Supplementary Figures

Supplementary Figure S1. Expression of ACP across *Neisseria gonorrhoeae* reference strains. (A) SDS-PAGE analysis of total protein lysates from *Neisseria gonorrhoeae* FA1090, isogenic *Δacp* mutant, and the 2016 WHO Ng reference strains (F, G, K, L, M, N, O, P, U, V, W, X, Y, Z). Purified recombinant ACP (rACP) was included as a control. (**B**) Western blot analysis with polyclonal rabbit serum against rACP detecting ACP expression in the same Ng whole cell lysates as in panel (A). rACP was used as a positive control. The *Δacp* mutant serves as a negative control, confirming specificity. Molecular weight markers (kDa) are indicated on the left.

Supplementary Figure S2. Serum antibody responses following intranasal immunization with rACP, rACP+CpG, or rMtrE+CpG. Serum antibody levels (AUC, $\log_{10}$) were measured in mice immunized intranasally with CpG, PBS, rACP, rACP+CpG, or rMtrE+CpG and assessed across retro-orbital (RO) and saphenous vein (SV) blood collection sites at days 31, 52, and 63 post-initial immunization. Data are presented as geometric means with interquartile ranges. Data are presented as log-transformed antibody titers, with individual data points from each mouse (green dots) and boxplots representing geometric mean values with interquartile ranges. (**A**) Total serum IgG titers, (**B**) Serum IgG1 titers, (**C**) Serum IgG2a titers, (**D**) Serum IgG3 titers, and (**E**) Serum IgA titers.

Supplementary Figure S3. Vaignal antibody responses following intranasal immunization with rACP, rACP+CpG, or rMtrE+CpG. Vaginal antibody levels (AUC, $\log_{10}$) were measured in mice immunized intranasally with CpG, PBS, rACP, rACP+CpG, or rMtrE+CpG and assessed at days 31, 52, and 63 post-initial immunization. Data are presented as geometric means with interquartile ranges. Data are presented as log-transformed antibody titers, with individual data points from each mouse (green dots) and boxplots representing geometric mean values with interquartile ranges. (**A**) Total vaginal IgG titers, (**B**) Vaginal IgA titers.

Supplementary Figure S4. Longitudinal and comparative analysis of serum IgG3 across antigen formulations and blood collection methods. Serum IgG3 levels (AUC, $\log_{10}$) were measured in mice immunized intranasally with rACP, rACP+CpG, or rMtrE+CpG and assessed across retro-orbital (RO) and saphenous vein (SV) blood collection sites. Data are presented as geometric means with interquartile ranges, and statistical significance was determined using a two-tailed paired t-test. (A) Serum IgG3 levels over time for RO and SV samples. (B) Comparison of serum IgG3 levels between RO and SV blood collection methods across different vaccine formulations. (C) Comparison of IgG3 responses among different vaccine formulations at different time points within RO and SV groups.

Supplementary Figure S5. Longitudinal and comparative analysis of serum IgA. Serum IgA levels (AUC, $\log_{10}$) were assessed in mice administered with rACP, rACP+CpG, or rMtrE+CpG and assessed across retro-orbital (RO) and saphenous vein

(SV) blood collection sites. Data are presented as geometric means with interquartile ranges, and statistical significance was determined using a two-tailed paired t-test. (**A**) Serum IgA levels over time for RO and SV samples. (**B**) Comparison of serum IgA levels between RO and SV blood collection methods across different vaccine formulations. (**C**) Comparison of IgA responses among different vaccine formulations at different time points within RO and SV groups.

Supplementary Figure S6. Original, unprocessed, and uncropped SDS-PAGE and immunoblot images supporting Figure 2, provided to ensure transparent data reporting.

(A) Coomassie-stained SDS-PAGE showing purified recombinant ACP (~11.3 kDa) and MtrE (~48.3 kDa) proteins, loaded at the indicated concentrations (μg/well). M: molecular weight marker.

