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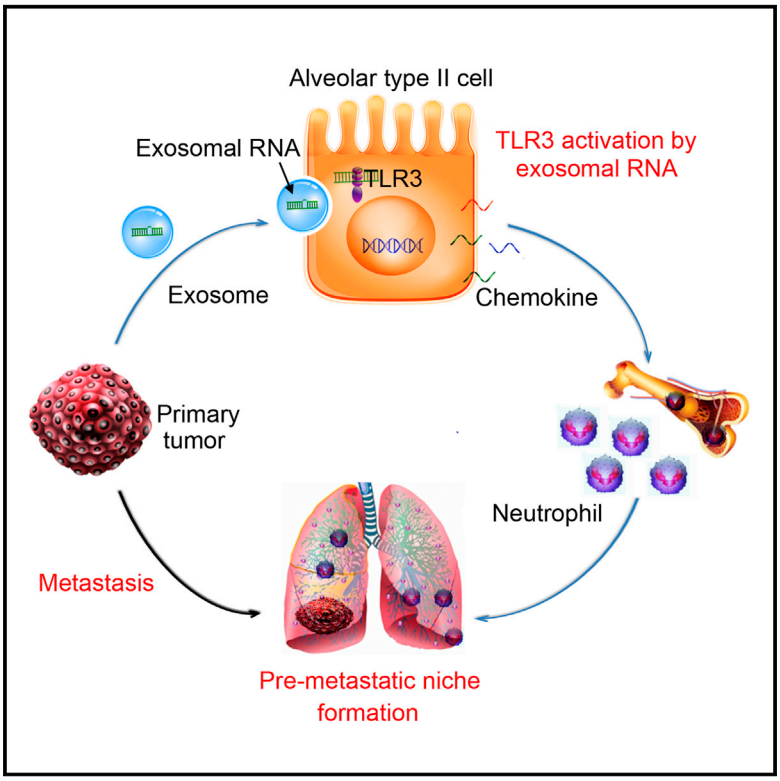
Cancer Cell

Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by Activating Alveolar Epithelial TLR3 to Recruit Neutrophils

Graphical Abstract



Authors

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In Brief

Liu et al. demonstrate that TLR3 in host alveolar epithelial cells is critical for neutrophil recruitment and lung pre-metastatic niche formation. Mechanistically, small nuclear RNAs from primary tumor-derived exosomes activate TLR3, which leads to chemokine secretion and neutrophil infiltration.

Highlights

- TLR3 deficiency reduces lung metastasis in the spontaneous cancer metastatic models
- Host TLR3 promotes lung pre-metastatic niche formation via neutrophil recruitment
- Tumor exosomal RNAs activate alveolar epithelial TLR3 to induce chemokines
- High TLR3 level and neutrophil infiltration in lung cancer predict poor prognosis

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Article

Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by Activating Alveolar Epithelial TLR3 to Recruit Neutrophils

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SUMMARY

The pre-metastatic niche educated by primary tumor-derived elements contributes to cancer metastasis. However, the role of host stromal cells in metastatic niche formation and organ-specific metastatic tropism is not clearly defined. Here, we demonstrate that lung epithelial cells are critical for initiating neutrophil recruitment and lung metastatic niche formation by sensing tumor exosomal RNAs via Toll-like receptor 3 (TLR3). TLR3-deficient mice show reduced lung metastasis in the spontaneous metastatic models. Mechanistically, primary tumor-derived exosomal RNAs, which are enriched in small nuclear RNAs, activate TLR3 in lung epithelial cells, consequently inducing chemokine secretion in the lung and promoting neutrophil recruitment. Identification of metastatic axis of tumor exosomal RNAs and host lung epithelial cell TLR3 activation provides potential targets to control cancer metastasis to the lung.

INTRODUCTION

Tumor metastasis is the leading cause of cancer-related mortality, which involves dissemination of cancer cells to distant organ sites and their adaptation to foreign environments (Hanahan and Weinberg, 2011). Each of these processes is driven by cooperation between tumors and their microenvironments. The primary tumor has been proposed to be able to educate the secondary sites by promoting the formation of supportive metastatic environments, termed the pre-metastatic niche (Kaplan et al., 2005). In detail, bone marrow-derived cells (BMDCs) are mobilized by primary tumor-derived soluble factors to the distinct organ, leading to the formation of a pre-metastatic niche as fertilized soil in preparation for the settlement and further colonization of the metastatic cells. This process involves crosstalk among various tumor-secreted factors, the host stromal microenvironment, and mobilized and recruited BMDCs within future metastatic sites (Erler et al., 2009; Peinado et al., 2012; Psaila and Lyden, 2009). The mechanisms for bilateral interaction between the primary tumor and distinct organs to prime a favorable local microenvironment as a pre-metastatic niche is an important issue that needs to be fully understood.

Different types of BMDCs have been identified as contributing to the stromal remodeling in the pre-metastatic niche (Hiratsuka et al., 2006; Kaplan et al., 2005). However, how host stromal cells in distinct organs, such as epithelial cells in the lung, can sense tumor-derived signals to trigger such BMDC recruitment

Significance

Formation of the pre-metastatic niche is critical for organ-specific metastatic tropism. Using spontaneous metastatic models, we uncovered a significant role of host lung epithelial cell TLR3 in promoting lung pre-metastatic niche formation via tumor exosome-mediated neutrophil recruitment. Small nuclear RNAs enriched in tumor exosomes activated TLR3 in alveolar type II epithelial cells, consequently inducing chemokine secretion and promoting neutrophil recruitment in the lung. In human lung cancers, high expression of TLR3 in adjacent tissues was closely correlated with a high level of neutrophil infiltration, both of which predicted poor survival of the patients. Thus, our findings provide mechanistic insight for pre-metastatic niche formation and organ-site-specific tropisms of metastasis.

for the pre-metastatic niche formation remains poorly defined. The epithelial surface of the lungs, lined by type I and type II epithelial cells, serves as a biological barrier in the respiratory tract (Wagner and Griffith, 2010). In addition to gas exchange and surface tension maintenance, lung epithelial cells also play an essential role in the recognition of pathogen- and injury-associated signals, orchestrating innate immunity in lung to maintain pulmonary homeostasis (Whitsett and Alenghat, 2015). Activation of various pattern-recognition receptors (PRRs) in these cells and subsequent production of cytokines and chemokines induces professional immune cell infiltration (Hartl et al., 2007; Juncadella et al., 2013). Aberrant pro-inflammatory responses to stimuli in lung epithelial cells also contribute to chronic inflammation, indicating a potential role in tumorigenesis (Brody and Steiling, 2011; Takahashi et al., 2010). However, little is known about the regulation of local tumor metastasis by lung epithelial cells.

PRRs are responsible for sensing the presence of molecular components from invading microorganisms or endogenous damaged cells, serving as the sensors for innate immunity. As one of the originally identified and best-characterized PRRs, Toll-like receptors (TLRs) have been shown to recognize various inflammatory innate signals and contribute to the innate defense (Akira and Takeda, 2004; Cao, 2016). Although both anti-tumor and tumor-promoting roles of TLRs have been reported, TLRs have gradually been found to promote multiple tumor process, including tumorigenesis, tumor growth, and metastasis (Pradere et al., 2014). Host TLRs initiate chronic inflammation to promote tumorigenesis (Dapito et al., 2012; Scheeren et al., 2014), while tumor cell TLRs provide intrinsic anti-apoptotic and proliferative signals to support tumor growth and invasion (Cherfils-Vicini et al., 2010; He et al., 2007; Wang et al., 2008). The mechanisms for how host TLRs modulate tumor metastasis attract much attention. For example, tumor-produced factors can activate TLR2:TLR6 complexes on myeloid cells to induce tumor necrosis factor α secretion and thus promote tumor lung metastasis (Kim et al., 2009). Tumor-secreted microRNAs bound to murine TLR7 and human TLR8 in immune cells to trigger tumor metastasis (Fabbri et al., 2012). However, owing to different metastatic models and distinct cells used, the roles of host TLRs in cancer metastasis are complex and even controversial (Pradere et al., 2014). Therefore, we set out to determine how host stromal cell TLRs, once activated by tumor-derived factors, contribute to initiate pro-metastatic inflammatory responses and pre-metastatic niche formation.

RESULTS

TLR3 Deficiency Prevents Lung Pre-metastatic Niche Formation and Metastasis

To obtain an overall view of TLRs except for well-defined TLR2 and TLR7/TLR8 in the metastatic process, we subcutaneously inoculated *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, and *Tlr9*$^{-/-}$ mice with Lewis lung carcinoma (LLC) or B16/F10 melanoma cells and then observed the lung metastasis in the spontaneous metastatic mouse model by surgically removing the primary tumors (Figures S1A and S1B). We found that *Tlr3*$^{-/-}$ mice, but not *Tlr4*$^{-/-}$ and *Tlr9*$^{-/-}$ mice, showed significant reduction in lung metastasis compared with wild-type (WT) littermates (Figures 1A, 1B, and S1C–S1E). Furthermore, *Tlr3*$^{-/-}$ mice survived much longer than WT littermates after tumor removal (Figure 1C). However, no difference was observed in early primary tumor growth in these mouse models (Figure 1D). This observation indicated that deficiency of TLR3 impairs cancer lung metastasis.

