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Supplementary Materials for

Microbial Hijacking of Complement–Toll-Like Receptor Crosstalk

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Table S1. Detection of *P. gingivalis* in blood and internal organs of wild-type and C5aR-deficient (*C5ar*$^{-/-}$) mice after intraperitoneal infection. Twenty-four hours post-intraperitoneal infection with 5x10^7 CFU, *P. gingivalis* bacterial loads were determined by plating serial dilutions of blood and tissue homogenates on blood agar plates subjected to anaerobic culture. Cultures were considered positive if at least three colonies of *P. gingivalis* were identified.

Organs	% mice with positive culture (n = 5 mice per genotype)	
	wild-type	*C5ar*$^{-/-}$
Blood	80	0
Spleen	0	0
Kidney	0	0
Liver	0	0
Lungs	0	0

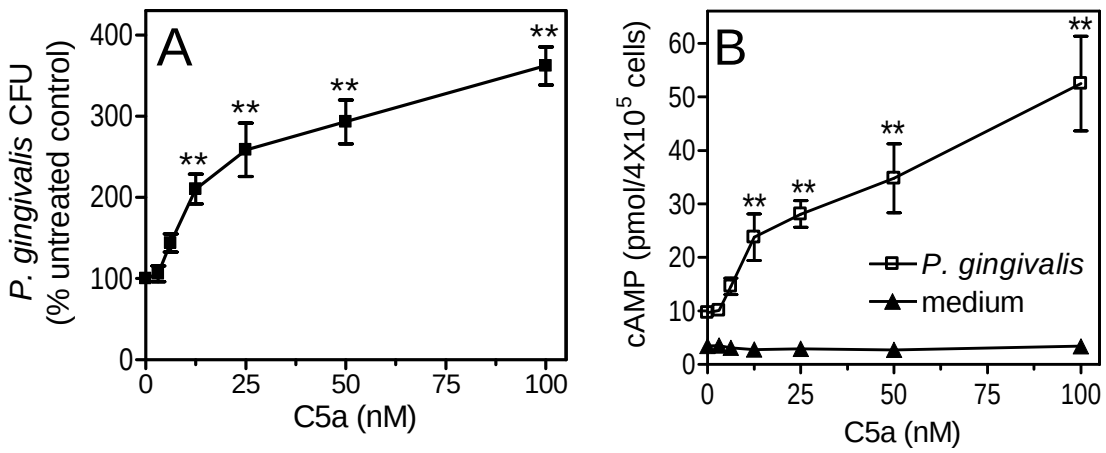

Figure S1. C5a dose-dependently promotes the intracellular survival of *P. gingivalis* and the cAMP response. (**A**) Peritoneal mouse macrophages were incubated with *P. gingivalis* in the presence of increasing concentrations of C5a and viable counts of internalized bacteria at 24 hours post-infection were determined by CFU enumeration. (**B**) *P. gingivalis*-induced cAMP responses in macrophages, assayed at 1 hour, in the presence of increasing concentrations of C5a. Data are means ± SD (n = 3 sets of macrophages) from typical experiments, each performed twice yielding consistent results. **, $P < 0.01$.

