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Supplementary Materials for

An ACE inhibitor reduces bactericidal activity of human neutrophils in vitro and impairs mouse neutrophil activity in vivo

Duo-Yao Cao *et al*.

Corresponding author: Zakir Khan, zakir.khan@csmc.edu

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The PDF file includes:

Figs. S1 to S8
Tables S1 to S5

Other Supplementary Material for this manuscript includes the following:

Data file S1

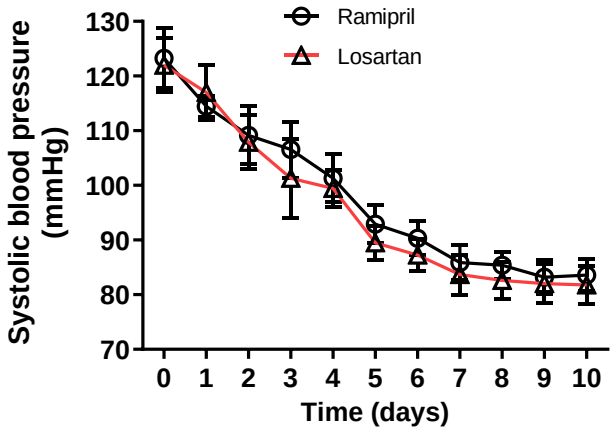


Fig. S1. Blood pressure was reduced as in mice treated with ramipril or losartan. Both ramipril and losartan were effective at the concentrations used as demonstrated by a reduction of about 35 mmHg of systolic blood pressure after 7 days, and then it is maintained at a steady state (n=10 per group). Blood pressure was measured by the tail-cuff method.

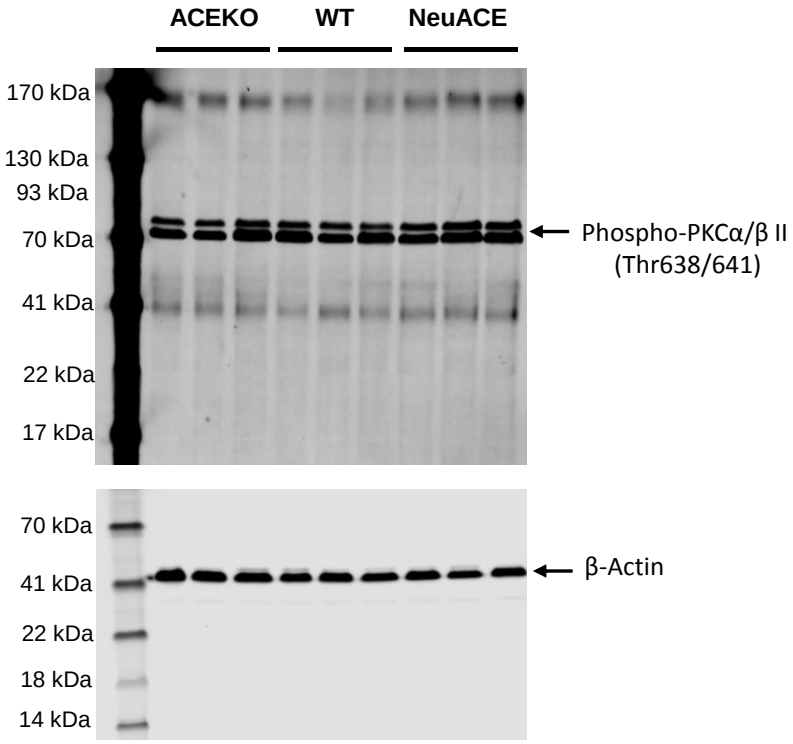


Fig. S2. Western blot analysis of phospho-PKCα/β II. Bone marrow-derived neutrophils were stimulated with 1µg/ml LPS for 30 minutes, lysed, and presence of phospho-PKCα/β II was evaluated.

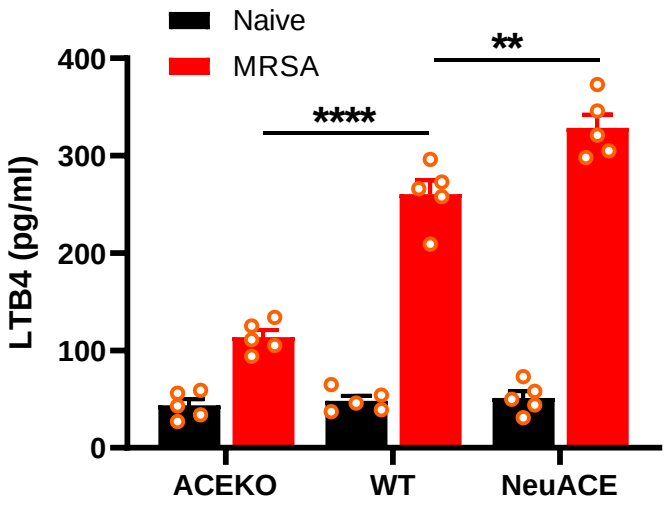


Fig. S3. In vitro exposure to MRSA induces LTB4 production by neutrophils. Bone marrow-derived neutrophils were challenged with MRSA at an MOI of 15 and, after 6 hours, LTB4 was measured in the supernatant by ELISA. Data were analyzed by using two-way ANOVA with Bonferroni's correction for multiple comparisons and presented as ± SEM (n=5). **p<0.01, ****p<0.0001.

