# CD137 signaling is critical in fungal clearance during systemic *Candida albicans* infection

**Running title: CD137 signaling in *C. albicans* infection**

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Abstract: Invasive fungal infections by *Candida albicans* frequently cause mortality in immunocompromised patients. Neutrophils are particularly important for fungal clearance at the early phase of infections, yet little has been known regarding which surface receptor controls neutrophil phagocytic activities during systemic *C. albican* infection. CD137, which is encoded by *Tnfrsf9*, belongs to the tumor necrosis receptor superfamily and has been shown to regulate neutrophils in Gram-positive bacterial infection. Here, we used genetic and immunological tools to probe the involvement of CD137 signaling in innate defense mechanisms against systemic *C. albicans* infection. We first found that *Tnfrsf9*$^{-/-}$ mice were susceptible to *C. albicans* infection, whereas injection of anti-CD137 agonistic antibody protected the host from infection, suggesting that CD137 signaling is indispensable for innate immunity against *C. albicans* infection. Priming of isolated neutrophils with anti-CD137 antibody promoted their phagocytic and fungicidal activities through phospholipase C. In addition, injection of anti-CD137 antibody significantly augmented restriction of fungal growth in *Tnfrsf9*$^{-/-}$ mice that received WT neutrophils. In conclusion, our results demonstrate that CD137 signaling contributes to defense mechanisms against systemic *C. albicans* infection by promoting rapid fungal clearance whereby harmful immunopathology-induced tissue injuries are minimalized.

Keywords: *Candida albicans*; CD137; neutrophil; fungal clearance

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## 1. Introduction

Neutrophils represent a major group of effector cells that are critical for defense against invasive fungal infection [1,2]. They employ several strategies to restrict fungi, including the machinery for capturing and killing, such as various cytotoxic enzymes and their products, neutrophil extracellular traps (NETs), and phagocytic receptors [2]. C-type lectin receptors such as Dectin-1 and Dectin-2 can directly recognize the cell wall components of *C. albicans* in macrophages, dendritic cells, and other phagocytes (3). Complement receptor 3 (CR3) and Fc-gamma receptors (FcγRs) are strongly expressed in neutrophils and represent phagocytic receptors recognizing opsonized *C. albicans* [4, 5]. Phagocytic receptors cooperate each other to enhance phagocytosis: For example, Mayada and colleagues [6] have demonstrated that β-glucan, a cell wall component of *C. albicans*, activates Dectin-1 to enhance the phagocytic activity of neutrophils through CR3. We have further demonstrated that IL-33 enforces Dectin-1 signaling to increase expression of CR3 and whereby IL-33 effectively restricts *C. albicans* [7]. These experimental outcomes may support it possible to design therapies that could selectively enhance neutrophil function in the context of fungal clearance. Accordingly, manipulation of neutrophil phagocytic function is considered to be an attractive avenue that could benefit patients during infections.

CD137 is well known for its costimulatory function for $CD8^+$ T cells [8]. However, its expression is detected in a broad range of cells, which has implications that CD137 could function in a variety of cells, including other types of lymphoid cells, myeloid cells, and non-hematopoietic cells. For example, there are reports showing that neutrophil-specific CD137 signaling plays an important role in defense against bacterial infections [9-12]. As yet, it remains to be clarified whether CD137 is involved in innate defense against invasive fungal infections. In this study, we specified CD137 function in neutrophils during systemic *C.*

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*albicans* infection. We also show that anti-CD137 agonistic antibody enhances neutrophil ability to restrict fungal growth and may provide an effective screen for mediators that impact neutrophil function.

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## 2. Materials and Methods

### *1.1. Mice, antibodies, and reagents*

C57BL/6 mice were purchased from Orient Bio-Charles River (Seongnam, Korea). *Tnfrsf9*$^{-/-}$ C57BL/6 [13] were maintained in a specific pathogen-free facility and used when 7-8 weeks old. All experiments were conducted according to the regulations issued by the Animal Committee of the University of Ulsan. Anti-CD137 (3H3) and anti-Gr-1 (RB6-8C5) monoclonal antibodies (mAbs) were purified from ascites fluid. Control rat IgG was purchased from Millipore Sigma Korea (Seoul, Korea). Anti-CD16/CD32 (2.4G2) antibody was purified from hybridoma culture. The following FITC-, PE-, APC-, PerCP-, APC-cy7- or biotin-conjugated mAbs to mouse proteins were purchased from BD Biosciences or e-Bioscience: CD45 (30-F11), CD11b (M1/70), Ly6G (1A8), and Ly6C (AL-21).

### *1.2. Fungal strains, growth conditions, and infection*

*C. albicans* (ATCC26555) was grown in peptone dextrose extract at 30 °C overnight and aliquots were frozen at -80 °C. To kill the *C. albicans* yeasts, the organisms were harvested by centrifugation, and pellets were washed twice in sterile PBS. After resuspension at a density of $1 \times 10^8$ cells/ml, heat killing was performed at 90 °C for 30 min. To induce experimental candidiasis, *C. albicans* was intravenously inoculated into the lateral caudal tail vein at a dose of $3 \times 10^5$ colony-forming units (CFUs). For counting CFUs, *m*ice were euthanized and kidneys were removed aseptically. Harvested kidneys were homogenized in 2 ml of PBS, and serial dilutions of homogenates were plated on Sabouraud agar and incubated at 37 $^0$C for 24 h. Colonies were counted and results were expressed as $\log_{10}$(CFUs/ml) or $\log_{10}$(CFUs/organ).