(B) Immunoblot using anti-ACP serum confirms specific detection of rACP at the expected molecular weight across increasing protein amounts.

(C) Immunoblot using anti-MtrE serum demonstrates specific detection of rMtrE at ~48.3 kDa.

The molecular weight of protein markers (in kDa) is indicated to the left of each panel.

Supplementary Figure S7. Immunoblotting analysis of ACP-specific IgG and IgA responses. Pooled sera and vaginal washes collected 63 days post-immunization from female BALB/c mice were used to detect ACP-specific antibody responses. Mice were immunized with recombinant ACP (rACP), rACP combined with CpG (rACP+CpG), CpG,

or PBS as a control. Sera and vaginal washes were used as the primary antibody in immunoblotting assays to detect specific IgG and IgA isotypes against rACP antigen. Unprocessed immunoblots are presented to support the data shown in Figure 3 and ensure transparent data reporting. The molecular weight of protein markers (in kDa) is indicated to the left of each panel. (**A**, **B**) Serum IgG; (**C**, **D**) Serum IgA; (**E**) Vaginal IgG; (**F**) Vaginal IgA.

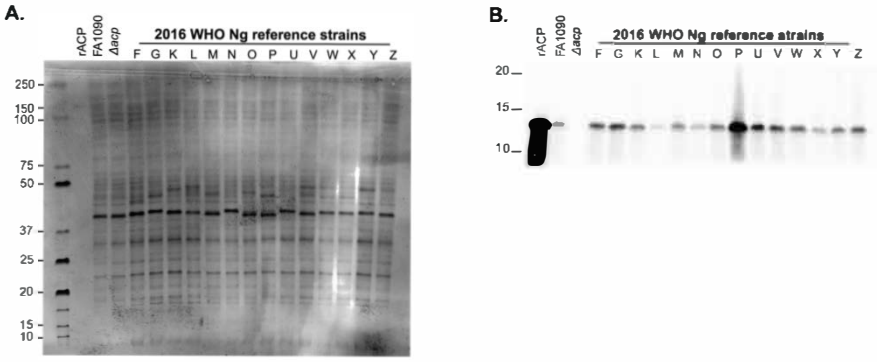
Blood was collected via RO (retro-orbital) or SV (saphenous vein) collection methods.

Supplementary Figure S8. Immunoblotting analysis of MtrE-specific IgG and IgA responses. Pooled sera and vaginal washes collected 63 days post-immunization from female BALB/c mice were used to detect MtrE-specific antibody responses. Mice were immunized with recombinant recombinant rMtrE combined with CpG (rMtrE+CpG), CpG alone, or PBS. Sera and vaginal washes were used as the primary antibody in immunoblotting assays to detect specific IgG and IgA isotypes against rMtrE. Unprocessed immunoblots are presented to support the data shown in Figure 3 and ensure transparent data reporting. The molecular weight of protein markers (in kDa) is indicated to the left of each panel. (**A**) Serum IgG; (**B**) Serum IgA; (**C**) Vaginal IgG; (**D**) Vaginal IgA.

