SEPSIS

Response to comment on "ALK is a therapeutic target for lethal sepsis"

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ALK inhibitors may exhibit STING-independent anti-inflammatory activity in macrophages and monocytes.

Excessive activation of STING (stimulator of interferon genes) signaling can trigger a lethal inflammatory response. Thus, defining STING regulatory pathways may help to develop novel strategies against inflammatory diseases. We recently screened a library of bioactive small molecules for activators and inhibitors of the STING pathway in macrophages. We demonstrated that ALK (anaplastic lymphoma kinase) inhibitors blocked STING activation in macrophages and their precursors, monocytes (*1*). Genetic suppression of ALK expression by RNA interference also limited STING activation in macrophages and monocytes (*1*). Blocking the ALK-STING pathway conferred resistance against polymicrobial sepsis in mice (*1*). We therefore concluded that ALK is a regulator of STING activation during bacterial infections (*1*). However, Blasco *et al.* (*2*) expressed concern with the conclusions drawn from our publication.

The first concern raised by Blasco *et al.* (*2*) was that the expression of ALK in macrophages was based on an online public database. ALK is a transmembrane receptor tyrosine kinase in the insulin receptor family. The ALK ligand pleiotrophin and the insulin receptor family are implicated in the interplay between metabolism and immunity (*3*). Blasco *et al.* (*2*) analyzed the Genotype-Tissue Expression database and found that ALK mRNA expression is undetectable in normal whole blood, spleen, and lung samples that are rich in monocytes or macrophages. We checked the BioGPS database and found that ALK mRNA is expressed in various tissues, including spleen and lung (http://biogps.org/#goto=genereport&id=238). We further assayed ALK mRNA expression in 34 independent macrophage-associated datasets from human samples or cell cultures in BioGPS. All datasets showed that macrophages or monocytes express ALK mRNA in various conditions. Moreover, analysis of the Human Protein Atlas confirmed that ALK protein is expressed in various tissues and cells including brain, testis, bone marrow, lung, and macrophages (www.proteinatlas.org/ENSG00000171094-ALK/tissue). Thus, our in silico analysis of ALK expression indicates that ALK is expressed in macrophages and monocytes.

The second concern Blasco *et al.* (*4*) raised regarded the specificity of ALK (F12) antibody (catalog no. sc-398791) from Santa Cruz Biotechnology. ALK (F-12) is a mouse monoclonal antibody specific for an epitope mapping between amino acids 117 to 145 (PAEARTLSRVLKGGSVRKLRRAKQLVLEL) within an N-terminal extracellular domain of ALK of human origin (UniProtKB-Q9UM73). Since human ALK amino acids 122 to 145 (TLSRVLKGGSVRKLRRAKQLVLEL) is 100% similar to mouse ALK amino acids 126 to 149 (N-terminal extracellular domain; UniProtKB-P97793), ALK (F-12) antibody is recommended for detection of ALK of mouse and human origin using Western blotting. This ALK (F-12) antibody is also used by other laboratories for Western blot (www.scbt.com/scbt/product/alk-antibody-f-12/). The molecular weight of normal human and mouse ALK is 176 and 175 kDa, respectively. Consistent with antibody datasheets, we saw a band around 170 to 190 kDa in human and mouse macrophages or monocytes using ALK (F-12) antibody (*1*). We knocked down ALK by specific short hairpin RNA–ALK and showed reduced expression using ALK (F-12) antibody (*1*). Additional Western blot results showed the absence of ALK protein expression about 170 to 190 kDa in peritoneal macrophages from septic ALK$^{-/-}$ mice, confirming the specificity