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*1.3. Isolation, depletion, and adoptive transfer of neutrophils*

Total bone marrow cells were collected from tibias and femurs of 8-week old mice by flushing with RPMI 1640 media, filtered through a sterile mesh (Corning, Glendale, Az), and washed. After erythrocytes were lysed in hemolysis buffer (144 mM $NH_4Cl$ and 17 mM Tris-HCl [pH 7.2]), the remaining cells were resuspended in MACS buffer (1x PBS containing EDTA and 3% calf serum). Neutrophils were isolated using anti-Ly6G MACS microbeads, according to manufacturer′s protocols (Miltenyi Biotech Korea, Seoul, Korea). The purity of neutrophils routinely reached >98%. For adoptive transfer, neutrophils ($5\times10^6$ cells/mouse) were then injected into *Tnfrsf9*$^{-/-}$ mice through the lateral tail vein. Neutrophil depletion was achieved by intraperitoneally injecting 200 μg of anti-Gr-1 (RB6-8C5) mAb into mice 2 day before *C. albicans infection.* `

*1.4. Preparation of kidney cells and flow cytometry*

Kidneys were perfused, minced, and placed in DMEM (Gibco) containing 1 μg/ml collagenase IA (Millipore Sigma Korea, Seoul, Korea) at 37 °C for 30 min. Digested kidney tissues were passed through a 40-μm cell strainer (Corning, Glendale, Az), and the cell suspensions obtained were centrifuged at 300 x g for 10 min. Cells were then washed in PBS containing 2% BSA, were suspended in 36% Percoll (GE Healthcare, Chalfont, UK), and were gently overlaid onto 72% Percoll. After centrifugation at 900 x gravity (g) for 30 min at room temperature, cells were retrieved from the Percoll interface and washed twice in DMEM medium and once with staining buffer (PBS containing 2% BSA and 0.1% sodium azide).

*1.9. Flow cytometry*

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Prepared cells were blocked with 2.4G2 mAb in staining buffer (PBS containing 0.2% BSA and 0.1% sodium azide) at 4 °C for 20 min, incubated with relevant mAbs at 4 °C for 30 min, and then rewashed twice with staining buffer. Flow cytometric analysis was performed using a FACS Canto II unit (BD Biosciences), and the data were analyzed using FACS Diva (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

*1.10. Analysis of renal function*

To determine kidney functions, concentrations of creatinine and BUN in sera were measured colorimetrically using the Quantichrom Urea Assay and the Quantichrom Creatine Assay kits (Bioassay Systems, Hayward, CA).

*1.11. Pathological scoring*

Kidneys were fixed in 10% (vol/vol) formalin, paraffin-embedded, sectioned (5 μm), stained with H&E or PAS, and analyzed. Kidney injury was scored by a single pathologist as the percentage of damaged tubules in the corticomedullary function. Criteria for kidney injury included tubular necrosis, cast formation, loss of brush border, tubular dilatation, and immune cell infiltration. Scoring for each category was as follows: 0, no change; 1, <10%; 2, 11-25%; 3, 25-45% area change. Scores for all the categories were added for the final injury scoring.

*1.12. Measurement of cytokines and chemokines*

Cytokines and chemokines present in total kidney homogenates were measured by ELISA (ThermoFisher Scientitic Korea, Seoul), according to the manufacturer's protocols.

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*1.13. Phagocytosis assay*

In vivo phagocytosis assays were performed as described previously [7]. In brief, mice were intraperitoneally injected with anti-CD137 antibody (200 µg per mouse) 1 hour before infusion of 5 x $10^8$ CFU FITC-labeled heat-killed (HK) C. albicans. One-hour later, peritoneal cells were harvested and stained with anti-CD11b and anti-Ly6G mAbs on ice. Percentages of neutrophils containing phagocytosed *C. albican*s were determined by flow cytometry. In vitro phagocytosis assays were performed as previously described [7, 14]. In brief, neutrophils were purified from the bone marrow using anti-Ly6G microbeads. HK *C. albicans* was labeled with FITC and opsonized, and then added to neutrophils at 37 $^0$C for 20 min (multiplicity of infection [MOI] = 10). Phagocytosis was stopped by transferring of cells into ice, and cells were then washed thoroughly with cold FACS buffer. Extracellular fluorescence was quenched by adding 200 µl of PBS containing 0.04% trypan blue and 1% formaldehyde. Cells containing fungi were counted by flow cytometry. Phagocytosis (%) was expressed as the percentage of neutrophils that phagocytosed FITC-labeled *C. albicans*.

*1.14. Fungicidal assay*

Live *C. albicans* was opsonized with mouse serum and added to neutrophils (MOI = 10). The mixture was incubated at 37 °C with shaking for 20 min to allow the phagocytosis of live *C. albicans*. Cells were then washed thoroughly in cold PBS, were resuspended in warm DMEM medium, and were further incubated at 37°C. At indicated times, a 200-ml sample was taken, cells were lysed in PBS containing 0.1% Triton X-100, and CFUs were enumerated by plating on agar. The killing (%) was calculated as [1 - (CFUs after incubation/phagocytized CFUs at the start of incubation)] x 100.

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*1.15. ROS (reactive oxygen species) production*

ROS were detected using the fluorescent probe DCF-DA (Invitrogen). Isolate neutrophils ($3 \times 10^5$) were seeded into 96-well plate and primed with anti-CD137 (5 μg/ml) for 2 h prior to being challenged by opsonized HK *C. albicans* (MOI = 10). After 30 min incubation, DCF-DA (2 μM) was added and fluorescence was measured by a fluorescent plate reader at an excitation/emission =485/530 nm at an interval of 10 min for 1 h. The fluorescence intensity was defined as the relative fluorescence units.

