WT mice. Statistical analysis for all gene comparison from two groups, P < 0.05; one-way ANOVA test. (K) A summary: increased IKKα-deficient KrasG12D ADC development is correlated with increased macrophage and Treg-cell numbers. Mφ, macrophage.
in CD4\(^+\)Foxp3\(^+\) cells (Fig. 3I), we analyzed nuclear c-Rel and expression of its target genes in CD4\(^+\) T cells isolated from Kras\(^{IKK\alpha}\) and Kras-CL lung ADCs. Kras\(^{IKK\alpha}\) ADC–derived CD4\(^+\) T cells showed elevated nuclear c-Rel (Fig. 3N and SI Appendix, Fig. S3 K and L), expressed higher amounts of c-Rel target mRNAs (20, 21), Foxp3 up-regulating molecules, TNF-family–related pathways, IL-1 signaling, and fewer apoptosis-related genes compared to CD4\(^+\) cells from Kras-CL ADC (Fig. 3O). These results suggest that intratumoral CD4\(^+\) T cells of IKK\alpha-deficient ADC have higher c-Rel-driven NF-κB activity.

**CD4\(^+\) T Cell c-Rel Signaling and Macrophages Promote Generation of Intratumoral Treg Cells.** To establish a link between intratumoral monocyte–derived macrophages and Treg generation, we

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**Fig. 3.** Macrophages paired with Foxp3\(^+\) T cells specifically promote IKK\alpha-deficient lung ADC development. (A and B) Lung ADC burden (% area) in Kras\(^{G12D}\) mice receiving WT or Nox2KO BM (n = 6 mice/group). Data represent mean ± SD. *P < 0.05; ***P < 0.001; Student’s t test. (C and D) IHC analyses of % Foxp3 Treg cells (C, Left, n = 3), % F4/80 macrophages (C, Right, n = 4), and % CD8 cells (D, n = 4) in lung ADCs of Kras\(^{G12D, ikk\alpha^{UL}}\) mice receiving WT BM or Nox2KO BM. Data represent mean ± SD (three repeats). **P < 0.01; ***P < 0.001; Student’s t test. (E) Lung ADC burden (% area) in Kras\(^{IKK\alpha}\) mice receiving WT BM (n = 5 mice) or Nox2KO BM (n = 6 mice). ns, not significant; Student’s t test. (F) IHC analyses of % F4/80 macrophages (Left) and % Foxp3 Treg cells (Right) in lung ADCs of chimeric Kras\(^{G12D}\) mice receiving WT BM or Nox2KO BM (n = 3 mice/group; three sections per mouse). Data represent mean ± SD, ns, not significant. Student’s t test. (G) Kras\(^{IKK\alpha}\) lung ADC burden (% area, Left) in WT mice treated with clodronate-loaded liposomes (Lipos) or a vehicle control (n = 4 for each group). Flow cytometric analyses of % F4/80 CD11\(b^+\) cells (Middle) and % CD8\(^+\) cells (Right) in CD4\(^+\) cells associated with Kras\(^{IKK\alpha}\) lung ADCs derived from WT mice treated with clodronate-loaded Lipos or a vehicle control (n = 5/group). Data represent mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; Student’s t test. (H) Flow cytometric analyses of % Treg cells (Left) in CD4\(^+\) T cells and c-Rel levels (Median fluorescence intensity, Right) in CD4\(^+\)Foxp3\(^+\) T cells associated with Kras\(^{IKK\alpha}\) lung ADCs of WT mice treated with clodronate-loaded Lipos or a vehicle control (n = 5/group). Data represent mean ± SD. *P < 0.05; ***P < 0.001; Student’s t test. (I) Kras\(^{IKK\alpha}\) lung ADC burden in WT and CD4\(^{−/−}\) (Cd4KO) mice (n = 5/group). *P < 0.05; Student’s t test. (J) Kras\(^{IKK\alpha}\) lung ADC burden in WT mice treated with an anti-Treg antibody (anti-CD25Ab) or a vehicle control (n = 5/group). Data represent mean ± SEM (three repeats). ***P < 0.001; ***P < 0.001; Student’s t test.
To rule out an effect of PTX on macrophages or ADC cells, we treated WT and ADC cells, which produced fewer ROS than WT macrophages and were less potent in Treg induction (Fig. 4 C, Left and Middle). To confirm the role of ROS in Treg cell induction, we added N-acetylcysteine (NAC) or apocynin, a ROS inhibitor, to the coculture experiments and found them to attenuate Treg-cell induction by WT macrophages (Fig. 4 C, Right). NAC treatment also reduced the burden of KrasIKKα- generated ADC and decreased the number of ADC-associated Treg cells, macrophages, macrophage ROS amounts, and c-Rel expression in intratumoral CD4+ T cells (Fig. 4D and SI Appendix, Fig. S4A). Reduced ADC IKKα expression is associated with increased TNF expression and Treg-cell scores (Fig. 1G), and TNF activates NF-κB (23). We therefore tested whether TNF enhances Treg induction in the coculture system by binding to TNFR2 and activating NF-κB in CD4+ T cells. Addition of either TNF or H2O2 to the macrophage-CD4+CD25+ T cell coculture system enhanced Treg-cell induction and increased c-Rel expression in both parental CD4+CD25+ T cells and CD4+CD25+Foxp3+ T cells (Fig. 4E).

