

ORIGINAL RESEARCH



NF- κ B RelA renders tumor-associated macrophages resistant to and capable of directly suppressing CD8⁺ T cells for tumor promotion

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ABSTRACT

Activation of the inflammatory transcription factor NF- κ B in tumor-associated macrophages (TAMs) is assumed to contribute to tumor promotion. However, whether and how NF- κ B drives the antitumor macrophages to become pro-tumorigenic have not been determined in any cancer type yet. Similarly, how TAMs repress CD8⁺ cytotoxic T lymphocytes (CTLs) remains largely unknown, although their importance in regulatory T (Treg) cell regulation and tumor promotion has been well appreciated. Here, using an endogenous lung cancer model we uncover a direct crosstalk between TAMs and CTLs. TAMs suppress CTLs through the T-cell inhibitory molecule B7x (B7-H4/B7S1) in a cell-cell contact manner, whereas CTLs kill TAMs in a tumor antigen-specific manner. Remarkably, TAMs secrete the known T-cell suppressive cytokine interleukin-10 (IL-10) to activate, but not to repress, CTLs. Notably, one major role of cell-intrinsic NF- κ B RelA is to drive TAMs to suppress CTLs for tumor promotion. It induces B7x expression in TAMs directly, and restricts IL-10 expression indirectly by repressing expression of the NF- κ B cofactor Bcl3 and subsequent Bcl3/NF- κ B1-mediated transcription of IL-10. It also renders TAMs resistant to CTLs by up-regulating anti-apoptotic genes. These studies help understand how immunity is shaped in lung tumorigenesis, and suggest a RelA-targeted immunotherapy for this deadliest cancer.

ARTICLE HISTORY

Received 24 January 2018
Accepted 27 January 2018

KEYWORDS

B7x/B7-H4/B7S1; immune checkpoint; IL-10; lung cancer; NF- κ B; RelA/p65; tumor-associated macrophage



Introduction

A causal link between inflammation and cancer has been recently established in several types of cancer including lung cancer, the leading cause of cancer-related deaths in both men and women.¹⁻⁴ However, the normal role of immunity is to clear pathogens and damaged or transformed cells. Although it remains largely unknown how the antitumor immunity becomes tumor-promoting inflammation during tumorigenesis, macrophages, which are mature myeloid cells and can be simply defined as F4/80⁺ cells in mice and CD68⁺ cells in humans, have recently been suggested to be main culprits.⁵ Macrophages are the most abundant immune cells around and within tumors. Increase of macrophages or total myeloid cells in lung tissues or even in uninvolved lymph nodes or blood has been found to correlate with the risk, progression, chemoresistance and poor prognosis of lung cancer in humans.⁶⁻⁸ On the other hand, depletion of macrophages decreases both the tumor number and size in a mouse model of lung cancer.⁹ It is generally believed that tumor-associated macrophages (TAMs) contribute to tumor development mainly through secretion of various cytokines/chemokines and many other


molecules, promoting tumor cell growth and protecting tumor cells from the immune system and in particular CD8⁺ cytotoxic T lymphocytes (CTLs).

Our current knowledge on TAM suppression of CTLs is largely derived from studies on the role of TAMs in secreting cytokines and chemokines to recruit and activate regulatory T (Treg) cells. Whether and how TAMs directly suppress CTLs are yet to be determined in lung or any other cancer type. Moreover, the intrinsic signaling pathways that switch TAMs from their original pro-inflammatory and anti-tumorigenic M1 phenotype to an anti-inflammatory and pro-tumorigenic M2 phenotype also remain largely unknown.

To address these fundamental questions in the fields of cancer and immunology, we investigated whether RelA functions as an intrinsic driver of macrophages for switching immunity from tumor immunosurveillance to tumor-promoting inflammation. RelA, also known as p65, is the prototypical member of the nuclear factor- κ B (NF- κ B) family of transcription factors, a central mediator of immune and inflammatory responses.¹⁰ Currently, the role of NF- κ B and in particular its prototypical member RelA, in tumor immunology has rarely been studied. So far, there is only one report directly studying

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the role of myeloid RelA in tumor. It showed that by inducing expression of the cytokine tumor necrosis factor alpha (TNF α), myeloid RelA is required to promote proliferation of a xenografted mouse lung cancer cell line.¹¹ However, the roles of RelA in TAM-mediated tumor development, progression, or immune evasion are yet to be investigated. Particularly, whether and how RelA transforms TAMs from anti-tumorigenic into pro-tumorigenic to repress CTLs is yet to be investigated. In addition, xenograft models using established cancer cell lines cannot recapitulate the natural coevolution of cancer and stromal cells as well as their intricate interplay in the complex microenvironment. Especially, they cannot address the roles of immune cells and how different immune cells interact in the early stages of tumorigenesis.

In this study, using endogenously arising lung tumor model, we examined the impact of selective deletion of RelA from myeloid cells on lung inflammation, tumor development and progression. We also investigated the molecular and cellular mechanisms by which RelA drives TAMs to transform immunity from tumor immunosurveillance to tumor-promoting inflammation, with a focus on the role of RelA in the TAM/CTL interaction. Moreover, we examined the activation status of macrophage RelA in human and mouse primary lung cancer tissues, as well as

the association between macrophage RelA activation and lung cancer patient survival.

Results

RelA activation in lung myeloid cells is associated with poor survival of lung cancer patients

To address the role of RelA in myeloid cell regulation and lung cancer, we performed immunocytochemistry (IHC) to examine whether RelA is activated in myeloid cells associated with mouse lung tumors induced by the tobacco carcinogen urethane, a well-accepted and widely used model for human lung cancer. As expected, myeloid cells were readily detected surrounding mouse lung tumors induced by urethane, whereas very few myeloid cells were found in the lung tissues from untreated mice (Fig. 1A). Notably, myeloid cells associated with lung tumors, but not those in normal lung tissues, exhibited strong nuclear expressions of RelA, indicating high RelA activation in those cells. This was confirmed by immunofluorescent (IF) staining of mouse bronchioalveolar lavage (BAL) cells using RelA and F4/80 antibodies (Fig. 1B). The finding is highly relevant to human lung cancer, because the same phenotypes were observed in human lung tissues (Fig. 1C).

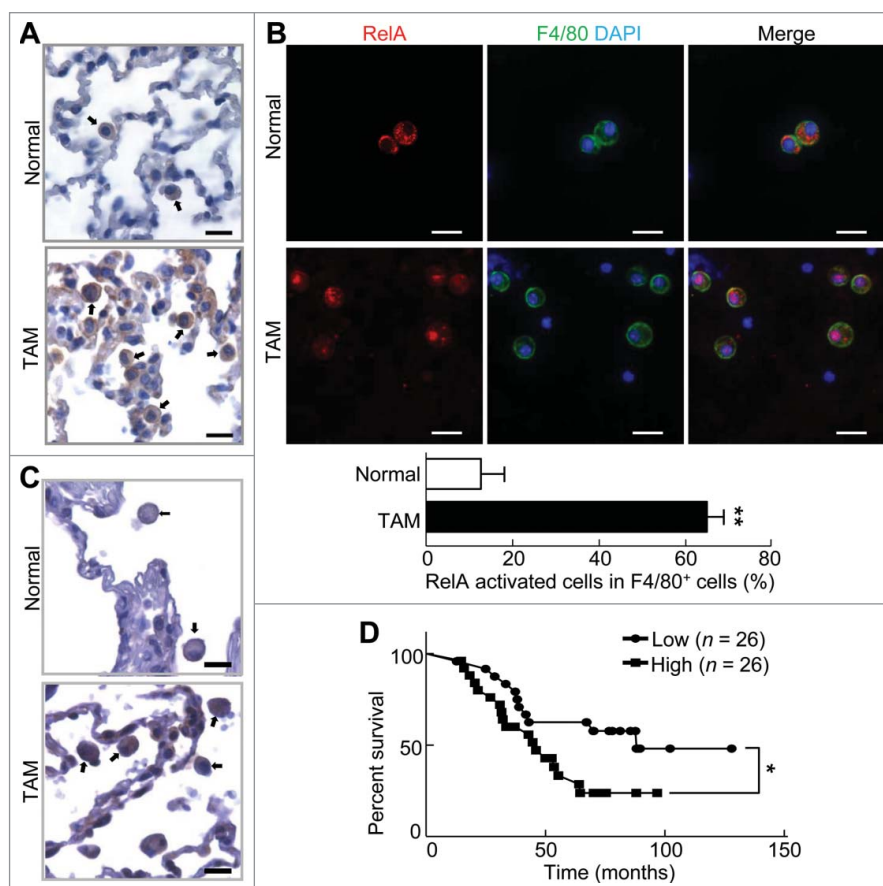


Figure 1. Myeloid RelA activation is associated with poor lung cancer patient survival. (A) IHC staining of RelA in lung tissues from untreated mice or mice with lung tumors induced by urethane. Arrows indicate myeloid cells. Scale bar: 20 μ m. (B) IF staining of RelA and F4/80 in BAL cells from untreated mice or mice with lung tumors induced by urethane. Scale bar: 20 μ m. Data are means \pm SD ($n = 3$ mice, 3–5 images per mouse). (C) IHC staining of RelA in human lung cancer tissues and matched normal human lung tissues. Arrows indicate myeloid cells. Scale bar: 20 μ m. (D) Kaplan-Meier survival curve showing the association between pulmonary myeloid RelA activation and lung cancer patient survival. $p = 0.033$ (Log-rank test); $p = 0.048$ (Gehan-Breslow-Wilcoxon test); $p = 0.046$ (Cox proportional hazards model).

