eLIFE
elifesciences.org

Figures and figure supplements

Female resistance to pneumonia identifies lung macrophage nitric oxide synthase-3 as a therapeutic target

Zhiping Yang, et al.

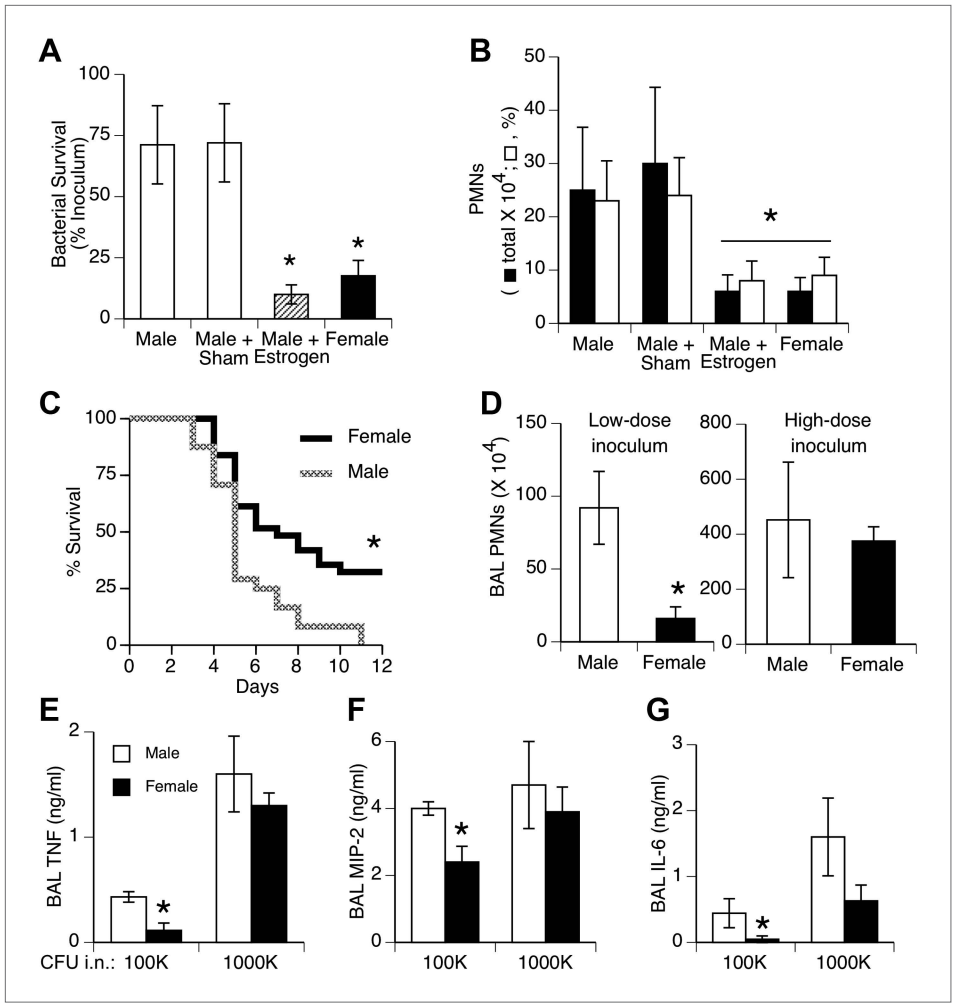

Figure 1. Females show greater resistance to pneumococcal pneumonia. (**A**) Twenty-four hours after intranasal (i.n.) inoculation of *S. pneumoniae* (~10^5 CFU), lung samples from female mice (and estrogen-treated male mice via subcutaneous slow-release 17-beta-estradiol pellets, ~70 μg/day) contain fewer live bacteria than seen in male mice ($n \geq 12$, * = $p < 0.01$ vs control or sham-treated males) and (**B**) show less acute inflammation (BAL neutrophils, $n \geq 12$, * = $p < 0.01$). (**C**) After i.n. pneumococcus, female mice show significantly greater survival than male mice (2.5×10^5 CFU, $n \geq 24$, * = $p < 0.01$). Gender differences in pneumonic inflammation are seen with low (4×10^5 CFU), but not high (11×10^5), bacterial inocula, measured as BAL neutrophilia (**D**) or BAL cytokines TNF (**E**), MIP-2 (**F**), or IL-6 (**G**), ($n \geq 3$, * = $p < 0.05$).