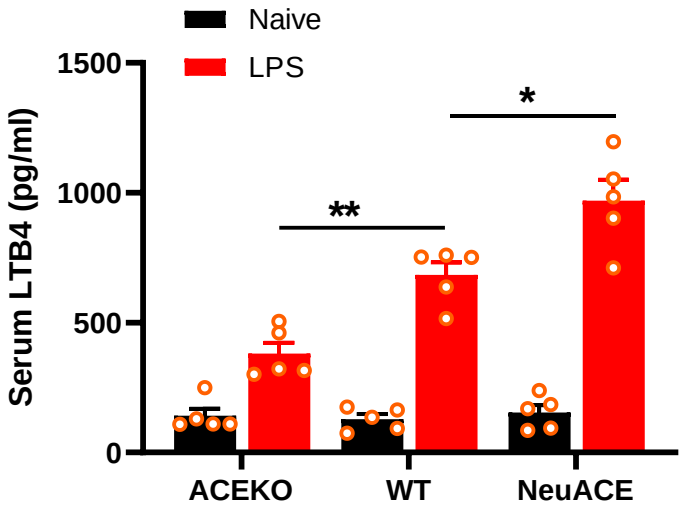


Fig. S4. LPS challenge increases serum LTB4. Mice were challenged i.p. with LPS (750 µg/Kg body weight) and, after 4 hours, blood was collected for analysis of LTB4 concentration in the serum. Data were analyzed using a two-way ANOVA with Bonferroni's correction for multiple comparisons and presented as ± SEM (n=5 mice per group). *p<0.05, **p<0.01