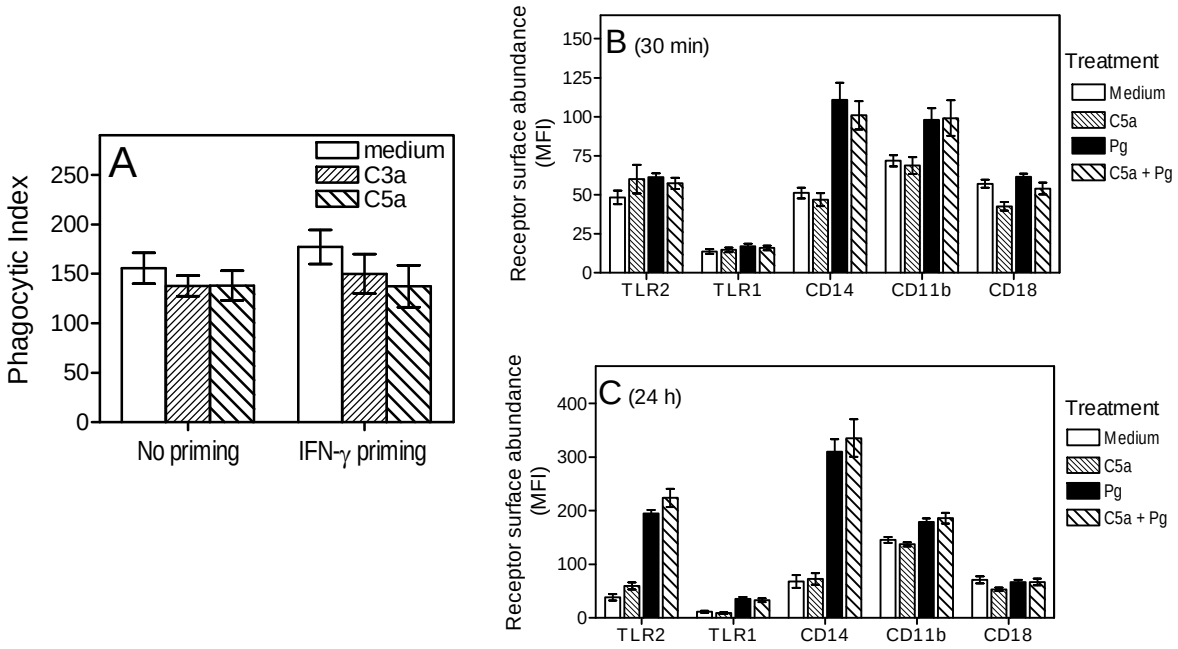

Figure S2. C5a does not affect *P. gingivalis* phagocytosis. (**A**) Effect of C5a (50 nM) or C3a (200 nM) on *P. gingivalis* phagocytosis by unprimed or IFN-γ–primed mouse peritoneal macrophages after 30 min incubation. The phagocytic index was calculated using the following formula: % positive cells for fluorescently labeled *P. gingivalis* x MFI/100 (extracellular fluorescence was quenched prior to flow cytometry). (**B** and **C**) Mouse macrophages were incubated at 37°C for 30 min (B) or 24 hours (C) with medium, C5a (50 nM) only, or *P. gingivalis* (MOI=10:1) with or without C5a (50 nM). The surface abundance of the indicated receptors, which coordinately mediate *P. gingivalis* uptake, were determined by flow cytometry after cell staining with appropriate fluorescently labeled antibodies. The 30 min timepoint was examined to determine possible induced increases in surface abundance of preformed receptors from intracellular pools. Mouse-specific mAbs to TLR2 (clone 6C2), TLR1 (TR23), CD14 (Sa2-8), CD11b (M1/70), and CD18 (M18/2) were from e-Bioscience. Data are means ± SD (*n* = 3 sets of macrophages) from one of two independent sets of experiments yielding consistent results. No significant differences were found between C5a+Pg compared to Pg alone (B and C). MFI; mean fluorescent intensity.