DOI: 10.7554/eLife.03711.003

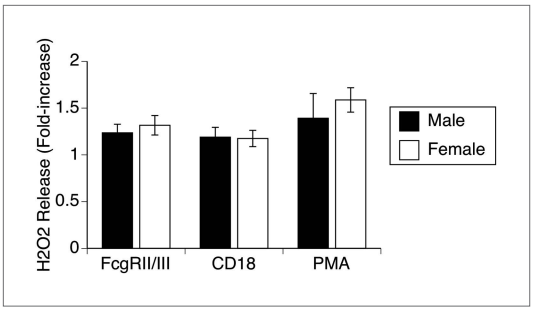

Figure 1—figure supplement 1. Respiratory burst by male and female alveolar macrophages. Stimulation of normal AMs by antibodies to 2 different surface receptors (FcR, CD18) or with PMA leads to approximately equal increases in H_2O_2 release in both male and female AMs, indicating absence of gender differences in production of reactive oxygen species.

DOI: 10.7554/eLife.03711.004

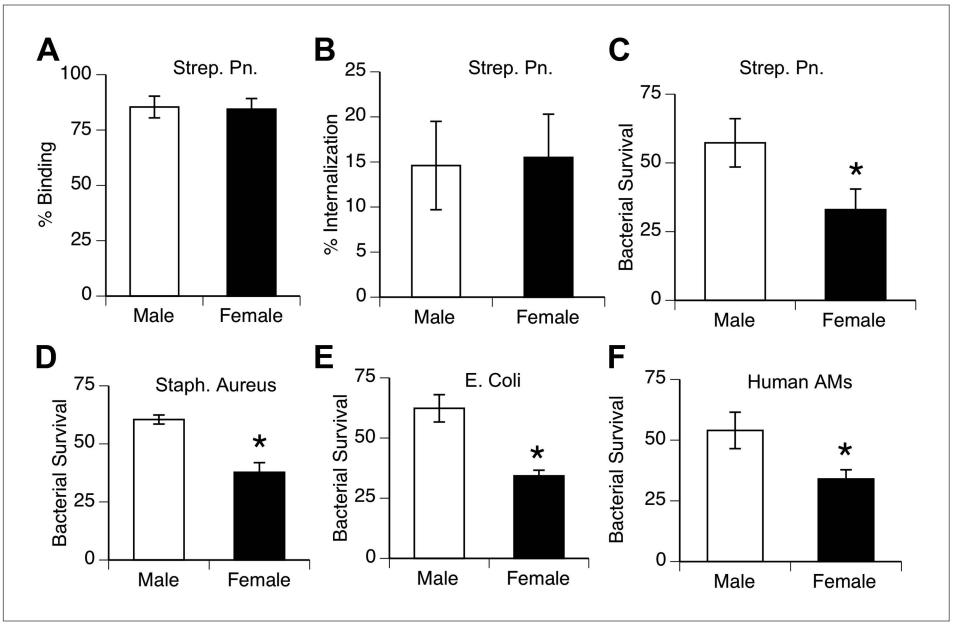

Figure 2. Female alveolar macrophages show better killing of ingested bacteria. Binding (**A**) and internalization (**B**) of *S. pneumoniae* in normal male and female AMs is similar. Female AMs kill more internalized bacteria than male AMs in assays using pneumococci (**C**) ($n \geq 11$, * = $p < 0.01$), *S. aureus* (**D**) or *E. coli* (**E**), ($n \geq 3$, * = $p < 0.01$). (**F**). Normal human female AMs also show greater killing of internalized pneumococci, ($n \geq 5$, * = $p < 0.01$).

