Supporting Information

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SI Materials and Methods

Construction, Expression, and Purification of Rhizavidin Fusion Antigens. The gene sequence encoding the rhizavidin (rhavi) fragment (aa 45–179) was synthesized by Genscript (Piscataway, NJ), using an optimized *Escherichia coli* expression codon (seq1). The expression construct contains a secretion signal-coding region (seq2) on the 5′ end of the rhavi gene and a flexible linker coding region (seq3) on the 3′ end of the *rhavi* gene. For preparation of lipidated rhavi, a DNA sequence encoding a lipidation box in *E. coli* (seq4) was added on the 5′ end of the *rhavi* gene. For fusion antigens, the DNA sequences encoding target proteins were amplified from the genomic DNA via PCR and then cloned to the 3′ end of the linker region. Table S1 lists the protein antigens used in this study. All constructs were cloned into a pET-21b vector and transformed into *E. coli* strain BL21 (DE3) for expression. His-tagged recombinant proteins were purified using Ni-nitriloriacetic acid (NTA) affinity chromatography. To improve purity, the eluents of the affinity column were then subjected to size-exclusion chromatography on a Superdex 200 column. The peak fractions containing the fusion proteins were collected, evaluated by SDS/PAGE, and then flash-frozen in liquid nitrogen for future use. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad).

seq1: 5-TTTGATGCGAGTAACTTTAAAGATTTCAGCTCT
ATTGCGAGCGCCAGTAGCTCTTGGCAGAATCA
GAGTGGCAGCACCATGATTATCCAGGTGGATT
CTTTCGGCAACGTTAGTGGCCAGTATGTTAATC
GTGCGCAGGGCACGGGTTGCCAGAACTCTCCG
TACCCGCTGACCGGCCGCGTGAATGGCACGTTT
ATCGCCTTCAGCGTTGGCTGGAACAATTCTACC
GAAAACTGTAATAGTGCAACCGGCTGGACGGG
TTATGCGCAGGTGAACGGTAACAATACCGAAA
TTGTTACGAGCTGGAATCTGGCCTATGAAGGCG
GTTCTGGCCCGGCAATCGAACAGGGTCAGGAT
ACCTTTCAGTACGTTCCGACCACGGAAAACAA
AAGCCTGCTGAAAGAT-3

seq2: 5-ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTA
GTTTTAGCGTTTAGCGCATCGGCGGCGCAGGAT
CCG-3

seq3: 5-GGAGGCGGAGGTTCGAGCTCC-3

seq4: 5-ATGAAGAAAGTAGCCGCGTTTGTTGCGCTAAG
CCTGCTGATGGCGGGATGTGTATCG-3

Biotinylation of Polysaccharides. Biotinylation of polysaccharides (PSs) containing hydroxyl groups was done using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as an activation reagent. The PS was dissolved in LPS-free water (cell culture grade; HyClone) for a final concentration of 1–5 mg/mL At time 0, a volume of CDAP (freshly made at a concentration of 100 mg/mL in acetonitrile) was added to a final ratio of 1 mg of CDAP per 1 mg of PS during vortexing. At 30 s, a volume of 0.2 M triethylamine (TEA; Sigma-Aldrich) was added to raise the pH to 8. (For neutral PS, the volume of TEA is equal to the volume of CDAP; for acidic PS, the volume of TEA is doubled.) At 2.5 min, a volume of biotin derivative (Pierce EZ-Link Amine-PEG3-Biotin, 20 mg/mL in water) was added to a final ratio of 1 mg of biotin to 1 mg of PS followed by incubation at room temperature for 1–4 h. For more diluted samples (<1 mg/mL), overnight incubation was used. The reaction was terminated by adding 25 mM of glycine, and the excess biotin was removed by extensive dialysis against PBS.