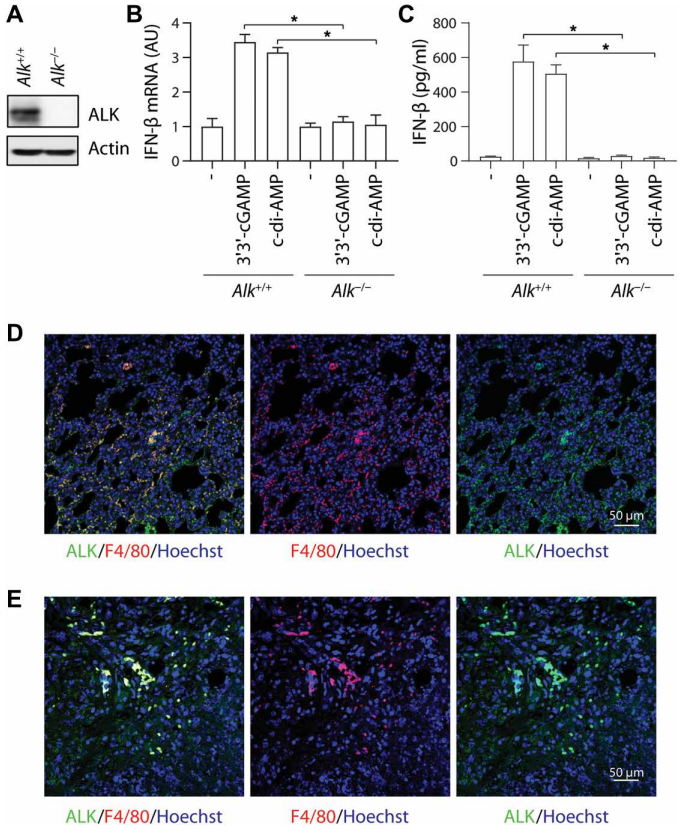

Fig. 1. Knockout of ALK limits STING activation in macrophages. (**A**) Western blot analysis of ALK expression in isolated peritoneal macrophages from septic *Alk*$^{+/+}$ or *Alk*$^{-/-}$ mice at 48 hours after cecal ligation and puncture (CLP). (**B** and **C**) Analysis of IFN-β mRNA (B) and release (C) in *Alk*$^{+/+}$ or *Alk*$^{-/-}$ peritoneal macrophages with or without indicated STING ligand (10 μg/ml) stimulation for 16 hours ($n = 3$, $^*P < 0.05$). (**D**) Confocal microscope imaging analysis of colocalization between ALK (green) and F4/80 (red) in lung from CLP-induced septic mice at 48 hours. (**E**) Confocal microscope imaging analysis of colocalization between ALK (green) and F4/80 (red) in lung tumor from a patient with NSCLC. Hoechst 33258 (blue) was used for nuclear staining. AU, arbitrary units.

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of ALK (F-12) antibody (Fig. 1A). Functionally, ALK depletion blocked 3′3′-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate) or c-di-AMP–induced interferon-β (IFN-β) mRNA expression (Fig. 1B) and release (Fig. 1C) in peritoneal macrophages from septic ALK$^{-/-}$ mice, also supporting our previous conclusion that ALK promotes STING activation in macrophages.

In contrast to our results showing the molecular weight of ALK at 175 to 175 kDa, Blasco *et al.* (*2*) claimed that 220- to 230-kDa full length is the normal form of ALK. This claim contradicts their previous observation that 170 to 190 kDa corresponds to full-length ALK in lung cancer cells (*4*). Another concern was the use of the Raji cell line as a negative control of ALK expression by Blasco *et al.* (*2*). In contrast, ALK (F-12) antibody datasheets indicate that the Raji cell line is a positive control of ALK expression. Thus, the claim of 220- to 230-kDa full length (*2*) could be nonspecific for other forms of ALK. ALK is cleaved by caspase in apoptosis (*5*). Blasco *et al.* (*2*) observed the cleaved ALK form in normal cells under normal culture conditions, indicating that their cells may suffer from apoptotic stresses or that the antibody they used produces an unspecific band. It is also possible that the overexpression of ALK complementary DNA used by Blasco *et al.* may produce gene fusion, which may affect the size of ALK.

Blasco *et al.* (*2*) claimed that "immunohistochemistry failed to detect ALK expression in normal macrophages surrounding ALK-rearranged NSCLC and ALCL." ALK-rearranged non–small cell lung cancer (NSCLC) patients often present with infection. Macrophages are among the most abundant normal cells in the tumor microenvironment. Tumor-associated macrophages are different from normal macrophages in activation and function (*6*). Unfortunately, Blasco *et al.* (*2*) did not stain for macrophage markers. Thus, it is difficult to distinguish macrophages from other cells, such as megakaryocytes, in the tumor microenvironment. F4/80 (also termed EMR1 in humans) is a macrophage marker. We observed a strong colocalization signal between ALK and F4/80 in lung from septic mice (Fig. 1D) and lung tumor from a patient with NSCLC (Fig. 1E), indicating that ALK is expressed by macrophages.