The pre-metastatic niche plays a critical role in the metastatic process as it prepares the ''congenial soil'' in distinct organs for tumor metastasis (Kaplan et al., 2005; Sceneay et al., 2013). To examine whether TLR3 could promote pre-metastatic niche formation in the lung, we assessed lung expression of the niche characteristic genes, including *Bv8* (also known as *Prok2*), *S100a8*, *S100a9*, and *Mmp9*, which are reported to promote tumor cell invasion, migration, and colonization in the metastatic site (Wu et al., 2015). We found that the lung from *Tlr3*$^{-/-}$ mice showed significantly lower expression of *Bv8*, *S100a8*, *S100a9*, and *Mmp9* after tumor inoculation (Figure 1E). Furthermore, fibronectin, a protein involved in the adhesion of BMDCs and tumor cells in the pre-metastatic niche (Erler et al., 2009), was downregulated in *Tlr3*$^{-/-}$ mice (Figure 1F). Thus, TLR3 may contribute to lung pre-metastatic niche formation.

TLR3 Deficiency Reduces Neutrophil Recruitment to the Lung

BMDCs are recruited to metastatic organ sites to form a metastatic niche (Kaplan et al., 2005). Thus, we analyzed the cellular composition of the lung in *Tlr3*$^{-/-}$ and WT littermates after tumor inoculation. In WT mice, fluorescence-activated cell sorting (FACS) analysis showed that CD11b^{+} myeloid cells were remarkably expanded in the pre-metastatic lung, among which CD45^{+} CD11b^{+} Ly6G^{+} Ly6C^{int} neutrophils were the most dominant (Figures 2A, 2B, and S2A). Ly6G^{-} Ly6C^{+} monocytes and F4/80^{+} macrophages showed a moderate accumulation in lung (Figures S2C and S2D), while CD11c^{+} Ia/e^{+} dendritic cells showed no difference (Figure S2E). However, both the percentage and number of neutrophils in pre-metastatic lung, as well as peripheral blood and spleen, were significantly reduced in *Tlr3*$^{-/-}$ mice compared with WT littermates (Figures 2A, 2B, and S2B). These data indicate that neutrophils may be the most prominent in pre-metastatic niche formation, although other myeloid cells may be also involved. Indeed, there was still an increase of neutrophils in the *Tlr3*$^{-/-}$ mice upon tumor inoculation compared with that without tumor inoculation (Figure 2A), suggesting that other TLRs, although less profound than TLR3, may also participate in the recruitment of neutrophils.

It has been shown that neutrophils may facilitate cancer lung metastasis (Bald et al., 2014). cKIT^{+} and VEGFR1^{+} myeloid cells are the main BMDCs identified in the metastatic niche (Coffelt et al., 2015; Kaplan et al., 2005). We found that VEGFR1 was expressed in neutrophils and monocytes, while cKIT^{+} neutrophils were significantly expanded in tumor-bearing mice (Figure S2F). BV8 is highly expressed in pro-metastatic neutrophils, which is one of the major components in the niche (Kowanetz et al., 2010). We detected much higher expression of *Bv8* in neutrophils (Ly6G^{+} Ly6C^{+}) than in Ly6G^{-} Ly6C^{+} or Ly6G^{-} Ly6C^{-} cells in tumor-bearing mice (Figure S2G). Therefore, reduced neutrophil recruitment and accumulation in lung may account for decreased lung metastasis in *Tlr3*$^{-/-}$ mice. Together, these data suggest that TLR3 may promote pre-metastatic niche formation by recruiting neutrophils to the lung.

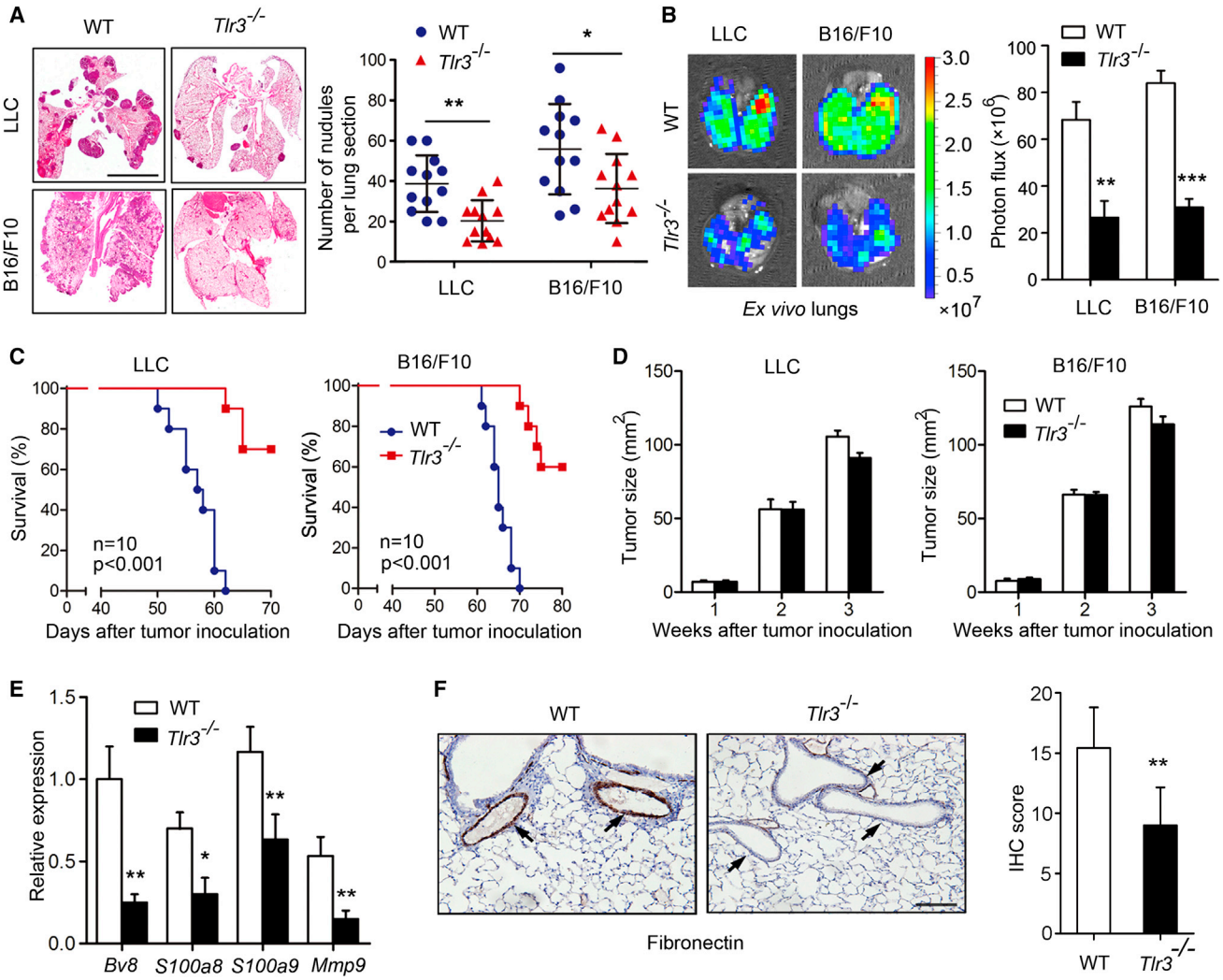

Figure 1. TLR3 Deficiency Prevents Lung Pre-metastatic Niche Formation

(A) H&E-stained lung sections and quantification of lung metastatic foci of *Tlr3*$^{-/-}$ mice or WT littermates (n = 12) after LLC and B16/F10 tumor inoculation. Scale bar, 5 mm.

(B) Representative images and quantitative analysis of lung metastasis of *Tlr3*$^{-/-}$ mice or WT littermates detected by ex vivo luciferase-based bioluminescence imaging after LLC and B16/F10 tumor inoculation.

(C) Survival of *Tlr3*$^{-/-}$ mice or WT littermates (n = 10 each) after LLC and B16/F10 tumor inoculation ($p < 0.001$; Kaplan-Meier test).

(D) Growth curves of tumors arising from inoculation with LLC or B16/F10 tumor cells. Tumor growth was measured with a digital caliper.

(E) Pro-metastatic gene expression in the lung of *Tlr3*$^{-/-}$ mice or WT littermates at 2 weeks after LLC tumor inoculation. The data were normalized to *Bv8* expression of WT mice as shown in the first column. β-Actin was assayed as a control.

(F) Representative images on fibronectin-stained lung sections and quantification of fibronectin expression of *Tlr3*$^{-/-}$ mice or WT littermates at 2 weeks after LLC tumor inoculation. The arrows indicate fibronectin staining. Scale bar, 100 μm.