We examined c-Rel’s involvement in Treg generation and tumorigenesis by using pentoxifylline (PTXF), which inhibits c-Rel activity, although it is not a c-Rel–specific inhibitor (20). PTXF treatment significantly reduced ex vivo Treg induction (Fig. 4F) and reduced KrasIKKα-generated lung ADC burden, as well as intratumoral Treg and macrophage numbers, macrophage ROS amounts, and CD4+ T cell c-Rel activity, while increasing CD8+ T cell numbers (Fig. 4 G and H and SI Appendix, Fig. S4 B–D). These results suggest that NF-κB/c-Rel enhances Treg-cell induction and contributes to tumorigenesis. To rule out an effect of PTXF on macrophages or ADC cells, we treated Rag1−/− mice inoculated with KrasIKKα cells with PTXF. The inhibitor did not alter lung weights and ADC burden in Rag1−/− mice (SI Appendix, Fig. S4 E and F), suggesting that PTXF acts on Tcells.

To test TNF’s involvement in Treg differentiation and tumorigenesis, we intratracheally injected KrasIKKα cells into lungs of WT and TNF−/− mice. Both lung ADC burden and intratumoral Treg-cell and macrophage numbers were reduced in TNF−/− mice along with increased CD8+ T cell numbers compared to WT (Fig. 4 I and J and SI Appendix, Fig. S4G). Notably, CD4+ T cells isolated from TNF−/− mice produced higher CD4+ Foxp3+ T cells numbers than WT (Fig. 4K). We compared ADC development in KrasIKKα mice along with increased CD8+ T cell responses poorly to TNF, exhibiting reduced Treg-cell induction and lower c-Rel expression than WT CD4+ T cells (Fig. 4 L and M). In addition, TNfsf1b−/−CD4+ T cells responded poorly to TNF, exhibiting reduced Treg-cell induction and lower c-Rel expression than WT CD4+ T cells (Fig. 4 L and M). Nonetheless, residual TNF-stimulated Treg induction in cocultures of WT macrophage and TNfsf1b−/−CD4+ T cells suggested that TNF may also work via TNFR1 in the absence of TNFR2. Because expression of CD4 and TNFSF11A, which encodes TNFR1, was not highly correlated in human lung ADCs (6), TNFR2 may be the more important TNFR in this setting.

Because TNF and H2O2 showed similar ability to enhance Treg induction in the coculture system, we hypothesized that TNF and ROS up-regulate each other’s production. Indeed, ROS levels were lower in TNF−/− than WT macrophages (Fig. 4N), and H2O2 enhanced TNF expression (Fig. 4O). Accordingly, TNF−/− macrophages cocultured with WT CD4+ T cells induced fewer Treg cells compared to WT macrophages, and the coculture of TNF−/− macrophages with TNfsf1b−/−CD4+ T cells led to a further reduction in Treg induction (Fig. 4P). To determine the role of macrophage-produced TNF in lungs ADC development, we injected CD45.2 WT or TNF−/− BM cells into irradiated CD45.1 WT mice that were inoculated with KrasIKKα lung ADC cells. The results showed decreased ADC burden, lung weights, and ADC-associated Treg cells in mice reconstituted with TNF−/− BM (Fig. 4 Q and R and SI Appendix, Fig. S4L). These experiments also suggested that ADC-associated monocye-derived F4/80+CD11b+high and F4/80−CD11b+low macrophages originate from the BM (SI Appendix, Fig. S4L), further supporting the role of macrophage ROS in TNF production at a level needed for Treg induction.

