Supporting Information

Vono et al. 10.1073/pnas.1319784110

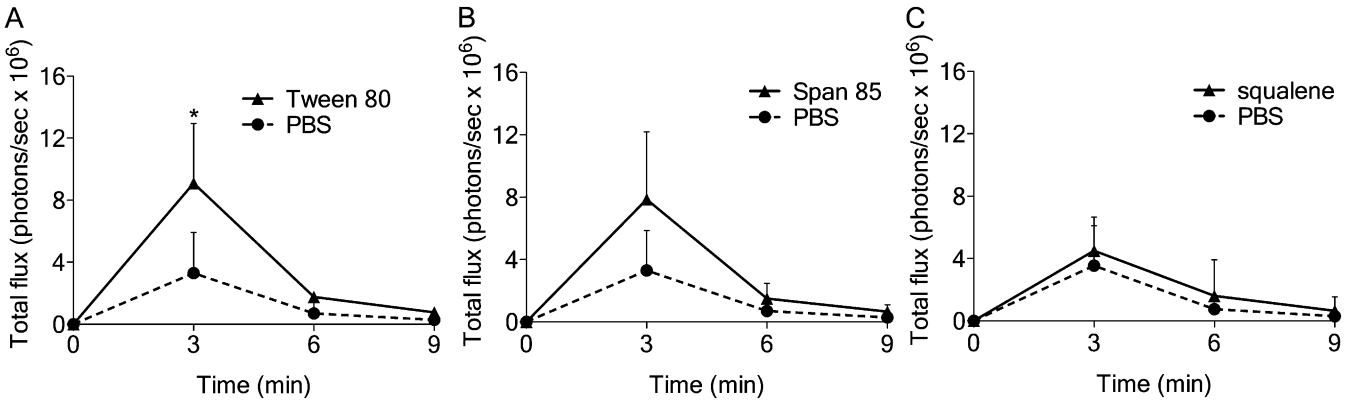

Fig. S1. The two surfactants, Tween 80 and Span 85, are responsible for MF59-induced ATP release. (*A–C*) Quantitative analyses of chemiluminescence emission over time (number of photons per second in the region of interest). (*A*) Tween 80 (0.5%), (*B*) Span 85 (0.5%), (*C*) squalene (5% vol/vol). Data show mean values + SD from at least four independent experiments. Unpaired, two-tailed Student's *t* test (T): $*P < 0.05$.