*1.16. Statistical analysis*

All data were analyzed using GraphPad Prism5 (GraphPad Software, San Diego, CA). Survivals and unpaired data were analyzed using the log rank test and the *t*-test, respectively. Results are expressed as means ± SEMs. Statistical significance was accepted for p values < 0.05.

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## 3. Results

### *3.1. CD137 signaling plays a protective role in systemic C. albicans infection*

To assess the function of CD137 signaling during systemic *C. albicans* infection, wild-type (WT) and *Tnfrsf9*$^{-/-}$ C57BL/6 mice were infected with 1 x $10^5$ CFU *C. albicans* per mouse. *Tnfrsf9*$^{-/-}$ mice displayed significantly increased mortality rates (Figure 1A). These mice also experienced more severe loss of body weight than WT mice (Figure 1B). By contrast, injection of anti-CD137 agonistic antibody resulted in enhanced survival time after lethal challenge and slowed down loss of body weight (Figure 1, C and D). There were significantly decreased levels of serum creatinine and BUN in anti-CD137 injected mice (Figure 1E). These results indicate that CD137 signaling protected the host from fatal renal injuries caused by *C. albicans* infection. Consistent with this interpretation, histopathological scores of the infected kidneys were significantly lower in anti-CD137-injected mice (Figure 1F).

### *3.2. CD137 signaling controls fungal growth and renal inflammation*

Susceptibility or resistance to systemic *C. albicans* infection is determined by the host's ability to repress growth of invaded fungi and lethal immunopathology []. Uncontrolled proliferation of pathogens is frequently linked to hyper-inflammatory responses that cause fatal tissue injuries. Accordingly, we found that anti-CD137-mediated resistance was associated with decreased outgrowth of *C. albicans* in all the organs examined, including the lung, liver, spleen, kidney and blood (Figure 2A). PAS staining of infected kidney sections revealed prominent hyphae within abscesses of control antibody-injected mouse kidneys but this was not evident in anti-CD137-injected mouse kidneys (Figure 2B). This result suggests that anti-CD137 antibody effectively restricted fungi in the kidney.

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Gross observations showed many more distinguishable nodules in control antibody-injected versus anti-CD137-injected mouse kidneys (Figure 3A). Consistent with this, histopathological analysis demonstrated that there were larger numbers of multifocal areas of abscess formation in control antibody-injected mouse kidneys (Figure 3B). These observations indicate that anti-CD137 antibody prevented extensive abscess formation by inhibiting fungal proliferation (also see Figure 2B). Furthermore, injection of anti-CD137 antibody slackened gain of kidney weight after infection (Figure 3C), an indication of less severe edema presumably as a result of lower degree of renal inflammation. To test this hypothesis, we measured levels of cytokines and chemokines and counted inflammatory cells in the kidneys at 3-day post-infection. Anti-CD137-injected mice had reduced levels of IL-6, TNF-α, CCL2, CXCL1, and CXCL2 in the infected kidneys (Figure 3D-F). There were smaller numbers of infiltrating inflammatory monocytes and neutrophils in the kidneys of mice that received anti-CD137 antibody (Fig. 3G). This result indicates that CD137 signaling decreased renal inflammation caused by *C. albicans* infection.

### *3.3. CD137 signaling in neutrophils is critical in fungal clearance*

The results presented so far seem to suggest that CD137 signaling play a key role in controlling fungal clearance and fungal proliferation linked directly to renal inflammatory responses to systemic *C. albicans* infection. As neutrophils are indispensable for these two processes during an early phase of *C. albicans* infection, we first performed in vivo phagocytosis assays to examine whether or not CD137 signaling could directly act on neutrophils and enhance their phagocytic activity. After a 1-hour priming with anti-CD137 antibody, FITC-labeled HK *C. albicans* were intraperitoneally challenged. Neutrophils primed with anti-CD137 antibody displayed increases in the percentage of FITC-positive

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neutrophils and the mean fluorescence intensity (MFI) of FITC-positive neutrophils at 30 minutes after incubation with *C. albicans* (Figure 4A). These results suggest that CD137 signaling not only broadened the pool of neutrophils with phagocytic capacity but also increased the phagocytic activity of individual neutrophils. We also performed in vitro phagocytic assays by challenging FITC-labeled HK *C. albicans* to neutrophils primed with anti-CD137 antibody for 1 hour. Consistent with the in vivo results, in vitro priming with anti-CD137 antibody significantly increased percentages of neutrophils engulfing *C. albicans* and the MFI of individual neutrophils compared to priming with control antibody (Figure 4B). Taken together, our results demonstrated that anti-CD137 antibody could directly stimulate neutrophils and increase their phagocytic activity for opsonized *C. albicans*. Pretreatment of U73122, a phospholipase C (PLC) inhibitor, abrogated the effect of anti-CD137 antibody on neutrophils' phagocytic activities to a large extent (Figure 4C), indicating that CD137 signaling may activate the Dectin-1-CR3 phagocytic pathway in which PLCγ functions as a central signaling molecule [5, 6].

Fungicidal assays demonstrated that phagocytosed *C. albicans* were killed more rapidly inside neutrophils primed by anti-CD137 antibody (Figure 4D), suggesting that priming with anti-CD137 antibody elevated the killing activity of neutrophils. Treatment of isolated neutrophils with *C. albicans* induced the production of higher levels of ROS after priming with anti-CD137 antibody (Figure 4E). Thus, CD137 signaling positively regulates a neutrophil's capacity for either phagocytosis or killing.

