The delicate balance in genetically engineering live vaccines.

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KEY WORDS

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ABBREVIATIONS

GFP; green fluorescent protein

LD50; 50% lethal dose

RDAP; regulated delayed attenuation phenotype

RDAS; regulated delayed antigen synthesis

ABSTRACT

Contemporary vaccine development relies less on empirical methods of vaccine construction, and now employs a powerful array of precise engineering strategies to construct immunogenic live vaccines. In this review, we will survey various engineering techniques used to create attenuated vaccines, with an emphasis on recent advances and insights. We will further explore the adaptation of attenuated strains to create multivalent vaccine platforms for immunization against multiple unrelated pathogens. These carrier vaccines are engineered to deliver sufficient levels of protective antigens to appropriate lymphoid inductive sites to elicit both carrier-specific and foreign antigen-specific immunity. Although many of these technologies were originally developed for use in *Salmonella* vaccines, application of the essential logic of these approaches will be extended to development of other enteric vaccines where possible. A central theme driving our discussion will stress that the ultimate success of an engineered vaccine rests on achieving the proper balance between attenuation and immunogenicity. Achieving this balance will avoid over-activation of inflammatory responses, which results in unacceptable reactogenicity, but will retain sufficient metabolic fitness to enable the live vaccine to reach deep tissue inductive sites and trigger protective immunity. The breadth of examples presented herein will clearly demonstrate that genetic engineering offers the potential for rapidly propelling vaccine development forward into novel applications and therapies which will significantly expand the role of vaccines in public health.

INTRODUCTION

The recent explosion in the availability of genomic sequences for a wide variety of pathogenic organisms, coupled with a rapid advance in powerful genetic engineering technologies, now offers the opportunity of efficiently developing highly immunogenic and protective vaccines against a wide variety of diseases. The pathogens against which these vaccines are developed may be of viral, bacterial, parasitic, or fungal origin, and the resulting vaccines can be engineered either for animal or human vaccination. In this review, we will focus on the engineering of live bacterial vaccines, and we will use the genus *Salmonella* to illustrate engineering strategies, which can in principle be applied to a variety of bacterial pathogens for which relevant molecular biology and pathogenicity data are available. A central theme of this review will be the importance of metabolic fitness and its impact on the immunogenicity and protective efficacy of live vaccines. The application of engineering technologies to pathogens without careful consideration of the balance between attenuation and immune responses can yield vaccine candidates that have excellent safety characteristics but have lost the capacity to reach immunological effector sites and consequently fail to induce protective immunity. Strategies that have been recently developed to address this critical balance between safety and immunogenicity will be emphasized within this context of metabolic fitness.

ENGINEERING of BACTERIA INTENDED AS HOMOLOGOUS VACCINES.

Attenuating strategies targeting virulence and metabolism. It is relatively easy to weaken pathogens and engineer safe candidate attenuated vaccines. Given that these pathogens are exquisitely adapted to grow and replicate within their hosts, engineering disruptions in their intricate balance of metabolic and virulence mechanisms will certainly not require an inordinate amount of technical prowess to create attenuated strains. However, assuring safety while still achieving the immunogenicity and protective efficacy required with live vaccines has proven to be a much more challenging proposition for vaccine development. In cases where virulence

factors such as toxins have been clearly defined, engineering deletions of such toxins has proven to be quite successful in creating effective vaccines. Complete deletion of virulence genes, rather than introduction of inactivating point mutations, is required to ensure that the likelihood of reversion of the vaccine candidate back to a wildtype pathogen is very low; to further reduce the possibility of reversion, introduction of one or more additional attenuating deletions is usually carried out as well. This early strategy for vaccine design was successfully applied by Tacket et al. almost a decade ago in the construction of an attenuated live cholera vaccine [1]. To accomplish this, the wildtype *V. cholerae* classical Inaba strain 569B was engineered for removal of both the catalytic subunit of cholera enterotoxin, as well as deletion of a putative hemolysin virulence factor. When tested in volunteers, this vaccine was found to be safe and highly immunogenic, with a protective efficacy of 91% against moderate to severe diarrhea and 80% against any diarrhea, after challenge with 10^5 colony forming units (CFUs) of fully virulent *Vibrio cholerae*.

In the case of *Salmonella* vaccines, attenuation of wildtype strains has focused both on deletion of virulence factors as well as disruption of metabolic pathways, and the two serovars of *Salmonella* with which most vaccine constructions have been carried out are *Salmonella enterica* serovars Typhimurium and Typhi. *S.* Typhimurium typically causes a self-limiting gastroenteritis in humans while *S.* Typhi is the etiologic agent of typhoid fever. In both serovars, virulence factors have been found to be chromosomally encoded within clusters called *Salmonella* Pathogenicity Islands (SPIs) which play critical roles in the manifestation of disease [2]. Much attention has been devoted in particular to two distinct pathogenicity islands that encode type III secretion systems (T3SS) that inject virulence proteins called effectors into target eukaryotic cells, disrupting normal host cellular functions and facilitating *Salmonella* invasion and systemic disease [3-5]. The SPI-1 T3SS externally targets eukaryotic host cells and injects effectors that trigger actin rearrangements to enhance uptake of *Salmonella*. Then

using the SPI-2 T3SS, internalized *Salmonella* are able to inject additional effector proteins into the cytoplasm essential for bacterial intracellular survival and replication [6].