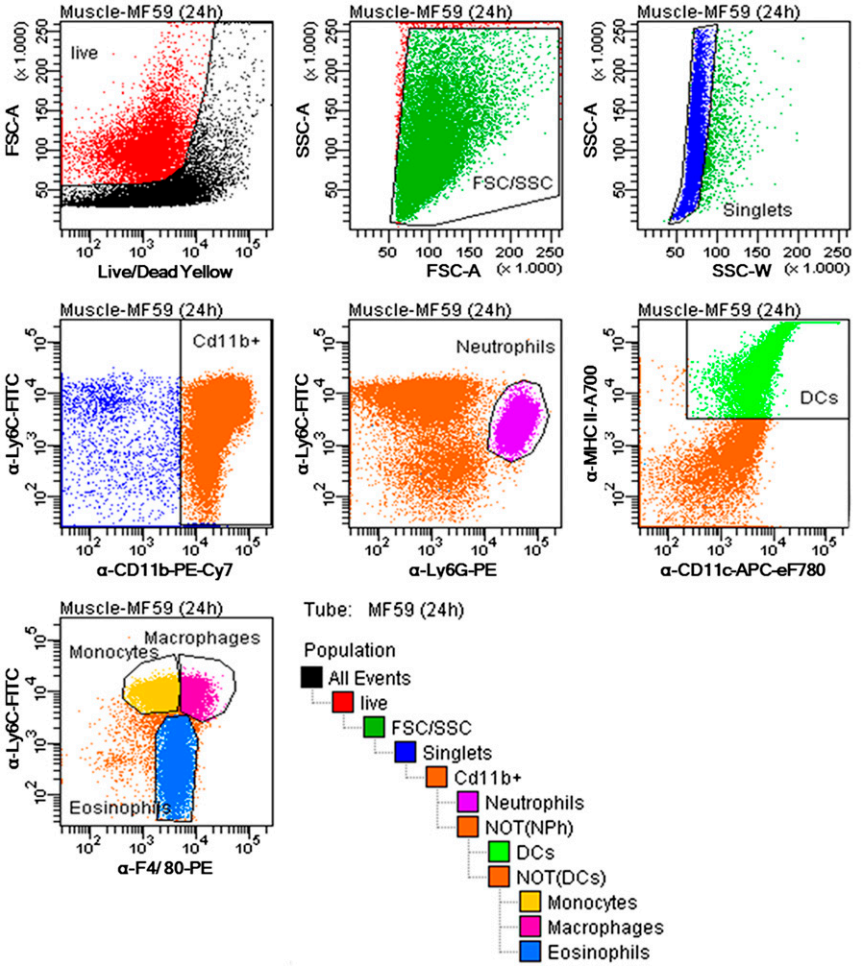

Fig. S2. Gating strategy of muscle-derived cells. Muscle single cell suspensions were prepared and analyzed by FACS, applying the depicted gating strategy.

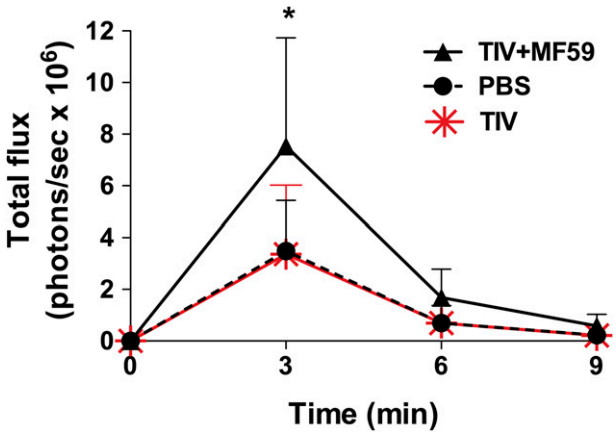

Fig. S3. The injection of a trivalent influenza vaccine (TIV) alone does not induce ATP release. Quantitative analyses of chemiluminescence emission over time (number of photons per second in the region of interest). For each mouse, one leg was injected intramuscularly with a mixture composed of the reporter and TIV antigens (0.1 μg each antigen) alone, whereas the contralateral leg was injected with the reporter solution plus PBS or TIV antigens adjuvanted with MF59 (20% vol/vol). Data show mean values + SD from eight to 11 mice per experimental condition in independent experiments. Unpaired, two-tailed Student's *t* test (T): $*P < 0.05$.