The third major concern raised by Blasco *et al.* (*2*) was the off-target effects of ALK inhibition. The use of ALK inhibitor concentration in cell culture is usually higher than in a cell-free system. In addition to using ALK inhibitors, we also observed that genetic knockdown or deletion of ALK blocks cyclic dinucleotide–induced STING activation, indicating that ALK plays a role in the regulation of STING activation in macrophages (*1*). We also observed that ALK inhibitor, ALK knockdown, and STING knockdown have overlapping and distinct immune functions in cytokine and chemokine release in macrophages (*1*).

Our bioinformatics, Western blot, real-time polymerase chain reaction, and image analysis confirmed ALK expression in human and mouse monocytes and macrophages. We observed that pharmacologic or genetic inhibition of ALK limited STING activation in monocytes and macrophages (*1*). Moreover, animal studies showed that *Alk*$^{-/-}$ mice are more resistant to polymicrobial sepsis (Fig. 2A).Biochemical measurement of tissue enzymes also revealed protective effects of ALK depletion against dysfunction of the heart (creatine kinase), pancreas (amylase), kidney (blood urea nitrogen), and liver (alanine aminotransferase) (Fig. 2B).These findings support a role for ALK in lethal infection. Notably, other independent groups also observed that ALK signaling affects macrophages. For example, a recent drug

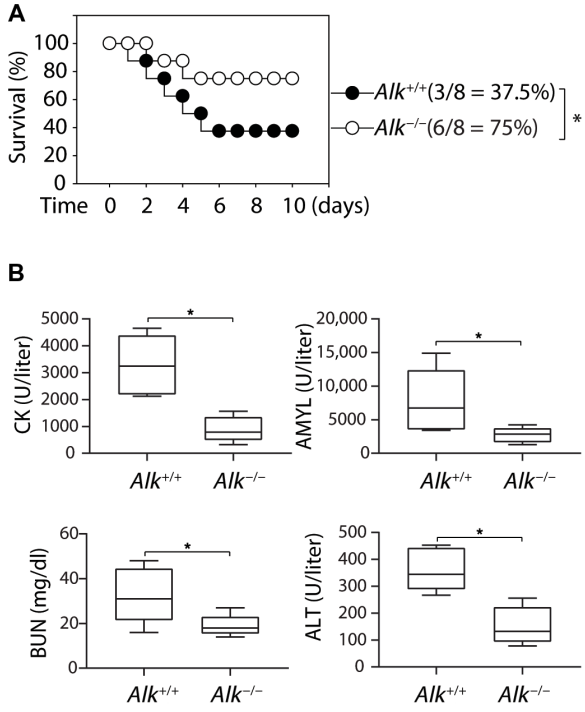

Fig. 2. ALK depletion protects mice against CLP-induced polymicrobial sepsis. (**A**) Depletion of ALK in mice prevented CLP (22-gauge needle)–induced animal death ($n = 8$ mice per group; *$P < 0.05$, Kaplan-Meier survival analysis). (**B**) In parallel, indicated serum enzyme activity (day 3) was assayed ($n = 5$ mice per group; *$P < 0.05$). CK, creatinine kinase; AMYL, amylase; BUN; blood urea nitrogen; ALT, alanine aminotransferase.

screen study identified ALK as a top activator of STING in macrophages and monocytes (*7*). This study found that 18 different ALK inhibitors could block STING activation in response to 2′3′-cGAMP in macrophages and monocytes (*7*). ALK in tumor-associated macrophages promoted tumor metastasis through sustaining inflammation (*8*). ALK also plays a role in promoting NALP3 (NLR family pyrin domain containing 3) inflammasome activation and pyroptosis in macrophages (*9*). The conclusions from these studies agree with our conclusion that ALK is a regulator of inflammation and innate immunity in macrophages and monocytes.

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