Figures and figure supplements

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

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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 > 12, * = p < 0.01 vs control or sham-treated males) and (B) show less acute inflammation (BAL neutrophils, n > 12, * = p < 0.01). (C) After i.n. pneumococcus, female mice show significantly greater survival than male mice (2.5 × 10^5 CFU, n > 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 ≥ 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$_2$O$_2$ 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 NOS3, 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 ≥ 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 led 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