DOI: 10.7554/eLife.03711.005

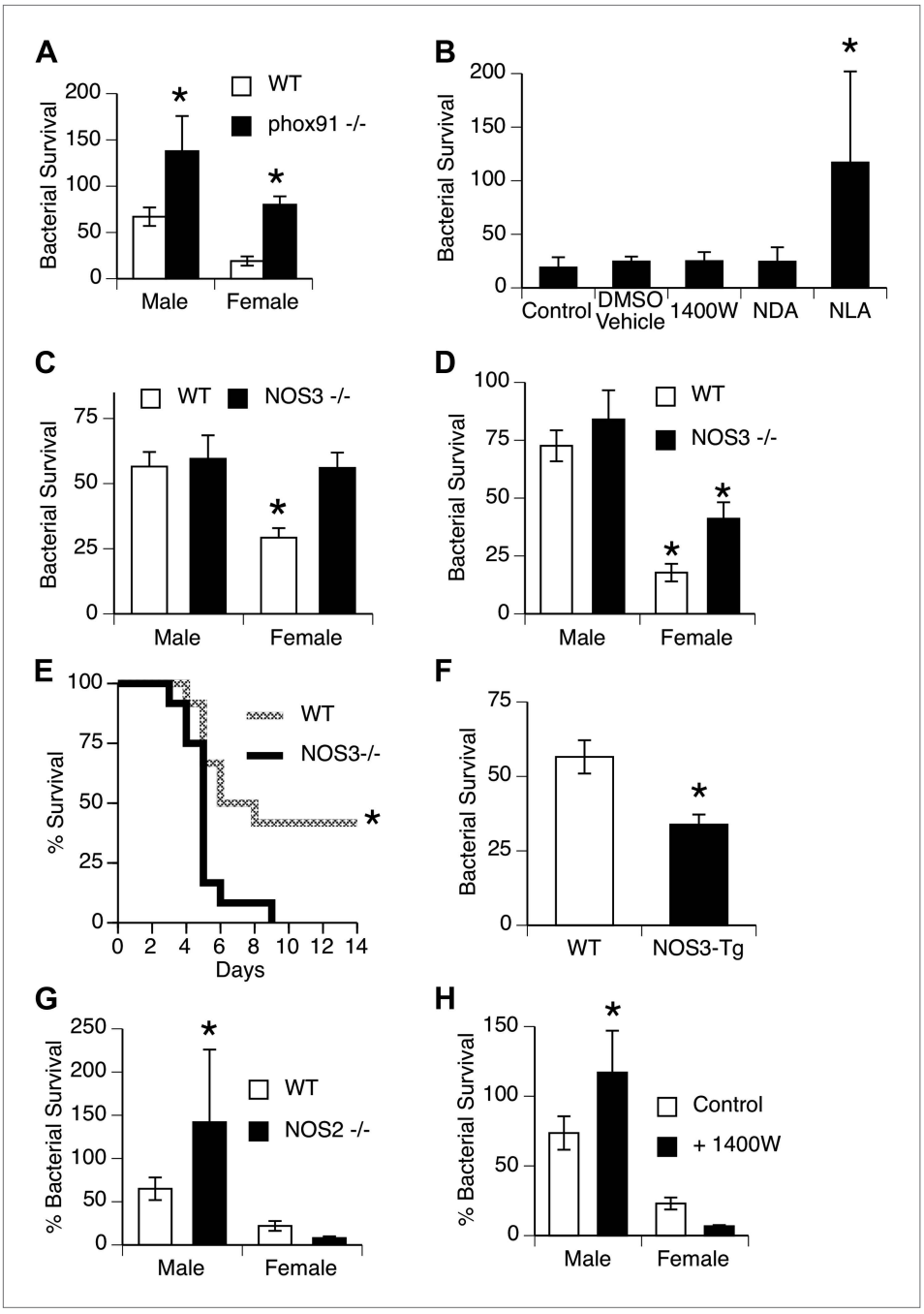

Figure 3. NOS3 and female resistance to pneumococcal pneumonia. (**A**) NADPH oxidase deficient (phox91$^{-/-}$) mice show comparable reduction in bacterial clearance in both male and female mice (n = 6, * = p < 0.01 vs wild-type). (**B**) In vitro killing of pneumococci by normal mouse female AMs is inhibited by the non-selective NOS inhibitor nitro-L-arginine (NLA), but not by its inactive stereo-isomer, nitro-D-arginine (NDA), nor by the type 2 NOS specific inhibitor 1400W (n = 3–4, * = p < 0.01). (**C**) Female AMs from *Nos3*$^{-/-}$ mice lose the in vitro killing advantage of wild-type female AMs and show the same killing rate as wild-type or NOS3 deficient male AMs (n = 3, * = p < 0.01 vs wild-type). (**D**) In vivo, absence of NOS3 reduces, but does not completely eliminate, the female advantage in bacterial clearance (n = 15, * = p<0.015 vs all 3 other groups) and results in increased mortality from pneumococcal pneumonia (**E**) (n = 12 female mice per group, * = p < 0.01). Conversely, transgenic male mice with increased expression of human NOS3 show enhanced killing of *S. pneumoniae* in vivo (**F**) (lower bacterial survival, n ≥ 5, * = p < 0.01). In this low-dose inoculum model, NOS2 deletion (**G**) or inhibition (**H**) causes reduced bacterial clearance in male, but not female mice (n = 8, * = p < 0.05).