To in vivo evaluate the contribution of neutrophil-specific CD137 signaling to innate defense against systemic *C. albicans* infection, we transferred WT neutrophils into $CD137^{-/-}$ mice and sequentially followed by injection of anti-CD137 antibody and infection with a lethal dose of *C. albicans*. Injection of anti-CD137 antibody significantly lowered fungal

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burden, while increasing survival time concomitantly (Figure 5, A and B). Depletion of neutrophils completely abolished the effect of anti-CD137 antibody on survival (Figure 5C), confirming that anti-CD137 antibody can increase survival by augmenting neutrophil activities for fungal clearance.

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## 4. Discussion

In this study, we demonstrate that CD137 signaling is critical for resistance mechanism against invading *C. albicans*. Our results indicate that CD137 expression on neutrophils is indispensable for fungal clearance. Considering that neutrophils express low levels of CD137 [9], it is surprising that CD137 signaling is required for such an early phase of infection. We suspect that tonic signaling through CD137 in neutrophils during differentiation processes in the bone marrow and during bloodstream circulation may continuously prime them. Without this priming, neutrophils may have impaired phagocytic activities. Two evidence support this interpretation: 1) *Tnfsf9*$^{-/-}$ neutrophils display a lower capacity of phagocytosis for *C. albicans* (data not shown); 2) Priming of neutrophils with anti-CD137 antibody augments their phagocytic activities. The priming effect of CD137 is likely to change the intracellular environment of neutrophils in such a way that the machinery for phagocytosis and killing can be operated more rapidly and potently. Regulatory networks for the expression and activation of phagocytic receptors seem to be critical in this process [6, 7, 15]. Further studies will be guaranteed to clarify this aspect.

CD137 is well known as a costimulatory receptor for T cells and possibly for myeloid cells [16]. Accordingly, CD137 signaling enforces primary signaling and thereby functions in a context-dependent way. In the case of neutrophils, the primary receptors seem to be a variety of pattern recognition receptors, immunoreceptors, and receptors for pro-inflammatory mediators [16, 17]. Thus, pathogens and inflammatory cells such as monocytes/macrophages, endothelial cells, and epithelial cells are a candidate to provide primary signals, including microbe-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and inflammatory cytokines [16, 17]. As the ligand of CD137 (referred to as CD137L) is a cell surface molecule, cell-cell interactions should

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occur to stimulate CD137. These interactions are important for neutrophil activation by two reasons: 1) Primary signals can be provided from the interacting neutrophil-binding partners in a paracrine fashion; 2) Reverse signaling via CD137L is triggered simultaneously during these interactions, which are likely to further activate neutrophils for fungal clearance. Since infiltrating inflammatory monocytes and macrophages derived from them are reported to express CD137L during tissue inflammation [18], we propose that the CD137/CD137L signaling system plays a pivotal role in a positive feedback loop of neutrophil activation to inhibit the outgrowth of *C. albicans* in the kidney. This hypothesis is currently under investigation in our laboratory.

Hyper-inflammatory responses can result in fatal renal injury during invasive candidiasis without affecting resistance mechanism [19]. In this study, we demonstrate that CD137 signaling suppresses renal inflammation during systemic *C. albicans* infection by limiting fungal growth. This seems to be caused by enhancement of neutrophils' abilities of phagocytosis, killing, and growth restriction for *C. albicans*. However, there is a possibility that potent stimulation of CD137 mobilizes regulatory types of immune cells such as dendritic cells, as seen in other tissue inflammatory models [20]. Nonetheless, our results show that CD137 signaling in neutrophils is critical for innate defense against *C. albicans*. Manipulation of neutrophils with anti-CD137 agonistic antibodies could be considered as a therapeutic design for infections.

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- Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Commitee of the Ulsan University Hospital (protocol code UUH-0120-03).
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- Data Availability Statement: None
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Reference

1. Brown, G.D.; Denning, D.W.; Gow, N.A.R.; Levitz, S.M.; Netea, M.G.; White, T.C. Hidden killers: human fungal infections. *Sci. Transl. Med.* 2012, 4, 165rv113.
2. Lionakis, M.S.; Levitz, S.M. Host control of fungal infections: lessons from basic studies and human cohorts. *Annu. Rev. Immunol.* 2018, 36, 157–191.
3. Brown, G.D.; Willment, J.A.; Whitehead, L. C-type lectins in immunity and homeostasis. *Nat. Rev. Immunol*. 2018. 18, 374-389.
4. Netea, M.G.; Brown, G.D.; Kullberg, B.J.; Gow, N.A. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol*. 2008, 6, 67–78.
5. Netea M.G.; Maródi, L. Innate immune mechanisms for recognition and uptake of Candida species. *Trends Immunol*. 2010, 31, 346–353.
6. Li, X.; Utomo, A.; Cullere, X.; Choi, M.M.; Milner Jr., A.; Venkatesh, D.; Yun, S.H.; Mayadas, T.N. The β-glucan receptor Dectin-1 activates the integrin Mac-1 in neutrophils via Vav protein signaling to promote *Candida albicans* clearance. *Cell Host Microbe* 2011, 10, 603–615.
7. Le, H.T.; Tran V.G.; Kim, W.; Kim, J.; Cho, H.R.; Kwon, B. IL-33 priming regulates multiple steps of the neutrophil-mediated anti-Candida albicans response by modulating TLR and dectin-1 signals. *J. Immunol*. 2012, 189, 287-295.
8. Shuford, W.W.; Klussman, K.; Tritchler, D.D.; Loo, D.T.; Chalupny, J.; Siadak, A.W.; Brown, T.J.; Emswiler, J.; Raecho, H.; Larsen, C.P.; Pearson, T.C.; Ledbetter, J.A.; Aruffo, A.; Mittler, R.S. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J. Exp. Med*. 1997, 186, 47-55.