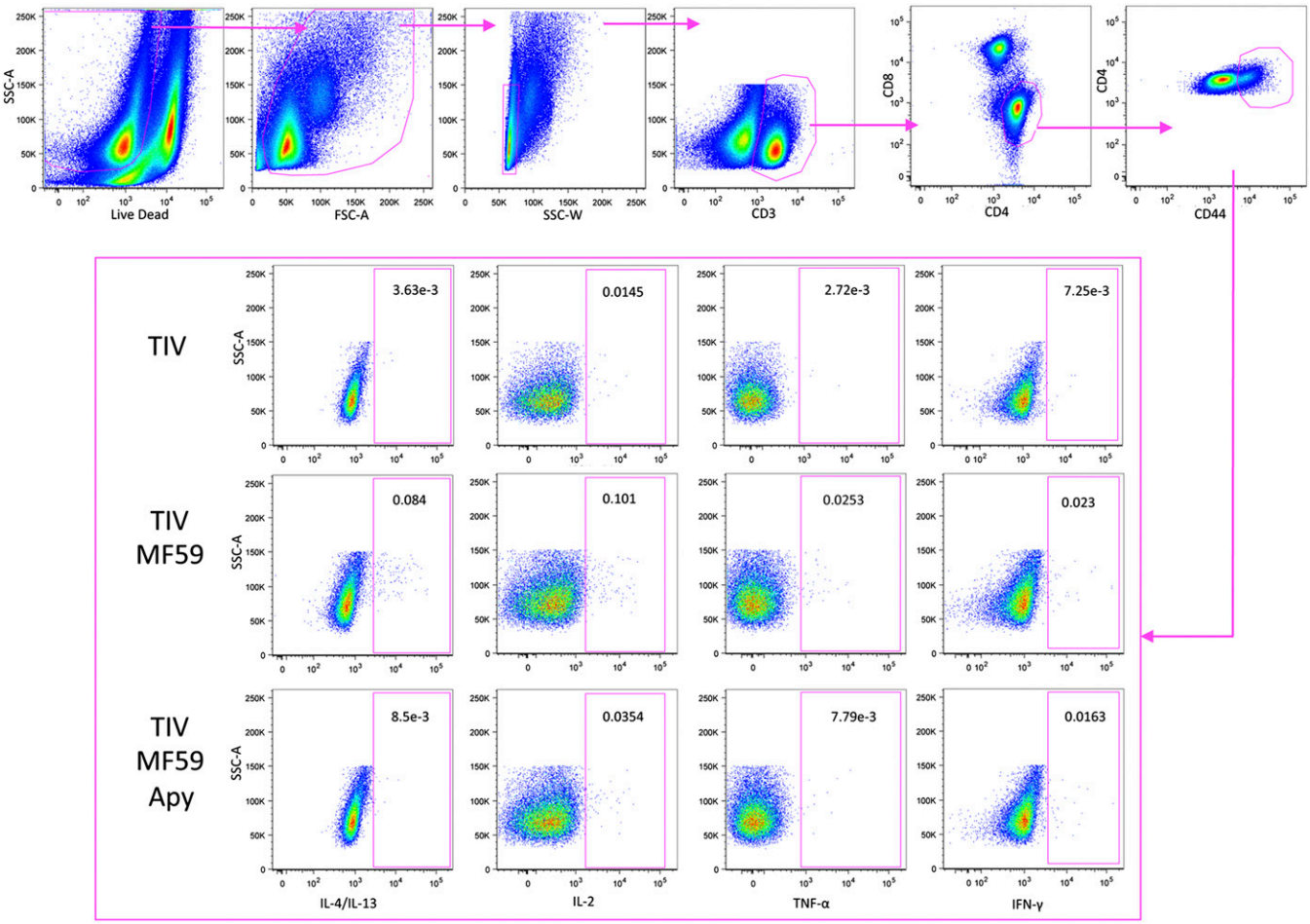

Fig. S4. Gating strategy of spleen-derived cells. Spleens from 4 mice per group were taken 2 wk after the first immunization, and vaccine-specific T helper cells were reactivated by in vitro stimulation. CD4+/CD44+ T-helper cells were identified by the depicted gating strategy. Their individual cytokine profile was assessed by intracellular cytokine staining and FACS analysis. Cytokine-producing cells were identified (numbers inside the gate refer to cytokine-positive cells per total CD4+ T cells), and multiple cytokine-expressing cells were calculated by Boolean gating. Shown are representative FACS blots from mice immunized with TIV, TIV+MF59 or TIV+MF59+apyrase, respectively.