DOI: 10.7554/eLife.03711.006

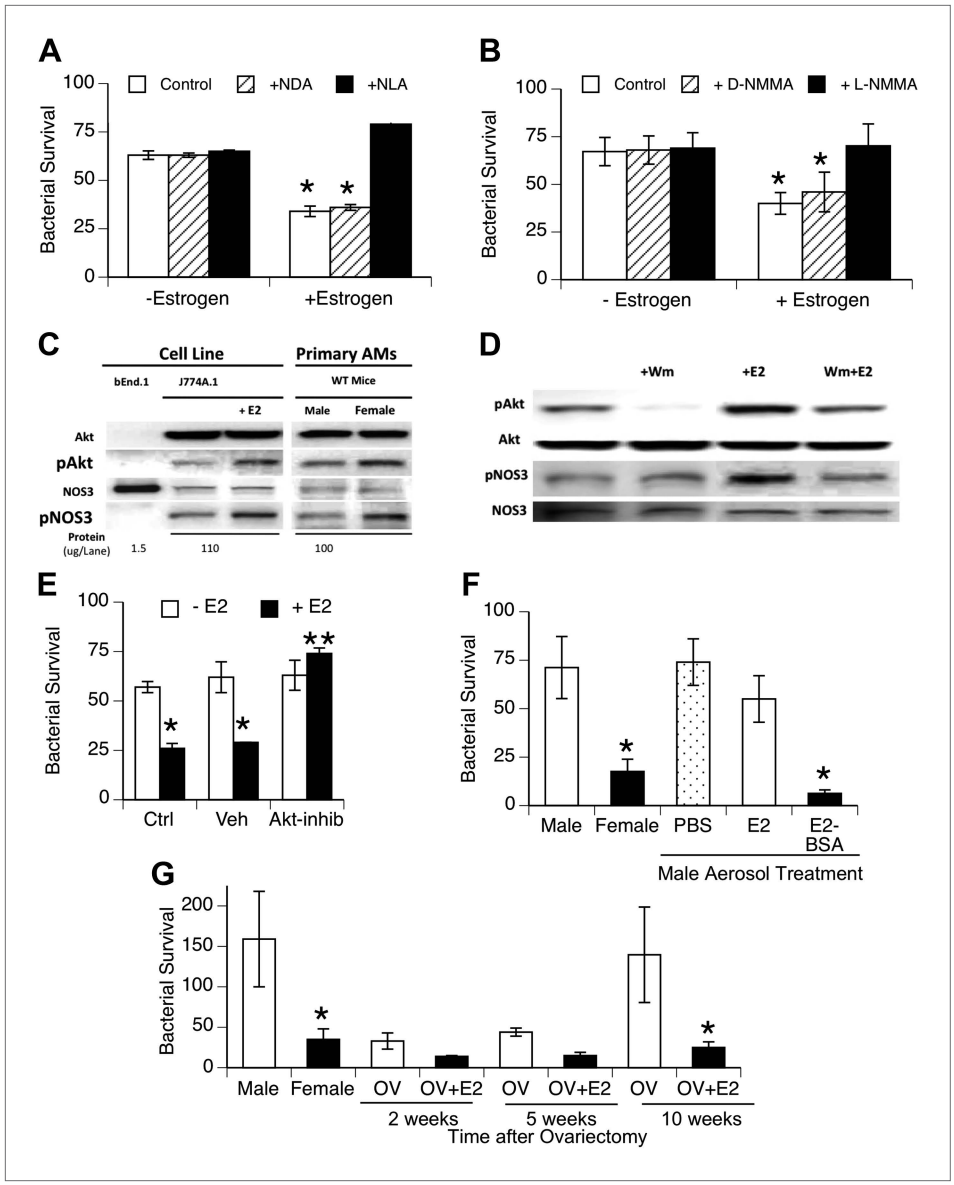

Figure 4. Estrogen-mediated activation of macrophage NOS3. Estrogen treatment of J774A.1 mouse or human U937 macrophages (**A** and **B**) increases killing of ingested pneumococci; this increased killing is prevented by the NOS inhibitors NLA or l-NMMA, but not control stereoisomers (n = 3–4, * = $p < 0.01$). (**C**) Western blot analysis shows >100-fold NOS3 in macrophages compared to the endothelial cell line bEnd.1; after 30 min, estrogen-treated (E2, estradiol, 0.2 ng/ml) J774A.1 mouse macrophages show increased phosphorylation of Akt and NOS 3, while normal female AMs show basally increased pAkt and pNOS3 compared to male AMs; (**D**) basal- and estrogen-enhanced phosphorylation of Akt and NOS3 are inhibited by wortmannin (Wm, 50 nM). (**E**) Inhibition of Akt with 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (10 μg/ml REF) prevents estrogen-mediated increased bacterial killing in J774A.1 cells (n = 3, * = $p < 0.01$). (**F**) Aerosol pre-treatment of male mice with albumin-conjugated estrogen 30 min before pneumococcal infection improves bacterial killing (n = 6, * = $p < 0.01$). (**G**) In ovariectomy-model of menopause, female mice lose their greater resistance to pneumococcal pneumonia after 10 weeks, an effect reversed by treatment with estrogen prior to infection, $n \geq 8$ for control, 10 week groups; n = 3 for 2 and 5 week groups; * = $p < 0.01$.

