Supporting Information

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SI Text

Plasmid Construction. Plasmids for ClyA, GFP, and ClyA-GFP were constructed with C-terminal 6×-histidine tags to facilitate purification of the protein products. The plasmids pClyA-His6, encoding the *E. coli* gene *clyA* fused to the 5′ end of a 6×-histidine tag, and pClyA-GFP, encoding *clyA* fused to the 5′ end of *gfp-mut2* (1), were previously described (2). To construct pClyA-GFP-His6, a ~1.7-kb fragment was amplified by PCR with plasmid pClyA-GFP as a template using primers (5′-TCGCAACTCTCTACTGTTTC-3′) and (5′-GCGATGAAGCTTTTAATGGTGATGGTGATGATGTTTGTATAGTTCATCCATGCC-3′). The resulting product was cloned in the *Xba*I and *Hind*III sites of pBAD18-Cm (3). For construction of pGFP-His6, a ~700-bp fragment was amplified by PCR with plasmid pClyA-GFP as a template and using primers (5′-GCGATGGAATTCGAGCTCTTAAAGAGGAGAAAGGTC ATGAGTAAAGGAGAAGAACTTTT-3′) and (5′-GCGATGAAGCTTTTA ATGGTGATGGT GATGATGTTTGTATAGTTCATCCATGCC-3′). The amplification product was cloned into pBAD18-Cm using *Sac*I and *Hind*III restriction sites. DNA constructs were verified by automated dideoxy chain-termination sequencing. Plasmids were transformed into *E. coli* DH5α and selected in LB medium containing chloramphenicol.

Fusion of GFP to ClyA Results in Expression of a 61-kDa Chimeric Protein that Retains the Native Activities of Its Components. Proper expression of ClyA, GFP, and ClyA-GFP was confirmed by polyacrylamide gel electrophoresis followed by Coomassie staining (Fig. S1*A*). The ClyA-GFP fusion protein was then examined for the characteristic hemolytic and fluorescence activities of its constituent proteins. The degree of hemolysis of sheep erythrocytes increased with increasing concentration of both ClyA and ClyA-GFP; ClyA-GFP exhibited lower hemolysis activity than native ClyA at all tested concentrations. Similarly, fluorescence-intensity measurements of ClyA-GFP showed an increase in fluorescence intensity with increasing concentration, but they diminished relative to free GFP. Together, these data showed that the intrinsic hemolysis and fluorescence activities of ClyA and GFP, respectively, were retained when the two proteins were fused together as ClyA-GFP, albeit to a lesser degree than the free proteins, which is likely because of protein proximity.

Inflammatory Responses to LPS in Engineered OMV Vaccines. Pairs of BALB/c mice were immunized with engineered OMVs or wild-type OMVs mixed with soluble ClyA-GFP, with or without LPS removal, to examine for any inflammatory responses to LPS in the formulations. Two days after injection, the animals were euthanized, and tissues were processed for histological examination. All animals showed slight superficial infiltrates of mast cells and eosinophils, which were considered background lesions and not significant. Lesions after injections extended from the deep dermis to the deep panniculus. In all treatment groups, lesions consisted of aggregates of degenerate and viable neutrophils, macrophages, some fibrin, and areas of edema. Overall, inflammation and edema were observed in all treatment groups, and no significant differences between comparable treatment groups with or without removal of LPS were identified. Because levels of tissue damage were low to moderate and virtually indistinguishable with or without reduction of LPS, we concluded that the adjuvant activity of the ClyA preparation was independent of LPS content. Examples of lesion grades 1 through 4 are provided for clarity in Figs. S2–S5.

1. Cormac BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33–38.
2. Kim J-Y, et al. (2008) Engineered bacterial outer membrane vesicles with enhanced functionality. *J Mol Biol* 380:51–66.
3. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177:4121–4130.