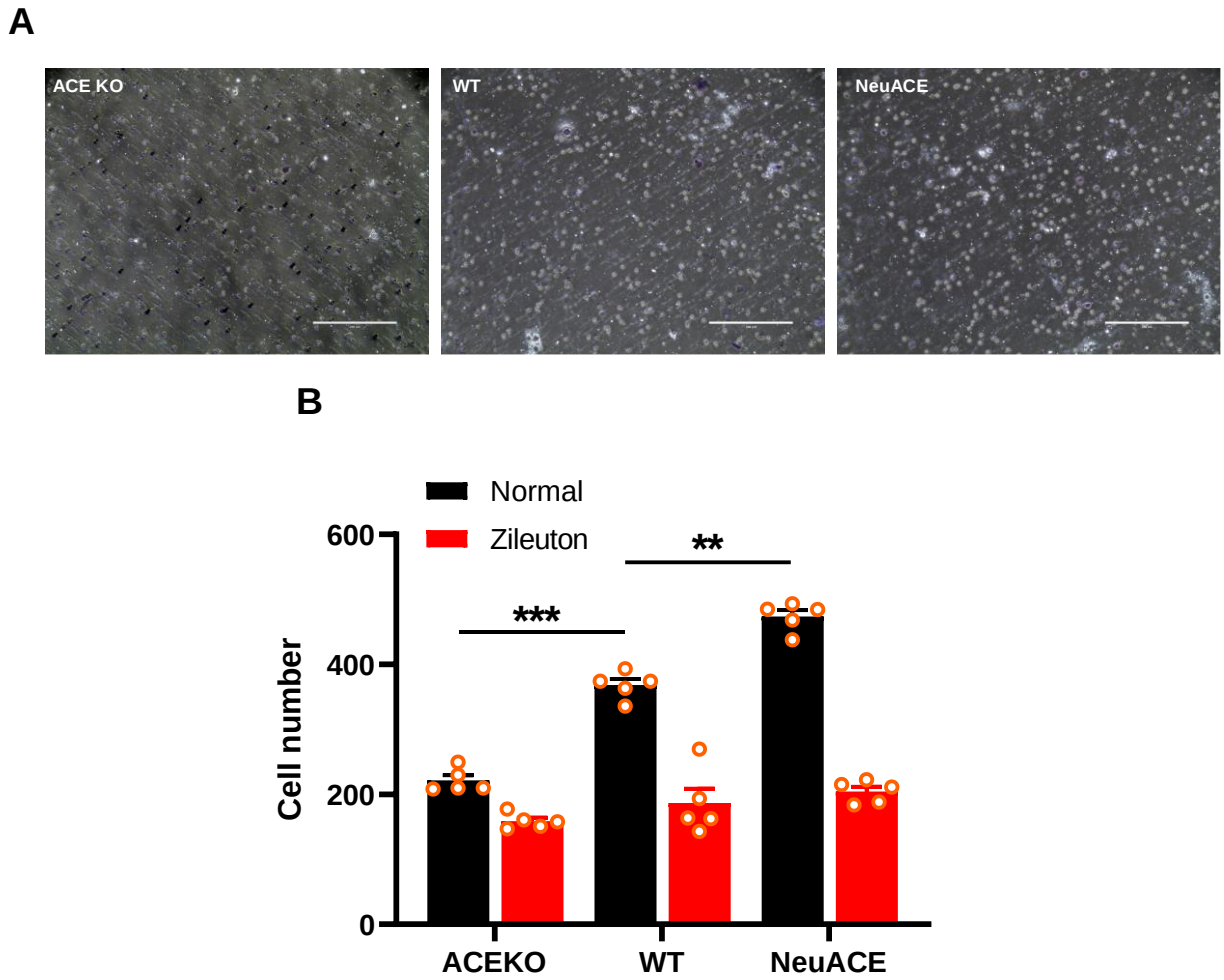


Fig. S5. Measurement of neutrophil chemotaxis. Neutrophils from ACE KO, WT and NeuACE mice were exposed to fMLP in a transwell chemotaxis assay, with or without 100 µM zileuton treatment. The cells that migrated from the upper insert surface to the lower surface were imaged and counted. **(A)** Representative images of the lower transwell membrane without zileuton for cell counting. Scale bar, 200 µm. **(B)** A graph shows the average number of cells per image. A two-way ANOVA with Bonferroni's correction for multiple comparisons was used to analyze group comparisons and data presented as ± SEM (n=5). **p<0.01, ***p<0.001.

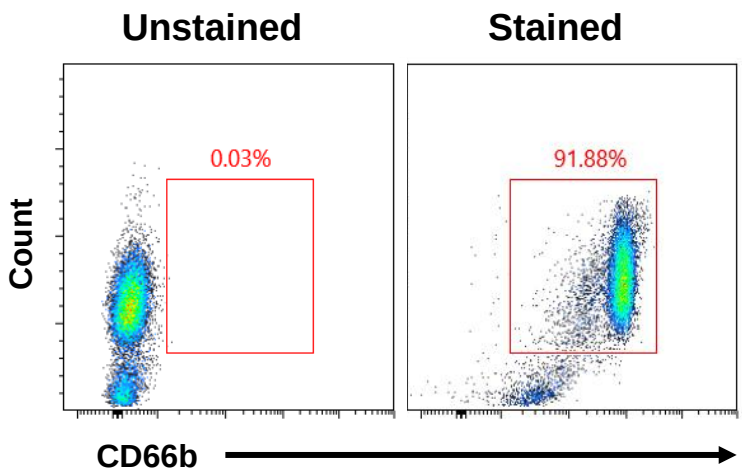


Fig. S6. Purity of human neutrophils by flow cytometry. Neutrophils were isolated from whole human blood and purity was determined by flow cytometry. Neutrophils are defined as $CD66b^{+}$.