Data are mean ± SD of one representative experiment. Similar results were seen in three independent experiments. Unpaired Student's t tests unless noted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S1.

Activation of Lung Epithelial TLR3 Induces Chemokine Production and Neutrophil Recruitment

To dissect the underlying mechanisms on impaired neutrophil recruitment in tumor-bearing *Tlr3*$^{-/-}$ mice, we first ruled out the possibility of a defect in neutrophils themselves in *Tlr3*$^{-/-}$ mice. Neutrophils in the bone marrow showed no significant differences in the mice with or without tumor inoculation (Figure S2B). It is known that chemokine and its receptor contribute to neutrophil mobilization and recruitment. However, the expression of chemokine receptors (CXCR1, CXCR2, CXCR4, and CCR2) on neutrophils was much higher in *Tlr3*$^{-/-}$ mice (Figures 2C and S3A). Next, we detected the levels of chemokines (CXCL1, CXCL2, CXCL5, and CXCL12) that are known to be required for neutrophil chemotaxis in the serum and bronchoalveolar lavage fluid (BALF), and found that chemokines from *Tlr3*$^{-/-}$ mice were much lower (Figures 2D, 2E, S3B, and S3C). Bone marrow reconstitution showed that *Tlr3*$^{-/-}$ mice receiving bone marrow of WT littermates exhibited lower chemokine

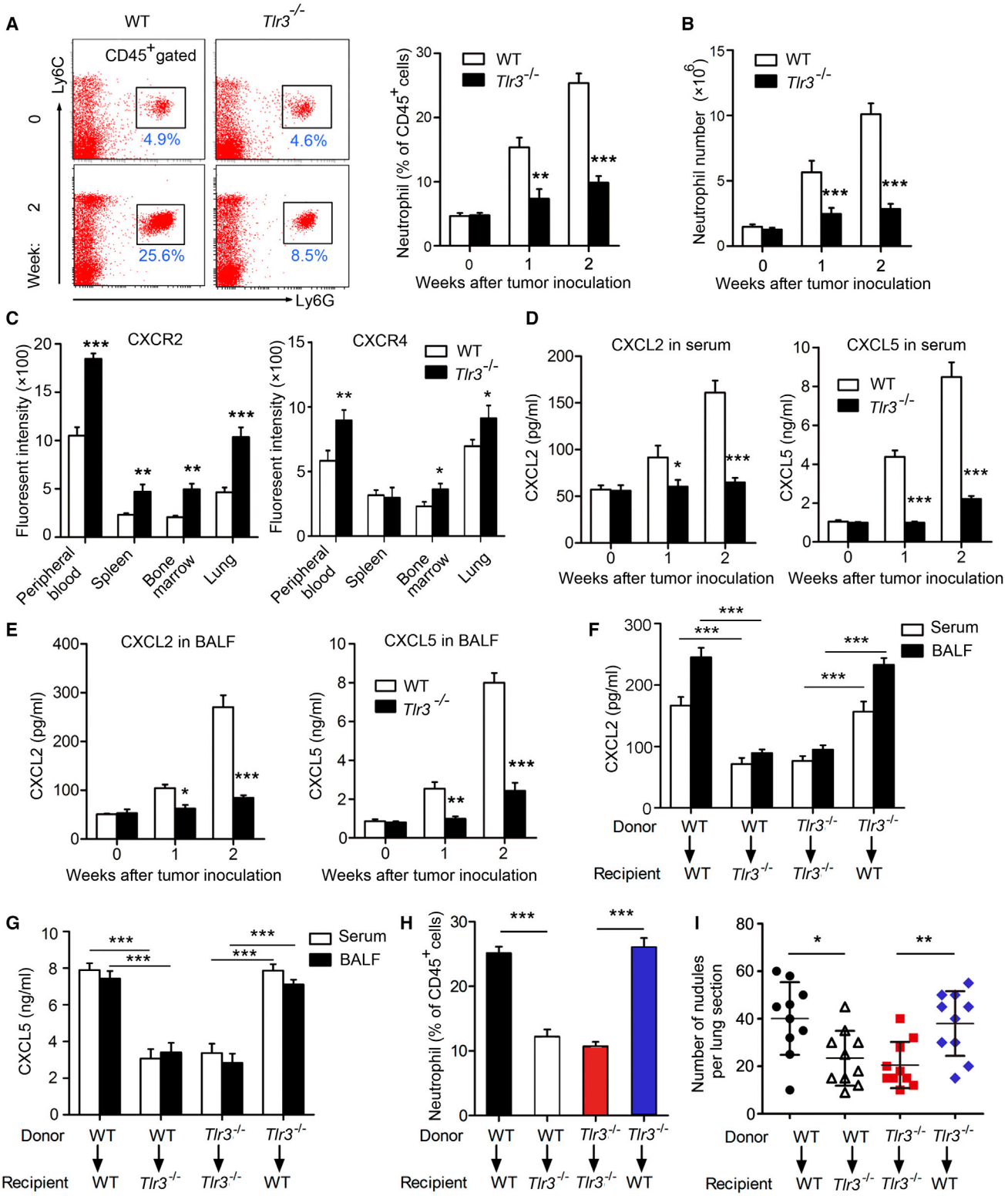

Figure 2. TLR3 Deficiency Reduces Neutrophil Recruitment to the Lung

(A and B) The proportions (A) and absolute numbers (B) of neutrophils in the lung were detected by flow cytometry in *Tlr3*$^{-/-}$ mice or WT littermates after LLC tumor inoculation.

(C) Fluorescence intensity of CXCR2 and CXCR4 expression on neutrophils of *Tlr3*$^{-/-}$ mice or WT littermates after LLC tumor inoculation was detected by flow cytometry.

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levels, less neutrophil recruitment, and less lung metastasis (Figures 2F–2I and S3D). These data suggest that TLR3 present on host stromal cells, but not the TLR3 on BMDCs, is critical for chemokine production and neutrophil recruitment.

qPCR analysis showed that *Tlr3* was highly expressed in lung epithelial cells, dendritic cells, and macrophages, but not in neutrophils and fibroblasts in the lung tissues (Figures 3A and 3B). Immunofluorescence staining demonstrated that TLR3 was mainly expressed in alveolar type II epithelial cells (AT-II) (Figure 3C). AT-II cells constitute 60% of all lung epithelial cells and contribute to respiratory immune regulation (Zanucco et al., 2014). Supporting the in vivo results, gene chip analysis revealed that the involved chemokine transcripts were lower in $Tlr3^{-/-}$ AT-II cells compared with WT AT-II cells in tumor-bearing mice (Figure 3D). Furthermore, the gene-expression pattern in AT-II cells from tumor-bearing mice showed significant overlap with the pattern from synthetic TLR3 ligand double-stranded RNA (dsRNA)-treated lungs (Figure 3E) (Harris et al., 2013), suggesting activation of the TLR3 pathway and its potential role in the pre-metastatic lung. Taken together, these data suggest that diminished capacity of chemokine production by AT-II cells in $Tlr3^{-/-}$ mice may account for the impaired neutrophil recruitment upon tumor inoculation.

To further evaluate the role of host lung epithelial cell TLR3 in the recruitment of neutrophils in the pre-metastatic niche, we delivered recombinant adenovirus to rescue *Tlr3* expression via respiratory tract and depleted neutrophils in vivo by administering anti-Ly6G antibody (Figures 3F and 3G). Adenovirus-mediated in vivo transfection of *Tlr3* in lung via respiratory tract inhalation rescued neutrophil infiltration (Figure 3H) and cancer lung metastasis (Figure 3I) in tumor-bearing $Tlr3^{-/-}$ mice. Depletion of neutrophils (Figure 3J) resulted in remarkable reduction of lung metastasis in the *Tlr3* rescue group (Figure 3K). Thus, TLR3 expression and activation in lung epithelial cells is critical for chemokine production and neutrophil recruitment in promoting pre-metastatic niche formation.

Tumor-Derived Exosomes Mediate Lung Epithelial TLR3 Activation

We then wondered how TLR3 in lung epithelial cells was activated in tumor-bearing mice and became involved in promoting pre-metastatic niche formation. Primary tumors can induce systemic changes by releasing exosomes (Couzin, 2005). Exosomes are lipid-bilayer vesicles containing diverse proteins, RNAs, and DNAs (Liu et al., 2015; Melo et al., 2014). These contents have been shown to be recognized by multiple PRRs (TLR2, TLR4, TLR7, TLR8, RIG-I) (Boelens et al., 2014; Chow et al., 2014; Fabbri et al., 2012). Furthermore, tumor-derived exosomes were able to stimulate BMDC mobilization to form the pre-metastatic niche and even determine organotropic metastasis via their membrane expression of integrins (Hoshino et al., 2015; Peinado et al., 2012). We hypothesized that tumor-derived exosomes might stimulate TLR3 activation and chemokine production in lung epithelial cells, thereby promoting the formation of a pre-metastatic niche. We first compared the content of exosomes collected from serum and lung tissues of WT mice with or without tumor inoculation (Figures S4A and S4B). Exosomes were dramatically enriched in serum and lung tissues after tumor inoculation (Figure 4A).