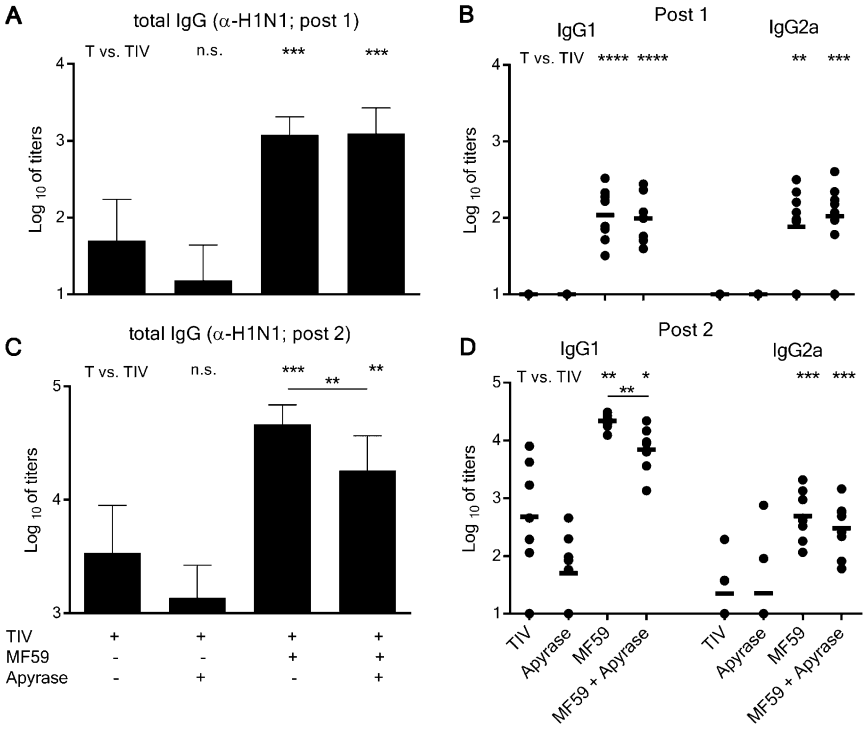

Fig. S5. Apyrase reduces vaccine-specific IgG1 levels induced by MF59. (*A–D*) Mice were immunized twice (4 wk apart), using the different formulations indicated in the abscissae. The following doses were used: MF59 (20% vol/vol), apyrase (10 U per leg), and TIV (0.1 μg of each antigen). Serum samples were drawn 2 wk after both immunizations, and the H1N1-specific total IgG, IgG1, and IgG2a levels in sera were measured by ELISA after the first (*A* and *B*) and second (*C* and *D*) immunization. Values represent mean logarithmic titers (log 10) of eight mice per group + SD. Unpaired, two-tailed Student's *t* test (T): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

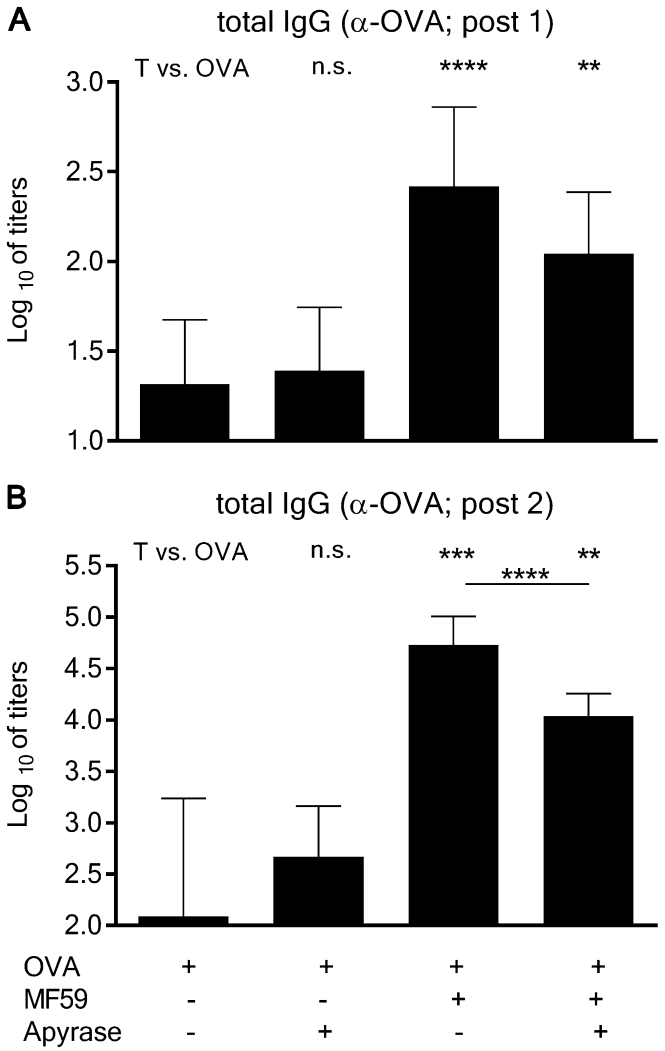

Fig. S6. Coinjection of apyrase reduces ovalbumin (OVA)-specific antibody titers enhanced by MF59. (*A* and *B*) Mice were immunized twice (4 wk apart) with endograde OVA and the indicated compounds. The following doses were used: MF59 (40% vol/vol), apyrase (10 U per leg), and OVA (10 μg per mouse). Serum samples were drawn 2 wk after each immunization, and OVA-specific antibody titers were measured by ELISA after the first (*A*) and second (*B*) immunization. Values represent mean logarithmic titers (log 10) of eight mice per group + SD. Unpaired, two-tailed Student's *t* test (T): $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