Remarkably, high RelA activation in tumor-associated myeloid cells correlated with poor survival of lung cancer patients (Fig. 1D). These data suggest that RelA activation may function as an intrinsic force of myeloid cells and in particular lung macrophages to promote lung cancer.

Deletion of myeloid RelA leads to decreased TAMs and increased CTL activation

To examine the role of myeloid RelA activation in lung inflammation and tumorigenesis, we generated RelA^{flx/flx}/lysozyme M-Cre (RelA^{ΔMye}) mice, in which RelA is selectively deleted from myeloid cells and in particular macrophages (Fig. 2A–C). RelA^{ΔMye} mice were healthy and showed no apparent abnormalities in myeloid cell development, lung size or morphology (data not shown). RelA^{ΔMye} mice and wildtype (WT) control mice were treated with urethane for lung carcinogenesis as

described in Materials and Methods and then subjected to careful examinations of lung inflammation and cancer.

Compared to WT mice, RelA^{ΔMye} mice had significantly fewer alveolar MACs (AMs) and interstitial MACs (IMs) in their lungs (Fig. 2D; and Supplementary Fig. S1A). On the other hand, other major myeloid cells, such as eosinophils, neutrophils and monocytes, were comparable in WT and RelA^{ΔMye} mice (Fig. 2D; and Supplementary Fig. S1A and B). Since over 95% of F4/80⁺ cells in the lungs were macrophages and all lung macrophages were F4/80⁺, we simply used F4/80 as a lung macrophage marker for further IF assays. In support of the FACS data, IF staining of F4/80 showed significant decrease of macrophages in the BAL fluids (BALF) from urethane-treated RelA^{ΔMye} mice (Fig. 2E). In line with the fact that TAMs mainly exhibit M2 phenotype, most lung macrophages in either the RelA^{ΔMye} or WT mice were positively stained for arginase, a hallmark of mouse M2 macrophages. However, arginase⁺ macrophages were significantly decreased in the RelA^{ΔMye} mice. These

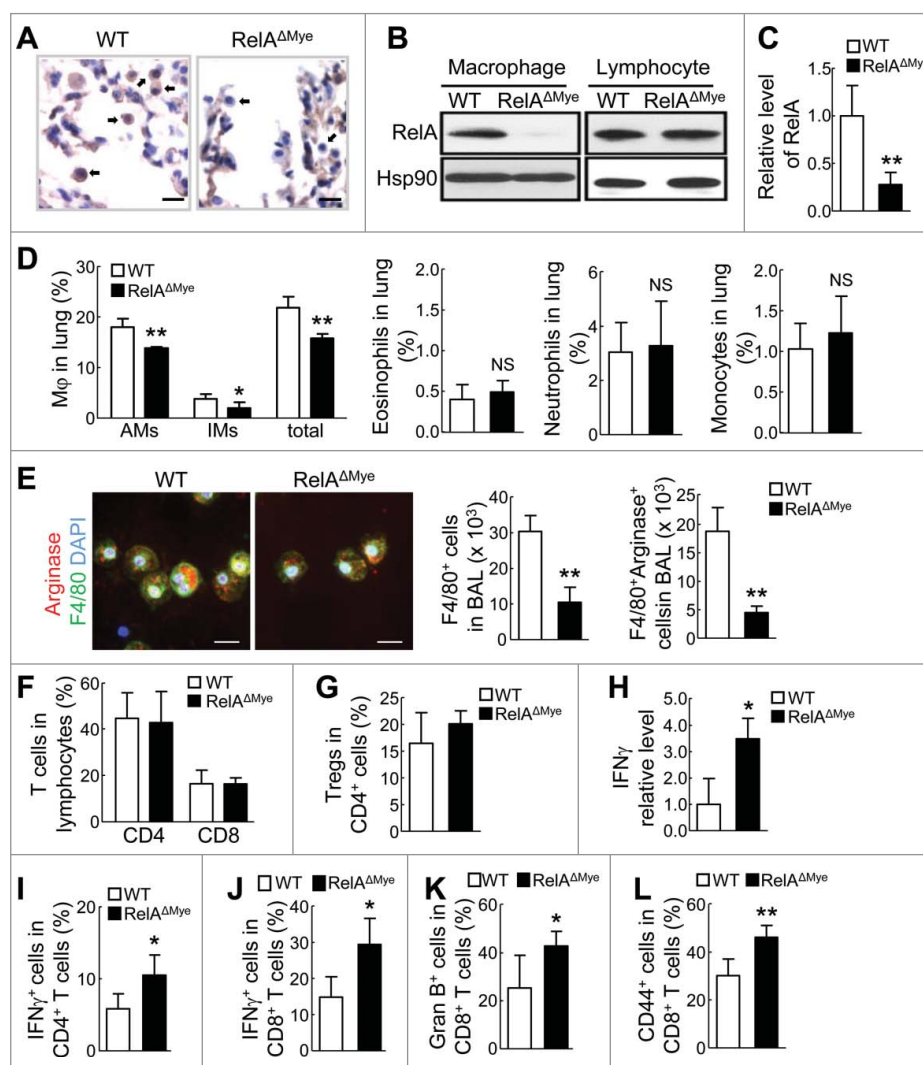


Figure 2. Macrophages are decreased and CTL activation is increased in lungs of RelA^{ΔMye} mice treated with urethane. (A) IHC staining of RelA in lung tissues from urethane-treated WT or RelA^{ΔMye} mice. Arrows indicate myeloid cells. Scale bar: 20 μ m. (B) Immunoblotting of RelA and Hsp90 in peritoneal macrophages and splenic lymphocytes from urethane-treated WT or RelA^{ΔMye} mice. (C) qPCR showing RelA mRNA levels in BAL cells from urethane-treated WT or RelA^{ΔMye} mice. Data are mean \pm SD ($n \geq 4$). (D) Flow cytometry (FACS) of alveolar MACs (CD11b⁺CD11c⁺F4/80⁺Siglec-F⁺), interstitial MACs (CD11b⁺CD11c⁻F4/80⁺Siglec-F⁻), eosinophils (CD11b⁺CD11c⁻F4/80⁺Siglec-F⁺), neutrophils (CD11b⁺Ly6G⁺) and monocytes (CD11b⁺Ly6G⁻Ly6C⁺) from urethane-treated WT or RelA^{ΔMye} mice. Data are means \pm SD ($n \geq 3$). (E) IF co-staining of F4/80 and arginase in BAL cells from urethane-treated WT or RelA^{ΔMye} mice. Scale bar: 20 μ m. Data are means \pm SD ($n = 3$ mice, 4–5 images per mouse). (F–G and I–L) FACS of CD4⁺ and CD8⁺ T cells (F), Treg cells (G), IFN γ ⁺ CD4⁺ T cells (I), IFN γ ⁺ CD8⁺ T cells (J), granzyme B⁺ CD8⁺ T cells (K), and CD44⁺ CD8⁺ T cells (L) in lungs of urethane-treated WT or RelA^{ΔMye} mice. Data are means \pm SD ($n \geq 3$ for (G) and (K), $n \geq 4$ for others). (H) qPCR showing IFN γ mRNA levels in BAL cells of urethane-treated WT or RelA^{ΔMye} mice. Data are means \pm SD ($n \geq 4$). For (C–L), * $p < 0.05$, ** $p < 0.01$, Student's t test.

data together suggest that RelA is mainly involved in the regulation of lung macrophages in lung tumorigenesis.