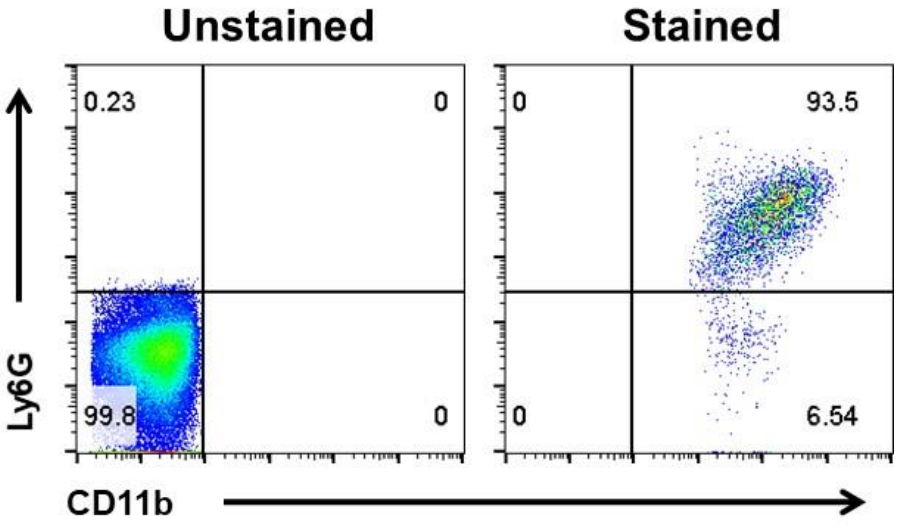
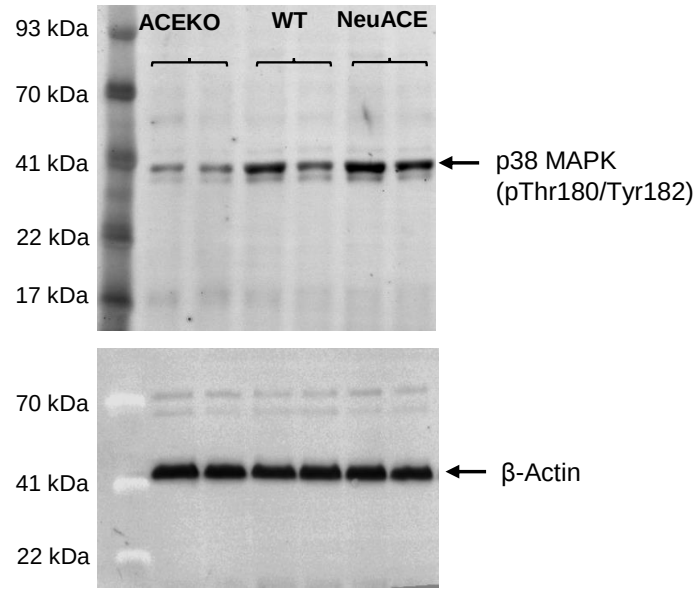
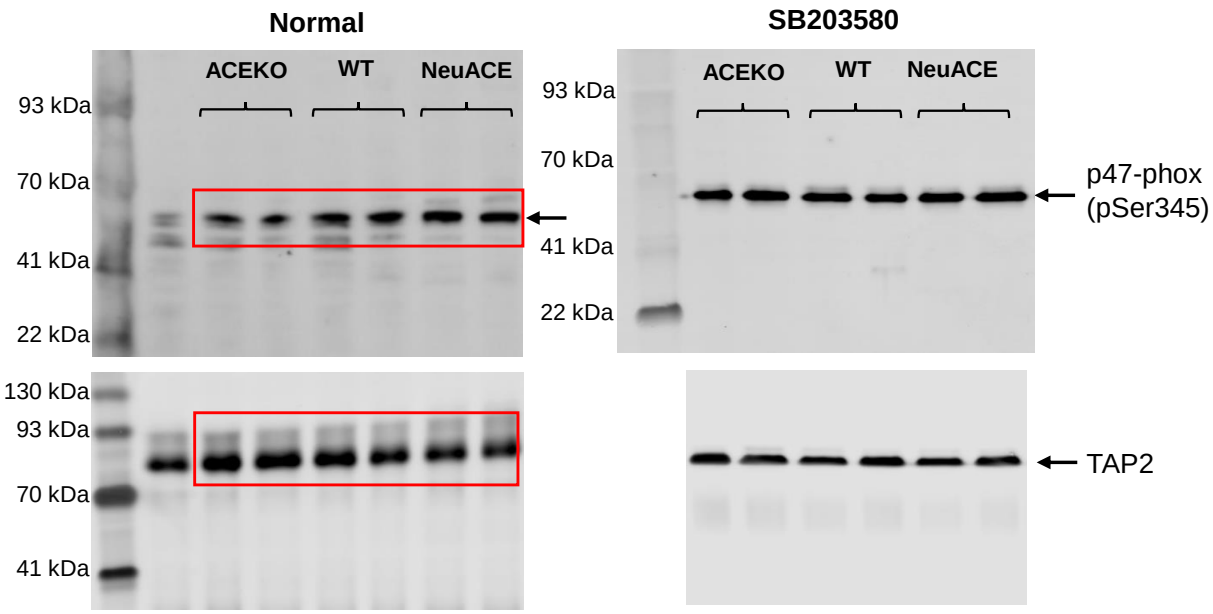


Figure S7. Purity of neutrophils. Neutrophils were isolated from mice bone marrow with STEMCELL Mouse Neutrophil Isolation Kit and purify was determined with flow cytometry. Neutrophils are defined as $Ly6G^{+}CD11b^{+}$.

A


ACEKO
WT
NeuACE
93 kDa
70 kDa
41 kDa
22 kDa
17 kDa
p38 MAPK
(pThr180/Tyr182)
70 kDa
41 kDa
22 kDa
β-Actin