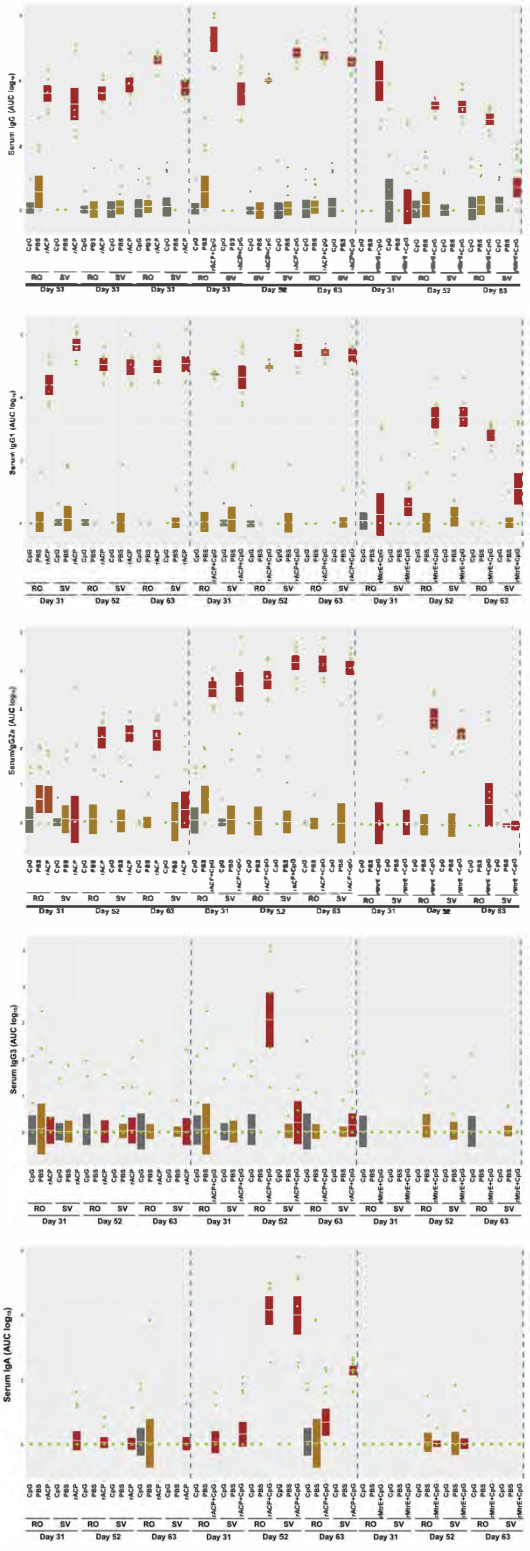
Blood was collected via RO (retro-orbital) or SV (saphenous vein) collection methods.

Supplementary Figure S9. Comparative immunoblot reactivity of total serum IgG to ACP and MtrE using pooled retro-orbital (RO) and saphenous vein (SV) serum samples. Immunoblottings show IgG binding to whole-cell lysates from 14 genetically and temporally diverse *Neisseria gonorrhoeae* (Ng) strains included in the 2016 WHO Ng reference panel. Blots were probed with pooled serum samples collected 63 days post-immunization from female BALB/c mice immunized with either recombinant ACP (rACP), ACP combined with CpG (ACP+CpG), or MtrE-CpG, and delivered via RO or SV routes. Each lane represents a different Ng strain (labeled F–Z). Panels A–D display anti-ACP IgG reactivity; panels E and F show anti-MtrE IgG reactivity. Unprocessed immunoblots are presented to support the data shown in Figure 3 and ensure transparent data reporting. Molecular weight markers (kDa) are indicated to the left of each panel. (**A**, **B**) ACP-specific IgG (RO vs SV); (**C**, **D**) ACP+CpG-specific IgG (RO vs SV); (**E**, **F**) MtrE+CpG-specific IgG (RO vs SV).

Supplementary Fig. S1

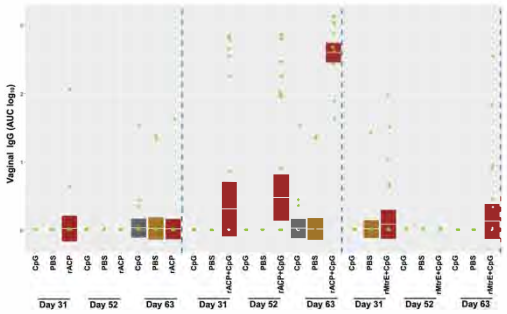


Supplementary Fig. S2



Supplementary Fig. S3

A.