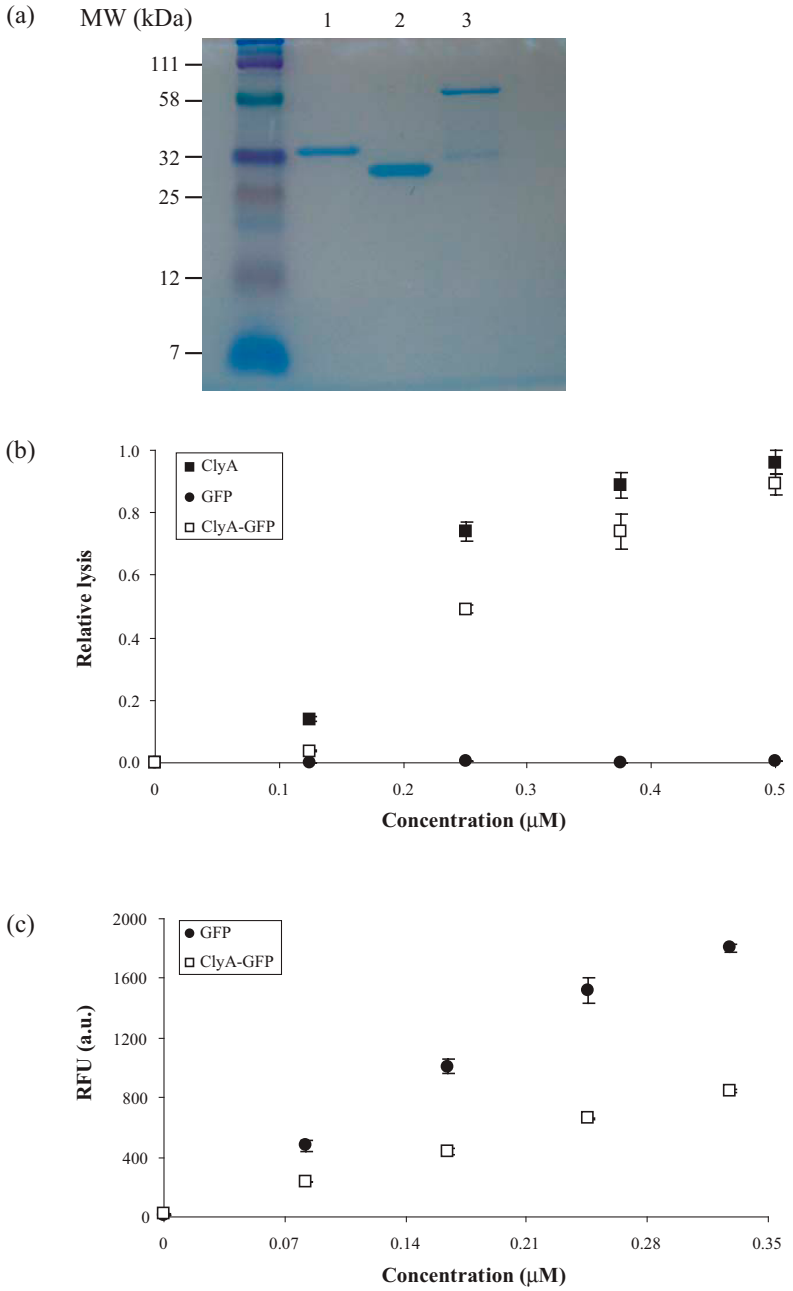

Fig. S1. Fusion of GFP to the C terminus of ClyA results in expression of a 61-kDa chimeric protein that exhibits fluorescence and hemolytic activity. (*A*) Polyacrylamide gel electrophoresis with Coomassie staining of the purified proteins (lane 1, soluble ClyA; lane 2, soluble GFP; lane 3, soluble ClyA-GFP). The expected molecular weights for ClyA, GFP, and the ClyA-GFP fusion are 34 kDa, 27 kDa, and 61 kDa, respectively. (*B*) Relative hemolysis activity of ClyA, ClyA-GFP, and GFP. The intrinsic hemolysis activity of ClyA is retained in the ClyA-GFP fusion protein, and it increases with increasing concentration. (*C*) Fluorescence intensity of GFP and recombinant ClyA-GFP in relative fluorescence units (RFUs; arbitrary units). The fluorescence intensity of ClyA-GFP and GFP increases linearly with increasing concentration.