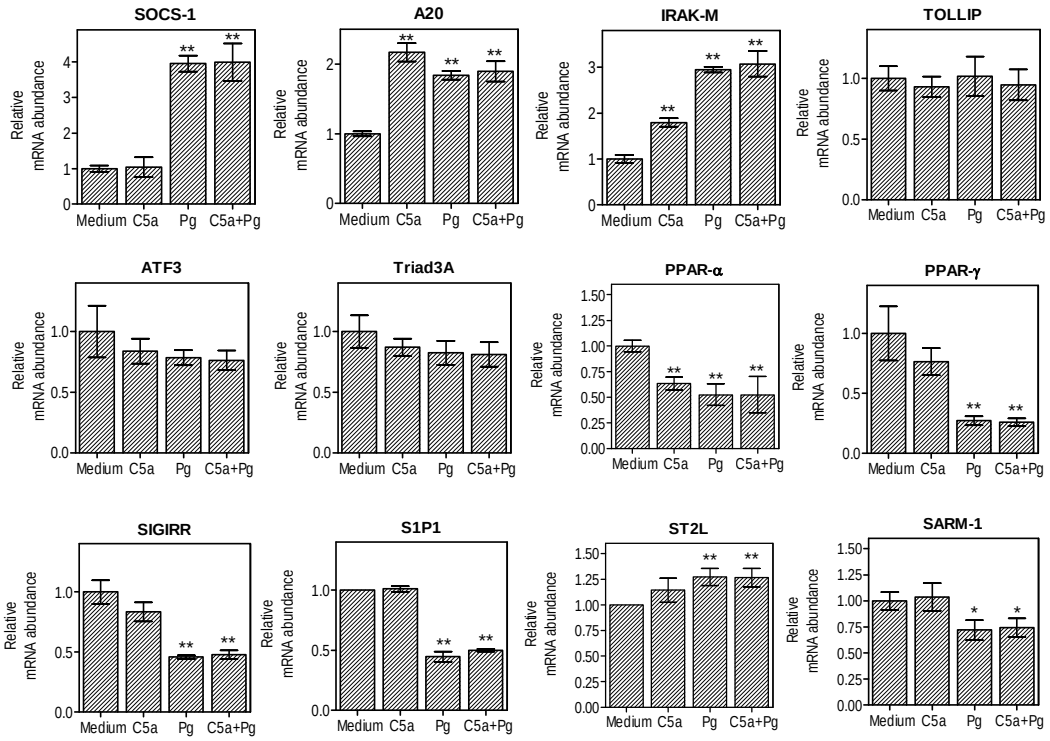

Figure S3. Relative mRNA abundance of negative regulators of TLR signaling in *P. gingivalis*-stimulated macrophages in the absence or presence of C5a. Mouse macrophages were stimulated with *P. gingivalis* (Pg; MOI=10:1) or medium control, with or without C5a (50 nM) for 4 hours. Quantitative real-time PCR (ABI 7500 Fast System; Applied Biosystems) was used to determine mRNA abundance for the indicated molecules (normalized against GAPDH mRNA abundance). Results are shown as fold induction relative to medium-only–treated macrophages. Data are means ± SD (n = 3 sets of macrophages) from one of two independent sets of experiments yielding consistent results. *, $P < 0.05$ and ** $P < 0.01$ compared to medium only control. No significant differences were observed between C5a+Pg and Pg alone.

SOCS-1, suppressor of cytokine signaling-1; IRAK-M, interleukin-1 receptor-associated kinase M; TOLLIP, Toll-interacting protein, ATF3, activating transcription factor-3; A20 is a ubiquitin-editing enzyme; Triad3A is an E3 ubiquitin ligase; PPAR-α, peroxisome proliferative activated receptor-α; PPAR-γ, peroxisome proliferative activated receptor-γ; SIGIRR, single immunoglobulin interleukin-1-related receptor; S1P1, sphingosine 1-phosphate receptor type 1;

ST2L is a type I transmembrane protein which sequesters MyD88 and MyD88 adaptor-like (Mal) protein; SARM-1, sterile alpha and HEAT/Armadillo motif protein-1.

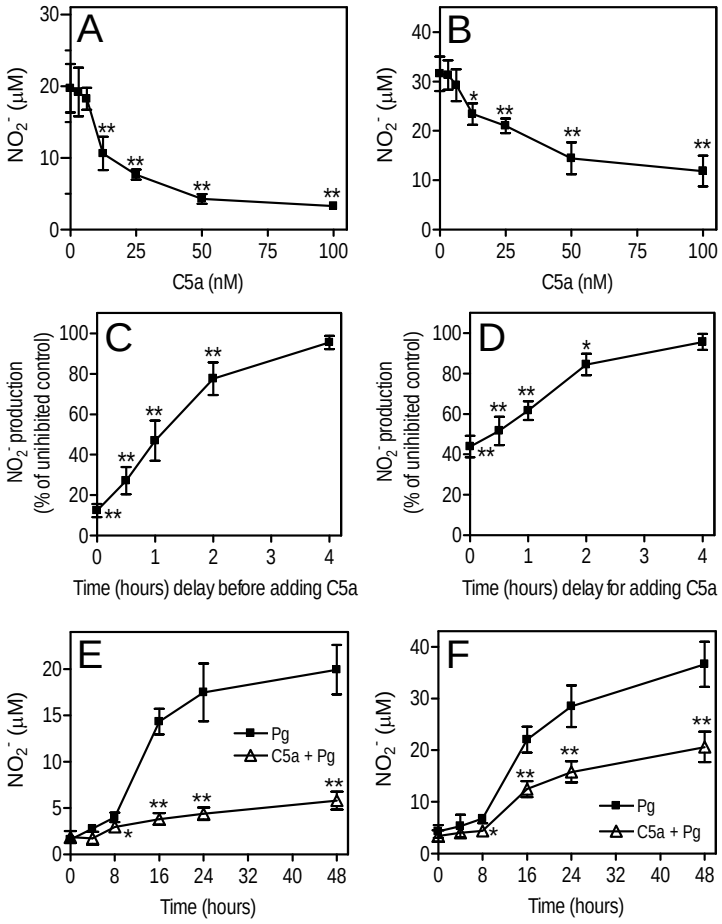

Figure S4. C5a inhibits nitric oxide production in a dose- and time-dependent way. Mouse peritoneal macrophages were left untreated (A, C, and E) or primed with 100 ng/ml IFN-γ (B, D, and F) overnight, washed, and incubated for 24 hours under the following conditions. In A and B the cells were incubated with *P. gingivalis* (Pg) in the presence of the indicated increasing concentrations of C5a. In C and D the cells were incubated with Pg with or without C5a (50 nM), which was added either together with the bacteria into the macrophage cultures (time "0") or was delayed for various times, as indicated ("uninhibited control" denotes the absence of C5a throughout the experiment). In E and F the cells were incubated with Pg, with or without C5a (50 nM), for the indicated time intervals. Pg was used at MOI=10:1 throughout and NO_2^- concentrations were assayed by the Griess reaction. Data are means ± SD (n = 3 sets of macrophages) from typical experiments that were performed twice yielding consistent results. Asterisks show significant (*, $P < 0.05$; **, $P < 0.01$) inhibition of NO_2^- production.