We compared ADC development in KrasIKKα;IkκαIKκα−/−;IkκβIKκβ−/− and IkκαIKκα−/−;KrasIKKα−/− mice and found that ADC burden was significantly lower in TNfsf1b−/−;IkκαIKκα−/−;KrasIKKα−/− mice (Figs. 5 S and T, Left). Treg numbers and c-Rel levels in Foxp3+ T cells were lower in lung ADCs of TNfsf1b−/−;IkκαIKκα−/−;KrasIKKα−/− mice compared to IkκαIKκα−/−;KrasIKKα−/− mice, which were consistent with the result obtained from the coculture system (Fig. 4 T, Middle and Right and SI Appendix, Fig. S4K). Patients with lung ADC with CHUK hemizygous deletions and TNFSF11B gain die earlier compared to patients doubly dyplod for CHUK and TNFSF11B (Fig. 4U). Patients with lung ADC with CHUK hemizygous deletions and FOXP3 gain showed a lower survival trend compared to patients with ADC who are double dyplod for CHUK and FOXP3 (SI Appendix, Fig. S4L). These results suggest that TNF2 activates c-Rel and stimulates Foxp3+ T cell differentiation and that immunosuppressive FOXP3+ Treg induction accelerates lung carcinogenesis. Accordingly, we postulated that low IKKα lung ADC generates a TNF-rich TME. Indeed, TNF expression was higher in KrasIKKα ADC than Kras-CL ADCs (SI Appendix, Fig. S4M).

IKKα Suppresses TNF, Csf1, CCL22, and IL-23A Expression in Lung ADC. To investigate how IKKα loss up-regulates expression of cytokines and chemokines that shape the immunosuppressive TME (iTMEM), we used bead-based flow cytometry and detected higher TNF, CCL22, IL-23A, and Csf1 mRNA levels in human lung ADCs and found that TNF-, CSF1, and CCL22 mRNA expression was significantly higher in TNF−/− mice and lung ADC cells (SI Appendix, Fig. S5A). We confirmed that KrasIKKα ADC cells expressed significantly higher levels of Csf1, CCL22, IL23A, and Adam8 mRNAs compared to Kras-CL cells (Fig. 5A). Likewise, low CHUK mRNA was correlated with elevated IL23A and ADAM8 mRNAs in human lung ADC (Fig. 5B). IL-23A inhibits CD8+ T cell infiltration, enhances inflammation, and increases tumor incidence (26, 27), and ADAMS promotes cell proliferation, invasion, and metastasis in human cancers (28, 29). Reduced IKKα also correlated with increased expression of CCL7, CCL8, and ADAM9 in mouse lung ADC cells (SI Appendix, Fig. S5B). Together, these results suggest that IKKα deficiency correlates with elevated expression of cytokines that are important for generation of Treg cells and iTME.

To determine how IKKα modulates cytokine expression, we silenced IKKα in either human or mouse lung ADC cells and found it to increase expression of IL23A, CCL22, CSF1, and TNF mRNAs (Fig. 5 C and D). In addition to being a
ADC burden (% area, Left) in WT mice receiving Kras^{Kdck} cells, treated with PTXF (50 mg/kg) or a vehicle control (n = 4/group). IHC analyses of % Treg cells (Middle) in ADCs and ROS (DCF) levels in ADC-associated macrophages (Right) from WT mice receiving Kras^{Kdck} cells, treated with PTXF or a vehicle control (n = 4/group). Data represent mean ± SEM (three repeats); *P < 0.05; **P < 0.01; ***P < 0.001; Student's t test. (B) Laminin A1, a nuclear protein loading control; α-tubulin, a cytosolic protein loading control. Data represent mean ± SEM (three repeats); ***P < 0.001; Student's t test. (C) Laminin A1 (KO) and c-Rel intensity (median fluorescence intensity, MFI) in these CD4 cells detected by Western blotting, from NAC-treated WT CD4 cells and CD25^{+}CD4 T cells from the coculture of WT PM and CD4^{+}CD25^{+} T cells, treated with or without NAC. **P < 0.01; ***P < 0.001; Student's t test. (D) Lung weight (% of total weight), analyzed by RT-PCR. (E) TNFR2/TNFRSF1B expression in CD4 T cells isolated from lung ADCs of WT and Kras^{Kdck} KO mice, analyzed by RT-PCR. Data represent mean ± SEM (three repeats); ***P < 0.001; Student's t test. (F) Human lung ADCs were analyzed by RT-PCR. Data represent mean ± SEM (three repeats); ***P < 0.001; Student's t test. (G) Flow cytometric analyses of % Treg cells in CD4 T cells from the coculture of WT PM with WT or TNfrsf1b^{−/−} CD4 T cells, analyzed by flow cytometry. Data represent mean ± SEM (three repeats); not significant; *P < 0.05; **P < 0.01; ***P < 0.001; Student's t test. (H) Lung weight (Left) and Kras^{Kdck} lung ADC burden (Right) in irradiated and WT CD45.1 mice receiving CD45.2 WT or TNfrsf1b^{−/−} (KO) BM (n = 4). Data represent mean ± SEM (three repeats). (I) Flow cytometric analyses of ADC-associated CD4^{+}Foxp3^{+} Treg cells (Middle) in WT and Kras^{Kdck} KO mice (n = 4). Data represent mean ± SEM (three experiments); *P < 0.05; **P < 0.01; Student's t test. (J) Flow cytometric analyses of CD4^{+}Foxp3^{+} Treg-cell numbers (Middle, n = 3) and CD4 T cell c-Rel levels (Right, n = 3) in lung ADCs of TNfrsf1b^{−/−}/Kras^{G12D} mice (n = 4/group). Data represent mean ± SEM (three experiments). **P < 0.01; Student's t test. (K) Survival rates for lung ADC patients expressing CHUK hemizygous deletions and TNFRSF1B (TNFR2) gain versus patients with ADC expressing CHUK and TNFRSF1B double diploid. The RNA-sequence data were obtained from cBioPortal, TCGA, ref. 6. Logrank test for P value analyses.