Although the numbers of lung CD4⁺ T cells or CD8⁺ T cells or Treg cells were comparable in those mice, interestingly, BAL cells from the RelA^{ΔMyc} mice expressed much higher level of the antitumor cytokine interferon- γ (IFN γ) (Fig. 2F-H; and Supplementary Fig. S1C and D). Consistent with the fact that activated type 1 CD4⁺ T-helper (Th1) cells and CD8⁺ T cells are major sources of IFN γ , significantly more lung CD4⁺ and CD8⁺ T cells in the RelA^{ΔMyc} mice were IFN γ ⁺ (Fig. 2I and J; and Supplementary Fig. S1E and F). Moreover, more lung CD8⁺ T cells in the RelA^{ΔMyc} mice expressed the activation markers granzyme B (Gran B) and CD44 (Fig. 2K and L; and Supplementary Fig. S1G and H). Altogether, these data suggest that RelA is required for lung macrophages to repress CTL activation in lung tumorigenesis.

Repression of CTL activation is a major mechanism underlying RelA-driven pro-tumorigenic action of macrophages

In association with decreased lung macrophages and increased CTL activation, interestingly, urethane-treated RelA^{ΔMyc} mice developed fewer and smaller lung tumors (Fig. 3A and B). Histopathological analysis indicated that compared to WT mice, RelA^{ΔMyc} mice had significantly fewer atypical adenomatous

hyperplasia (AAH), adenomas (AD) and adenocarcinomas (AC) in their lungs (Fig. 3C). Not surprisingly, the decreased lung tumor induction in RelA^{ΔMyc} mice was associated with increased apoptosis of tumor cells (Fig. 3D). These data suggest that through repressing tumor cell apoptosis by CTLs, macrophage RelA contributes to both the initiation and progression of lung cancer.

To directly examine the role of the increased CTL activity in the decreased lung tumorigenesis in RelA^{ΔMyc} mice, we generated RelA^{ΔMyc}/CD8^{KO} mice. Consistent with previous studies showing that CD8 is needed only for the development of CTLs but not CD4⁺ T cells,¹² CD8 deletion had no obvious effect on lung CD4⁺ T cells (data not shown). Notably, deletion of CD8⁺ T cells led to a complete recovery of lung tumorigenesis by urethane in RelA^{ΔMyc} mice, as evidenced by the increased lung tumor number and size in urethane-treated RelA^{ΔMyc}/CD8^{KO} mice (Fig. 3A and B). The apoptosis rate of tumor cells in the urethane-treated RelA^{ΔMyc}/CD8^{KO} mice was also reversed to the level in the WT control mice (Fig. 3D). It should be pointed out that CD8⁺ T-cell deletion had no obvious effect on lung tumorigenesis or tumor cell survival in the WT mice (Fig. 3A and B), which further supports our previous finding that CTLs are repressed by RelA-driven macrophages in lung tumorigenesis (Fig. 2 above and Fig. 4–7 below). It should also be pointed out that although the tumor sizes were comparable,

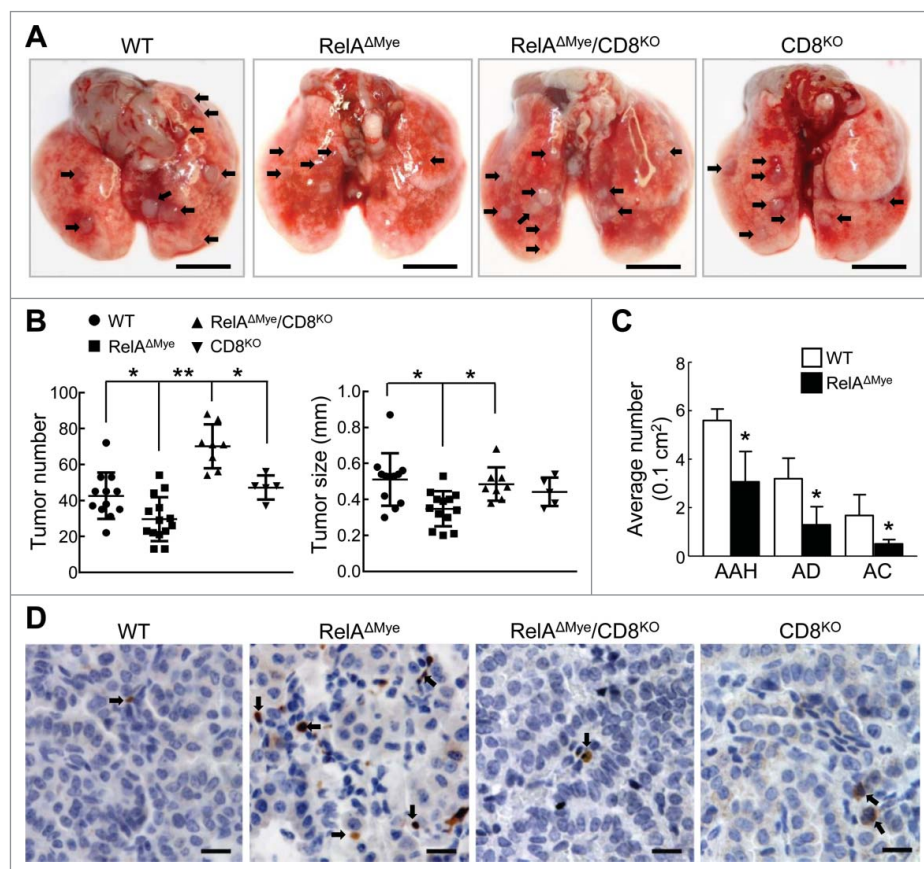


Figure 3. CTL activation plays a causative role in decreased lung tumorigenesis in RelA^{ΔMyc} mice treated with urethane. (A) Representatives of lungs from the indicated mice treated with urethane. Arrows indicate tumors. Scale bar: 5 mm. (B) Lung tumor number and size in the indicated mice treated with urethane. Data are means \pm SD. * p < 0.05, ** p < 0.01, one way ANOVA/Tukey's multiple comparisons test. (C) H&E staining showing numbers of AAH, AD and AC in lung sections (per 0.1 cm²) of the indicated mice treated with urethane. Data shown are means \pm SD ($n \geq 3$; *, p < 0.05, Student's t test). (D) IHC staining of cleaved caspase-3 in lung tumors from the indicated mice treated with urethane. Scale bar: 20 μ m.

RelA^{ΔMyc}/CD8^{KO} mice developed more lung tumors than CD8^{KO} mice, suggesting a CTL-independent, tumor suppressive role for RelA in macrophages. Nevertheless, our data clearly indicate that one major role of macrophage-intrinsic RelA in tumor promotion is to drive these important immune cells to suppress CTL activation.

RelA protects TAMs from CTL killing in lung tumorigenesis by repressing CTL activation and inducing cell survival genes

In line with the decrease in lung macrophages in urethane-treated RelA^{ΔMyc} mice, our IHC and IF staining of cleaved caspase 3 (marker of apoptosis) as well as FACS assays showed dramatically increased apoptosis of lung macrophages in those mice (Fig. 4A-D; and Supplementary Fig. S2). On the other hand, RelA knockout (RelA^{KO}) or WT macrophages exhibited high but comparable migration toward lung tumor cells (Fig. 4E). These data suggest that RelA is required for the survival but not migration of lung macrophages in lung tumorigenesis.

We assumed that the high lung CTL activation accounts for the high apoptosis of TAMs in urethane-treated RelA^{ΔMyc} mice, given the potent cytotoxic activity of CTLs. Currently, it remains unknown whether and how CTLs kill TAMs in any cancer type. Indeed, CD8 deletion completely blocked the increased apoptosis of lung macrophages in urethane-treated RelA^{ΔMyc} mice, leading to a full recovery of lung macrophages (Fig. 4A-D; and Supplementary Fig. S2). These data suggest that the decrease of lung macrophages in urethane-treated RelA^{ΔMyc} mice was fully mediated by CTL killing.