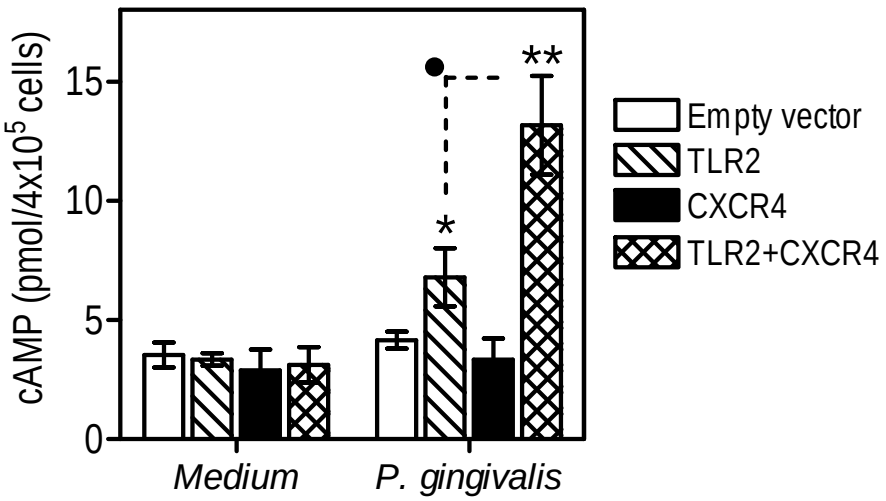

Figure S5. TLR2-dependent cAMP production by *P. gingivalis*. CHO-K1 cells, transfected with the indicated receptors (using expression plasmids from InVivogen and the PolyFect transfection reagent from Qiagen), were stimulated or not with *P. gingivalis* for 1 hour and assayed for intracellular cAMP concentrations. Data are means ± SD (n = 3 sets of transfected cells) from a typical experiment performed three times yielding consistent results. *, $P < 0.05$ and **, $P < 0.01$ compared to empty vector control. •, $P < 0.01$ between the indicated groups.