Next, we examined the role of tumor-derived exosomes in the formation of the metastatic niche in our system. To this end, we first evaluated whether the exogenously administered exosomes could be enriched in lung and taken up by AT-II cells in vivo. Fluorescently labeled exosomes were quickly detected in lung after intravenous injection (Figure 4B). When the exosomes were injected into the site of primary tumors, the exosomes could also be detected in the lung (Figure S4C). Next we analyzed exosome-positive cells in the lung after in vivo administration of PKH67-labeled exosomes. Previous study has shown that lung epithelial cells and fibroblasts were the main cells taking up exosomes in the lung (Hoshino et al., 2015). Our FACS results showed that 41.2% of exosome-positive cells were $SftpD^{+}$ AT-II cells (Figures 4C and S4D).

In vivo administration of exosomes but not control liposomes induced high expression of *Bv8*, *S100a8*, *S100a9*, and *Mmp9*, as well as fibronectin, in lung (Figures 4E–4G). In addition, we found a marked increase in the level of chemokines (CXCL1, CXCL2, CXCL5, and CXCL12) in serum and BALF after administration of exosomes (Figures 4H and S4E). Accordingly, neutrophils were accumulated in the pre-metastatic lung (Figure 4I). Two weeks after administration of exosomes, LLC cells were injected via tail vein and tumor metastasis was evaluated. $Tlr3^{-/-}$ mice had less neutrophil accumulation as well as significantly reduced lung metastasis (Figures 4I and 4J). These data suggest that activation of lung epithelial TLR3 by tumor exosomes is critical for tumor exosome-mediated neutrophil recruitment and pre-metastatic niche formation in the lung.

Exosomal RNAs Activate Lung Epithelial Cell TLR3

Next, we explored how tumor-derived exosomes activate TLR3 in lung epithelial cells to induce chemokine production. We collected exosomes from primary LLC tumors to stimulate the freshly purified AT-II cells and AT-II cell line MLE-12. *Tlr3* and chemokine gene expression can be upregulated after exosome stimulation (Figures 5A–5D), while deficiency of *Tlr3* in AT-II cells or silencing of *Tlr3* in MLE-12 cells diminished the upregulation of chemokines (Figures 5C, 5D, S5A, and S5B).

Exosomes have been shown to contain RNAs (Boelens et al., 2014). Given the role of TLR3 in the recognition of dsRNA from not only virus but also tissue damage (Cavassani et al., 2008; Liu et al., 2008; Nelson et al., 2015), we extracted RNAs from tumor-derived exosomes or primary tumors to identify mediators that stimulated TLR3 activation. Total RNAs were isolated from tumor-derived exosomes or primary tumors, pre-treated with RNase or DNase, and added into the culture system of purified AT-II cells in the presence of RNase inhibitor. *Tlr3* and

(D and E) Quantification of CXCL2 and CXCL5 in serum (D) and bronchoalveolar lavage fluid (BALF) (E) of $Tlr3^{-/-}$ mice or WT littermates after LLC tumor inoculation.

(F–I) Quantification of CXCL2 (F) and CXCL5 (G), proportions of neutrophils in the lung (H), and quantification of lung metastasis (I) of four different groups of bone marrow transplantation (n = 10 each). Data are mean ± SD of one representative experiment. Similar results were seen in three independent experiments. Unpaired Student's t tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figures S2 and S3.

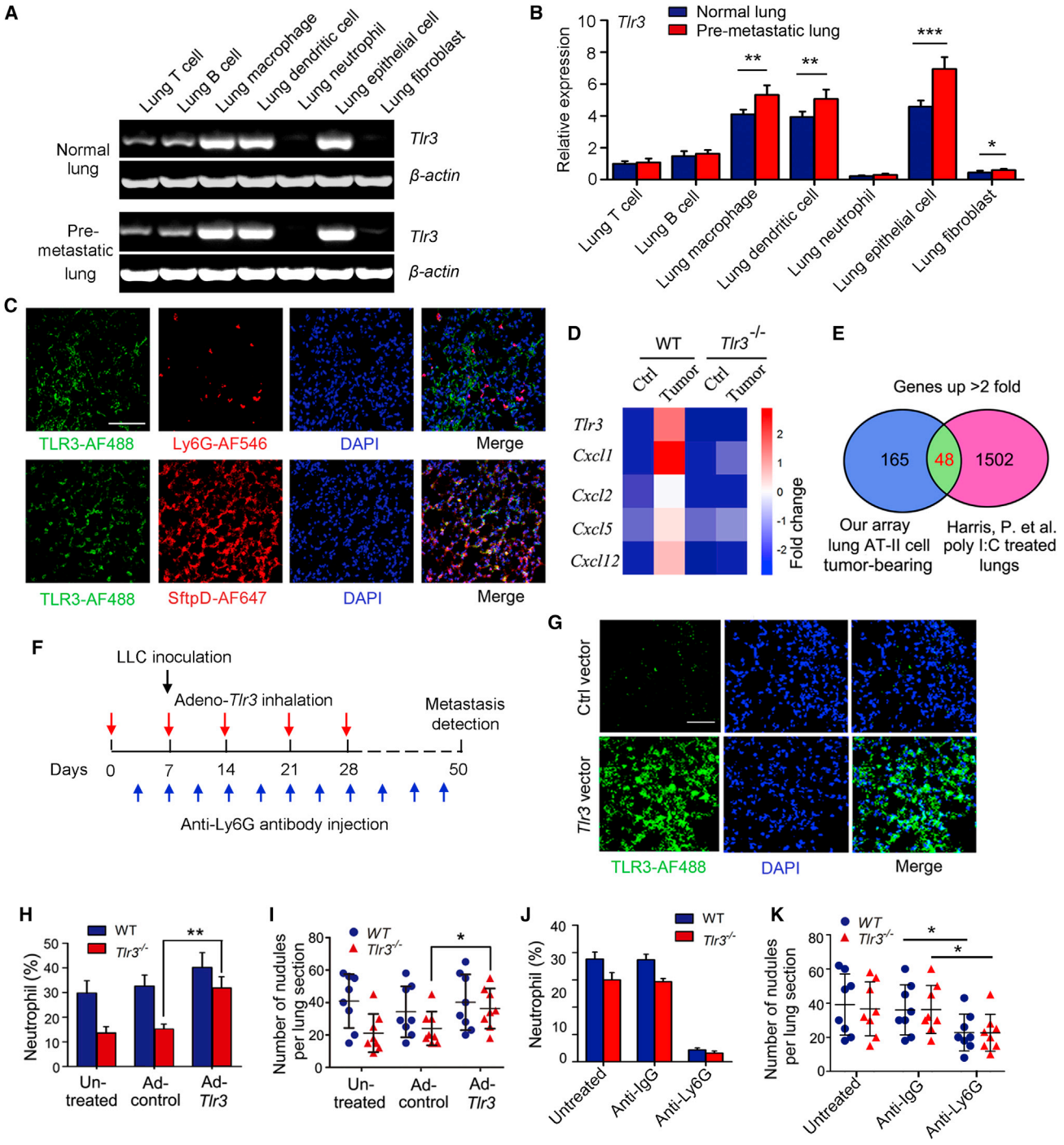

Figure 3. Activation of Lung Epithelial TLR3 Recruits Neutrophils to Lung and Promotes Lung Cancer Metastasis

(A and B) Semi-qPCR (A) and quantification (B) of *Tlr3* expression in different cells purified from normal or pre-metastatic lung collected at 2 weeks after tumor inoculation.

(C) Immunofluorescent analysis of TLR3, Ly6G, and SftpD expression in lung of WT mice. Scale bar, 100 μm.

(D) Gene chip analysis of purified AT-II cells from lung of *Tlr3*$^{-/-}$ mice or WT littermates at 0 or 2 weeks after LLC tumor subcutaneous inoculation.

(E) Venn diagram depicting significant overlap between genes of lung AT-II cells from WT tumor-bearing mice and the lungs treated with poly(I:C) in vivo (Harris et al., 2013 under GEO: GSM960563-68 versus GSM960533-38).

(F) Schematic illustration for the adenovirus-mediated in vivo transfection of *Tlr3* and neutrophil depletion by anti-ly6G antibodies.

(G) Representative immunofluorescent analysis of TLR3 expression in the lung of *Tlr3*$^{-/-}$ mice with or without adenovirus-mediated in vivo transfection of *Tlr3*. Scale bar, 50 μm.

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chemokine gene expression of AT-II cells was upregulated after exosomal RNA (exoRNA) stimulation, which could be blocked by RNase treatment but not by DNase treatment. However, tumor RNA had no effect on these gene expressions (Figures 5E–5G, S5C, and S5D).