To validate the *in vivo* studies and to investigate the molecular mechanisms by which CTLs specifically recognize and kill TAMs, we tested the potential role of tumor antigens, because one important function of macrophages is to act as professional antigen-presenting cells to induce the antigen-specific cytotoxicity of CTLs. Indeed, macrophages pulsed with lung tumor lysate (Ag-Mφ) could induce strong activation of CD8⁺ T cells *in vitro* (Supplementary Fig. S3A). We used Ag-Mφ-activated CD8⁺ T cells (Ag-T cells) to directly co-culture with RelA^{KO} and WT macrophages with or without pulse treatments. Ag-T cells did not significantly increase the apoptosis of macrophages without tumor antigen pulse, no matter of RelA expression

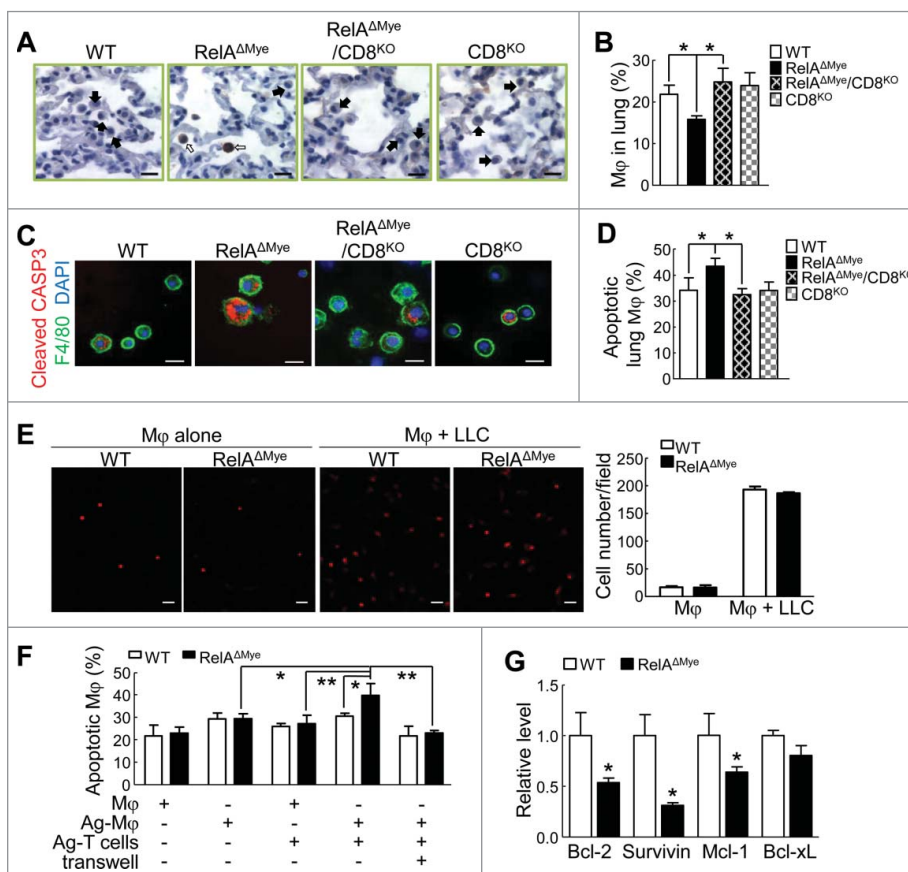


Figure 4. RelA is required for protection of pulmonary macrophages from CTLs in lung tumorigenesis. (A) IHC staining of cleaved caspase-3 in lung tissues from the indicated mice treated with urethane. Filled arrows and open arrows indicate normal and apoptotic myeloid cells, respectively. Scale bar: 20 μ m. (B) FACS of macrophages in the lungs of the indicated mice treated with urethane. (C) IF co-staining of cleaved caspase-3 and F4/80 in BAL cells from the indicated mice treated with urethane. Scale bar: 20 μ m. (D) FACS assays showing apoptosis rates of lung macrophages in the lungs from the indicated mice treated with urethane. Apoptotic macrophages (Annexin V⁺F4/80⁺ cells) were represented as the percentage of total macrophages (F4/80⁺ cells). (E) *In vitro* cell transwell migration assays showing similar migration to lung cancer cells for RelA^{KO} and WT macrophages. PI-stained migrated macrophages were counted and represented as cell number per field. Scale bar: 100 μ m. (F) FACS showing the apoptosis of tumor antigen pulsed or un-pulsed WT or RelA^{KO} macrophages co-cultured with tumor antigen activated CD8⁺ T cells in normal plates or transwell plates. (G) qPCR showing the mRNA levels of the indicated genes in WT or RelA^{KO} macrophages. For (B) and (D-G), data are means \pm SD ($n \geq 3$), * $p < 0.05$, ** $p < 0.01$, one way ANOVA/Tukey's multiple comparisons test (B, D, and F) or Student's t test (E and G).

status (Fig. 4F; and Supplementary Fig. S3B). They also failed to significantly increase the apoptosis of WT macrophages pulsed with tumor antigens. However, they did significantly increase the apoptosis of tumor antigen-pulsed RelA^{KO} macrophages. The apoptosis of tumor antigen-pulsed RelA^{KO} macrophages induced by co-cultured Ag-T cells was completely blocked when their direct contact was blocked using transwell chambers for their co-culture. These data suggest that in addition to killing tumor cells, CTLs exert tumor antigen-specific cytotoxicity toward TAMs, which is inhibited by RelA activation in TAMs.

Given its transcription activity, we examined whether RelA induces anti-apoptotic genes to make TAMs resistant to CTLs. Indeed, several important cell survival genes that are known transcription targets of NF- κ B, including Bcl-2, survivin and Mcl-1, were significantly decreased in RelA^{KO} macrophages, in comparison to WT cells (Fig. 4G). Another well-known NF- κ B target survival gene, Bcl-xL, however, was not significantly decreased. These data suggest that in addition to arming TAMs to suppress CTL activation, RelA also renders those important pro-tumorigenic immune cells resistant to CTL cytotoxicity by inducing expression of multiple cell survival genes.

RelA promotes TAMs to directly repress CTL activation in lung tumorigenesis by inducing B7x and restricting IL-10 expression

To investigate the molecular mechanisms by which RelA drives TAMs to repress CTL activation for lung cancer promotion, we compared the effect of RelA^{KO} and WT macrophage co-culture on CD8⁺ T-cell activation. We used both peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs) for the studies. To mimic lung tumor environment, lung cancer conditioned medium (LCCM) was used for the *in vitro* co-culture. We first tested whether the *in vitro* system recapitulates the *in vivo* situation. Indeed, direct co-culture with WT PMs or BMDMs decreased while with RelA^{KO} PMs or BMDMs increased CD8⁺ T-cell activation stimulated by CD3 and CD28 antibodies (Fig. 5A and Supplementary Fig. S4). Similarly, RelA^{KO} macrophages pulsed with tumor antigens induced higher activation of naïve CD8⁺ T cells, in comparison to tumor antigen-pulsed WT macrophages (Supplementary Fig. S5).

We then used the authentic *in vitro* system to examine the contributions of direct cell-cell contact and paracrine signaling in TAM repression of CTL activation. Remarkably, blockade of direct contact between LCCM-polarized WT PMs or BMDMs and CD3/CD28-stimulated CD8⁺ T cells by culturing them in different transwell chambers led to dramatically increased activation of CD8⁺ T cells (Fig. 5A and Supplementary Fig. S4). These data suggest that the net outcome of paracrine signals from macrophages is actually to induce CD8⁺ T-cell activation, and that cell-cell contact signals from macrophages are able to overturn the paracrine activation resulting in an overall repression of CD8⁺ T cells. It seems that RelA is involved in both confining the paracrine activation and inducing the cell-cell contact inhibition of CD8⁺ T cells by TAMs. Although blockade of RelA^{KO} macrophage/CD8⁺ T-cell contact further increased CD8⁺ T-cell activation, also in a dramatic way, the increased CD8⁺ T-cell activation was significantly lower than that by WT macrophages (Fig. 5B). The

difference in CD8⁺ T-cell activation by RelA^{KO} macrophages and WT macrophages was also much smaller under this cell-cell contact free condition (Fig. 5C).

To investigate the molecular mechanisms of how RelA induces the cell-cell contact inhibition of CTLs by TAMs, we examined the expression levels of PD-L1 (also known as B7-H1), PD-L2, B7x (also called B7-H4 or B7S1) and VSIG4, known cell surface inhibitors of T-cell activation, in the TAMs isolated from urethane-treated WT or RelA^{ΔMye} mice. While PD-L1 and PD-L2 expression was comparable and VSIG4 was undetectable (Fig. 5D, and data not shown), B7x was significantly decreased in RelA^{KO} TAMs (Fig. 5E). The same phenomena were observed in PMs and BMDMs cultured with LCCM *in vitro* (Fig. 5E). Importantly, B7x blockade by a specific neutralizing antibody significantly released CD8⁺ T cells from WT macrophage-mediated suppression (Fig. 5F). To validate the *in vitro* data and also to further determine the significance of B7x induction by RelA in lung tumorigenesis, we used specific neutralizing antibodies to block B7x in WT mice in lung tumorigenesis by urethane (Supplementary Fig. S6). Blockade of B7x led to significantly fewer lung tumors in WT mice (Fig. 5G). In inverse association with the lung tumorigenesis, blockade of B7x significantly increased the activation of pulmonary CD8⁺ T cells in the WT mice (Fig. 5H). These data indicate that RelA induces B7x expression on the cell surface of TAMs to repress CTL activation in lung tumorigenesis.