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Fig. 5. IKKα reduction up-regulates transcription of cytokine genes in human and mouse lung ADC cells. (A) Expression of CCL2, CSF1, TNF, ADAM8, and IL-23A in Kras-CL and KrasIKKαL ADC cells, analyzed by RT-PCR. Data represent mean ± SEM (three repeats). ***P < 0.001; **P < 0.01; Student's t test. (B) Correlation of CHUK (IKKα) expression levels (using quartiles’ analysis to divide IKKα expression groups) with the expression levels of IL-23A or ADAM8 genes in human lung ADCs by analyzing the TCGA data (PanCancer Atlas, cBioPortal). H-CHUK, high CHUK; L-CHUK, low CHUK; P value at the Top of panels, Student's t test; n, ADC numbers. (C–D) Expression of CHUK (IKKα), IL-23A (IL-23a), CCL22, CSF1, and TNF in human A549 (C) and mouse Kras-CL (D) lung ADC cells treated with Si-control (Si-Cont) or Si-IKKα RNA, analyzed by RT-PCR. Data represent mean ± SEM (three repeats). *P < 0.05; **P < 0.01; ***P < 0.001; Student's t test. (E–F) ChIP analyses for binding of IKKα to the promoter regions of TNF (Tnf), CSF1 (Csf1), CCL22 (Ccl22), and IL-23A (IL-23a) genes by using an anti-IKKα antibody for immunoprecipitation, followed by PCR with primers for these genes in A549 cells (E) and in Kras-CL cells (F) treated with Si-control (Si-Cont) or Si-IKKα RNA. Data represent mean ± SEM (three repeats). *P < 0.05; **P < 0.01; ***P < 0.001; Student's t test. (G) ChIP-seq analyses for IKKα enrichment on the TNF gene in A549 cells. The enrichment peaks are marked by red lines. bp, nucleotide base pair; red arrow, 6,066 bp; Ab, antibody for immunoprecipitation. TNF gene: thickest lines, exons; second-thickest lines, untranslated sequences; thin lines, introns; red line, consensus sequences containing eight nucleotides. (H) A working model showing that ADC-IKKα reduction up-regulates the expression of TNF, CSF1, CCL22, and IL-23A and macrophage recruitment, which generate a TNF/TNFRSF1B/c-Rel pathway for CD4 T cells to stimulate Treg-cell induction, accelerating KrasG12D ADC development. Arrows, promotion; lines, inhibition; Mϕ, monocyte-derived macrophage.
cytoplasmic protein kinase, IKKα was reported to act in the nucleus (30–32). We therefore hypothesized that IKKα may directly or indirectly suppress expression of these cytokine genes. We conducted chromatin immunoprecipitation (ChIP) assays with an IKKα antibody and found enrichment for IKKα on the IL-23A, CCL22, CSF1, and TNF promoter regions in both human A549 and mouse Kras-CL cells, and this was attenuated by IKKα silencing (Fig. 5E and F and SI Appendix, Fig. S3C). Of note, the correlation between IKKα enrichment at these cytokine genes and their low expression in IKKα-deficient cells, suggests that IKKα act as a transcriptional suppressor of these particular genes. Because an interaction between IKKα and SMAD3/4 regulates expression of certain genes in keratinocytes (33–35), we tested whether IKKα suppresses the above cytokine gene through SMAD transcription factors. We performed a ChIP assay with SMAD3 or SMAD4 antibodies and could not detect SMAD3/4 enrichment on the IKKα-bound genes (SI Appendix, Fig. SSD). We further performed unbiased ChIP-seq experiments with an IKKα antibody (Fig. 5G). Sequence analysis of the regions at which IKKα was enriched revealed an 8-bp-long consensus sequence on the TNF, CSF1, CCL22, and IL-23A genes (Fig. 5G and SI Appendix, Fig. SSE). Future studies will probe the regulatory importance of this consensus sequence and whether it is recognized by IKKα or another transcription factor or chromatin protein with which IKKα interacts.