Moreover, we analyzed the mechanism for exoRNA-mediated upregulation of TLR3. TLR3 could be induced and upregulated by exogenous and endogenous stimulators through nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Zhu et al., 2015). We found that exoRNA can induce the phosphorylation of the NF-κB p65 subunit and of the MAPKs Erk, Jnk, and p38, in accordance with the effects of poly(I:C) but not tumor RNA (Figure 5H). Inhibition of these signaling pathways indicated that exoRNA-mediated upregulation of TLR3 was dependent on the activation of ERK, p38, and NF-κB signals (Figure 5I). Thus, RNAs in tumor-derived exosomes upregulate TLR3 expression via NF-κB and MAPK pathways in AT-II cells, leading to chemokine production and consequent neutrophil recruitment to form the pre-metastatic niche.

snRNAs Enriched in Tumor Exosomes Are Potential Ligands for TLR3

TLR3 is well known for its role in recognizing dsRNA in viral pathogens and even damaged tissues (Alexopoulou et al., 2001; Cavassani et al., 2008). To identify specific alterations in exoRNA that are responsible for TLR3 activation, we performed a comprehensive sequencing (HiSeq2500) of RNAs isolated from tumor exosomes (exoRNA), taking tumor RNA as a control. Interestingly, distribution of exoRNA fragments was mainly below 200 bp (Figure 6A). Firstly, we systematically analyzed the classification of the sequenced RNA pools. In the exoRNA group more than 70% of the RNAs belong to non-coding RNAs, totally different from those in the tumor group (Figure 6B, left panel). Among the known non-coding RNA classes in the Rfam database, the proportion of tRNA and small nuclear RNA (snRNA), the top two origins of exoRNA, was significantly higher in exoRNA group than in the tumor group (Figure S6). Meanwhile, repeat sequences-derived RNAs (SINE, LINE, LTR, etc.) contributed to a large proportion of the sequenced RNAs in both groups. Secondly, we analyzed in detail the classification of the non-coding RNAs without the tRNA, which mainly localized in cytoplasm, and the results further confirmed that snRNAs were one of the most enriched subtypes (Figure 6B, right panel). TLR3 is shown to be necessary for UV radiation-induced immune responses in skin through recognition of U1 snRNAs (Bernard et al., 2012). Surprisingly, we found that several U1 snRNAs markedly increased in tumor exosomes by more than 1,000-fold compared with that from primary tumor (Figure 6C). These snRNAs show similar secondary structure with stem loops that can form double strands (Figure 6D). Therefore, we identify the highly enriched snRNAs in tumor exosomes as potential ligands for TLR3, which provides an explanation for tumor exosome-triggering TLR3 activation in the alveolar epithelial cells.

Higher TLR3 Expression and Neutrophil Infiltration Predict Poor Prognosis of Lung Cancer Patients

To extend our findings to human cancers, we investigated the relationship between TLR3 expression and neutrophil infiltration in tumor-adjacent tissues and clinical outcomes in a cohort of 90 lung cancer patients with non-small cell lung carcinoma (NSCLC) (Table S1). High expression of TLR3 in adjacent tissues of lung cancers was closely related to massive neutrophil infiltration (Figures 7A–7C). Also, high levels of both TLR3 and neutrophils were related to enhanced expression of pro-metastatic S100A8 and S100A9, indicating their roles in pre-metastatic niche formation (Figures 7D and 7E). Furthermore, reduced overall survival was found in lung cancer patients with higher TLR3 expression or more neutrophil infiltration in tumor-adjacent tissues, which were also independent prognostic parameters indicated by multivariate analysis (Figure 7F and Table S2). These clinical data suggest that two parameters, increased TLR3 expression and massive neutrophil infiltration, may predict the poor prognosis of lung cancer patients. Further analysis of TLR3 expression and neutrophil infiltration in metastatic tissues and their correlations with the prognosis of cancer patients is warranted.

In sum, we show that activation of TLR3 in host lung epithelial cells by tumor exoRNAs can initiate neutrophil recruitment and pro-metastatic inflammation (Figure 7G), indicating a profound and selective role of host stromal TLRs in inducing the inflammatory cascade in distinct organ and pre-metastatic niche formation.

DISCUSSION

Recognition and sensing of primary tumor-derived signals to initiate subsequent immune responses in distinct organs is a critical but poorly defined process in pre-metastatic niche formation. TLRs can recognize exogenous and endogenous stimulators to prime immune responses, and certain types of TLRs have been shown to contribute to multiple tumor progression. Despite their roles in anti-tumor innate immunity, TLRs are critical for priming tumor-promoting inflammation and providing cancer survival signals (Pradere et al., 2014). In this respect, TLR-mediated chronic inflammation is highly related to tumorigenesis (Dapito et al., 2012; Scheeren et al., 2014). As the sensors for dsRNA, the function of TLR3 in tumor cells is well defined due to its high intrinsic expression. Tumor TLR3 can improve the sensitivity to poly(I:C)-mediated cancer therapy (Chin et al., 2010; Forte et al., 2012). However, others show that activation of TLR3 and its downstream pathway in tumors promotes tumor cell survival, proliferation, and chemotherapy resistance (Hasan et al., 2007; Sistigu et al., 2014). In this study, we focused on the

(H and I) Neutrophil proportions in lung (H) and quantification of lung metastasis (I) collected from *Tlr3*$^{-/-}$ mice or WT littermates with or without adenovirus-mediated in vivo transfection of *Tlr3* (n = 8 each).

(J and K) Neutrophil proportions in lung (J) and quantification of lung metastasis (K) of WT or *Tlr3*$^{-/-}$ mice with adenovirus-mediated in vivo transfection of *Tlr3* in the absence or presence of neutrophil depletion (n = 8 each).

Data are mean ± SD of one representative experiment. Similar results were seen in three independent experiments. Unpaired Student's t tests unless noted. *p < 0.05, **p < 0.01, ***p < 0.001.

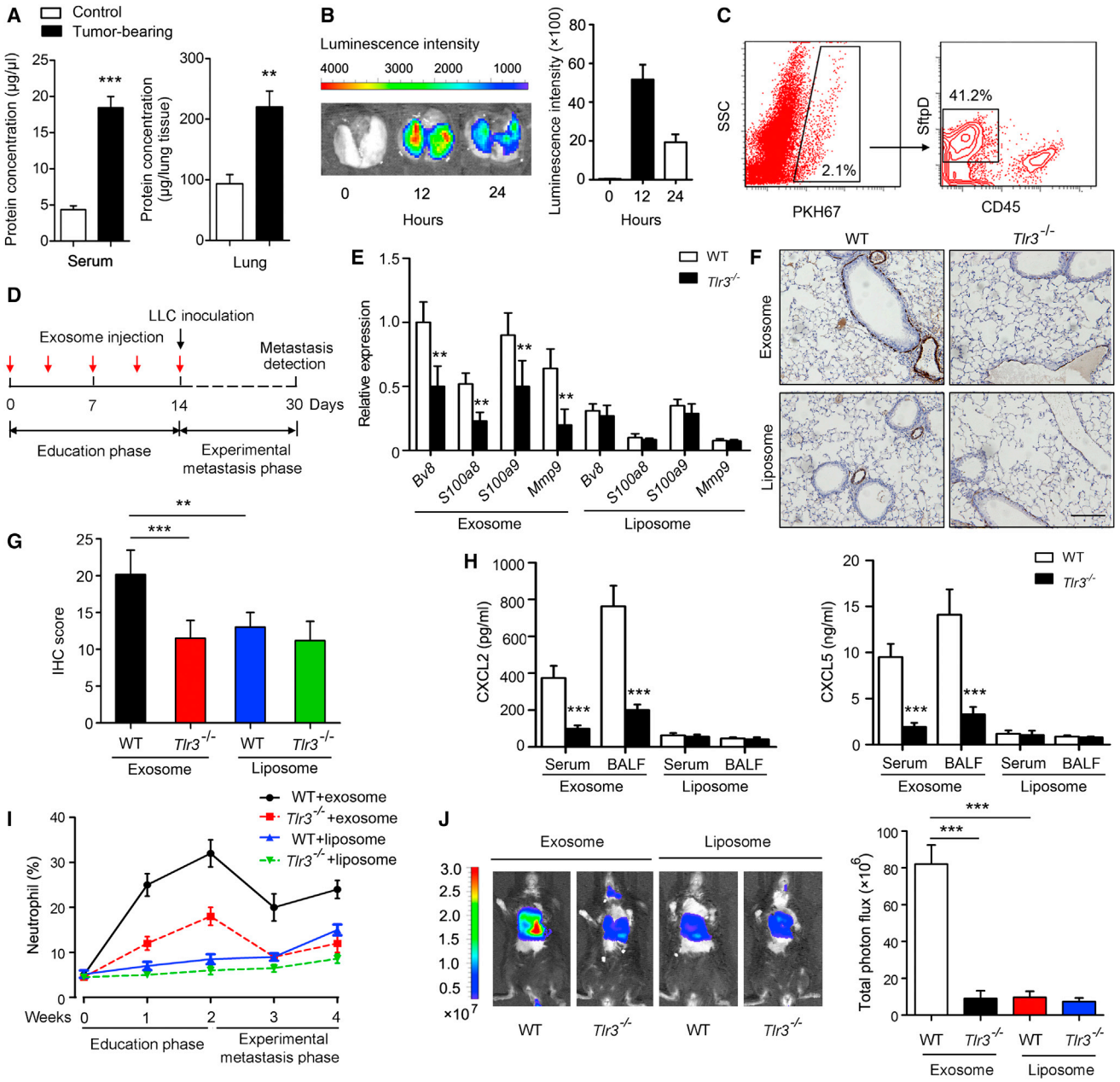

Figure 4. In Vivo Administration of Exosomes Activates TLR3 in Lung Epithelial Cells to Induce Chemokine Production and Neutrophil Recruitment

(A) Protein concentrations of purified exosomes from serum and lung of the mice with or without LLC tumor inoculation.