To identify RelA-targeted secretory molecule(s) in TAMs that are involved in restricting TAM paracrine activation of CTLs, we compared the expression levels of a large panel of cytokines in WT and RelA^{KO} TAMs from urethane-treated mice, including the T-cell activating cytokine interleukin-12 (IL-12) and the T-cell suppressing cytokine IL-10. Among those cytokines we examined, only IL-10 was statistically differentially expressed in RelA^{KO} and WT TAMs (Fig. 6A and B). However, IL-10 was increased, instead of being decreased, in RelA^{KO} macrophages, suggesting that IL-10 may actually contribute to CD8⁺ T-cell activation. Indeed, biological inhibition of IL-10 using a specific neutralizing antibody prevented the activation and tumoricidal activity of CD8⁺ T cells by RelA^{KO} macrophages (Fig. 6C and D). On the other hand, IL-10 activated CD8⁺ T cells and increased their tumoricidal activity (Supplementary Fig. S7). Consistent with the *in vitro* data, *in vivo* blockade of IL-10 led to significantly decreased pulmonary CTL activation and increased lung tumors in RelA^{ΔMye} mice but not in WT mice (Fig. 6E and F). Taken together, these data clearly suggest that RelA induces B7x and represses IL-10 in TAMs to prevent CTL activation respectively in cell-cell contact dependent and independent manners, thereby promoting lung tumorigenesis.

RelA directly binds to the b7x promoter for B7x induction in TAMs and restricts IL-10 expression indirectly via repressing Bcl-3/p50

To examine the molecular mechanisms by which RelA induces B7x and represses IL-10 expression in TAMs for CTL suppression and lung tumor promotion, we performed chromatin immunoprecipitation (ChIP) assays to examine whether RelA directly binds to the *b7x* and *il-10* promoters. Our computer-based promoter analysis revealed that both *b7x* and *il-10*

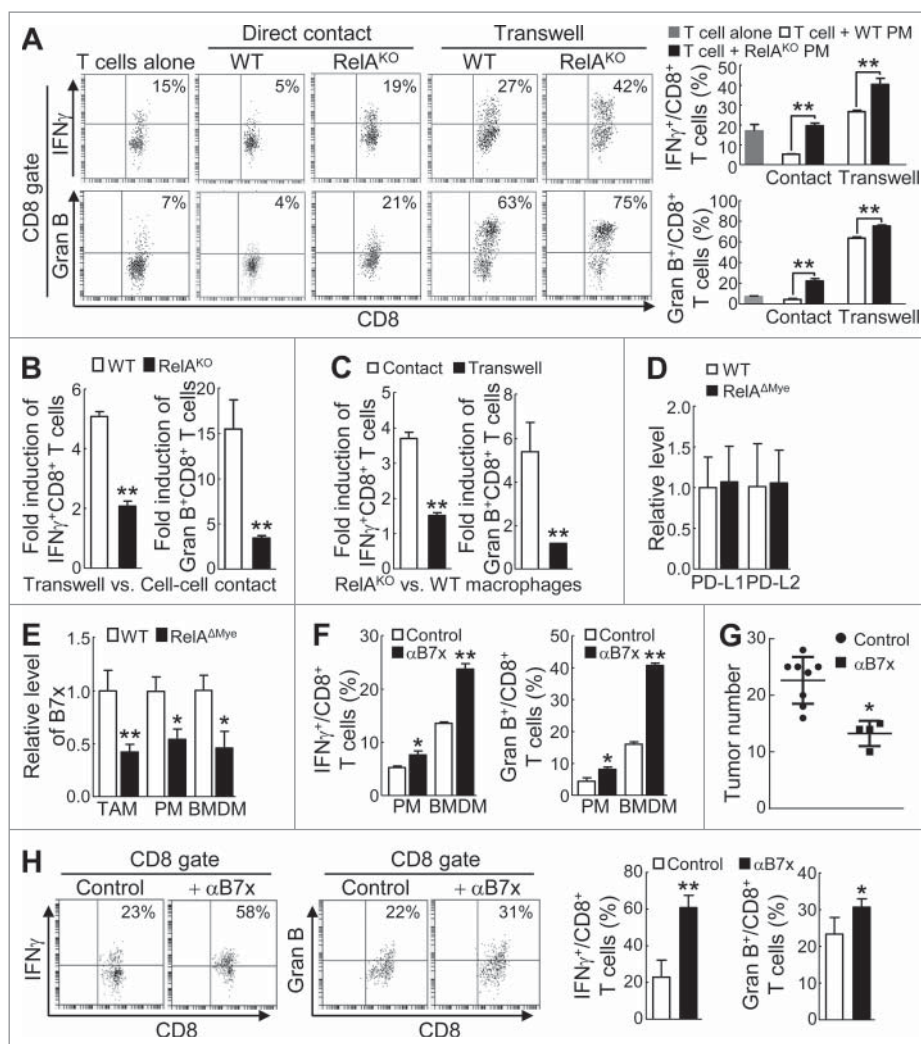


Figure 5. RelA induces B7x to arm macrophages for cell-cell contact inhibition of CTLs in lung tumorigenesis. (A) FACS of IFN γ ⁺ or granzyme B⁺ cells in CD8⁺ T cells co-cultured with WT or RelA^{KO} PMs in normal plates or transwell plates with LCCM containing anti-CD3 and anti-CD28 antibodies. (B) Fold induction of IFN γ ⁺ or granzyme B⁺ CD8⁺ T cells by co-cultured WT or RelA^{KO} macrophages under transwell versus normal cell-cell contact culture conditions. (C) RelA^{KO} versus WT macrophage induction (fold) of IFN γ ⁺ or granzyme B⁺ CD8⁺ T cells under normal cell-cell contact or transwell culture conditions. (D) qPCR showing PD-L1 and PD-L2 mRNA levels in TAMs from urethane-treated WT or RelA ^{Δ Mye} mice. (E) qPCR showing B7x mRNA levels in WT or RelA^{KO} TAMs, LCCM cultured PMs and BMDMs. (F) FACS of IFN γ ⁺ or granzyme B⁺ CD8⁺ T cells in T cells co-cultured with WT PMs or BMDMs in LCCM in normal plates with or without B7x neutralizing antibodies. (G and H) Lung tumor numbers (G) and FACS of IFN γ ⁺ CD8⁺ T cells and granzyme B⁺ CD8⁺ T cells in lungs (H) of WT mice administered with B7x neutralizing antibodies or control antibodies after urethane treatment. In (A-H), data are means \pm SD ($n \geq 4$ for (G), $n \geq 3$ for others). *, $p < 0.05$, **, $p < 0.01$, Student's t test.

promoters contained a putative NF- κ B-binding site (Fig. 7A). We also included NF- κ B1 p50, NF- κ B2 p52, and Bcl3 in our assays. p50 and p52 usually form heterodimers with and are functional partners of RelA, while Bcl3 is the functional partner of p50 or p52 homodimers in inducing gene transcription.¹³ We found that RelA and p50, but not p52 or Bcl3, bound to the NF- κ B-binding site within the *b7x* promoter in WT macrophages cultured with LCCM (Fig. 7B). However, like p52 and Bcl3, p50 also failed to do so in RelA^{KO} macrophages cultured with LCCM. Consistently, RNA polymerase II (Pol II) bound to the *b7x* promoter only in WT but not in RelA^{KO} macrophages. These data suggest that RelA forms heterodimers with and recruits p50 onto the *b7x* promoter to induce B7x expression in TAMs for CTL suppression and lung cancer promotion.

Whereas abundant p52 and relatively low levels of Bcl3 and p50 bound to the *il-10* promoter, RelA was hardly detected at

the promoter in the LCCM-cultured WT macrophages (Fig. 7C). These data were consistent with previous studies showing that the p52/Bcl3 or p50/Bcl3 complex, but not RelA, bound to the *il-10* promoter in a murine macrophage cell line in response to bacterial lipopolysaccharide.¹⁴ In the RelA^{KO} macrophages cultured with LCCM, however, p50, but not p52, was significantly increased at the *il-10* promoter. Bcl3 was also increased at the *il-10* promoter. Further, significantly more Pol II occupied the *il-10* promoter in the RelA^{KO} macrophages. These data suggest that RelA represses p50/Bcl3 promoter recruitment and thereby indirectly restricts IL-10 expression in TAMs in lung tumorigenesis.