For PSs that lack hydroxyls but contain carboxyl groups, such as Vi PS, activation was accomplished with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Pierce). The PS was dissolved in MES buffer (0.1 M, pH 5.8, 150 mM NaCl) for a final concentration of 5–10 mg/mL A volume of biotin derivative was added to a final ratio of 2 mg of biotin to 1 mg of PS. A volume of EDC solution (freshly made at 100 mg/mL in MES buffer) was added to a ratio of 1 mg of EDC to 1 mg of PS. The mixture was incubated overnight at room temperature, after which the reaction was terminated by the addition of 25 mM glycine. Excess biotin and EDC were removed by dialysis against PBS.

The level of biotinylation was determined using a biotin quantification kit (Pierce). The concentrations of CPSs (except Vi) were determined by an anthrone assay (1). The concentration of Vi was measured as described previously (2).

Antibody Measurement. Serum antibody titer was measured by ELISA in 96-well plates (Immulon 2 HB) coated with target PS or protein antigens. Plates were blocked with PBS containing 1% BSA for 1 h at room temperature. Mouse or rabbit serum was diluted in PBS containing 0.005% Tween 20 (PBS-T), added to the plate, and incubated at room temperature for 2 h. For measurement of antibody against pneumococcal CPS, serum was diluted with PBS-T containing 5 μg/mL purified pneumococcal cell wall PS (CWPS). After a wash with PBS-T, a secondary HRP-conjugated antibody against mouse or rabbit IgG was added, followed by incubation at room temperature for 1 h. The plates were washed and then developed with SureBlue TMB Microwell Peroxidase Substrate (KPL). The reactions were terminated by the addition of 1 M HCl, and the A_{450} was analyzed using an ELISA reader. Antibody titers were expressed relative to a standard serum.

In Vitro Stimulation of Whole Blood and Splenocytes. In vitro stimulation of peripheral blood was performed in 96-well round-bottom plates. All stimulants were diluted in stimulation medium (DMEM F-12; 10% FBS, 50 μM 2-mercaptoethanol, 10 μg/mL ciprofloxacin) at final concentration of 10 μg/mL as described previously (3). In each well, 25 μL of heparinized blood was added to 225 μL of stimulation medium containing indicated stimulants, followed by incubation at 37 °C for 6 d. Supernatants were collected after centrifugation, and cytokine concentration was analyzed using IFN-γ and IL-17A ELISA kits (R&D Systems).

Mouse splenocytes were isolated as described previously (3). Isolated splenocytes were resuspended in stimulation medium and seeded into 48-well plates at 3×10^6 cells/well in a volume of 300 μL for incubation at 37 °C for 2 h. Stimulants were added at the indicated concentration, and the cells were incubated at 37 °C for 3 d, followed by collection of supernatants for cytokine measurements. Depletion of specific T-cell subsets in splenocytes was done using $CD4^+$ or $CD8a^+$ microbeads (Miltenyi Biotech). Flow cytometry confirmed ≥ 95% depletion of the $CD4^+$ or $CD8^+$ T-cell populations.

In Vitro Stimulation of Peritoneal Macrophages and Human Embryonic Kidney (HEK293) Cells. Mouse peritoneal macrophages were obtained by i.p. injection of 3% (wt/vol) Brewer thioglycollate medium as described previously (4). Isolated cells were plated into 24-well plates at a density of 5×10^6 cells per well in a volume of 500 μL (DMEM F-12, 10% FBS) for 4 h, followed by the addition of 50 μL of stimulants premixed with aluminum hydroxide at final concentration of 1.2 mg/mL. Supernatants were collected after 12–16 h of incubation at 37 °C and subjected to cytokine analysis using TNF-α, IL-12, IL-23, and IL-1β ELISA kits (R&D Systems). HEK293 cells with or without stable human TLR2 transfection were maintained in DMEM supplemented with 10% FBS and 10 μg/mL ciprofloxacin. Stimulation of HEK293 cells was performed for 18 h, after which supernatants were collected and assayed for IL-8 concentration (R&D Systems).