(B) Tracing Vivotrack 680-labeled LLC-derived exosomes in lung via tail vein injection by ex vivo luciferase-based bioluminescence imaging.

(C) PKH67-labeled LLC-derived exosome incorporation by SftpD$^+$ AT-II cells of lung was detected by flow cytometry.

(D) Schematic illustration for the phases of exosome education and experimental metastasis.

(E) Pro-metastatic gene expression in the lung of *Tlr3*$^{-/-}$ mice or WT littermates at 2 weeks after exosome or liposome administration was quantified by qPCR. The data were normalized to *Bv8* expression of WT mice treated with exosomes as shown in the first column. β-Actin was assayed as a control.

(F and G) Representative images on fibronectin-stained lung sections (F) and quantification of fibronectin expression (G) of *Tlr3*$^{-/-}$ mice or WT littermates at 2 weeks after exosome or liposome administration. Scale bar, 100 μm.

(H) Quantification of serum or BALF levels of CXCL2 and CXCL5 in *Tlr3*$^{-/-}$ mice or WT littermates after exosome or liposome administration.

(I) Proportions of neutrophils in lung of *Tlr3*$^{-/-}$ mice or WT littermates after exosome or liposome administration and LLC injection.

(J) Quantification of lung metastasis of *Tlr3*$^{-/-}$ mice or WT littermates after exosome or liposome administration by luciferase-based bioluminescence imaging.

Data are mean ± SD of one representative experiment. Similar results were seen in three independent experiments. Unpaired Student's t tests. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.

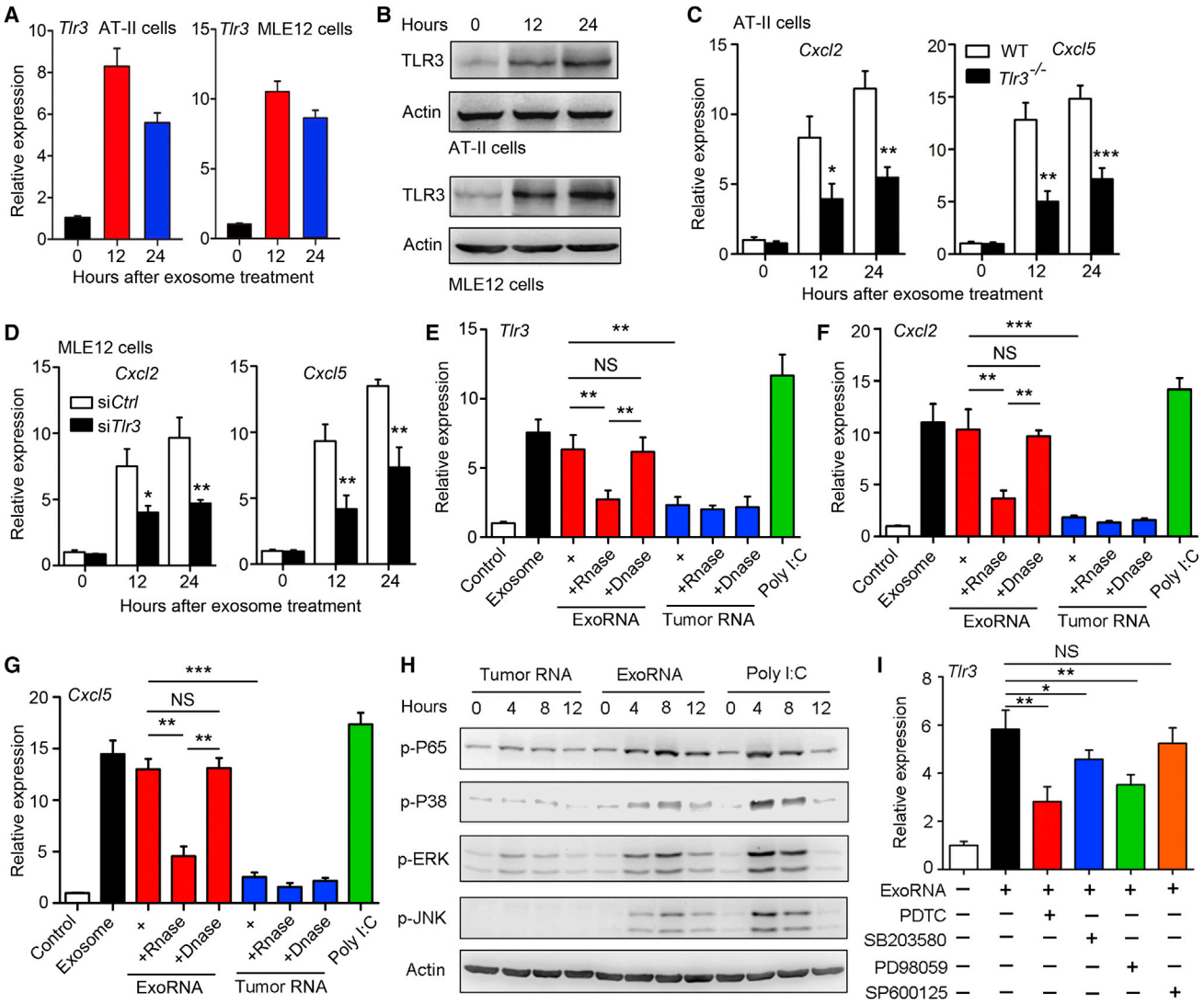

Figure 5. Exosomal RNAs Activate TLR3 in Lung Epithelial Cells to Induce Chemokine Expression

(A and B) mRNA expression (A) and protein level (B) of TLR3 of purified AT-II or MLE-12 cells stimulated by tumor exosomes.

(C) mRNA expression of *Cxcl2* and *Cxcl5* of AT-II cells purified from *Tlr3*$^{-/-}$ mice or WT littermates after exosome stimulation.

(D) mRNA expression of *Cxcl2* and *Cxcl5* of MLE-12 cells with or without *Tlr3* silencing after exosome stimulation.

(E–G) mRNA expression of *Tlr3* (E), *Cxcl2* (F), and *Cxcl5* (G) in the purified AT-II cells stimulated with tumor exosomal RNAs (exoRNA), tumor RNA, or poly(I:C), pre-treated with or without RNase or DNase.

(H) Phosphorylation of p65, p38, ERK, and JNK in purified AT-II cells stimulated with exoRNA, tumor RNA, or poly(I:C) was detected by immunoblot. β-Actin was used as control.

(I) Purified AT-II cells were pre-treated with PDTC, SB203580, PD98059, or SP600125, followed by stimulation with exoRNA. Expression of *Tlr3* was analyzed by qPCR.

Data are mean ± SD of one representative experiment. Similar results were seen in three independent experiments. Unpaired Student's t tests, NS, not significant. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S5.

role of host stromal (lung epithelial) TLR3 in cancer metastasis. TLR3 is highly expressed in lung epithelial cells (Handke et al., 2009), and the lung epithelial TLR3 pathway is activated on exposure to virus, fungus, and even cigarettes (Babiceanu et al., 2013; Wortham et al., 2013). Interestingly, high epithelial TLR3 expression is correlated with smoke-induced chronic inflammation in the lung (Milara et al., 2015). Given the tumor-promoting role of chronic inflammation, host TLR3 may participate in local immune responses to tumors. Using a gene microarray, we found that the TLR3 pathway in lung epithelial cells was activated after subcutaneous tumor inoculation. In addition, tumor-derived exosomes were the stimulator.