To investigate the mechanisms by which RelA represses the promoter recruitment of the p50/Bcl3 complex for IL-10 repression in TAMs, we compared the expression levels of p50 and Bcl3 in WT and RelA^{KO} macrophages cultured in LCCM.

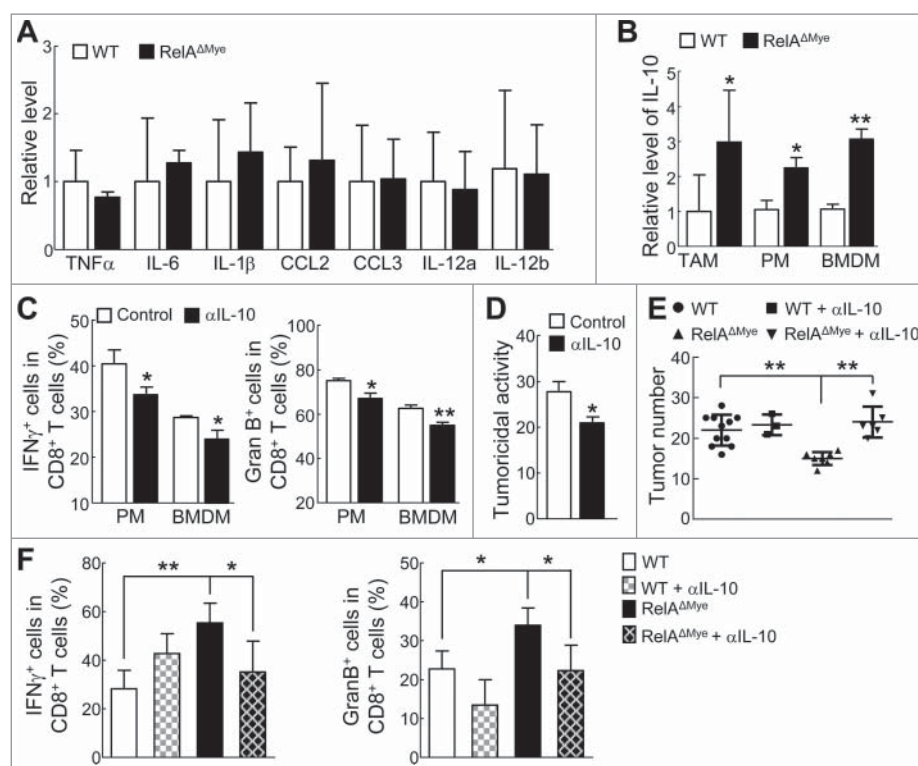


Figure 6. IL-10 activates CTL and RelA represses IL-10 to restrict macrophage-mediated paracrine activation of CTLs in lung tumorigenesis. (A) qPCR analysis showing RNA levels of the indicated cytokine/chemokine in TAMs from urethane-treated WT or RelA^{ΔMyc} mice. (B) qPCR showing IL-10 RNA levels in WT or RelA^{KO} TAMs, as well as PMs and BMDMs cultured with LCCM. (C) FACS of IFN γ ⁺ or granzyme B⁺ cells in CD8⁺ T cells co-cultured in LCCM in transwell plates with RelA^{KO} macrophages in the presence or absence of IL-10 neutralizing antibodies. (D) *In vitro* tumoricidal activity of T cells co-cultured in LCCM in transwell plates with RelA^{KO} macrophages in the presence or absence of IL-10 neutralizing antibodies. (E) Lung tumor numbers in WT and RelA^{ΔMyc} mice administered with IL-10 neutralizing antibodies or control antibodies after urethane treatment. (F) FACS of IFN γ ⁺ CD8⁺ T cells and granzyme B⁺ CD8⁺ T cells in the lungs from WT and RelA^{ΔMyc} mice administered with IL-10 neutralizing antibodies or control antibodies after urethane treatment. In (A-F), data are means \pm SD ($n \geq 3$). *, $p < 0.05$, **, $p < 0.01$, Student's *t* test (A-D), or one way ANOVA/Tukey's multiple comparisons test (E and F).

Different from previous reports showing that over-expressed NF- κ B, particularly RelA, induces p50 and Bcl3 expression,^{15, 16} we found that while the expression of p50 was not statistically different, Bcl3 was significantly increased in RelA^{KO} macrophages in comparison to WT macrophages (Fig. 7D-F). These data suggest that RelA restriction of Bcl3/p50-dependent IL-10 transcription in TAMs for CTL suppression in lung tumorigenesis is indirectly through repressing Bcl3 expression.

Discussion

One of the most fundamental and challenging questions in the cancer and immunology fields is how the immunity in cancer patients is transformed from tumor immunosurveillance to tumor-promoting inflammation. In particular, it remains largely unknown how the antitumor macrophages become pro-tumorigenic to repress the antitumor CTLs, and whether and how this pathogenic process can be blocked or even reversed in cancer patients for cancer treatment. The knowledge is direly needed for successful development of effective cancer immunotherapy. Currently, the overall clinical benefit of cancer immunotherapy is still very limited, particularly for solid tumors, although it is being touted as a breakthrough in cancer treatment.¹⁷ Our studies uncover a direct counterbalance between TAMs and CTLs through previously unidentified mechanisms, and identify RelA as a main culprit tipping the balance toward tumor development. Notably, deletion of RelA from macrophages boosts CTL

antitumor immunity and suppresses lung cancer in a mouse model. These findings are highly relevant to human lung cancer, because RelA is constitutively activated in lung macrophages in lung cancer patients and the RelA activation correlates with poor patient survival.

Most studies on tumor immunology have used tumor implants. Although informative, these studies cannot resemble the natural coevolution of cancer and immune cells. They also cannot address how different immune cells interact to control tumor initiation and progression. Using an endogenously arising mouse lung cancer model, our studies reveal some important but previously unidentified functions for CTLs and TAMs. We find that in addition to their well-known tumoricidal activity, CTLs also kill pro-tumorigenic TAMs in a tumor antigen-specific manner to suppress lung cancer indirectly. On the other hand, TAMs exert a complex role in CTL activation under lung cancer conditions, although the final effect is to repress CTLs. Our current knowledge on macrophage repression of CTLs is very limited and largely relies on the paracrine induction of Treg cells through secretion of IL-10 and transforming growth factor β (TGF β). Notably, we find that IL-10 actually activates CTLs to suppress lung cancer. Currently, this important function of IL-10 has been commonly ignored and drowned out by its well-known anti-inflammatory function, although previous studies from us and others also showed that IL-10 has the potential to stimulate CD8⁺ T cells.^{3, 18–20} Interestingly, TAMs repress CTL activation in a cell-cell contact