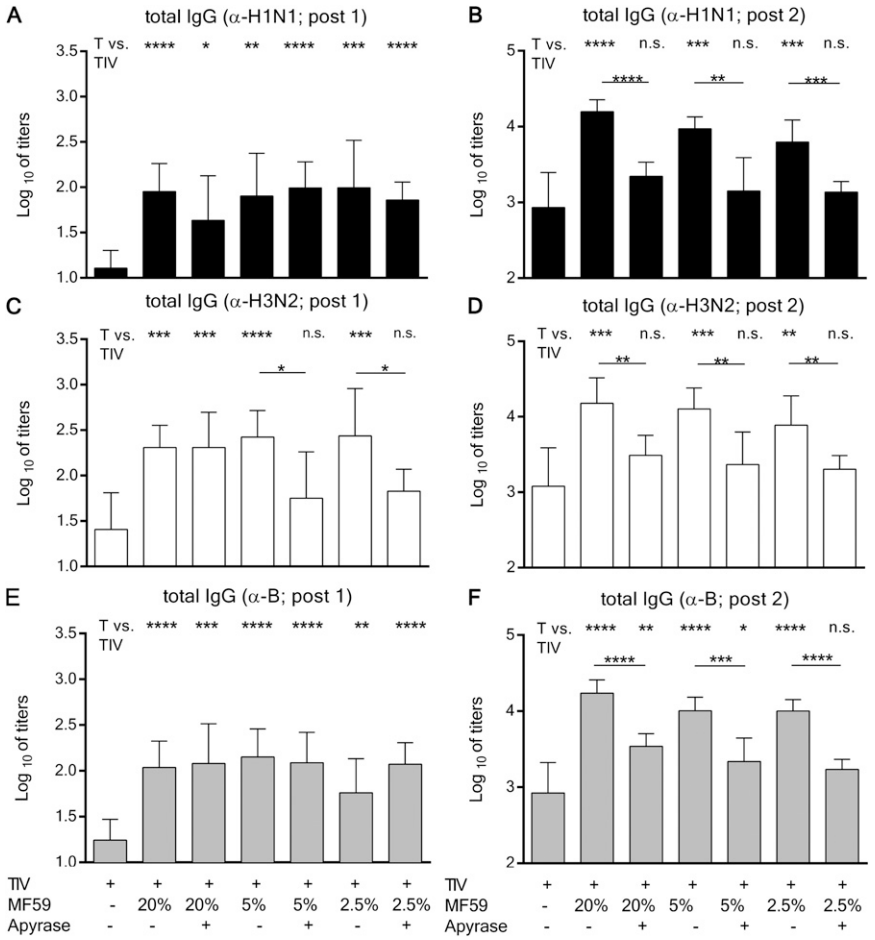

Fig. S7. Effect of apyrase on antibody responses induced by decreasing doses of MF59. (*A*–*F*) Mice were vaccinated with TIV and different doses of MF59 (20% vol/vol, 5% vol/vol, and 2.5% vol/vol) with or without apyrase (10 U per leg). Serum samples were drawn 2 wk after each immunization, and the total IgG antibody titers toward (*A* and *B*) H1N1/California, (*C* and *D*) H3N2/Perth, and (*E* and *F*) B/Brisbane were measured by ELISA after the prime (post 1) and the booster vaccination (post 2). Values are the mean of logarithmic titers (log 10) of eight mice per group + SD. Unpaired, two-tailed Student's *t* test (T): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