Based on the above findings, we propose a working model that explains how IKKα deficiency in lung ADC generates iTME that dismantles immune surveillance and accelerates tumor progression. Key to this model is the up-regulation of TNF, CSF1, CCL22, and IL-23A in IKKα-deficient lung ADC, which enhances macrophage recruitment and Treg differentiation (Fig. 5H).

Discussion

Human lung ADC and SCC, which originate from different cell types, show distinct histological features and genomic alterations (cBioPortal, The Cancer Genome Atlas [TCGA], refs. 6 and 36, PanCancer Atlas). KRAS mutations are common in human lung ADC but rare in lung SCC. Previous studies conducted by other investigators had focused on the role of canonical, IKKβ-dependent, NF-κB signaling in mouse Kras-initiated lung ADC (37, 38). We, on the other hand, had focused on the role of IKKα, which unlike IKKβ, activates noncanonical NF-κB signaling (39) and has several other, NF-κB unrelated, functions (31, 32, 40, 41). Previously, we reported that Iκκα ablation in mice promotes KrasG12D-initiated lung ADC development and results in spontaneous SCC formation associated with increased macrophage infiltration (4, 17, 42). In the present study we followed on the significant correlation between monocyte-derived macrophages and Treg scores and reduced IKKα expression in human lung ADC cohorts. Low IKKα expression also correlated with elevated expression of cytokines and chemokines (CSF1R, CSF1, TNFRSF1B, TNF, CCL2, FOXP3, and CCL22) that regulate macrophage recruitment and Treg-cell development (24, 25, 43–45). Similar correlations were observed in the mouse lung ADC model we have investigated.

Although macrophage-produced ROS support Treg-cell differentiation in vitro (22), the mechanism by which macrophage ROS production stimulates tumorigenesis is unknown. Our results suggest that ROS are required for maintaining high macrophage TNF expression, which is needed for stimulation of Treg-cell differentiation via a TNF/TNFFR2/c-Rel signaling cascade. Ablation of TNF or the ROS-producing enzyme Nox2 inhibited lung ADC pathogenesis and reduced tumor monocyte-derived macrophage and Treg infiltration. Moreover, in human lung ADC cohorts (TCGA, ref. 6 and PanCancer Atlas, ebioPortal), TNFR2 expression is significantly correlated with CD4 expression. Also, Trnfsf1b−/− CD4+ T cells gave rise to fewer Treg cells than WT CD4+ T cells when cocultured with macrophages. Correspondingly, lung ADC burden and intratumoral Treg-cell numbers were reduced in KrasG12D, Ikkκ−/−Trnfsf1b−/− mice compared to KrasG12D, Ikkκ−/− mice. However, TNF still led to residual Treg induction in cocultures of Trnfsf1b−/− CD4+ T cells and macrophages, suggesting that in the absence of TNFR2, TNF may stimulate Treg differentiation via TNFR1, even though TNFR1 expression is not significantly correlated with CD4 expression in human lung ADC. Even in the complete absence of TNF, we still observed a basal level of Treg induction in the coculture system, suggesting that other macrophage-generated stimuli can drive Treg differentiation. Regardless of the underlying mechanism, our results demonstrate that Treg cells are key components of the iTME that accompanies IKKα-deficient lung ADC.