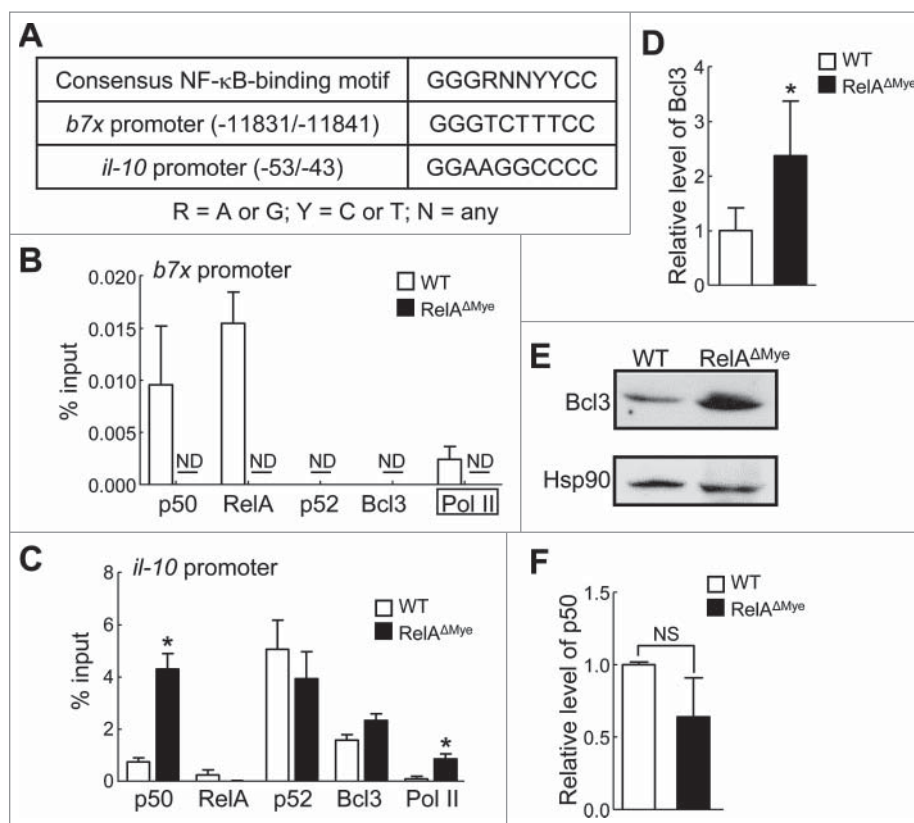


Figure 7. In TAMs RelA induces B7x directly by binding to B7x promoter while represses IL-10 indirectly via repressing Bcl3 transcription and Bcl3/p50-mediated IL-10 transcription. (A) Putative NF- κ B-binding motifs within the *b7x* and *il-10* promoters. (B and C) ChIP of the indicated proteins bound to the *b7x* (B) and *il-10* (C) promoters in WT or RelA Δ Myc macrophages cultured in LCCM. ND, not detected. Primers used for Pol II pull-down in (B) were different, as the NF- κ B-binding site within the *b7x* promoter was far upstream from the transcription site, see (A). (D and F) qPCR showing Bcl3 (D) and p50 (F) mRNA levels in pulmonary macrophages from WT or RelA Δ Myc mice treated with urethane. (E) Immunoblotting of Bcl3 and Hsp90 in WT and RelA Δ Myc macrophages cultured in LCCM. In (B-D) and (F), data are means \pm SD ($n \geq 3$). *, $p < 0.05$, **, $p < 0.01$, Student's *t* test.

manner and this cell-cell contact inhibition dominates TAM paracrine activation of CTLs resulting in the net repression of CTLs during lung tumorigenesis.

We also uncover, for the first time, the detailed mechanisms by which RelA drives TAMs to prevent CTL activation for lung cancer promotion. We find that while directly inducing B7x in TAMs to mediate the cell-cell contact inhibition of CTLs, RelA limits IL-10 expression in TAMs to prevent the paracrine activation of CTLs indirectly via repressing Bcl3 expression and subsequent Bcl3/p50-mediated IL-10 transcription. Of note, RelA is dispensable for the expressions of several other well-known T-cell activation inhibitors, such as PD-L1, PD-L2 and VSIG4, in the TAMs. This identifies B7x as a unique RelA target gene that is important for TAMs to directly suppress CTLs for lung tumorigenesis. RelA also induces the expressions of the cell survival genes Bcl-2, Mcl-1 and survivin, rendering TAMs resistant to CTLs and cancer stress. Although the main role of cell intrinsic RelA is to drive macrophages to suppress CTLs for tumor promotion, RelA also has CTL-independent and even antitumor functions in macrophages. In this regard, after urethane treatment RelA Δ Myc/CD8 Δ KO mice developed lung tumors not only more than RelA Δ Myc mice but also more than WT and CD8 Δ KO mice. Mechanistically, we found that RelA is almost absolutely required for TAMs to express inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of the tumoricidal molecule nitric oxide (NO) (data not shown). Decreased NO production by RelA Δ Myc TAMs

could contribute to the increased lung tumorigenesis in RelA Δ Myc/CD8 Δ KO mice. It is noteworthy that RelA Δ Myc/CD8 Δ KO mice have comparable numbers of TAMs relative to WT and CD8 Δ KO mice. Moreover, increased IL-10 due to RelA deletion in macrophages may also contribute to the increased lung tumorigenesis in RelA Δ Myc/CD8 Δ KO mice. While one key function of IL-10 is to activate CTLs for tumor suppression, particularly when the immune checkpoint inhibitory signal is lost (such as in RelA Δ Myc mice in which the expression of B7x in TAMs is repressed), it also stimulates the survival and proliferation of pre-cancerous and cancer cells in RelA Δ Myc/CD8 Δ KO mice and other conditions that CTLs are inhibited.

It should be pointed out that it currently remains largely unknown whether and how RelA and other NF- κ B members regulate immune cells and in particular macrophages in tumor pathogenesis. Except the xenograft study suggesting that macrophage RelA is required to induce TNF α expression for the proliferation of an established mouse lung cancer cell line,¹¹ only two studies have indirectly linked macrophage NF- κ B to tumorigenesis by using mice in which IKK β , a known NF- κ B-activating kinase,¹³ is selectively deleted from myeloid cells. Similar to the xenograft study, however, these two studies only suggested that macrophage IKK β is indispensable for the carcinogenesis of colon and ovarian cancers by inducing IL-6 or repressing IL-12, respectively.^{21, 22} They did not examine the role of macrophage IKK β in tumor immunology. Moreover, IKK β activates many other signaling pathways important for

immunity and cancer, besides RelA and other NF- κ B members.²³ Indeed, different from those studies, we find that RelA is not required for IL-6 or IL-12 regulation but controls a different set of genes in TAMs for lung cancer promotion. This could be due to the redundant functions with RelA of other NF- κ B members or the roles of NF- κ B-unrelated targets of IKK β in IL-6 and IL-12 regulation. It could also be that IKK β and RelA work as an integrated signaling unit but regulate different genes in different cancers.

Our findings provide a molecular and cellular basis to target RelA for the prevention and treatment of lung cancer. RelA has long been a target of great interest for cancer therapy, given its causative role in lung and many other cancers. However, no clinically practical way is currently available to specifically target NF- κ B in cancer cells. Systemic inhibition of NF- κ B will be too toxic to cancer patients, given the physiological importance of the NF- κ B, particularly RelA. In fact, the function of NF- κ B is very complex, and individual NF- κ B members have different or even opposite functions, although functional redundancy does exist among them. In this regard, we have recently shown that in contrast to RelA, the NF- κ B1 precursor p105 functions as a lung tumor suppressor.²⁴ Since deletion of RelA from myeloid cells suppresses both the initiation and progression of lung cancer, targeting myeloid RelA may provide a novel, feasible and effective approach for lung cancer therapy. It should be pointed out that myeloid RelA could be efficiently knocked down in cancer patients by RelA siRNAs conjugated with the Toll-like Receptor 9 (TLR9) agonists CpG oligonucleotides.²⁵ RelA-based immunotherapy can be used independently or combined with conventional chemoradiotherapies and immunotherapies to treat lung cancer. This is particularly important and interesting, given the critical role of tumor-associated myeloid cells and in particular macrophages in the resistance to chemo- and radio- and immuno-therapies of cancer cells as well as our findings showing the central role of RelA in the functions of TAMs. Moreover, lung tumors are known to be highly resistant to these classical therapies.

In summary, the data presented here demonstrate several novel mechanisms underlying the counterattack between pulmonary macrophages and CD8⁺ T cells, and identify RelA as a major culprit to drive lung macrophages to tilt the balance toward lung cancer development and progression (Fig. 8). Whereas activated CD8⁺ T cells have the potential to kill TAMs and tumor cells in a tumor antigen-specific manner, RelA arms TAMs to suppress CD8⁺ T-cell activation by directly inducing B7x and indirectly restricting IL-10 expression through repressing Bcl3 expression and subsequent Bcl3/p50 transcription of IL-10. RelA also induces several cell survival genes to render TAMs resistant to CD8⁺ T cells. Remarkably, myeloid RelA deletion reverts these pathogenic processes, leading to lung cancer suppression. These preclinical studies greatly increase our understanding of lung cancer immunology and more importantly, suggest a novel, feasible and effective RelA-based immunotherapy for lung cancer prevention and treatment. Given the important roles of tumor-associated myeloid cells in many other solid tumors, these studies are highly relevant to the cancer field at large.