9. Lee, S.C.; Ju, S.A; Pack, H.N.; Heo, S.K.; Suh, J.H.; Park, S.M.; Choi, B.K.; Kwon, B.S.; Kim, B.S. *4-1BB (CD137) is required for rapid clearance of Listeria monocytogenes* infection. *Infect. Immun*. 2005, 73, 5144-5151.

10. Lee, S.C.; Ju, S.A.; Sung, B.H.; Heo, S.K.; Cho, H.R.; Lee, E.A.; Kim, J.D.; Lee, I.H.; Park, S.M.; Nguyen, Q.T.; Suh, J.H.; Kim, B.S. Stimulation of the molecule 4-1BB enhances host defense against Listeria monocytogenes infection in mice by inducing rapid infiltration and activation of neutrophils and monocytes. *Infect. Immun*. 2009, May;77(5):2168-2176.

11. Nguyen, Q.T.; Ju, S.A.; Park, S.M.; Lee, S.C.; Yagita, H.; Lee, I.H.; Kim, B.S. Blockade of CD137 signaling counteracts polymicrobial sepsis induced by cecal ligation and puncture. *Infect. Immun*. 2009, 77, 3932-2938.

12. Nguyen, Q.T.; Nguyen, T.H.; Ju, S.A.; Lee, Y.S.; Han, S.H.; Lee, S.C.; Kwon, B.S.; Yu, R.; Kim, G.Y.; Lee, B.J.; Kim, B.S. CD137 expressed on neutrophils plays dual roles in antibacterial responses against Gram-positive and Gram-negative bacterial infections. *Infect. Immun*. 2013, 81, 2168-2177.

13. Kwon, B.S.; Hurtado, J.C.; Lee, Z.H.; Kwack, K.B.; Seo, S.K.; Choi, B.K.; Koller, B.H.; Wolisi, G.; Broxmeyer, H.E.; Vinay, D.S. Immune responses in 4-1BB (CD137)-deficient mice. *J. Immunol*. 2002. 168, 5483-5490.

14. Tran, V.G.; Kim, H.J.; Kim, J.; Kang, S.W.; Moon, U.J.; Cho, H.R.; Kwon, B. IL-33 Enhances Host Tolerance to Candida albicans Kidney Infections through Induction of IL-13 Production by CD4+ T Cells. *J. Immunol*. 2015, 194, 4871-4879.

15. Zhao, X,; Guo, Y.; Jiang, C.; Chang, Q.; Zhang, S.; Luo, T.; Zhang, B.; Jia, X.; Hung, M.C.; Dong, C.; Lin, X. JNK1 negatively controls antifungal innate immunity by suppressing CD23 expression. *Nat. Med*. 2017, 23, 337-346.

16. van Rees, D.J.; Szilagyi, K.; Kuijpers, T.W.; Matlung, H.L.; van den Berg, T.K. Immunoreceptors on neutrophils. *Semin. Immunol*. 2016, 28, 94-108.

17. Amulic, B.; Cazalet, C.; Hayes, G.L.; Metzler, K.D.; Zychlinsky, A. Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol*. 2012, 30, 459-489.

18. Shao, Z.; Schwarz, H. CD137 ligand, a member of the tumor necrosis factor family, regulates immune responses via reverse signal transduction. *J. Leukoc. Biol*. 2011, 89, 21-29.

19. Majer, O.; Bourgeois, C.; Zwolanek, F.; Lassnig, C.; Kerjaschki, D.; Mack, M.; Müller, M.; Kuchler, K. Type I interferons promote fatal immunopathology by regulating inflammatory monocytes and neutrophils during Candida infections. *PLoS Pathog*. 2012, 8, e1002811.

20. Jin, J.; Jung, I.H.; Moon, S.H.; Jeon, S.; Jeong, S.J.; Sonn, S.K.; Seo, S.; Lee, M.N.; Song, E.J.; Kweon, H.Y.; Kim, S.; Kim, T.K.; Kim, J.; Cho, H.R.; Choi, J.H.; Kwon, B.; Oh, G.T. CD137 Signaling Regulates Acute Colitis via RALDH2-Expressing CD11b(-)CD103(+) DCs. *Cell Rep*. 2020, 30, 4124-4136.e5.

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Figure Legends

Figure 1. CD137 signaling protects the host from systemic *C. albicans* infection.

(A-D) WT or *Tnfrsf9*$^{-/-}$ C57BL/6 mice were infected with 3 x $10^5$ CFU *C. albicans*. (A) Survival rate (n = 12 mice per group). (B) Changes in body weight (n = 6 mice per group). (C, D) Mice were intraperitoneally injected with 200 μg of anti-CD137 or control rat IgG antibody 1 day before infection with 3 x $10^5$ CFU *C. albicans*. (D) Survival rate (n = 6 mice per group). (D) Changes in body weight (n = 6 mice per group). (E) Serum creatinine and BUN levels (n = 5 mice per group). (F) Histopathological scores of 3-d post-infection kidney sections (n = 5 mice per group). Results are representative of at least three experiments. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001 between the two groups.

Figure 2. CD137 stimulation inhibits the outgrowth of *C. albicans*.

(A-B) Mice were intraperitoneally injected with 200 μg of anti-CD137 or control rat IgG antibody 1 day before infection with 3x$10^5$ CFU of *C. albicans*. Organs were harvested at 3-d post-infection. (A) Counting of CFUs (n = 5 mice per group). (B) PAS staining of kidney sections. Magnification: 200X. Results are representative of 3 experiments. *$P$ < 0.05; **$P$ < 0.01 between the two groups.