B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(pSer345)
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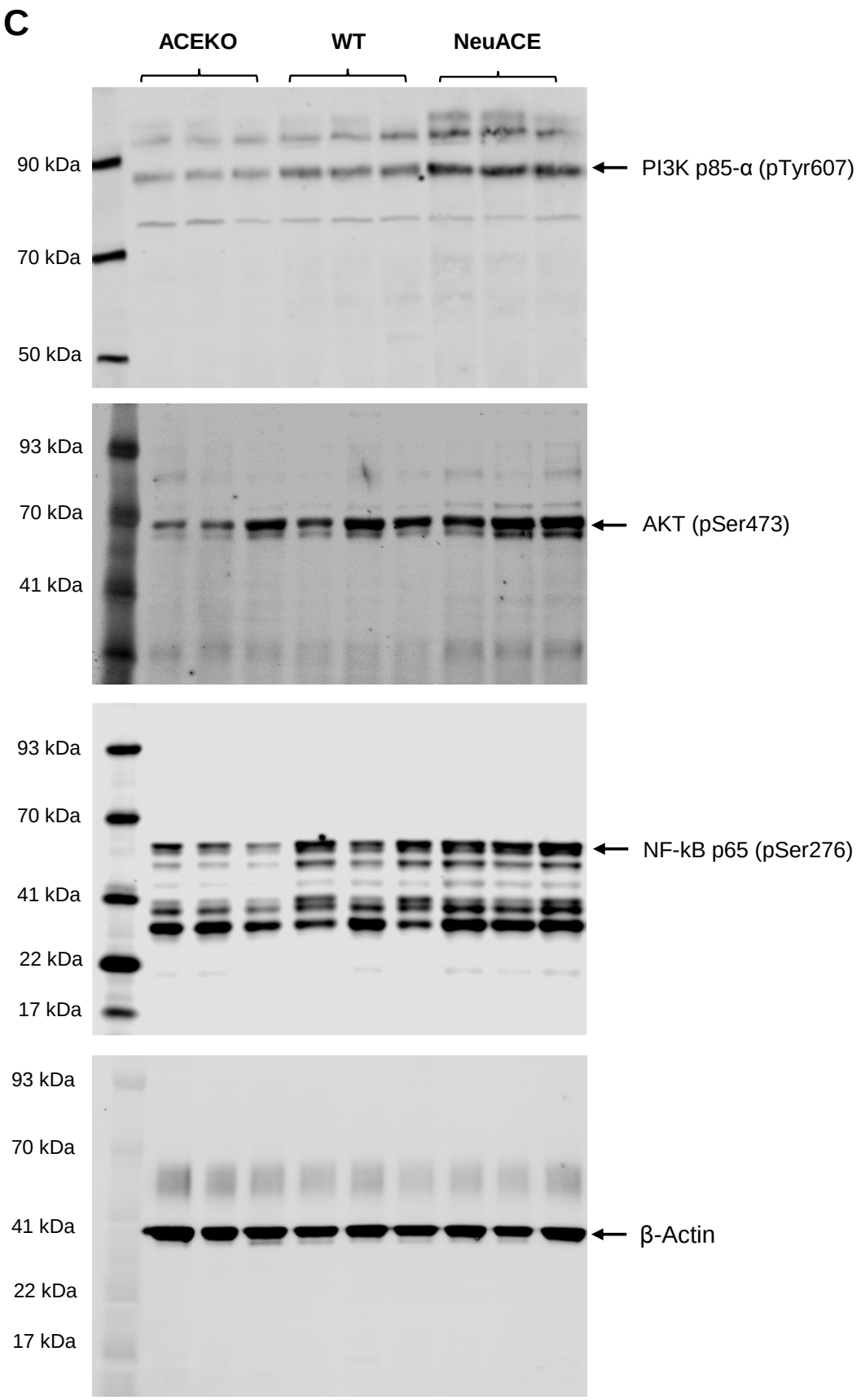
70 kDa
41 kDa
TAP2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WT