DOI: 10.7554/eLife.03711.007

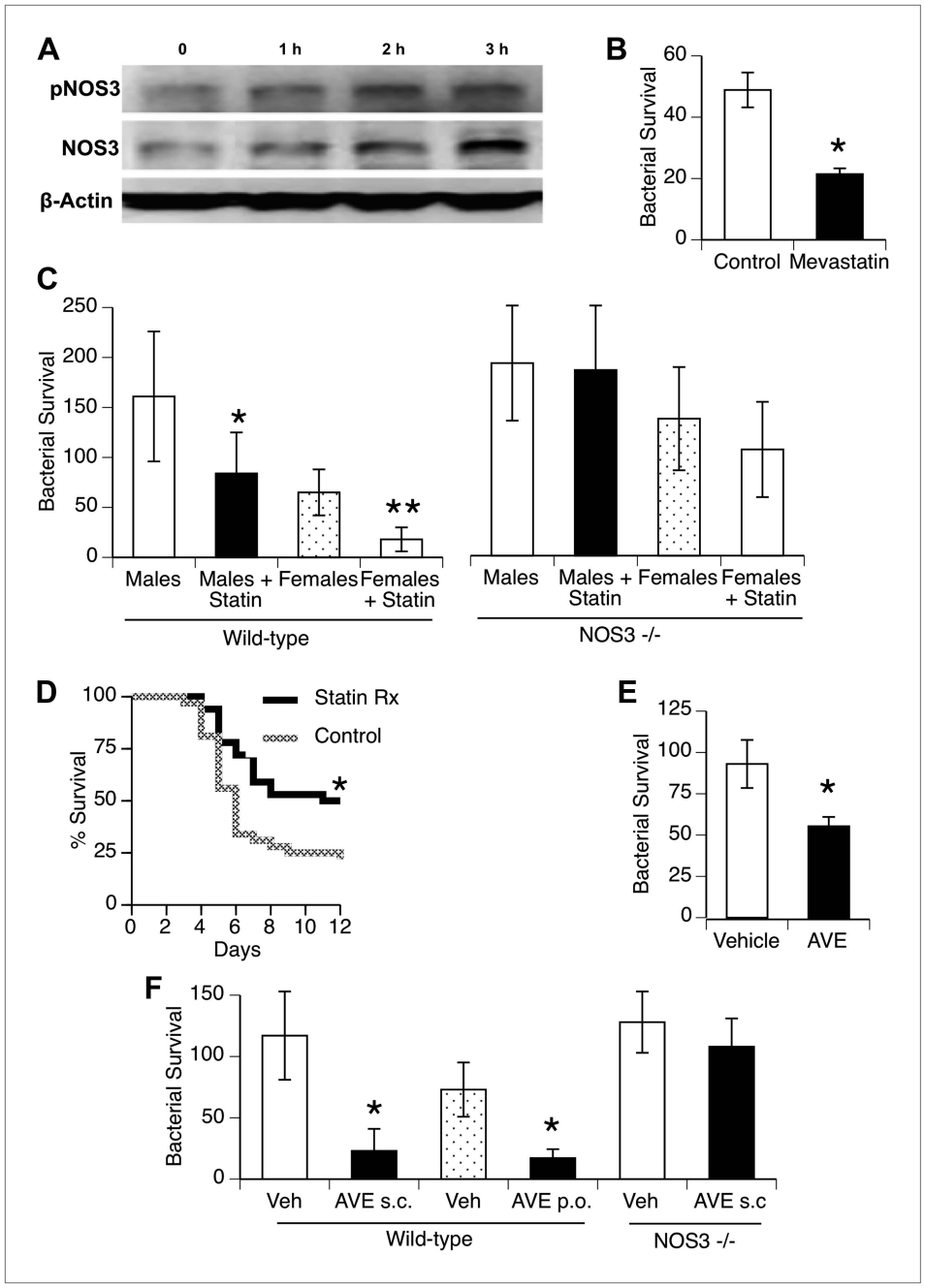

Figure 5. Statins enhance innate immune resistance to *S. pneumoniae* via NOS3. (**A**) In vitro treatment of J774A.1 mouse macrophages with mevastatin (5 μM) increases levels of pNOS3 and NOS3 and (**B**) concomitantly increases killing of internalized bacteria (n = 4, * = $p < 0.01$). (**C**) In vivo, pre-treatment of mice with pravastatin (50 mg/kg) significantly improves bacterial clearance in wild-type mice (n = 8, * = $p < 0.01$ vs male controls; ** = $p < 0.01$ vs males, males + statin), but has no significant effect on either male or female $NOS3^{-/-}$ mice. (**D**) Statin-treated male mice with pneumococcal pneumonia show improved survival (n = 8, * = $p < 0.01$). (**E**) AVE3085, a small molecule activator of NOS3, increases bacterial killing by mouse macrophages in vitro (n = 