In Vitro Killing Assays. In vitro killing of Vi-expressing *Salmonella typhimurium* strains was performed as described previously (5). In brief, the *S. typhimurium* C5 (non–Vi PS-expressing) or C5.507 (Vi-expressing) strain was grown to log phase and then frozen in LB/10% glycerol at −80 °C. HL-60 cells were differentiated by incubation with 100 mM N, *N*-dimethylformamide (DMSF) for 5 d. On the day of the experiment, bacteria were thawed and then washed twice with PBS. Opsonization of bacteria was performed by incubating 3×10^4 CFU with a 1: 10 dilution of serum in a total volume of 30 μL for 20 min at room temperature, followed by the addition of 3×10^6 differentiated HL-60 cells in a total final volume of 300 μL. Samples were incubated at 37 °C on a rotator, and the number of viable bacteria was measured after 60, 120, and 180 min of incubation by serial dilution on LB agar plates.

Opsonophagocytic killing of serotype-1 (strain 101), -5 (strain 501), or -14 (strain 1401) pneumococcus was performed using a standard protocol (6), with serum pooled from mice ($n = 10$) that had received two immunizations of adjuvant alone, PCV13 (200 μL per immunization), or multiple antigen-presenting system (MAPS) complex. Opsonophagocytic activity was defined as the serum dilution at which 50% of input bacteria were killed in an opsonophagocytic killing assay (OPA).

1. Roe JH (1955) The determination of sugar in blood and spinal fluid with anthrone reagent. *J Biol Chem* 212(1):335–343.
2. Lu YJ, et al. (2012) A bivalent vaccine to protect against *Streptococcus pneumoniae* and *Salmonella typhi*. *Vaccine* 30(23):3405–3412.
3. Lu YJ, et al. (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog* 4(9):e1000159.
4. Ray A, Dittel BN (2010) Isolation of mouse peritoneal cavity cells. *J Vis Exp* 35:e1488, doi:10.3791/1488.
5. Hale C, et al. (2006) Evaluation of a novel Vi conjugate vaccine in a murine model of salmonellosis. *Vaccine* 24(20):4312–4320.
6. Burton RL, Nahm MH (2006) Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. *Clin Vaccine Immunol* 13(9):1004–1009.

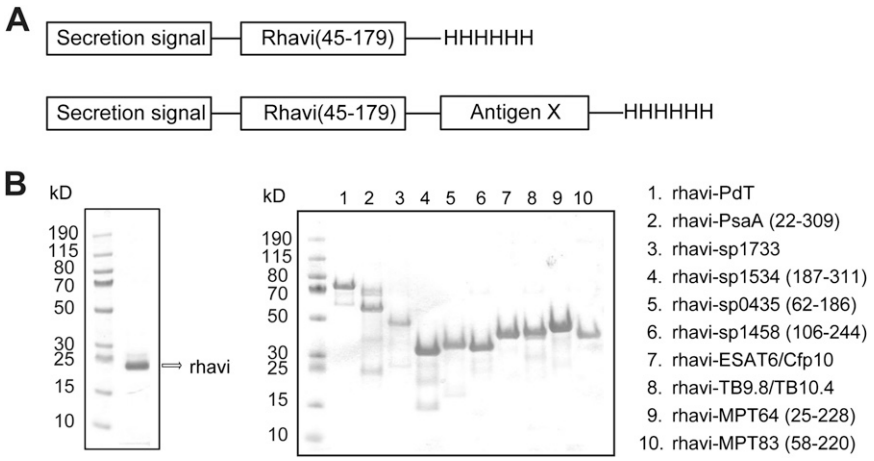

Fig. S1. Recombinant rhavi and rhavi fusion antigens. (*A*) Construction of recombinant rhavi (*Upper*) and rhavi fusion antigens (*Lower*). An N-terminal truncated fragment of rhavi (aa 45–179) was used in all constructs. A secretion signal sequence (consisting of MKKIWLALAGLVLAFSASA) was introduced to the N terminus of rhavi (aa 45–179). In all fusion proteins, a flexible linker (consisting of GGGGSSSS) was used between the rhavi moiety and the target antigens. (*B*) SDS/PAGE of purified rhavi (*Left*) and rhavi fusion antigens (*Right*).