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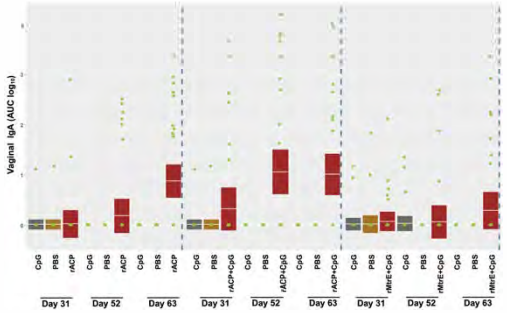

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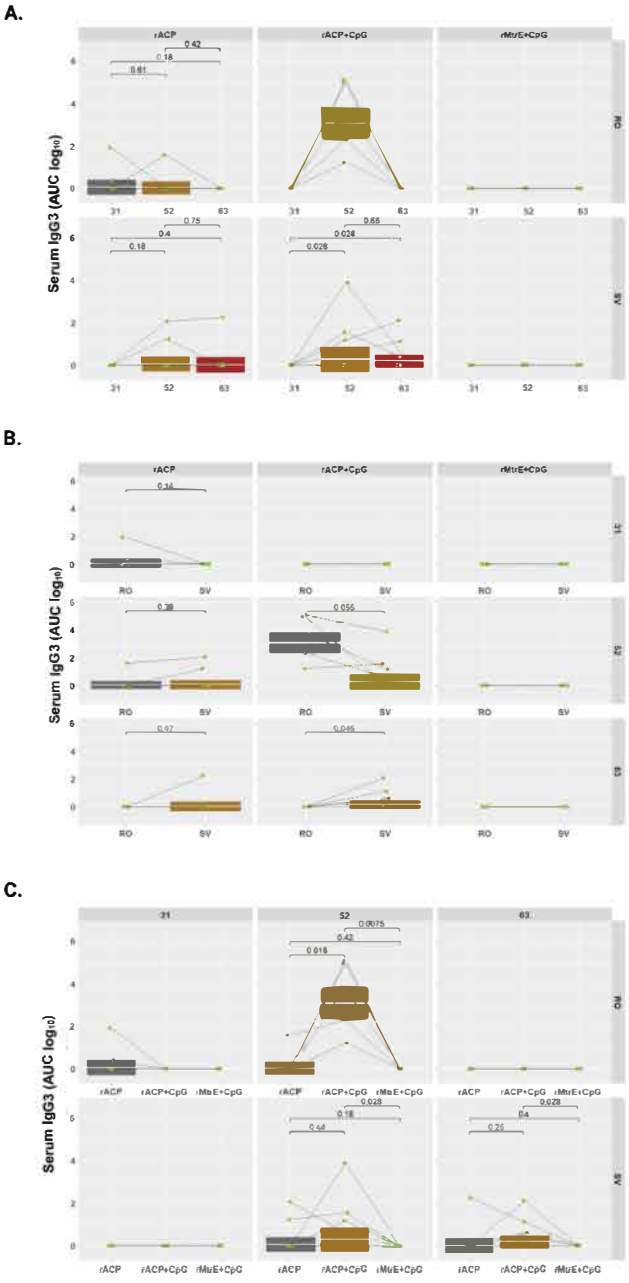