Figure 3. CD137 stimulation reduces renal inflammation after *C. albicans* infection.

(A-G) C57BL/6 mice were injected intraperitoneally with 200 μg of anti-CD137 or control rat IgG antibody 1 day before infection with 3 x $10^5$ CFU of *C. albicans*. (A) Gross morphology. (B) Representative H&E staining of 3-d post-infection kidneys. Magnification: 40X. (C) Changes in kidney weight (n = 6 mice per group). (D-F) Levels of IL-6 and TNF-α (D), CCL2 (E), and CXCL1 and CXCL2 (F) in kidney lysates at 3-d post-infection (n = 6

mice per group). (G) Numbers of infiltrating inflammatory monocytes and neutrophils in 3-d post-infection kidneys (n = 6 per group). Results are representative of 3 experiments. *$P$<0.05; **$P$<0.01 between the two groups at the indicated time points.

Figure 4. CD137 stimulation enhances the phagocytic and fungicidal activities of neutrophils. (A) In vivo analysis of phagocytosis. C57BL/6 mice were intraperitoneally injected with 200 μg of anti-CD137 or control rat IgG antibody 1 hour before challenge with FITC-labeled HK *C. albicans*. One-hour later, harvested cells were stained and percentages of phagocytosis were determined by counting $CD11b^{+}Ly6G^{hi}$ neutrophils containing *C. albicans*. The MFI was also presented for the extent of phagocytosis (n = 4 mice per group). (B) In vitro analysis of phagocytosis. Purified neutrophils were preincubated with anti-CD137 mAb (5 μg/ml) for 2 hours and challenged with opsonized, FITC-labeled, HK *C. albiccans* (MOI = 10) for 30 min. (C) In vitro phagocytosis assays were performed in neutrophils preincubated with 2 μM U73122 (PLC inhibitor) for 3 hours. The percentages of $FITC^{+}$ cells and MFIs were calculated using FACS. (D) Fungicidal assays for neutrophils primed with anti-CD137 or control antibody. (F) Measurement of ROS in neutrophils primed with anti-CD137 or control antibody. Results are representative of 3 experiments (n = 4 per group). *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001 between the two groups.

Figure 5. Neutrophils are required for anti-CD137-mediated enhancement of fungal clearance.

(A, B) Neutrophils were adoptively transferred to WT or *Tnfrsf9*$^{-/-}$ mice immediately before infection with 3 x $10^5$ CFU *C. albicans*. (A) Survival rates (n = 6-8 mice per group). (B) Fungal burdens in 3-d post-infection kidneys. (n = 5-6 mice per group). (C) Survival rates of

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mice depleted of neutrophils (n = 6 per group). Fungal burdens in kidneys (n = 5 mice per group). (D) Levels of IL-6 and TNF-α in kidney lysates. Results are representative of 3 experiments. *$P < 0.05$; ***$P < 0.001$ between the two groups.