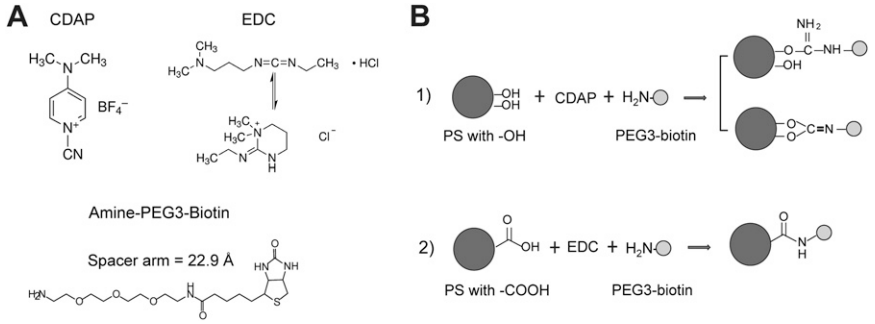

Fig. S2. Biotinylation of PSs. (*A*) Chemical structure of CDAP, EDC, and amine-PEG3-biotin. (*B*) Schematic diagram of the biotinylation process. Biotinylation was done on the hydroxyl groups of PS using CDAP as the activating reagent (reaction 1) or on the carboxyl groups of PS using EDC as the activating reagent (reaction 2).

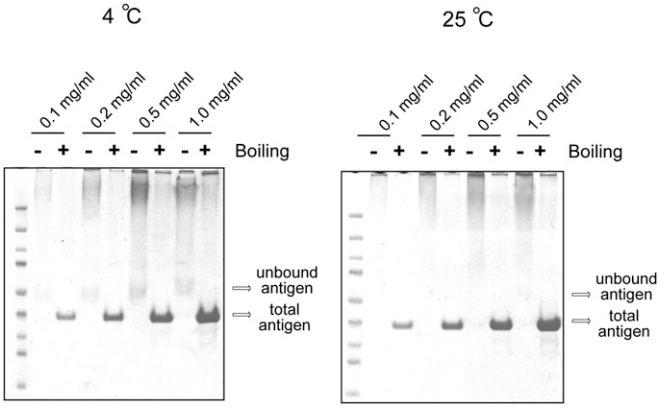

Fig. S3. Assembly of MAPS complexes at different temperature and concentrations. MAPS complexes were prepared by overnight incubation of BD500 and rhavi-sp0435 at different concentrations (0.1–1 mg/mL) at 4 °C (*Left*) or 25 °C (*Right*). The assembly of MAPS complexes was evaluated by SDS/PAGE of the mixture after incubation. The fusion antigens that were incorporated into the MAPS complexes remained associated with the PS unless the sample was boiled. Only the unbound antigens were detectable in the unboiled samples. The assembling efficiency was estimated by comparing the antigen bands in samples with boiling and without boiling. In general, more than 90% of antigens could be incorporated into MAPS complexes after overnight incubation at 4 °C or 25 °C, even with starting concentrations as low as 0.1 mg/mL.