Exosomes are rich in multiple RNAs and contain different transcript patterns with primary tumor cells as determined by exoRNA sequencing, and TLR3 was identified as one of the top two contributors in PRRs for the sensing of exosomes to stimulate downstream pathways in breast cancer cells (Boelens et al., 2014). Here, we showed that exosomes could be taken up

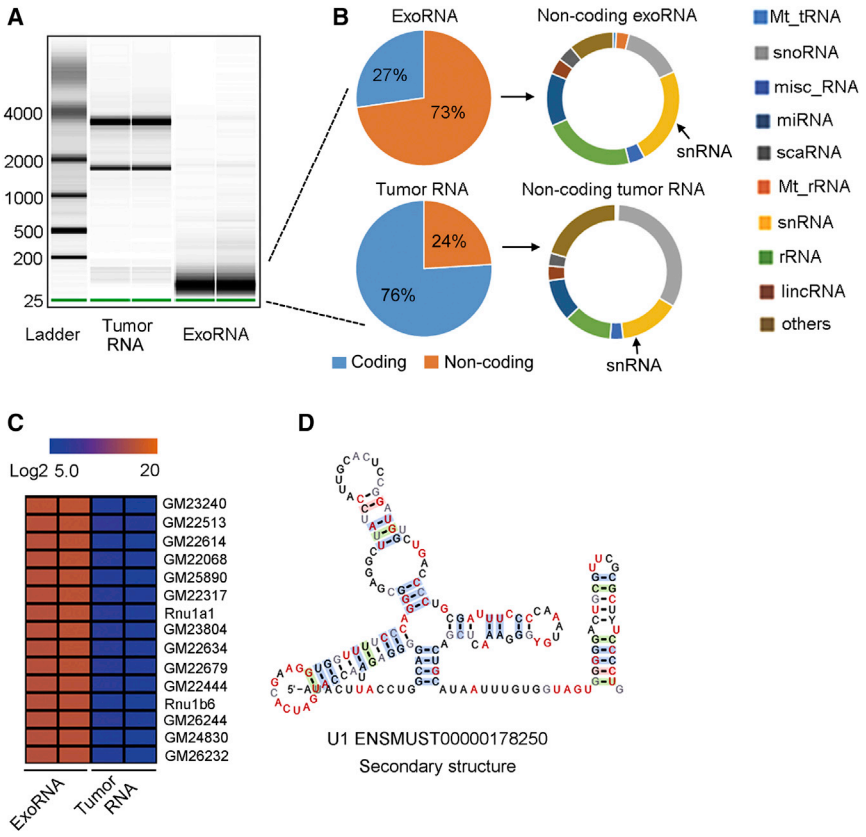

Figure 6. snRNAs Enriched in Tumor Exosomes Are Potential Ligands for TLR3

(A) Expression pattern of exoRNA and tumor RNA was shown by electropherogram.

(B) Distribution of the coding and non-coding gene transcripts from exoRNA or tumor RNA (left) and major classes of non-coding elements (right).

(C) Heatmap of the top 15 snRNA enriched in exoRNA, compared with that in tumor RNA.

(D) Representative secondary structure of U1 snRNA (gene ID: ENSMUST00000178250) is downloaded from http://asia.ensembl.org/.

See also Figure S6.

by lung epithelial cells and that exosome-mediated chemokine gene expression was dependent on TLR3 as confirmed by in vivo and in vitro studies. TLR3 can be activated by mRNAs derived from damaged tissues and necrotic cells (Cavassani et al., 2008). However, little is known about the specific RNAs and structure bases for TLR3 activation independent of viral infection. Recent evidence showed that TLR3 may recognize U1 snRNA released from keratinocytes after UV radiation exposure (Bernard et al., 2012). Interestingly, through RNA sequencing we found significant enrichment of non-coding transcripts, especially snRNAs, in tumor-derived exosomes. These snRNAs contain stem loops that can form double strands, the structural bases for TLR3 recognition. These data provide evidence to support our findings about tumor exoRNA-mediated TLR3 activation. Thus, we identify an important role of host stromal TLR3, but not tumor TLR3, in sensing tumor exoRNAs, initiating the inflammatory cascade and promoting pre-metastatic niche formation.

TLRs are functionally plastic in the tumor microenvironment. TLR3 has been proposed to inhibit tumor metastasis by activating natural killer (NK) cell responses in an experimental metastatic model (Guillerey et al., 2015). However, the immune response or status in the experimental metastatic mouse model via intravenous injection of tumor cells was different from that in the spontaneous metastatic mouse model (Khanna and Hunter, 2005). TLR3-activated NK cells are involved in the resistance of the experimental metastatic mice in response to the stressful tumor challenge via intravenous injection. There is also the possibility that TLR3 may exert its function in an alternative tumor microenvironment of different metastatic models and different tumor stage. It is generally accepted that TLRs, along with tumor progression, are critical for the induction of the inflammatory cascade in the metastatic niche and creation of an immunosuppressive microenvironment for tumor metastasis (Fabbri et al., 2012; Kim et al., 2009).

As the physiological barrier of the respiratory tract, lung epithelial cells may also orchestrate pulmonary immunity in response to infection and injury (Whitsett and Alenghat, 2015). However, little is known about their roles in modulating the local environment for tumor metastasis. Here, we reported that lung epithelial cells were essential for primary tumor-induced neutrophil recruitment in lung and subsequent pre-metastatic niche formation. An explanation of this phenomenon may lie in the innate immune function and high phagocytic ability of lung epithelial cells (Korfhagen et al., 2012). We and others found that they were one of the main cells that can take up exosomes in lung upon in vivo administration of exosomes (Hoshino et al., 2015). Therefore, lung epithelial cells can be activated via its PRRs to sense the signals from tumor exosomes, consequently initiating neutrophil recruitment and pro-metastatic inflammatory responses. It would be interesting to investigate the detailed function and mechanism of the remodeling of local metastatic environments elicited by lung epithelial cells.

Activation of TLR3 in lung epithelial cells is found to promote neutrophil recruitment in the pre-metastatic lung. Neutrophils, which are recruited in the niche, can alter their polarization state in the tumor-bearing host, switching from suppressing to promoting roles in tumor metastasis (Fridlender et al., 2009; Liu and Cao, 2016). Efficient neutrophil chemoattraction and cytotoxicity to tumor cells is important for the control of tumor growth and metastasis (Granot et al., 2011). However, increasing evidence suggests that neutrophils can elicit a pro-metastatic inflammatory microenvironment by suppressing innate and adaptive anti-tumor immunity (Coffelt et al., 2015; Cools-Lartigue et al., 2013; Wu et al., 2015). Neutrophils were recently shown to be the main driver of metastasis in the pre-metastatic lung by supporting lung colonization of metastasis-initiating cancer cells (Wculek and Malanchi, 2015). By depletion of

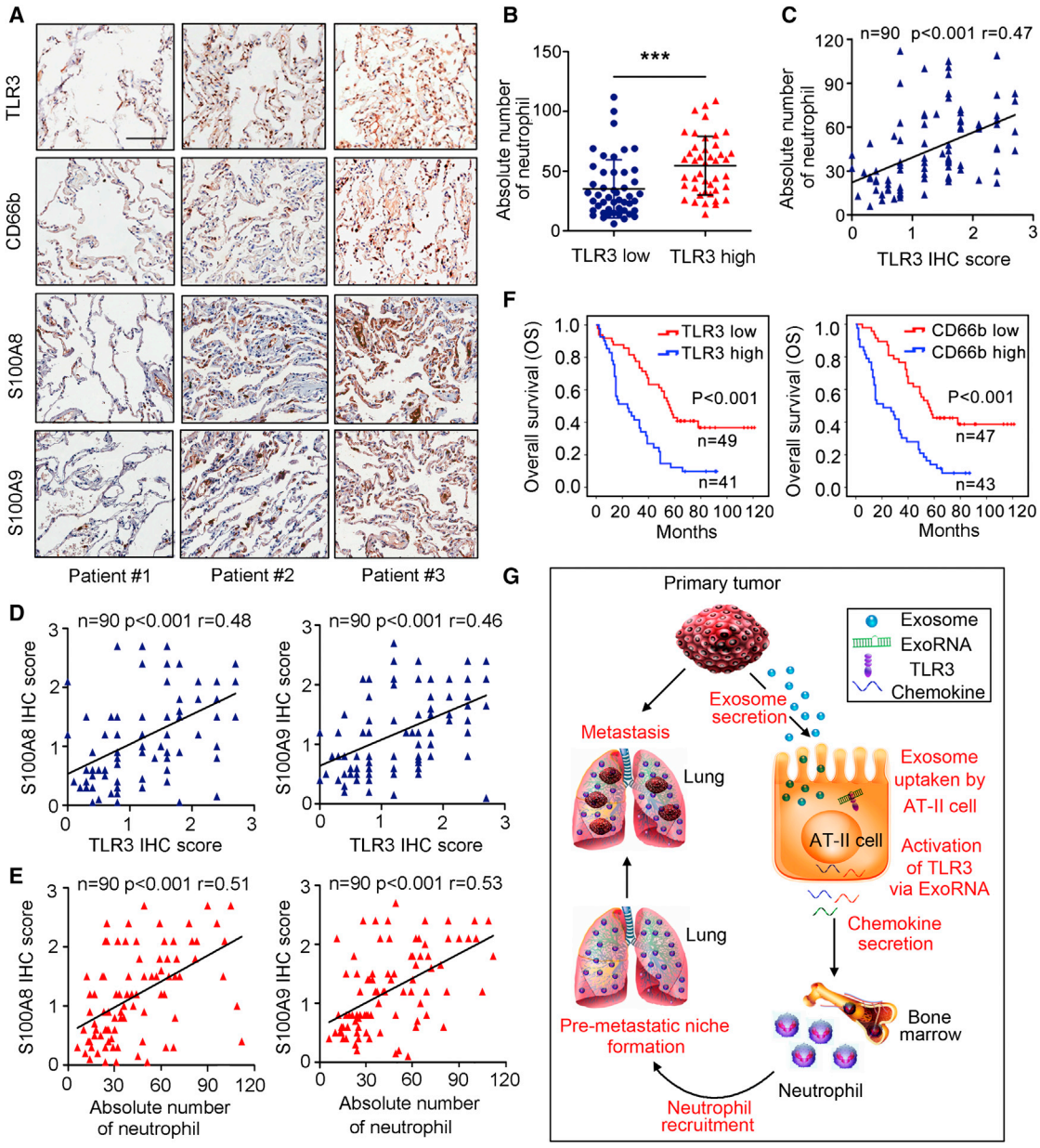

Figure 7. Massive TLR3 Expression and Neutrophil Infiltration Predict Poor Prognosis for Lung Cancer Patients

(A) Representative images of TLR3, CD66b, S100A8, and S100A9 staining in tumor-adjacent tissues from three lung cancer patients. Scale bar, 100 μm.