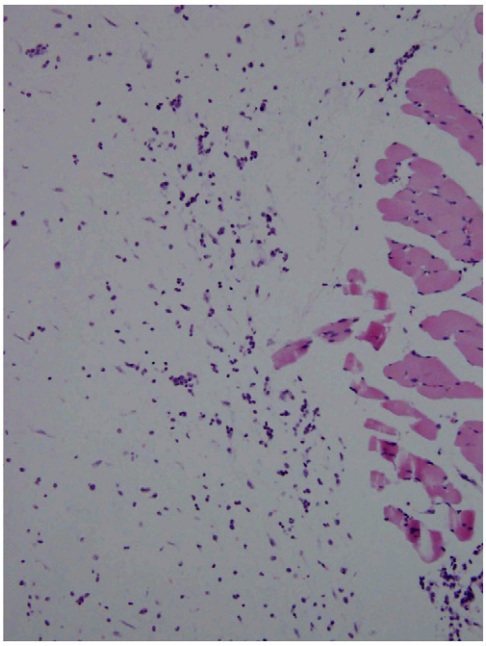

Fig. S2. Example of lesion grade 1.

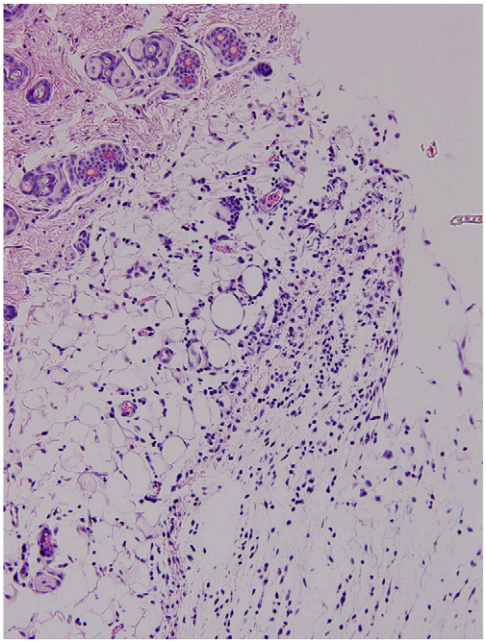

Fig. S3. Example of lesion grade 2.

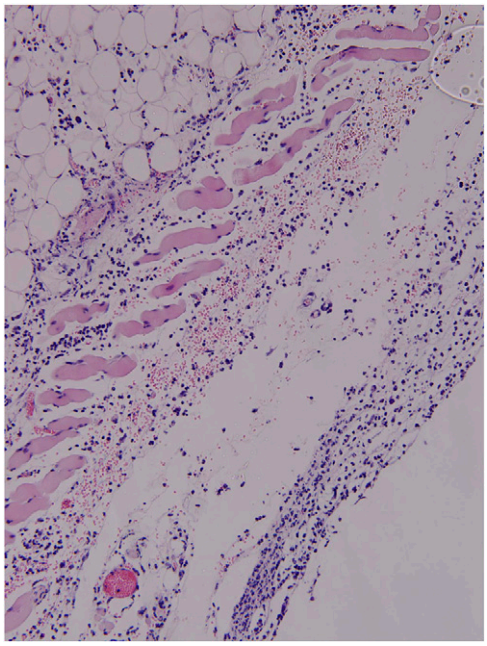

Fig. S4. Example of lesion grade 3.

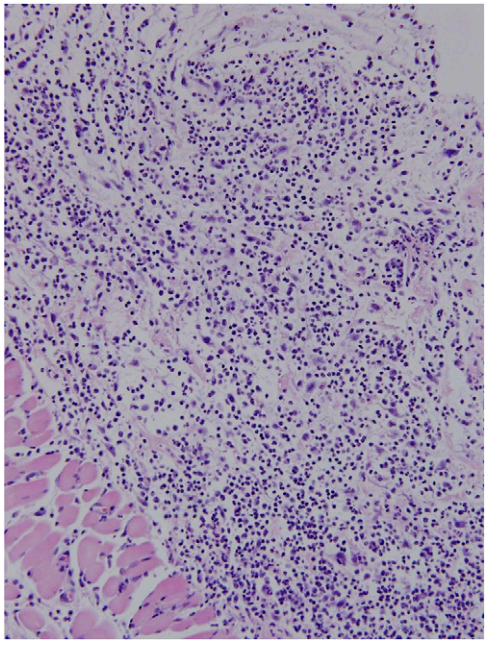

Fig. S5. Example of lesion grade 4.

Table S1. Inflammatory responses in immunized BALB/c mice

Sample	LPS, nmol/μg protein	Lesion grades
Engineered OMVs	0.28	2
	0.15	3
Wild-type (empty) OMVs	0.35	1
	0.01	1.5 (1–2)
Soluble ClyA-GFP	n.d.	1.5 (1–2)
PBS	n.d.	2

n.d., not detectable.