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**Figure 1**

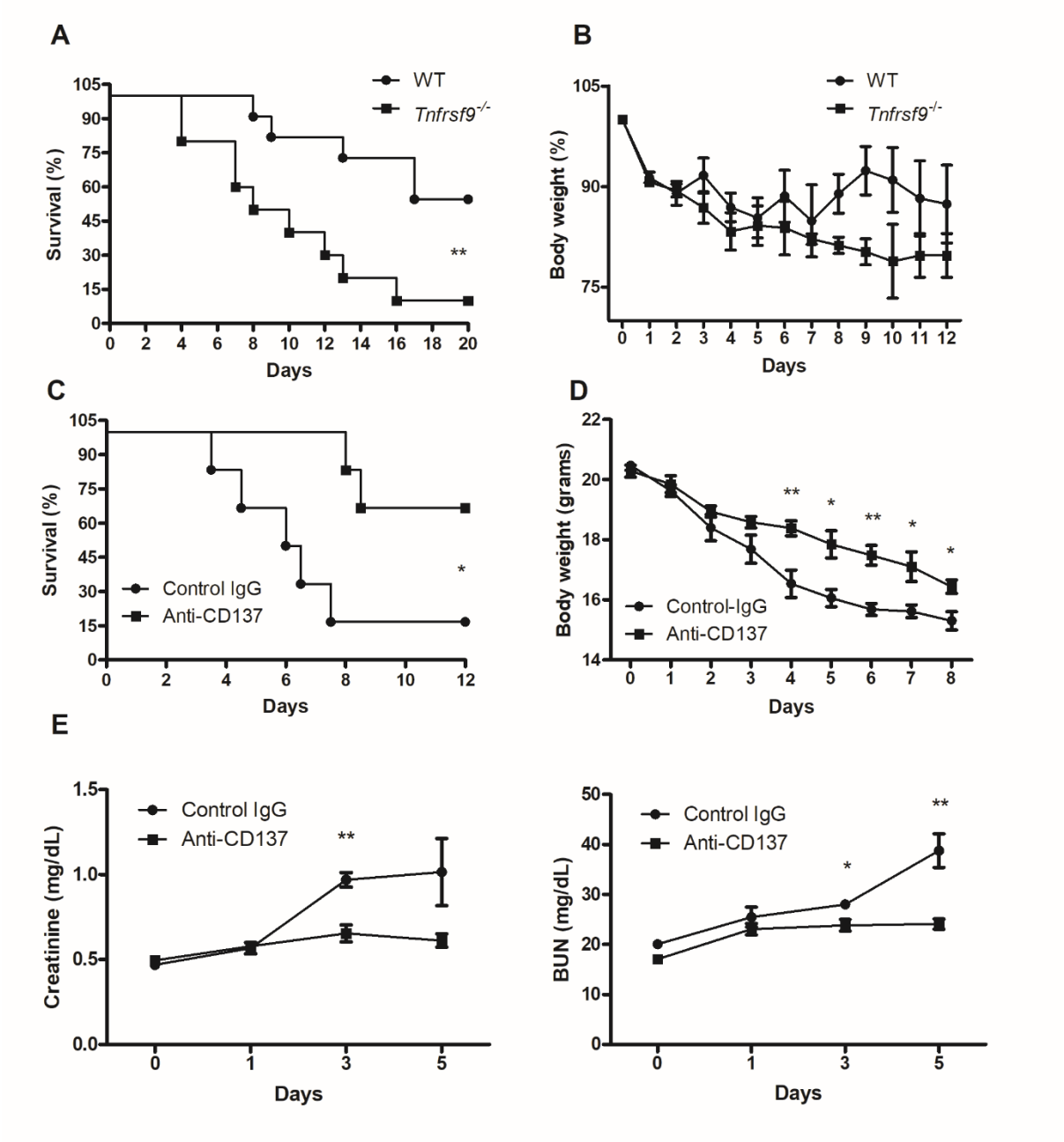

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**Figure 2**

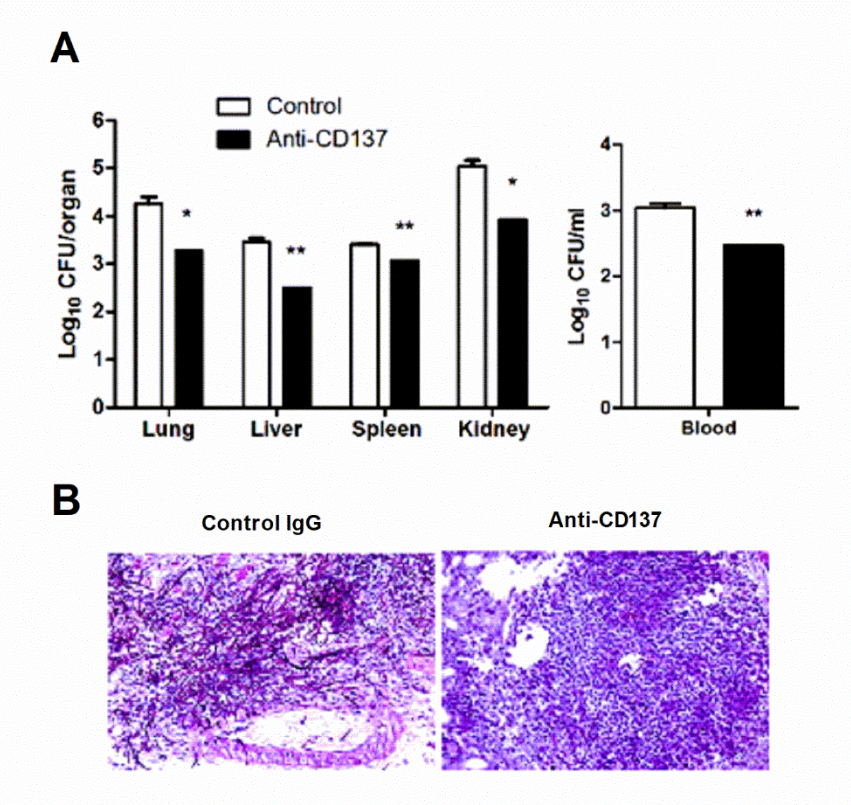