Together with IKKβ, IKKα is one of the two catalytic subunits of the IKK complex. Curiously, however, in human lung ADC, the expression patterns of IKKα and IKKβ associated with expression of macrophage- and Treg-cell-regulating cytokines and chemokines are reciprocal. Ours and other animal studies are consistent with the findings in human lung ADC (4, 7, 38, 46, 47). However, a recent report showed that Iκκα, but not IKKβ, deletion in lung epithelial cells attenuated Kras-initiated lung ADC development (48). The cause of this discrepancy is not clear, but it may be due to unknown microenvironmental conditions. Consistent with the results described herein, transgenic Tg-K5.Iκκα and Tg-Lori.IKKα mice develop normally and are resistant to carcinogen-induced tumorigenesis and metastasis (15, 49, 50), whereas transgenic Tg-K5.IKKβ and Tg-EDL-2.IKKβ mice develop epidermal and esophageal hyperplasia and oral carcinomas (51–53). Our results are also consistent with previous studies showing that while IKKβ is the critical IKK catalytic subunit responsible for NF-κB activation, IKKα has numerous other functions. IKKα ablation in human and mouse lung ADC cells results in up-regulation of the CSF1, CCL22, TNF, and IL-23A genes, to whose promoter regions IKKα is recruited, suggesting that in lung ADC, IKKα works in the nucleus as a transcriptional repressor. However, ChIP assays cannot determine whether IKKα directly recognizes specific DNA sequences or interacts with transcription factors that bind to these sequences, a question that needs to be addressed in future studies. In past studies IKKα was reported to interact with SMAD transcription factors to determine expression of Myc antagonists (33–35) and was shown to modulate methylation of histone proteins (17, 31, 54). Whether these mechanisms apply to lung ADC cells remains to be determined.

Overall, Iκκα ablation promotes KrasG12D-initiated lung ADC development in mice. In previous studies we showed that increased tumor-intrinsic ROS are associated with KrasG12D/Ikkκ−/− ADC progression (4). We now extend these results to show that Iκκα-deficient lung ADC generates an iTME by up-regulating Treg-cell induction and that ROS produced by intratumoral macrophage facilitate Treg differentiation through the TNF/TNFFR2/c-Rel pathway. Given the present findings, tumor cell-intrinsic ROS may also contribute to iTME generation through potentiation of TNF signaling.

Materials and Methods

Mice, Human Tissue Array, and Cell Lines. All mice used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the NIH. All animal experiments (Protocols 17-051 and 17-052) were approved by the IACUC. The following mice were of a C57BL/6 background: Ikkκ−/−; KrasG12D, Ikkκ−/− (stock No. 002620; The Jackson Laboratory), Rag1−/−, KrasG12D (KrasG12D, stock No. 008179; The Jackson Laboratory), Cd4−/− (stock No. 002663; The Jackson Laboratory), Tnf−/− (stock No. 003008; The Jackson Laboratory), Trnf2−/− (stock No. 0002620; The Jackson Laboratory), with CD4 expression. Also, Trnfsf1b−/− CD4+ T cells gave rise to fewer Treg cells than WT CD4+ T cells when cocultured with macrophages. Correspondingly, lung ADC burden and intratumoral Treg-cell numbers were reduced in KrasG12D, Ikkκ−/−Trnfsf1b−/− mice compared to KrasG12D, Ikkκ−/− mice. However, TNF still led to residual Treg induction in cocultures of Trnfsf1b−/− CD4+ T cells and macrophages, suggesting that in the absence of TNFR2, TNF may stimulate Treg differentiation via TNFR1, even though TNFR1 expression is not significantly correlated with CD4 expression in human lung ADC. Even in the complete absence of TNF, we still observed a basal level of Treg induction in the coculture system, suggesting that other macrophage-generated stimuli can drive Treg differentiation. Regardless of the underlying mechanism, our results demonstrate that Treg cells are key components of the iTME that accompanies IKKα-deficient lung ADC.
Laboratory), CD51.5 B6 (stock No. 002016; The Jackson Laboratory), and B6-Cg-Foxp3 sf/J (Foxp3-DTR-gfp-DREG, stock No. 32050; The Jackson Laboratory).

Human lung ADC tissue arrays (BCS04017a and HLug03PG02) were purchased from US Biomax, Inc. We used an A549 human lung ADC cell line (ATCC). Human lung ADC tissue arrays (BCS04017a and HLug03PG02) were purchased from US Biomax, Inc. We used an A549 human lung ADC cell line (ATCC). We used antibodies to:

**Antibodies.** For Western blotting and immunostaining, we used antibodies to:

- Lamin B (sc-6216), c-Rel (sc-6955), NOX2 (sc-5827), IKK \( \alpha \).
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