NeuACE
90 kDa
70 kDa
50 kDa
PI3K p85-α (pTyr607)
93 kDa
70 kDa
41 kDa
AKT (pSer473)
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22 kDa
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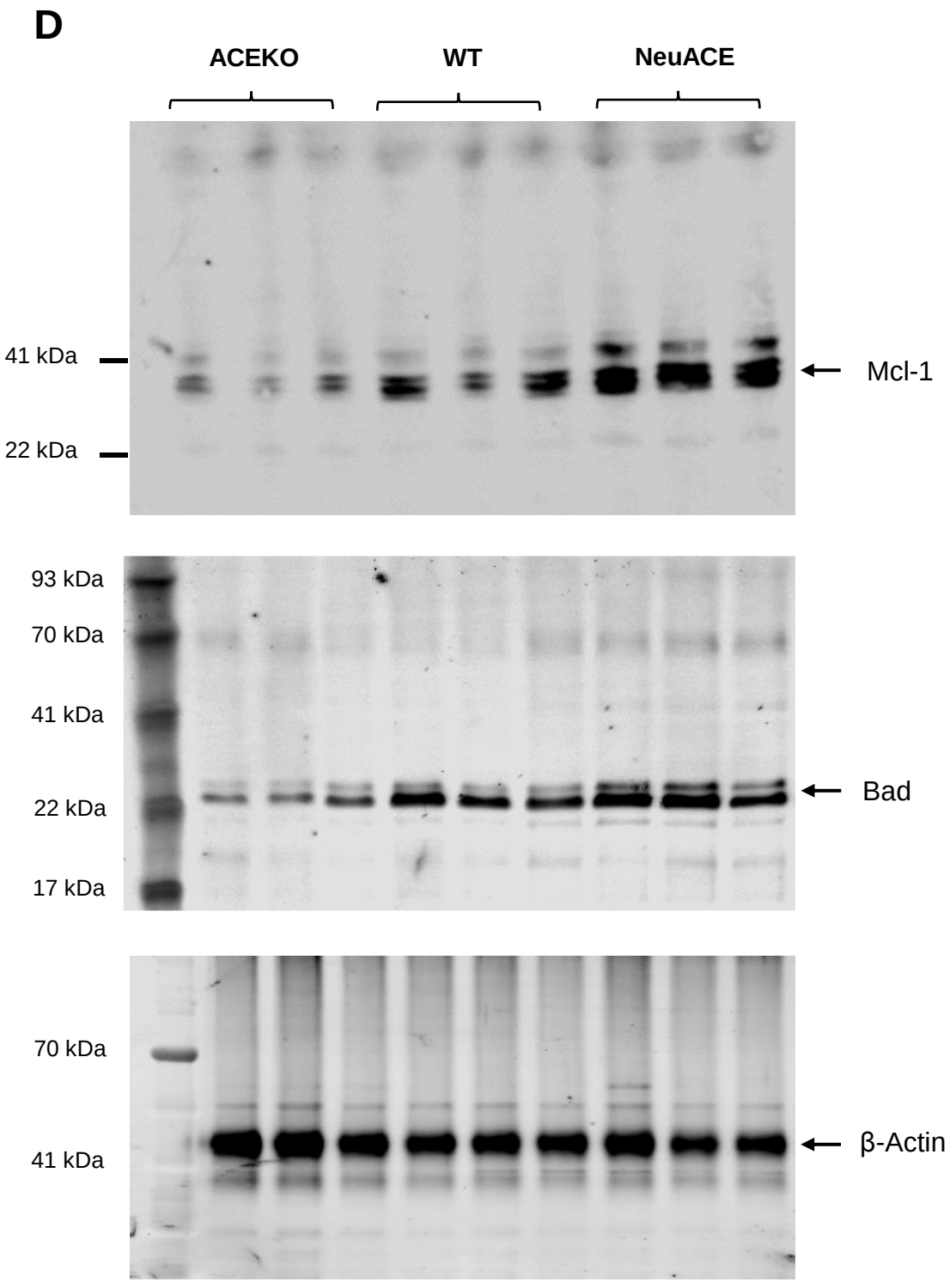
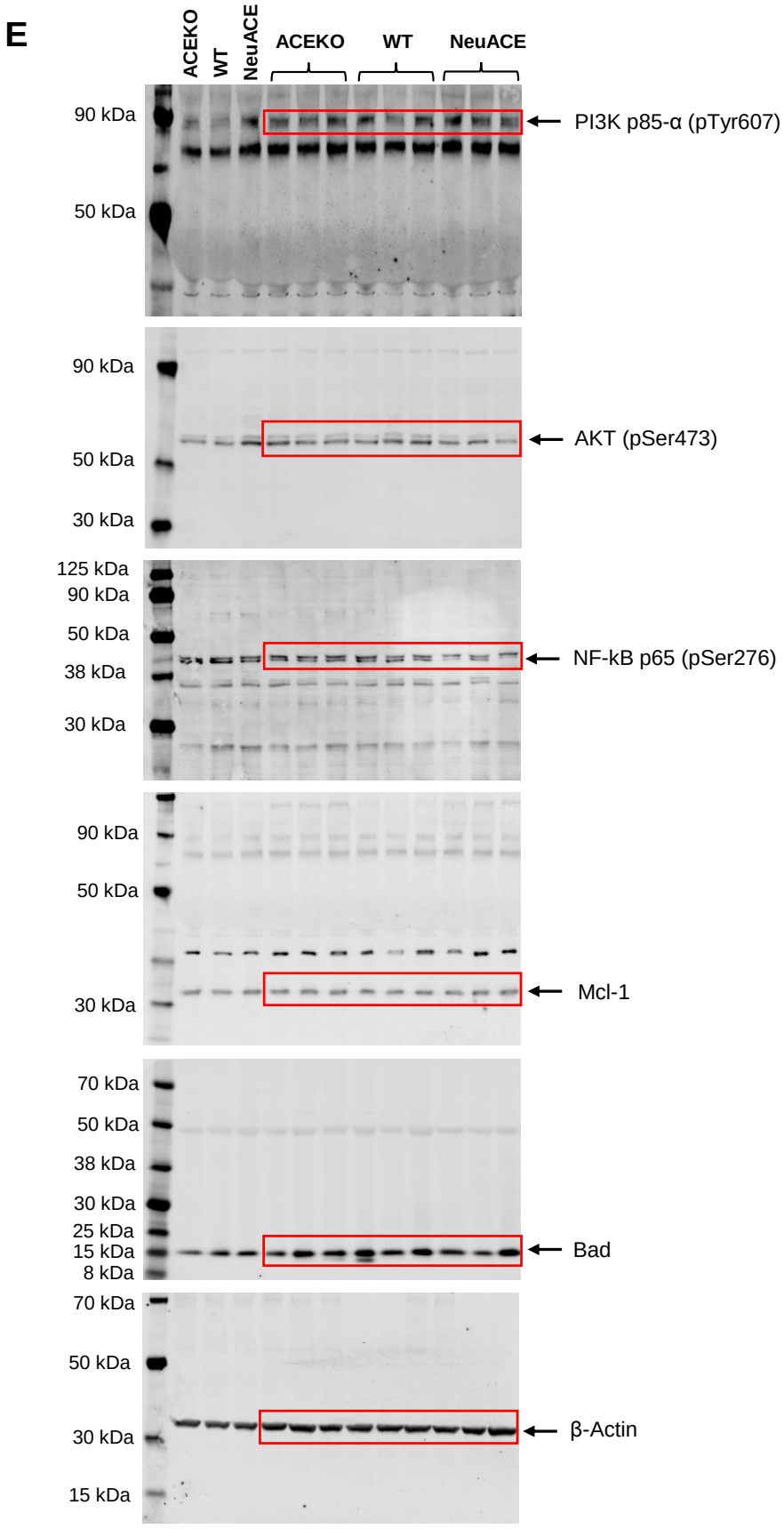
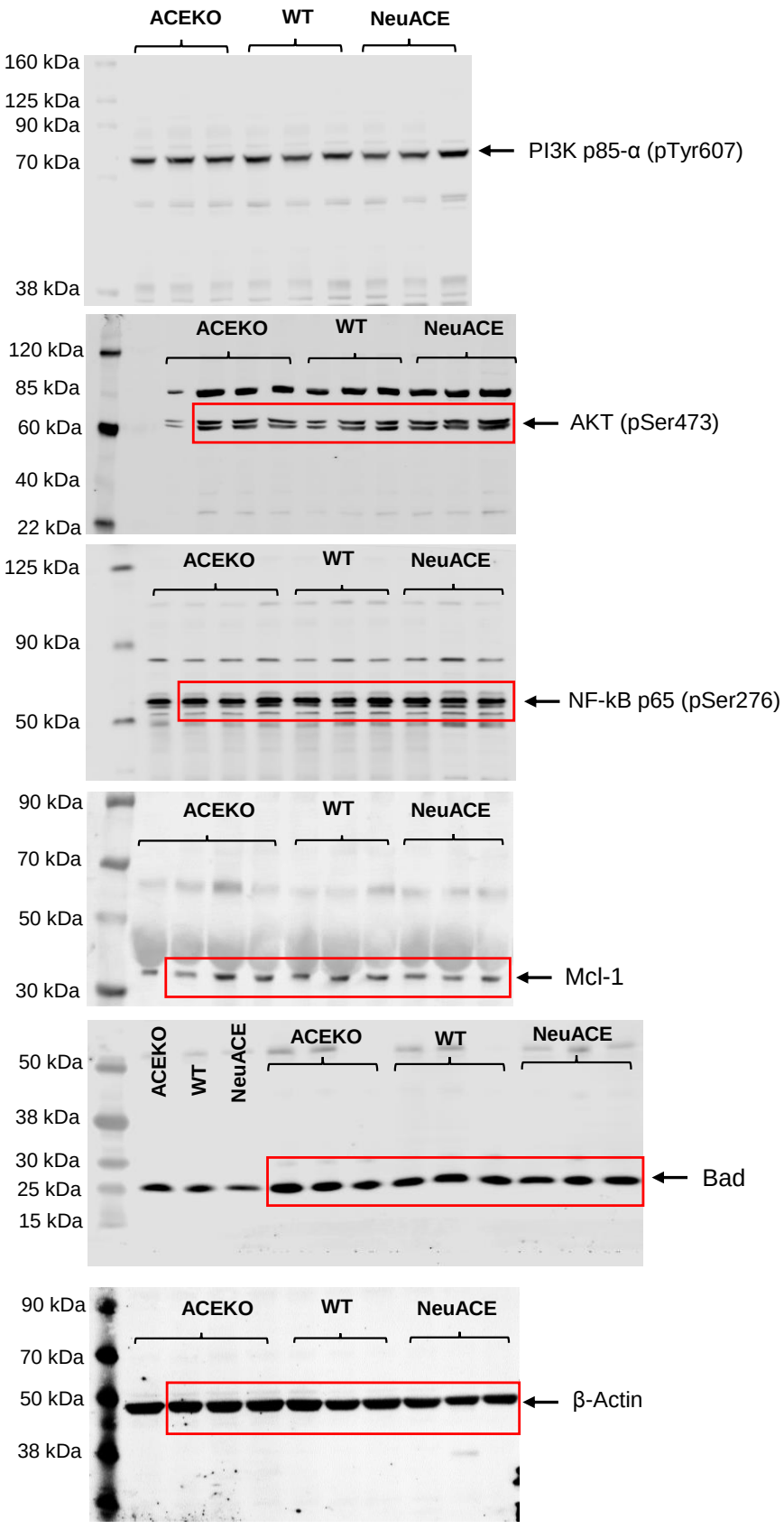
NF-kB p65 (pSer276)
93 kDa
70 kDa
41 kDa
22 kDa
17 kDa
β-Actin