3, * = $p < 0.01$) (**F**) Pre-treatment of male mice with AVE3085 by either subcutaneous or oral route improves in vivo bacterial clearance, an effect not seen in $NOS3^{-/-}$ male mice (n = 3–8, * = $p < 0.01$).

DOI: 10.7554/eLife.03711.010

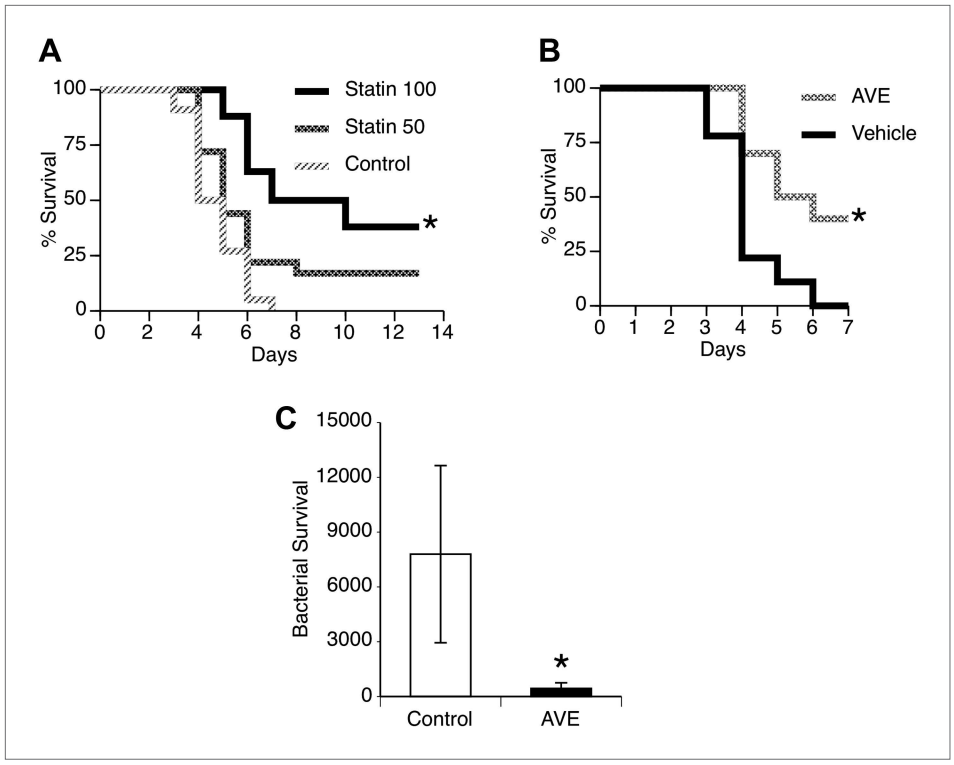

Figure 6. Statins and AVE3085 improve survival from post-influenza secondary pneumococcal pneumonia. Male mice were allowed to recover 7 days from mild influenza (PR8 1 PFU i.n.) and then challenged with *S. pneumoniae* (500 CFU i.n.). Pre-treatment with (**A**) pravastatin (50 or 100 mg/kg) or (**B**) AVE3085 (0.75 mg, s.c.) caused a significant improvement in survival (n = 10, * = $p < 0.01$). (**C**) AVE3085 treatment also lead to improved bacterial clearance 24 hr after pneumococcal challenge in this post-influenza model (n = 6, * = $p < 0.01$).

DOI: 10.7554/eLife.03711.011