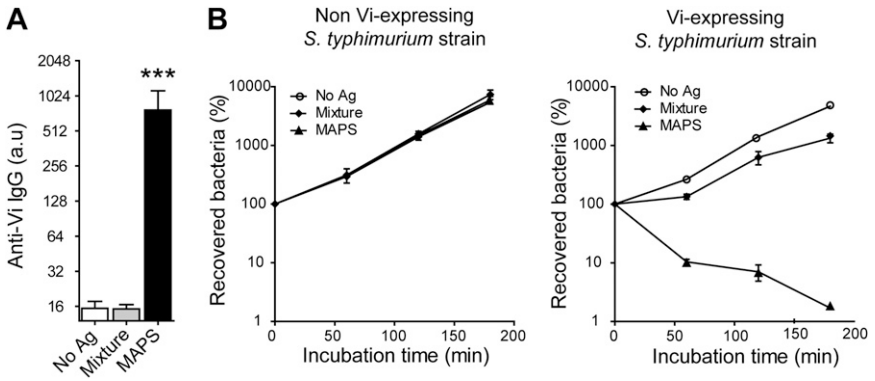

Fig. S4. Generation of anti-Vi antibody by immunization with MAPS complexes. MAPS complexes were created with biotinylated Vi coupled with five pneumococcal fusion antigens: rhavi-PdT, rhavi-PsaA, rhavi-sp0435, rhavi-sp1458, and rhavi-sp1534. Mice were immunized s.c. three times at 2-wk intervals, with 10 μg of Vi per dose. Control groups received adjuvant alone or a mixture of uncoupled Vi and the five pneumococcal proteins. (*A*) Serum anti-Vi IgG antibody was measured by ELISA. (*B*) The in vitro killing assay was done by mixing non–Vi-expressing (*Left*) or Vi-expressing *S. typhimurium* strain (*Right*) with sera (1:10 dilution) from different immunization groups in the presence of differentiated HL-60 cells. An aliquot of the mixture was obtained after incubation at 37 °C for 1–3 h and plated for evaluation of surviving bacteria. Serum from MAPS-immunized mice, but not from the other mice, showed strong and specific killing of the Vi-expressing strain, with >90% killing within 1 h of incubation. Bars represent mean ± SEM. ***$P < 0.0001$. Statistical comparisons are between groups that received immunization with a mixture and those immunized with a MAPS complex.

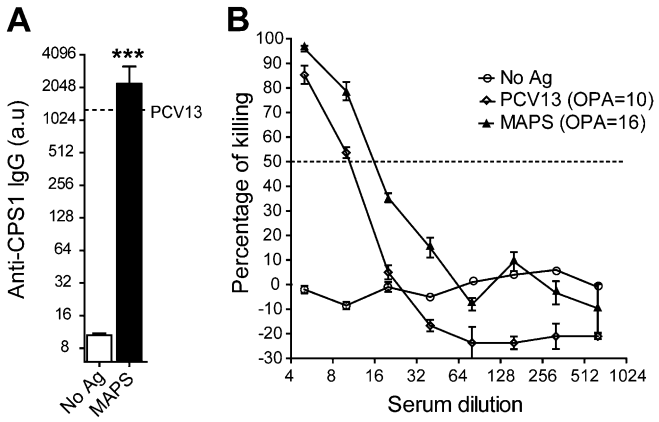

Fig. S5. Generation of anti-CPS1 antibody by immunization with MAPS complexes. MAPS complexes were created with biotinylated CPS1 coupled with rhavi-PdT. Two s.c. immunizations with 1 μg of CPS1 per dose were administered. (*A*) Serum anti-CPS1 IgG was measured by ELISA. The dashed line indicates the anti-CPS1 IgG titer in PCV13 standard serum. (*B*) An OPA was performed using serotype-1 pneumococci (strain 101). Sera from MAPS-immunized mice demonstrated slightly higher anti-CPS1 antibody titers and OPA values compared with PCV13 standard serum. Bars represent mean ± SEM. ***$P < 0.0001$. Statistical comparisons are between groups that received an adjuvant alone and those that received a MAPS complex.