Materials and Methods

Animals and lung carcinogenesis

Lysozyme M-Cre mice, CD8^{KO} mice (Jackson laboratory), and RelA^{flx/flx} mice²⁶ were backcrossed to FVB/N mice for more than ten generations, and then used to generate the experimental mice for lung carcinogenesis as described previously.²⁷ Briefly, mice were intraperitoneally (i.p.) injected with urethane (1 mg/g body weight, Sigma-Aldrich, St. Louis, MO, USA) once a week for six weeks. Mice were sacrificed for lung inflammation and tumor examinations at eight weeks post urethane treatment, except for those also treated with B7x and IL-10 neutralizing antibodies or their control antibodies, which were sacrificed at three weeks post urethane treatment. Surface tumors in mouse lungs were counted by three blinded readers

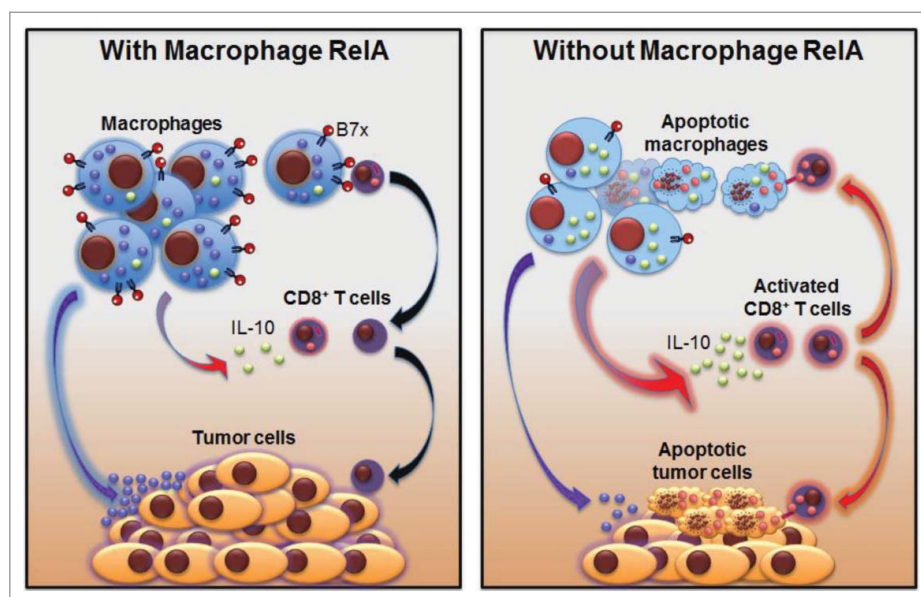


Figure 8. Modeling the roles of macrophage RelA in lung cancer biology and therapy.

under a dissecting microscope, and tumor diameters were measured by microcalipers. For B7x antibody treatment, WT mice were i.p. injected with B7x or control antibodies (200 μg) three times in the first, second and sixth week and two times in the third, fourth and fifth week after the third urethane injection. For IL-10 antibody treatment, RelA ^{ΔMye} mice were i.p. injected with IL-10 or control antibodies (500 μg) twice weekly after the third urethane injection. All animals were maintained and used according to protocols approved by Institutional Animal Care and Use Committee of the University of Pittsburgh. B7x antibodies were generated and purified from the hybridoma cell line 1H3 as described previously.²⁸ IL-10 antibodies were purchased from BioXCell, West Lebanon, NH, USA.

BAL

Upon sacrifice, mice lungs were lavaged with phosphate buffered saline as described.²⁹ The recovered BAL fluids were centrifuged.

IF analysis

Cells were fixed, permeabilized, and subsequently incubated with the indicated primary antibodies, followed by FITC- or TRITC-conjugated secondary antibodies.³⁰ Cells were also counterstained with DAPI for nuclear staining. Stained proteins and their subcellular localizations were detected using a fluorescence microscope.³¹ Antibodies used are listed in Supplementary Table S1.

Histology and IHC and human lung tumor tissue array assays

Lung tissues were excised, fixed in formalin, embedded in paraffin, and cut into 4- μm -thick sections. Sections were stained with H&E, or subjected to sequential incubations with the indicated primary antibodies, biotinylated secondary antibodies and streptavidin-HRP.²⁴ The human lung tumor tissue arrays were described previously.⁴ Briefly, at least 50 myeloid cells were counted in randomly selected microscopic fields in each sample. Cells with obvious nuclear staining of RelA were scored as 2, otherwise, scored as 1. Scores were averaged and used for the cutoff of high (≥ 1.5) and low (< 1.5) RelA activation. Antibodies used are listed in Supplementary Table S1.

Quantitative polymerase chain reaction (qPCR) analysis

The indicated cells were subjected to RNA extraction, RNA reverse transcription and qPCR as described.^{32, 33} Primers for qPCR are listed in Supplementary Table S2.

FACS analysis

The indicated cells were fixed with paraformaldehyde (2%) and permeabilized with saponin (0.5%), or directly treated with the indicated antibodies. Data were acquired using FACSCalibur (BD Biosciences) and analyzed using CellQuest software (Becton Dickinson) as described.³⁴ Antibodies used are listed in Supplementary Table S1.

Peritoneal macrophage (PM) and bone marrow-derived macrophage (BMDM) generation

Five days after intraperitoneal (i.p.) injection of 2 mL of 3% thio-glycollate (Sigma-Aldrich, St. Louis, MO, USA), ice-cold phosphate-buffered saline (PBS) was injected into peritoneal cavity. Peritoneum was gently and completely massaged, and PBS was then recovered from peritoneal cavity. Peritoneal cells were centrifuged and cultured for 24 hours. Non-adherent cells were washed away and the remaining cells were PMs. For BMDM preparation, bone marrow cells were flushed from femurs of mice and cultured for 8 days with 10 ng/mL macrophage colony-stimulating factor (M-CSF). Adherent cells were BMDMs and non-attached cells were washed away. The purities of PMs and BMDMs were $> 90\%$ as confirmed by FACS analysis.

In vitro transwell migration assays

Essentially as described before,⁴ the indicated macrophages were plated in the upper chamber of transwell (with 8- μm pore size membrane) coated with Matrigel (BD Biosciences, Bedford, MA, USA). The lower chambers were seeded with murine Lewis lung carcinoma (LLC) cells. Cells were incubated for 24 hours. Nonmigrated cells were scraped from the upper surface of the membrane with a cotton swab, and migrated cells remaining on the lower surface were stained with propidium iodide (PI).

In vitro tumor antigen-dependent and -independent activation of T cells by macrophages

For tumor antigen-dependent T-cell activation assay, BMDMs from WT or RelA ^{ΔMye} mice were pulsed with LLC lysate (1:3 ratio) for 18 hours, and then co-cultured with splenic CD3⁺ or CD8⁺ T cells from WT mice (1:5 ratio) in the presence of CD40L (0.5 $\mu\text{g}/\text{mL}$) and IL-2 (50 U/mL) for 4 days, followed by FACS analysis to detect granzyme B⁺ and IFN γ ⁺ CD8⁺ T cells. For tumor antigen-independent T-cell activation assay, splenic CD3⁺ or CD8⁺ T cells from WT mice and macrophages from WT or RelA ^{ΔMye} mice (2:1 ratio) were co-cultured in LLC cell-conditioned medium with or without B7x or IL-10 neutralizing antibodies in normal plates or in different chambers of transwell plates (0.4 μm pore size) for 3 days, followed by FACS analysis to detect granzyme B⁺ and IFN γ ⁺ CD8⁺ T cells. CD3e (10 $\mu\text{g}/\text{mL}$) and CD28 (2 $\mu\text{g}/\text{mL}$) antibodies were also added in the co-culture.

In vitro CTL killing of macrophages and tumor cells

RelA^{KO} or WT BMDMs with or without tumor antigen pulse were co-cultured with T cells activated by tumor antigen-pulsed BMDMs (1:2 ratio) for 2 days, followed by FACS analysis to detect apoptotic macrophages. For T-cell tumoricidal activity assays, T cells activated by CD3 and CD28 antibodies were treated *in vitro* with IL-10 or co-cultured with RelA^{KO} macrophages in transwell plates in the presence or absence of IL-10 neutralizing antibodies for three days and then co-cultured with LLC cells stably expressing luciferase (LLC-Luc) in 10:1 ratio in normal plates for 8 hours, followed by detection of luciferase in the culture medium.

ChIP assays

ChIP assays were performed essentially as described.³⁵ Primers for ChIP assays are listed in Supplementary Table S3.

Statistical analysis

Data were reported as mean \pm standard deviation (SD). Student's *t* test (two tailed) and one way ANOVA/Tukey's multiple comparisons test were used to assess significance of differences between two groups and multiple comparisons, respectively. Gehan-Breslow-Wilcoxon test and log-rank test were used to compare overall patient survival between high and low myeloid RelA activation groups. Multivariate survival analysis was also performed using Cox's proportional hazards model to statistically consider and adjust the potential effect of other clinical factors, such as age and tumor stage. The *p* values < 0.05 and 0.01 were considered statistically significant and highly statistically significant, respectively.³⁶

Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

This study was financially supported in part by the National Institute of Health (NIH)/National Cancer Institute (NCI) grants R01 CA172090, R21 CA175252, R21 CA189703, P30 CA047904, and P50 CA090440, as well as the American Lung Association (ALA) Lung Cancer Discovery Award.

Funding

This work was supported by the HHS | National Institutes of Health (NIH).

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Development of methodology: L. Li, L. Han, F. Sun, J. Zhou, Z. Qu, G. Xiao

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Li, L. Han, F. Sun, J. Zhou

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Li, L. Han, F. Sun, J. Zhou, Z. Qu, G. Xiao

Writing, review, and/or revision of the manuscript: Z. Qu, G. Xiao

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