D
ACEKO
WT
NeuACE
41 kDa
22 kDa
Mcl-1
93 kDa
70 kDa
41 kDa
22 kDa
17 kDa
Bad
70 kDa
41 kDa
β-Actin


E
ACEKO
WT
NeuACE
ACEKO
WT
NeuACE
90 kDa
50 kDa
PI3K p85-α (pTyr607)
90 kDa
50 kDa
30 kDa
AKT (pSer473)
125 kDa
90 kDa
50 kDa
38 kDa
30 kDa
NF-kB p65 (pSer276)
90 kDa
50 kDa
30 kDa
Mcl-1
70 kDa
50 kDa
38 kDa
30 kDa
25 kDa
15 kDa
8 kDa
Bad
70 kDa
50 kDa
30 kDa
15 kDa
β-Actin

F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WT
NeuACE
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125 kDa
90 kDa
70 kDa
38 kDa
PI3K p85-α (pTyr607)
120 kDa
85 kDa
60 kDa
40 kDa
22 kDa
AKT (pSer473)
125 kDa
90 kDa
50 kDa
NF-kB p65 (pSer276)
90 kDa
70 kDa
50 kDa
30 kDa
Mcl-1
50 kDa
38 kDa
30 kDa
25 kDa
15 kDa
Bad
β-Actin

Fig. S8. All original uncropped western blots. (A) Phospho-p38-MAPK (Fig. 4C). **(B)** Phospho-p47-phox (Fig. 4E)**. (C)** Phospho-PI3K, phospho-AKT, and phospho-NF-kB p65 (Fig. 7B). **(D)** Mcl-1 and Bad (Fig. 7C)**. (E)** Western blot analyses with ramipril treatment: phospho-PI3K, phospho-AKT, phospho-NF-kB p65, Mcl-1, and Bad (Fig. 7D). **(F)** Western blot analyses with LTB4 inhibition: phospho-PI3K, phospho-AKT, phospho-NF-kB p65, Mcl-1, and Bad (Fig. 7E).

Table S1. Details of human volunteers.

Volunteer	#1	#2	#3	#4	#5	#6	#7
Age	69 years	46 years	40 years	31 years	34 years	52 years	39 years
Sex	M	M	M	M	M	M	M
Ethnicity	Caucasian	Caucasian	Caucasian	Asian	Asian	Asian	Caucasian
Blood Pressure	125/73	128/71	131/83	128/76	110/64	124/83	116/71
Disease	None	None	None	None	None	Type 2 diabetes	None
Medications	None	None	None	Vitamin D2	Zinc	Metformin Lipitor	Levothyroxine

Table S2. Global statistical analyses of bacterial killing during incubation with whole blood isolated from humans treated with ramipril. Significant differences were observed in the percent survival of bacteria based on ramipril treatment ($p<0.0001$) and by bacterial strain ($p<0.0001$; *P. aeruginosa* has the overall highest percent survival). There was no difference in the percent bacterial survival by the interaction term of bacterial strain and treatment ($p=0.1181$) suggesting the effect of ramipril on bacteria killing was similar across all three strains. Globally, there was no difference either between 2 hour and 5 hour timepoints in the assay ($p=0.2232$).

Global Testing: Type 3 Tests of Fixed Effects	
Effect	**p-value**
Bacteria	<.0001
Treatment	<.0001
Time	0.2232
Bacteria*Treatment	0.1181
Bacteria*Time	<.0001
Time*Treatment	0.4571

Table S3. Pairwise statistical analyses of bacterial killing during incubation with whole blood isolated from humans treated with ramipril. Within each bacterial killing assay, the effects of ramipril (R) on whole blood-mediated killing were significantly different from untreated (U) or the washout (W) phase except at the 5 hour time point for P. aeruginosa.

Differences between treatments	
Label	**Bonferroni (p-value)**
MRSA 2h RvU	0.0006
MRSA 2h RvW	0.0192
MRSA 5h RvU	0.0006
MRSA 5h RvW	0.0060
Kleb 2h RvU	0.0360
Kleb 2h RvW	0.0024
Kleb 5h RvU	0.0006
Kleb 5h RvW	0.0006
Pseu 2h RvU	0.0012
Pseu 2h RvW	0.0036
Pseu 5h RvU	0.3672
Pseu 5h RvW	1.0000

Table S4. Global statistical analyses of intracellular killing by human neutrophils in vitro. Differences were observed in the percent survival of bacteria exposed to neutrophils isolated from humans receiving ramipril treatment ($p<0.0001$) and by bacterial strain ($p=0.0010$; *P. aeruginosa* has the overall highest percent survival) and by time ($p<0.0001$; 2 hours had higher percent survival than 5 hours overall). There was no difference for the interaction of bacterial strain with treatment ($p=0.0916$) suggesting the response to ramipril is similar across all three strains.

Global Testing: Type 3 Tests of Fixed Effects	
Effect	**p-value**
Bacteria	0.0010
Treatment	<.0001
Time	<.0001
Bacteria*Treatment	0.0916
Bacteria*Time	0.4756
Time*Treatment	0.0047

Table S5. Pairwise statistical analyses of intracellular killing by human neutrophils in vitro. Within each bacterial killing assay, the reduced killing of neutrophils following ramipril (R) exposure was significantly different from untreated (U) or the washout (W) phase except in *P. aeruginosa* groups at the 2 hour timepoint.

Pairwise testing: Differences between Treatments	
Label	**p-value (Bonferroni)**
MRSA 2h RvU	0.0006
MRSA 2h RvW	0.0024
MRSA 5h RvU	0.0006
MRSA 5h RvW	0.0006
Kleb 2h RvU	0.0006
Kleb 2h RvW	0.0480
Kleb 5h RvU	0.0006
Kleb 5h RvW	0.0006
Pseu 2h RvU	0.1620
Pseu 2h RvW	0.2688
Pseu 5h RvU	0.0012
Pseu 5h RvW	0.0006