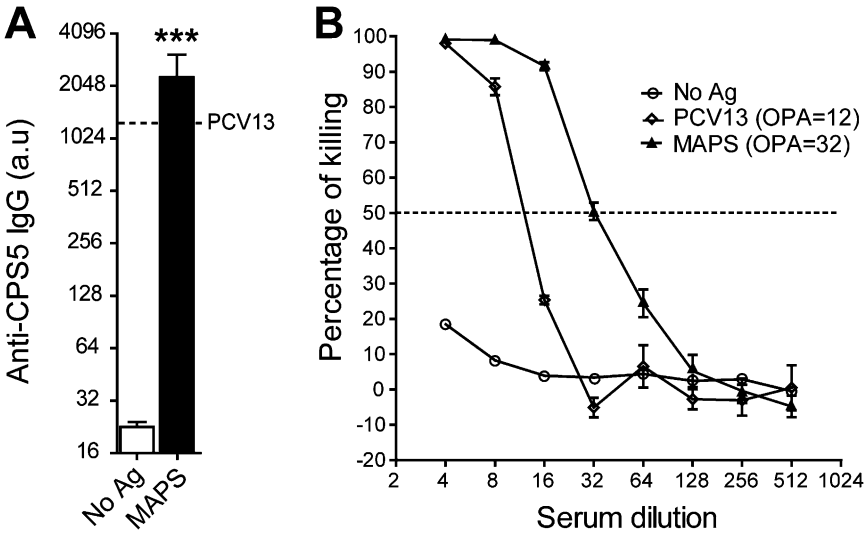

Fig. S6. Generation of anti-CPS5 antibody by immunization with MAPS complexes. MAPS complexes were prepared with biotinylated CPS5 coupled with rhavi-PdT. Two s.c. immunizations with 1 μg of CPS5 per dose were administered. (*A*) Serum anti-CPS5 IgG was measured by ELISA. The dashed line indicates the anti-CPS5 IgG titer in PCV13 standard serum. (*B*) An OPA was performed against serotype-5 pneumococci (strain 501). Sera from MAPS-immunized mice demonstrated twofold higher anti-CPS5 antibody titers and OPA values compared with PCV13 standard serum. Bars represent mean ± SEM. ***$P < 0.0001$. Statistical comparisons are between groups that received an adjuvant alone and those that received a MAPS complex.

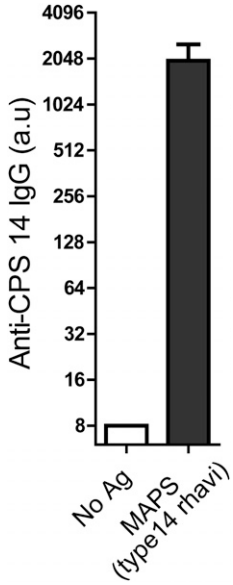

Fig. S7. Immunization of mice with a MAPS complex consisting of biotinylated CPS14 and the rhavi protein induces robust anti-CPS14 IgG responses. Mice (n = 10 per group) received three s.c. immunizations with adjuvant alone (no Ag) or with MAPS complexes formed with CPS14 and the rhavi protein (type 14 rhavi, 1.5 μg of PS per dose). Serum anti-CPS14 IgG titers after three immunizations were measured by ELISA.