(B) Numbers of $CD66b^+$ neutrophils in tumor-adjacent tissues of lung cancer patients subgrouped by TLR3 expression ($n = 90$; ***$p < 0.001$, unpaired Student's t test).

(C) The Pearson correlation between TLR3 expression and numbers of $CD66b^+$ neutrophils ($n = 90$; $p < 0.001$, $r = 0.47$).

(D and E) The Pearson correlation (shown above graphs) between S100A8, S100A9, and TLR3 expression (D) or numbers of $CD66b^+$ neutrophils (E).

(F) The relationship between overall survival of lung cancer patients and TLR3 expression (left) and $CD66b^+$ neutrophils (right) in tumor-adjacent tissues ($p < 0.001$, Kaplan-Meier test).

(G) Proposed working model of tumor exosomal RNA-mediated lung epithelial cell TLR3 activation and neutrophil recruitment in promoting lung pre-metastatic niche formation.

See also Tables S1 and S2.

neutrophils in vivo, we further confirmed that recruited neutrophils in the lung were critical for pre-metastatic niche formation. Moreover, we identified host stromal TLR3, activated by tumor-derived exoRNAs, as an initiator of neutrophil recruitment. Understanding bidirectional communication between neutrophils and their microenvironment as well as the regulation of neutrophil recruitment may provide more effective strategies for neutrophil-based cancer therapy.

In sum, we identify a role of host lung epithelial cell TLR3 in promoting lung pre-metastatic niche formation via neutrophil recruitment. We show that tumor-derived exoRNAs exert the driving forces to "fertilize" the distinct organ for metastasis via

snRNA-mediated TLR3 activation inside the lung epithelial cells. Thus, our findings provide mechanistic insight regarding pre-metastatic niche formation and organ site-specific tropisms of metastasis. More research on the metastatic axis of tumor-derived exoRNAs and host lung epithelial cell TLR3 activation may reveal effective targets to prevent and treat tumor metastasis to the lung.

EXPERIMENTAL PROCEDURES

Mice and Tumor Models

Tlr3$^{-/-}$ mice were obtained from the Jackson Laboratory. *Tlr4*$^{-/-}$ and *Tlr9*$^{-/-}$ mice were a gift from Dr. Shizuo Akira (Osaka University, Japan). All of these mice were backcrossed for at least eight generations onto C57BL/6 background. C57BL/6 mice were obtained from Joint Ventures Sipper BK Experimental Animal Company. Animal experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. All mice in our experiments were gender and age matched (range between 6 and 10 weeks).

For the generation of spontaneous lung metastatic models, 1×10^6 luciferase-labeled LLC or B16/F10 cells were subcutaneously injected in the shaved right flank of mice. The tumors were surgically removed when they reached 100 mm^2 (range from 18 to 20 days). Lung metastasis were measured by H&E staining on lung paraffin sections, or detected by ex vivo luciferase-based noninvasive bioluminescence imaging using the platform of IVIS Lumina II (PerkinElmer).

Lung Tissue Dissociation and Cell Isolation

Lung tissues were collected, cut into small pieces, and incubated in dissociation solution with 2 mg/ml collagenase type I (Sigma), 2 mg/ml collagenase type IV (Sigma), and 1 mg/ml DNase (Sigma). The solution was pipetting every 10 min during the incubation and suspension was dispersed through a 70-mm cell strainer.

For purification of cells from lung tissues, single-cell suspensions from lungs were stained with antibodies, and T cells (CD45$^+$ CD3$^+$), B cells (CD45$^+$ CD19$^+$), macrophages (CD45$^+$ F4/80$^+$), dendritic cells (CD45$^+$ CD11c$^+$ MHC-II$^+$), neutrophils (CD45$^+$ Ly6G$^+$ Ly6C$^+$), epithelial cells (CD45$^-$ Epcam$^+$), and AT-II cells (CD45$^-$ SftpD$^+$) were sorted using a MoFlo XDP flow cytometer (Beckman Coulter) with purities of >95%.

Tumor Exosome Isolation and Application

Exosomes from tumor tissues were isolated as described by Chen et al. (2011). In brief, tissue was cut into small pieces and cultured in RPMI medium without fetal bovine serum for 12 hr, after which the medium was collected and centrifuged at 800 × *g* for 10 min, followed by a centrifugation step of 20,000 × *g* for 20 min to remove cellular debris. Next, the supernatant was filtered using a 0.2-μm filter (Pall Corporation). The collected media was then ultracentrifuged at 100,000 × *g* for 90 min at 4°C and the supernatant was discarded. Exosomes used for RNA and protein extraction were isolated using Exosome Precipitation Solution (ExoQuick-TC, System Biosciences) without ultracentrifugation. Identification of exosomes was processed according to the protocol described in Exosome Antibody Array (System Biosciences).

For in vivo exosome administration, 5 mg of exosomes in 100 μl of RPMI 1640 was intravenously injected via tail vein every 3 days, and 14 days later the tumor cells were intravenously injected to the mice. The control liposomes were prepared and quantitated as previously described (Hood et al., 2011). For in vivo exosome-tracking experiments, purified exosomes were fluorescently labeled with PKH67 membrane dye (Sigma). Labeled exosomes were washed, collected by ultracentrifugation, and resuspended in RPMI 1640. Labeled exosomes (5 mg) were intravenously injected to mice. Lungs were collected and analyzed by luciferase-based noninvasive bioluminescence imaging (PerkinElmer) at 12 and 24 hr after exosome injection.

In Vivo Transfection of *Tlr3* and Depletion of Neutrophil

In vivo administration of *Tlr3*-expressing recombinant adenovirus and depletion of neutrophils by specific antibody were conducted as described previously (Coffelt et al., 2015; DuPage et al., 2009). Adeno-GFP or Adeno-*Tlr3* (50 μl, MOI 1×10^9) was inhaled into mice through the airway once weekly for 4 weeks. LLC tumor was inoculated before the second inhalation. Antibody-mediated depletion of Ly6G$^+$ cells was performed by twice-weekly intraperitoneal injections of 100 μg per mouse anti-Ly6G (Clone 1A8), with IgG2a (Clone 2A3, both from Becton Dickinson) as the control.

Exosomal RNA Sequencing

Total RNA was extracted and purified using an miRNeasy Mini Kit (Qiagen) and checked for an RNA integrity number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent Technologies). The 5′ and 3′ adaptors were ligated sequentially to the RNAs and amplified by RT-PCR. The amplification products were excised from 6% TBE urea gel (Invitrogen), and purified DNA fragments were clustered and sequenced by an Illumina HiSeq 2500 from Shanghai Biotechnology Corporation.

Cases

Tumor tissues were obtained from 90 patients with lung adenocarcinoma in 2004. The surgical specimens were obtained from Shanghai Outdo Biotech (for patient characteristics, see Table S1). All lung cancer samples we used were NSCLC, and no chemotherapy or radiotherapy was given to patients before surgery. All surgical pathology materials were reviewed independently by two pathologists. Written informed consent was obtained from all patients, and the Institutional Review Board of the Second Military Medical University approved the study.

Statistical Analysis

The data analysis was performed using SPSS software for Windows. Unpaired Student's t test was used for comparisons of the mean between two groups and the Kaplan-Meier test for survival rates in the animal study. Statistics comparing different parameters in human subjects were determined by chi-square test, and overall survival was assessed by a Kaplan-Meier test. Statistical significance was two-tailed and set at <0.05.

ACCESSION NUMBERS

The gene microarray data for alveolar type II epithelial cells and sequencing data for tumor exosomal RNAs have been deposited in the Gene Expression Omnibus under accession numbers GEO: GSE76397 and GSE80678, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2016.06.021.

AUTHOR CONTRIBUTIONS

X.C. designed the experiments; Y.L., Y.G., Y.H., Q.Z., Z.J., X.Z., X.X., and J.Z. conducted the experiments; B.H. provided the reagent; X.C., Y.L., and Y.G. analyzed data and wrote the paper; X.C. is responsible for research supervision, coordination, and strategy.

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