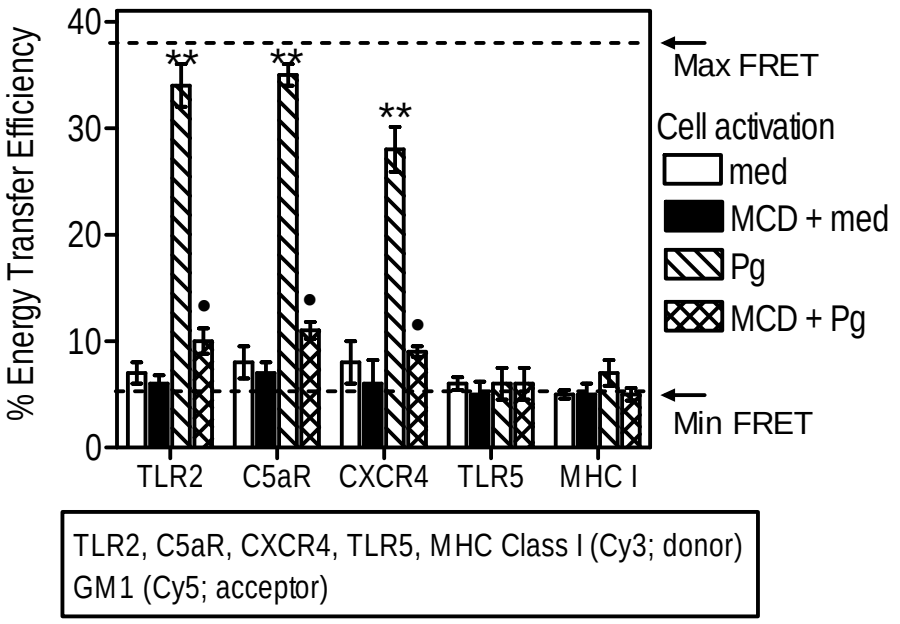

Figure S6. Association of TLR2, C5aR, and CXCR4 with GM1 (lipid raft marker) in *P. gingivalis*-stimulated macrophages. Mouse macrophages were pretreated or not with methyl-β-cyclodextrin (MCD; 10 mM for 30 min) and then stimulated for 10 min with *P. gingivalis* (Pg; MOI = 10:1) or medium only (med). Fluorescence resonance energy transfer (FRET) between TLR2, C5aR, CXCR4, TLR5, or MHC Class I (Cy3-labeled) and the GM1 ganglioside (Cy5-labeled) was measured from the increase in donor (Cy3) fluorescence after acceptor (Cy5) photobleaching. TLR5 and MHC Class I served as negative controls. The indicated maximum (Max) and minimum (Min) FRET efficiencies in the system were determined, respectively, as the energy transfer between two different epitopes on the same molecule (TLR2) or between molecules that do not engage in heterotypic associations (TLR2 and MHC Class I). As expected, max FRET values (38 ± 1.2) were not affected by the cell activation status (med compared to Pg) or the use or not of MCD. Data are means ± SD (n = 3 sets of macrophages). **, significant (P < 0.01) FRET increase compared to medium-only control. •, significant (P < 0.01) reversal of FRET increase by MCD.

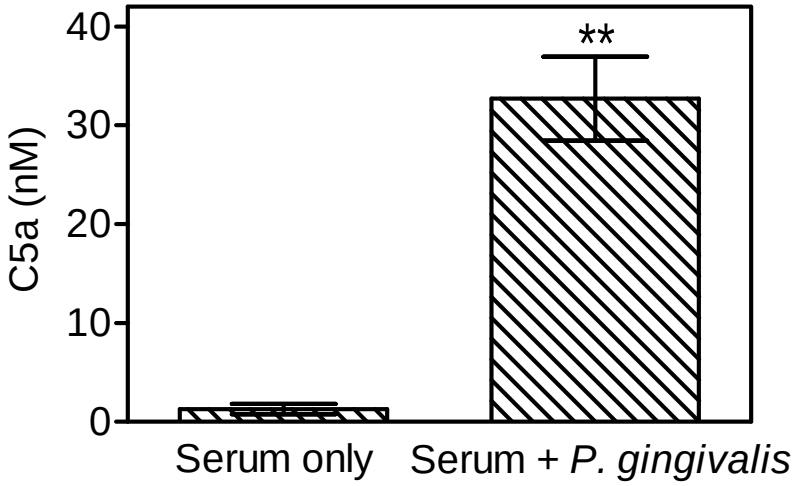

Figure S7. Generation of C5a by *P. gingivalis* from heat-inactivated human serum. Heat-inactivated human serum was incubated with or without *P. gingivalis* (10^8 bacterial cells per ml) for 30 min at 37°C and C5a generation was determined using a Human C5a ELISA Kit (BD Biosciences). Data are means ± SD (n = 3 sets of treatments) from one of two similar experiments yielding consistent results. **, $P < 0.01$ compared to serum-only control.

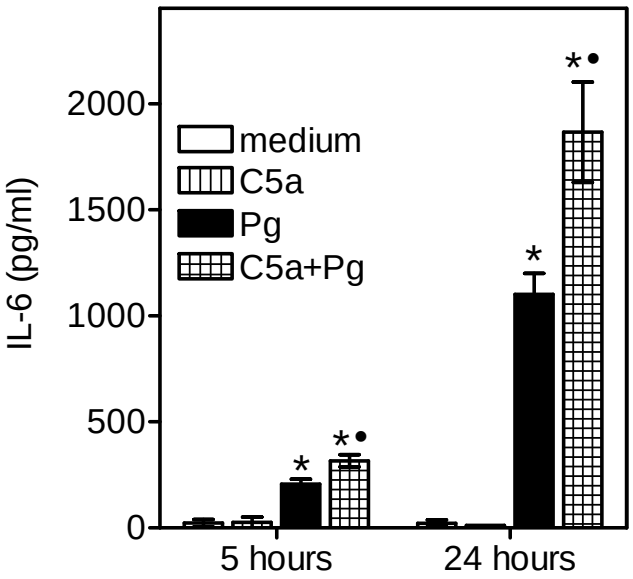

Figure S8. Up-regulation of IL-6 production by C5a in *P. gingivalis*-stimulated macrophages. Mouse peritoneal macrophages were incubated for 5 or 24 hours at 37°C with *P. gingivalis* (Pg; MOI = 10:1) in the absence or presence of C5a (50 nM) and culture supernatants were assayed for IL-6 by ELISA. Data are means ± SD (n = 3 sets of macrophages) from a typical experiment performed three times with consistent results. *, $P < 0.01$ compared to medium control. •, $P < 0.01$ in C5a+Pg compared to Pg alone.