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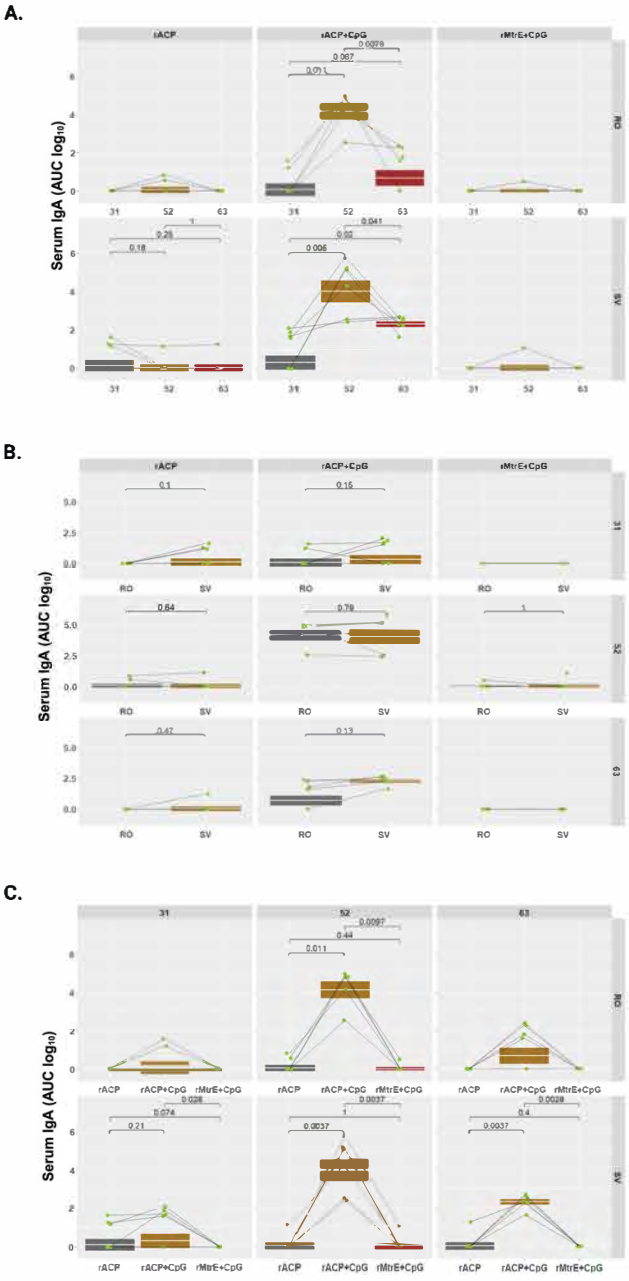

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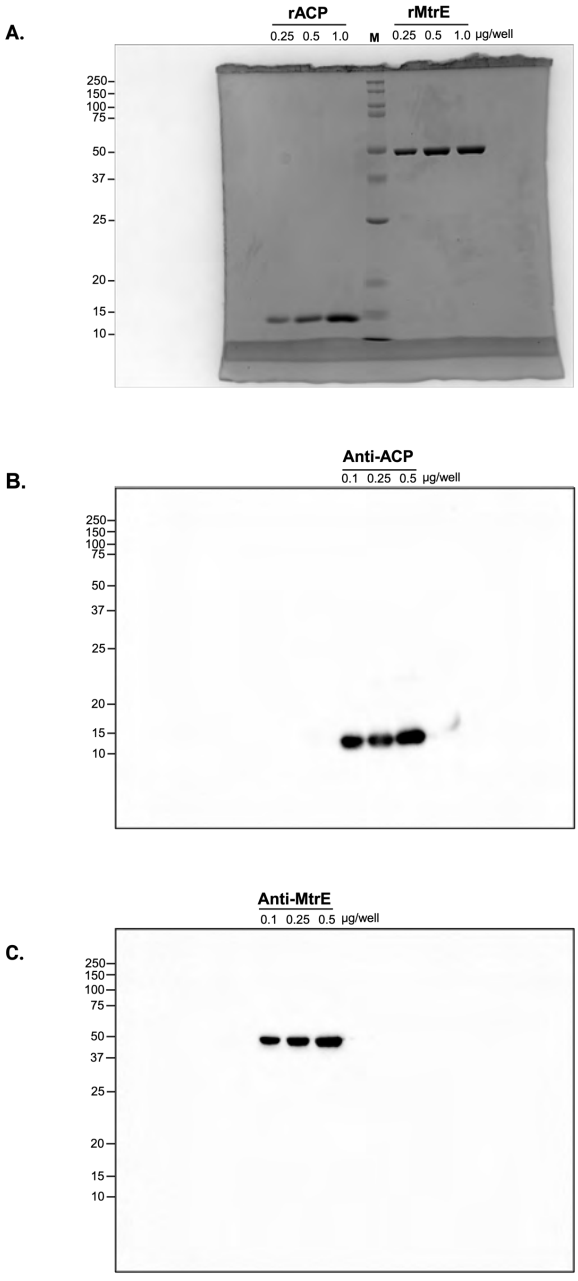