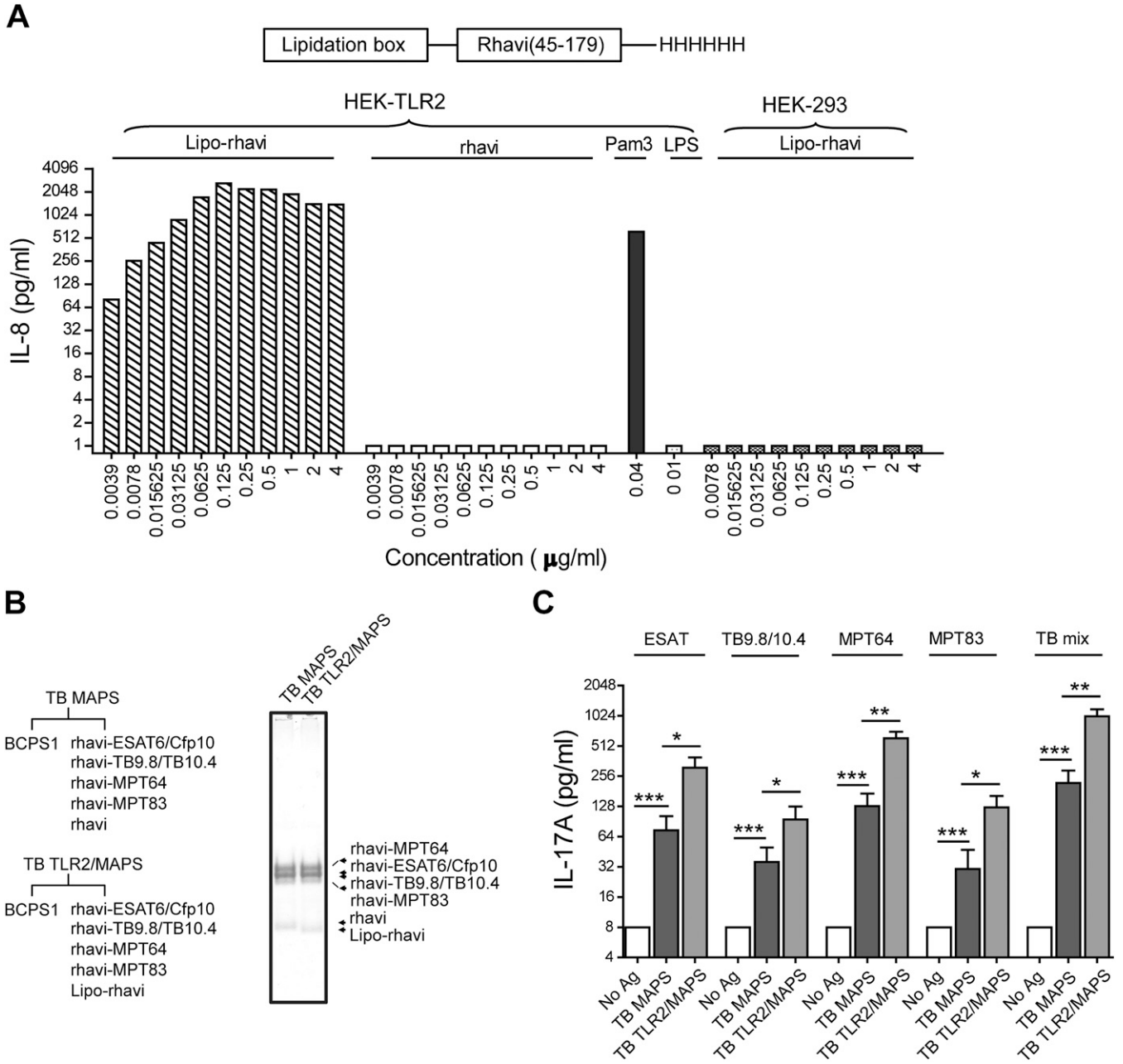

Fig. S8. TLR2 activity enhances T-cell immunogenicity of MAPS constructs. (*A*) MAPS constructs were prepared with biotinylated CPS1 (BCPS1) and either a lipidated (Lipo-rhavi) or nonlipidated rhavi (rhavi) and used to stimulate HEK cells that express (HEK-TLR2) or do not express (HEK-293) TLR2. The Lipo-rhavi MAPS potently activated HEK-TLR2 cells, but not HEK-293 cells, whereas the rhavi MAPS did not activate either type of HEK cells. Pam_3Cysk_4 (Pam3, a synthetic TLR2 agonist) and LPS (a TLR4 agonist) served as controls. (*B*) *Mycobacterium tuberculosis* (TB) MAPS constructs were created by combining BCPS1 with fusion proteins of rhavi and TB antigens, as well as rhavi (TB MAPS) or lipo-rhavi (TB TLR2/MAPS). SDS/PAGE of the two MAPS constructs confirmed similar protein content in all constructs. (*C*) Mice were immunized three times with either of the two TB MAPS constructs and aluminum hydroxide. At 2 wk after the last immunization, blood samples were obtained and stimulated with the four TB proteins individually or as a mixture (TB mix). Six days later, supernatants were collected and assayed for IL-17A concentration by ELISA. In all cases, the presence of lipo-rhavi was associated with significantly higher IL-17A responses. Bars represent mean ± SEM. $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$.

