antigens in the appropriate bacterial compartment can have a profound impact on immunogenicity.

Engineering Immune Responses by Targeted Antigen Expression

The type of immune response elicited by recombinant antigens expressed by live vectors largely depends both on the efficiency of antigen presentation and the capacity of the organism to target specific immune cells. Accessibility of sufficient amounts of antigen to the appropriate cellular compartment will directly influence the nature and strength of immune responses induced. Depending on whether humoral or cellular immune responses are required to provide protection against a given pathogen, targeting antigen delivery to the appropriate cellular compartment becomes critical. While surface-expressed antigens are known to preferentially stimulate humoral responses, refinements in antigen expression and delivery technology have allowed targeting other cellular compartments to enhance cellular immunity. Hly-mediated (via HlyA) antigen secretion into the phagosome was shown to enhance priming of CD4 and CD8 T-cell responses (37). A series of studies led by Russmann et al. showed that SPI-1 and SPI-2-mediated antigen delivery into the cytoplasm of antigen-presenting cells (APC) enhances T cell–mediated immunity (38,50,51). SPI-1-dependent translocation of *Listeria* peptides into the cytosol led to efficient major histocompatibility complex (MHC) I–restricted antigen presentation demonstrated by IFN-γ production and cytotoxic responses by peptide-specific CD8 T cells, which conferred protection against lethal challenge with wild-type *Listeria* (38). Similarly, SPI-2-mediated antigen delivery resulted in efficient priming of central and effector memory CD8 T cells (51). Because of the efficiency in priming T cells, this live vector-based strategy has been explored for prophylactic treatment of tumors (52).

Optimization of Plasmid Stability

Further refinements to improving the immunogenicity of plasmid-based foreign antigens delivered by live vectors have addressed the inheritance of expression plasmids within dividing live vectors. To prevent plasmidless daughter cells from overtaking a growing population, conditionally lethal systems were engineered such that plasmid loss quickly led to cell death (53–55). One such system is based on the expression of the *asd* gene encoding aspartate β-semialdehyde dehydrogenase (Asd), an enzyme critical to synthesis of the cell wall and several amino acids (56). Loss of plasmids encoding Asd is lethal for any bacterium incapable of synthesizing Asd from the chromosome, resulting in lysis of the bacterium due to an inability to correctly assemble the peptidoglycan layer of the cell wall. The *asd* system thus improves the apparent stability of expression plasmids by removing plasmid-cured bacteria from the population (i.e., a post-segregational killing system).

The *asd* system has been successfully employed in attenuated *S.* Typhimurium live vector strains (57) expressing a variety of antigens including tetanus toxin frag C (58), *E. coli* heat-labile enterotoxin (LT) (59), synthetic hepatitis B viral peptides, (60) and, more recently, *Yersinia pestis* F1 and LcrV antigens (61). Mice immunized mucosally with these recombinant strains elicited potent immune responses including serum IgG and secretory IgA. However, results were disappointing when the *asd* system was introduced into attenuated *S.* Typhi strains and tested in clinical trials. Volunteers immunized with *S.* Typhi *asd* mutants expressing hepatitis B viral peptides from *asd*-stabilized plasmids failed to elicit responses to the foreign antigen (62).

A variation of the conditionally lethal system to enhance plasmid retention involves expression plasmids that encode a self-contained toxin-antitoxin system in which the protective antitoxin is unstable and requires constant synthesis from resident expression plasmids; plasmid loss activates the toxin, again leading to cell lysis (63,64). To remove the random partitioning of multicopy plasmids during cell division, plasmid segregation functions were also introduced to ensure nonrandom inheritance of plasmids into all daughter cells (64). Quantitative in vitro analysis of plasmid retention clearly demonstrated that as toxin-antitoxin and partitioning maintenance functions were incrementally introduced, plasmid stability improved accordingly. Use of this plasmid maintenance system has recently progressed into preclinical trials in nonhuman primates, where expression plasmids have combined this maintenance system with the ClyA antigen export system to test the immunogenicity of ClyA-PA83 fusions. Monkeys primed mucosally (i.n.) with attenuated *S.* Typhi live vector CVD 908-*htrA* expressing ClyA-PA83 fusions were boosted three months later with a single parenteral dose of BioThrax® vaccine. Notably, within seven days after administration of the single parenteral booster, robust toxin-neutralizing antibody levels were detected in serum (49).

Another approach to improving plasmid stability that shows promise for improving the immunogenicity of foreign antigens borrows from motifs observed in nature that reduce the multimerization of plasmids. The *cer*-Xer recombination system of *E. coli* ColE1 replicons was the first site-specific multimer resolution system proven to decatenate plasmids and promote stability by increasing the number of functionally inheritable replicons (65,66). However, this locus depends on four chromosomally encoded host functions for multimer resolution (67), and the efficiency of this system will likely depend on the host background. Several analogously functioning but apparently self-contained resolution systems have since been identified in self-transmissible factors isolated from a variety of enteric strains (68,69). Using the *crs-rsd* site-specific resolution system, originally identified in the virulence plasmid pSDL2 from *S. enterica* serovar Dublin, Stephens et al. (70) observed that incorporation of this stability module into ColE1 replicons dramatically improved plasmid retention in *S.* Typhi vaccine strain CVD 908-*htrA*. Interestingly, the highest retention frequencies were observed only after additional transcription elements were incorporated into these expression plasmids to tightly regulate foreign antigen expression levels (70). This system awaits further immunogenicity testing in animal models.

Development of Nonantibiotic Plasmid Selection Systems

As described above, one method for accomplishing both the selection and retention of expression plasmids, without the use of antibiotic selection, involves the construction of a conditionally lethal system. A clever variation of the balanced lethal nonantibiotic strategy for plasmid selection and maintenance involves construction of a conditionally lethal transcriptional control circuit in which the *lacO-lacI* operator-repressor genes controlling the *E. coli* lactose operon are engineered to control the synthesis of a chromosomally encoded protein critical for bacterial survival. Introduction of multicopy expression