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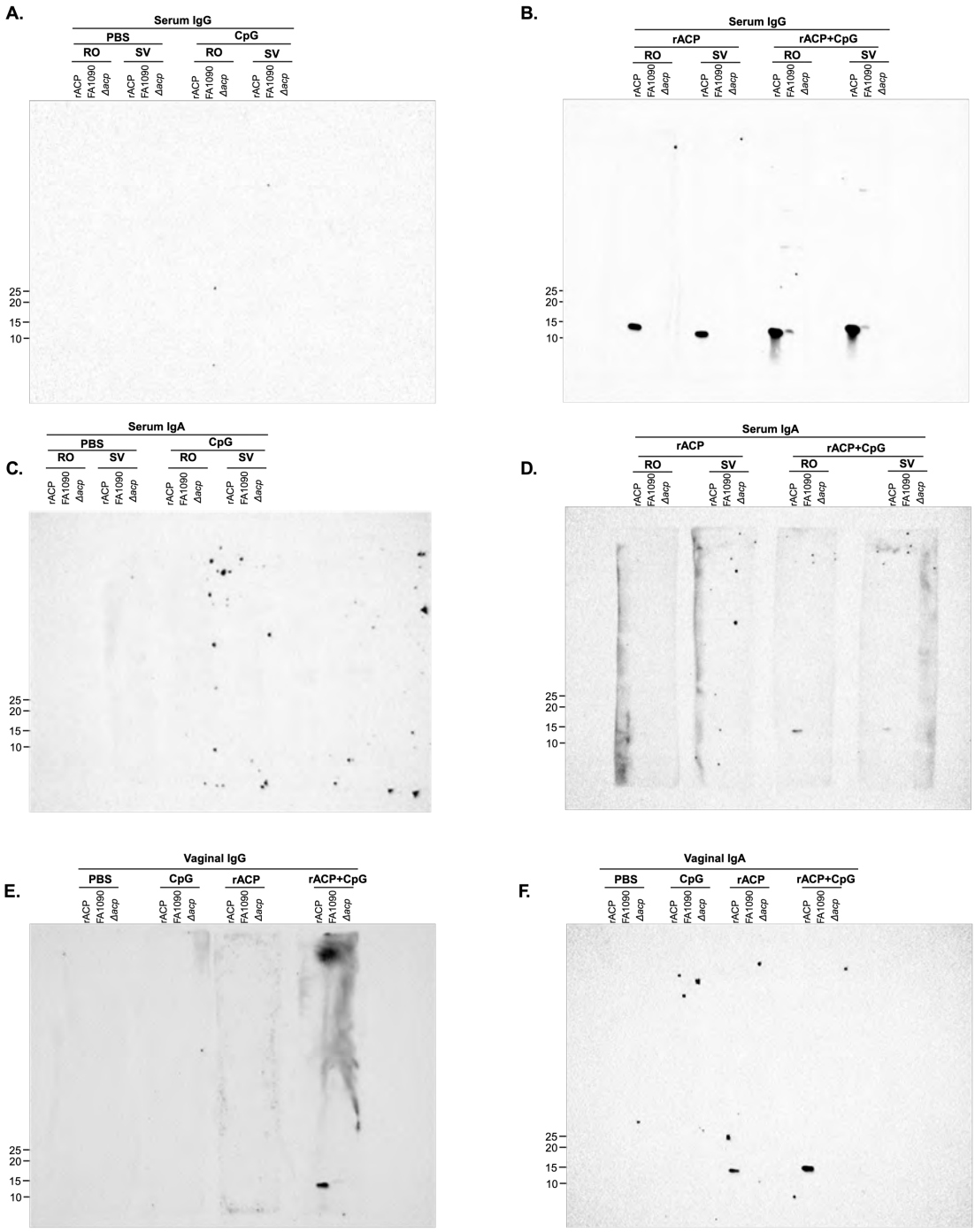