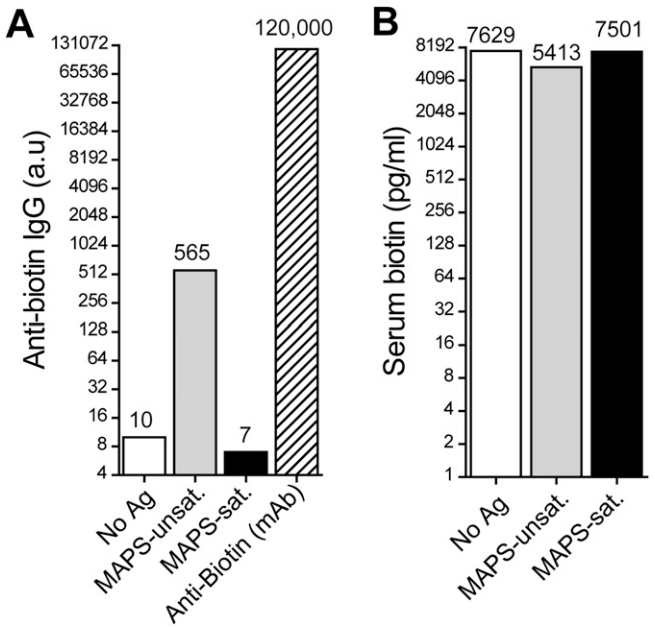

Fig. S9. Saturation of biotin sites in MAPS abrogates biotin antibody responses. Mice (n = 10 per group) received three immunizations with MAPS complexes that were saturated (MAPS-sat) or not saturated (MAPS-unsat) with protein antigen. (*A*) Serum (pooled serum from 10 mice) antibodies to biotin were measured after three immunizations by ELISA, using an antibiotin mAb as a standard. The titer of the standard was set arbitrarily as 120,000 units. (*B*) Serum (pooled from 10 mice) biotin concentration was measured by inhibition ELISA.

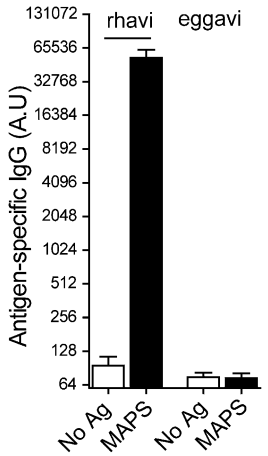

Fig. S10. Immunization with MAPS complexes elicits rhavi antibodies, which do not cross-react with egg avidin. Mice received three immunizations with MAPS complexes, after which serum antibodies against rhavi or against egg avidin (egg avi) were measured by ELISA.

Table S1. Protein antigens used in this study

Antigen	Origin	Description	Construct
PdT	SP	Pneumolysin toxoid	Full length with mutations of W433F, D385N, and C428G
PsaA	SP	Pneumococcal surface adhesin A	Fragment (22–309)
Sp1534	SP	Putative manganese-dependent inorganic pyrophosphatase	Fragment (187–311)
Sp0435	SP	Elongation factor P	Fragment (62–186)
Sp1458	SP	Thioredoxin reductase	Fragment (106–244)
Sp1733	SP	Putative phosphatase	Full length
ESAT6	MTB	6 -Da early secretory antigen	Fusion protein [full-length ESAT6 followed by CFP10 fragment (45–80) and CFP10 fragment (1–41)]
CFP10	MTB	10-kDa culture filtrate antigen	
TB9.8	MTB	ESAT-6–like hypothetical protein	Fusion protein (full-length TB9.8 followed by full-length TB10.4)
TB10.4	MTB	Low molecular weight protein antigen 7 (CFP7)	
MPT64	MTB	Immunogenic protein	Fragment (25–228)
MPT83	MTB	Lipoprotein P23	Fragment (58–220)

SP, *Streptococcus pneumoniae*; MTB, *Mycobacterium tuberculosis.*