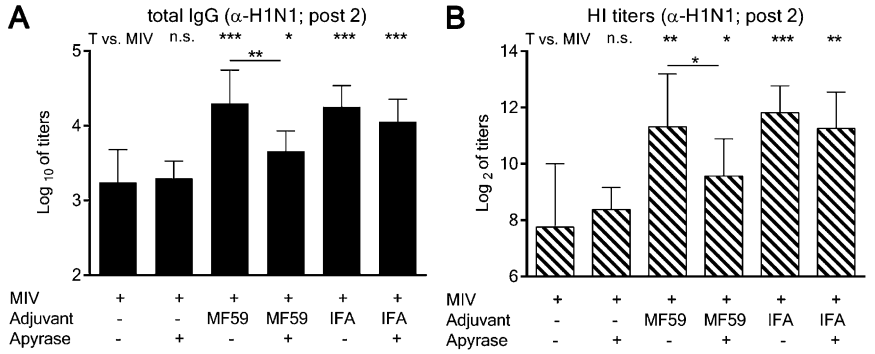

Fig. S8. Coinjection of apyrase abrogates adjuvanticity of MF59, but not incomplete Freund's adjuvant. Mice were vaccinated with a monovalent influenza vaccine (MIV) and different adjuvants (MF59 20% vol/vol or incomplete Freund's adjuvant 40% vol/vol) with or without apyrase (10 U per leg). (*A*) Total IgG antibody titers toward H1N1/California. Values represent the mean logarithmic titers (log 10) of eight mice per group + SD. (*B*) Hemagglutination inhibition titers toward H1N1/California; values represent means of Log2 titers of eight mice/group + SD. Unpaired, two-tailed Student's *t* test (T): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

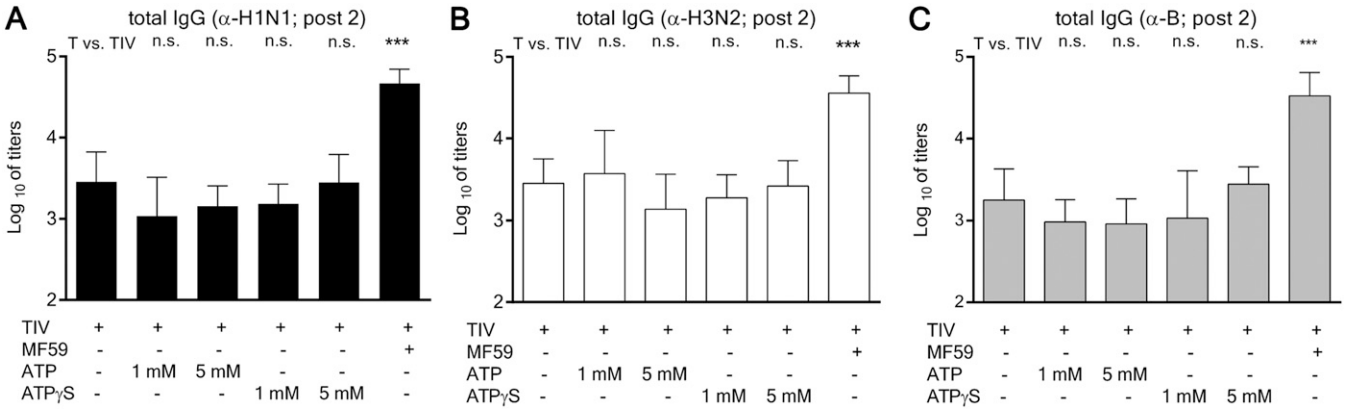

Fig. S9. Injection of ATP or ATP-γS does not have any adjuvant effect. Groups of mice were immunized twice with a 4-wk interval, using the different formulations indicated in the abscissae. The following doses were used: MF59 (20% vol/vol), ATP or ATP-γS (1 and 5 mM), and TIV (0.1 μg of each antigen). Serum samples were drawn 2 wk after the second immunization, and the total IgG antibody titers toward (*A*) H1N1/California, (*B*) H3N2/Perth, and (*C*) B/Brisbane were measured by ELISA. Values are the mean of logarithmic titers (log 10) of eight mice per group + SD. Unpaired, two-tailed Student's *t* test (T): $***P < 0.001$.