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**Figure 3**

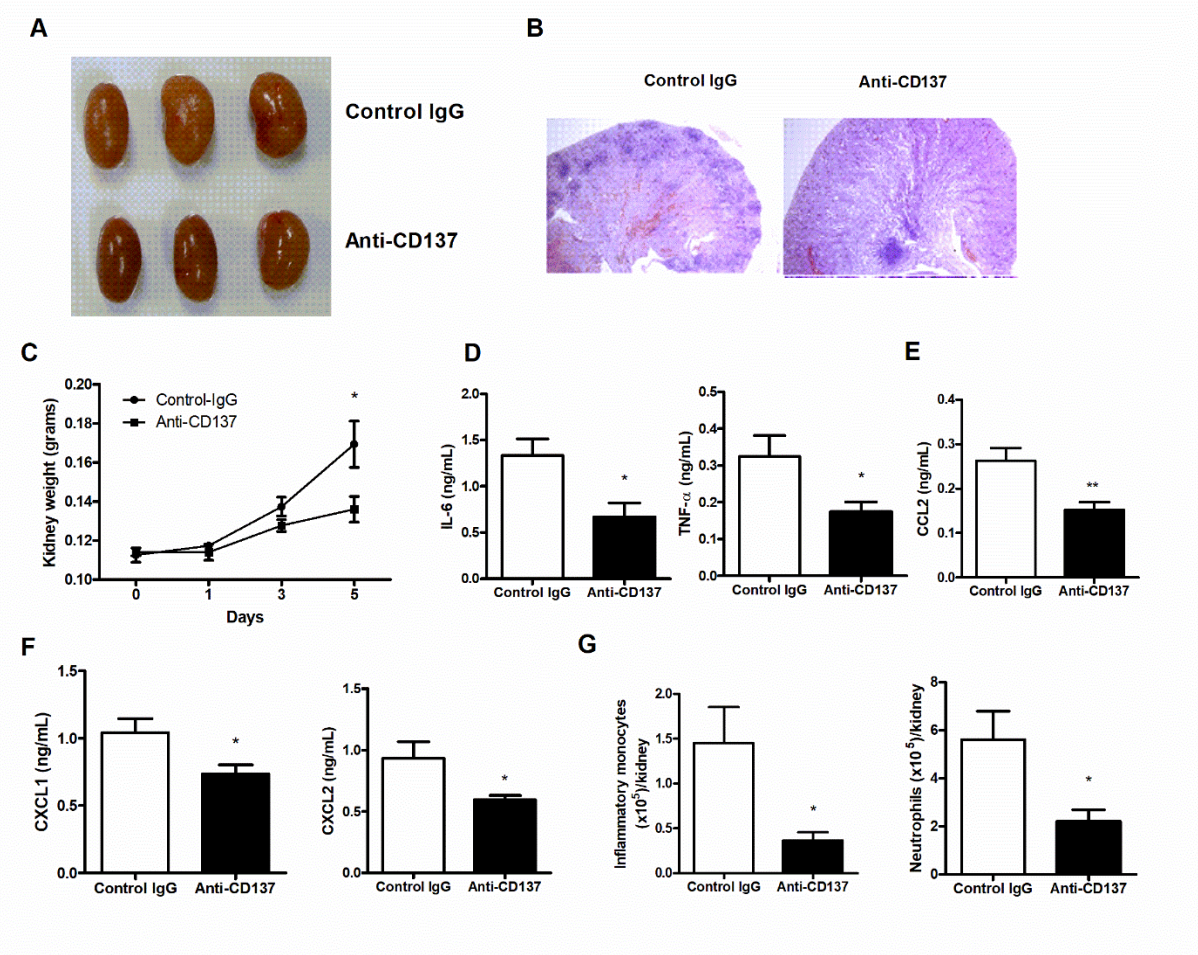

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**Figure 4**

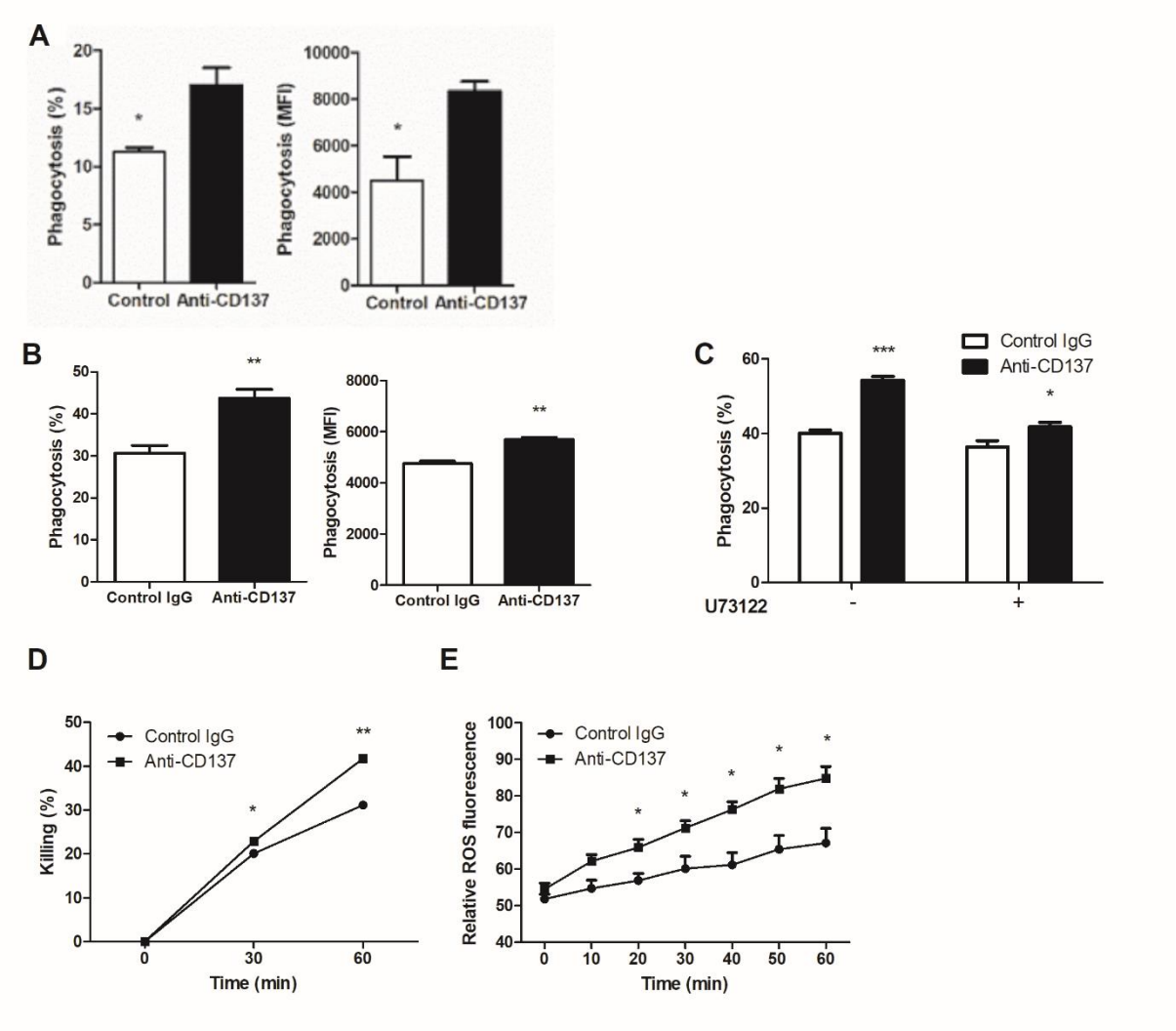

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**Figure 5**