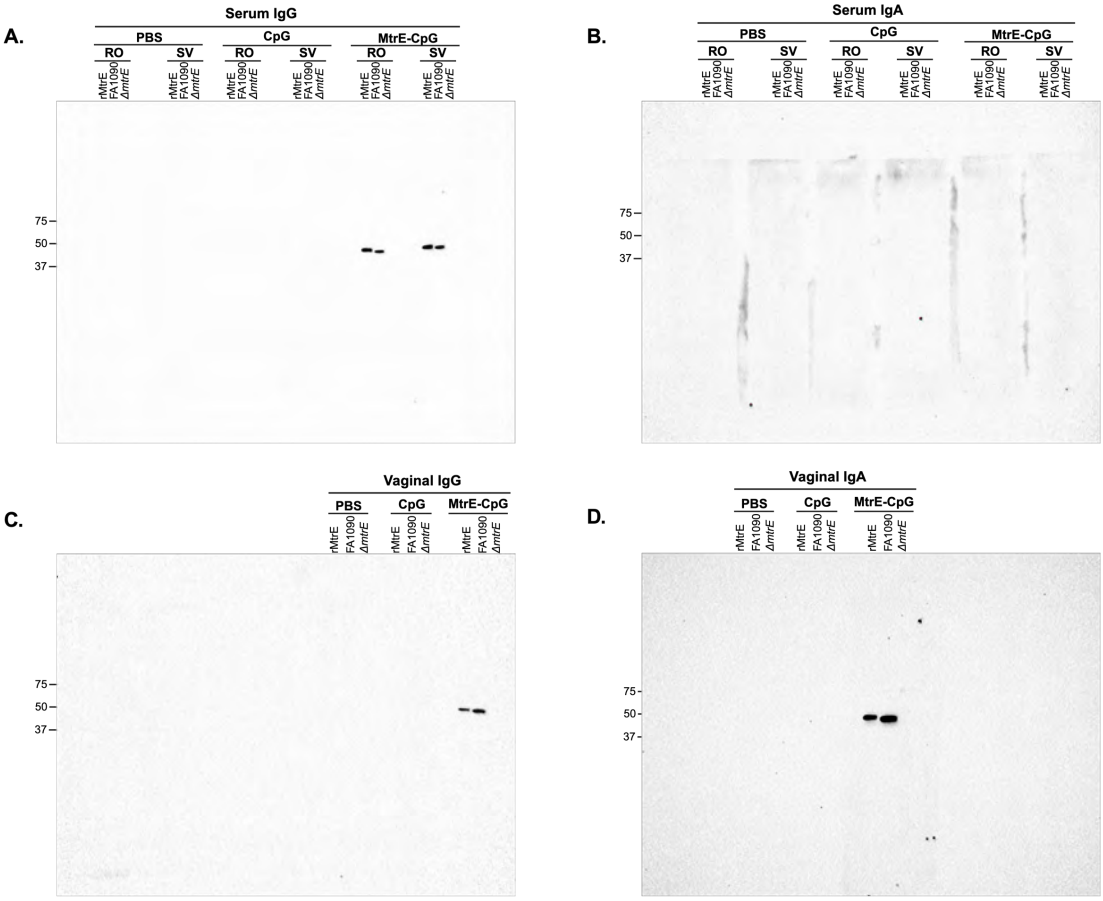


Supplementary Fig. S8



Supplementary Fig. S9