In a study reported by Hindle et al. [7], attenuated vaccine candidates from both *S.* Typhimurium (designated WT05) and *S.* Typhi (designated M01ZH09) were engineered such that delivery of all SPI-2 effectors was disrupted by deletion of a critical structural protein *ssaV* involved in the assembly of the effector injectisome apparatus. This deletion mutation was accompanied by a further deletion in *aroC* involved in the aromatic amino acid biosynthesis pathway, creating candidate vaccine strains which were then compared in a Phase 1 dose-escalating clinical trial [7]. Both strains were shown to be safe, with negligible clinical symptoms and no vaccine organisms detected in the blood. The *S*. Typhi M01ZH09 vaccine was shed from the majority of volunteers for 3 days. However, the attenuated *S.* Typhimurium WT05 strain established an unacceptably persistent colonization of volunteers with shedding for up to 3 weeks, and this strain was not pursued further. When evaluated in Phase 2 clinical trials [8], single oral doses of M01ZH09 up to 1.7×10^{10} CFUs were found to be safe and immunogenic, with 97.4% of subjects responding to vaccination with either IgG or IgA responses to *S.* Typhi LPS, and 92.1% of those receiving a dose of 7.5×10^{9} CFUs having a positive *S.* Typhi LPS-specific ELISPOT response.

Balancing safety and immunogenicity. Live vaccines that are insufficiently attenuated elicit unacceptable clinically defined adverse events in vaccinees and are considered unacceptably reactogenic. As work with the attenuated M01ZH09 *S.* Typhi vaccine illustrates, attenuation strategies targeting both virulence determinants and metabolic factors can be quite effective for constructing safe and immunogenic live vaccines that perform well in clinical trials. However, care must be taken to ensure that metabolic attenuation strategies do not result in the over-attenuation of vaccines, with subsequent loss of immunogenicity resulting from the crippling of metabolic fitness of the live vaccine. Genetic inactivation of too many critical genes, or inappropriate selection of targets, can result in vaccine candidates that fail to colonize a host

sufficiently to engage innate and acquired immunity, and elicit durable protection. We have previously reviewed the results of clinical trials conducted with attenuated *S.* Typhi candidate vaccines, and noticed a striking relationship between reactogenicity and immunogenicity [9], which we illustrate schematically in **Figure 1**. Fully virulent strains, as well as vaccine candidates, which are insufficiently attenuated elicit unacceptable clinical symptoms (i.e. highly reactogenic) but also tend to be highly immunogenic (**Figure 1A**). Vaccines that have been genetically engineered to minimize reactogenicity may become insufficiently immunogenic (**Figure 1B**). Ideally, the most promising live vaccines that perform well in clinical trials will achieve a delicate balance between reactogenicity and immunogenicity (**Figure 1C**).

This concept is clearly illustrated by a series of attenuated *S*. Typhi candidate oral vaccines engineered from the wildtype strain CDC10-80, all carrying a deletion in the *aroA* gene critical to the aromatic amino acid biosynthesis pathway. When coupled with an additional mutation in *aroD*, the resulting Δ*aroA* Δ*aroD* strain proved to be insufficiently attenuated but highly immunogenic (Figure 1A) [10] . When the triple deletion mutant Δ*aroA* Δ*aroD* Δ*htrA* was constructed (by further deletion of *htrA* encoding a heat-shock serine protease), safety improved at lower oral dosage levels but immunogenicity declined (Figure 1B); at higher oral doses which improved immunogenicity, reactogenicity (i.e. fever and bacteremia) was unacceptably high (Figure 1A) [10]. Combining Δ*aroA* with deletions in either *purA* (involved in the purine biosynthesis pathway), or *phoP*/*phoQ* (a two-component environmental regulatory system of virulence in *Salmonella*) dramatically reduced both reactogenicity and immunogenicity (Figure 1B) [11;12]. It was only when the *phoP*/*phoQ* deletion mutation alone was introduced into a different parent strain of *S*. Typhi (Ty2) that it became possible to balance reactogenicity with immunogenicity at high oral dosage levels (Figure 1C) to induce vaccine-specific immunity [13]. These observations clearly illustrate that the engineering of an attenuated live bacterial vaccine requires a carefully crafted balance between attenuation and immunogenicity that is not always attainable by deliberate engineering and may sometimes only be achieved by trial and error,

with clinical trials ultimately determining the fate of vaccines that animal models can only suggest as promising candidates.

Over-attenuation and the subsequent failure of engineered strains to reach appropriate immune inductive sites was encountered by Kong et al. [14] with efforts to construct attenuated strains of *S.* Typhimurium by engineering modifications to lipopolysaccharide (LPS). LPS is the major component of the outer membrane of *Salmonella*, and is a key virulence determinant that confers protection against complement activation and killing by macrophages [15]. LPS is comprised of a lipid A membrane anchor, a core oligosaccharide, and the outer O-antigen which defines the various serovars of *Salmonella*. Since the enzymatic pathways involved in LPS synthesis are well characterized for *Salmonella* [16], Kong et al. undertook a systematic analysis of the effects of engineering truncations in O-antigen and core sugars of LPS on the virulence and immunogenicity of *S*. Typhimurium. All of the resulting mutants tested were shown to be avirulent in mice; the lethal dose of organisms resulting in death for 50% of a group of orally challenged mice (LD50) was determined to be $>10^9$ CFU for all engineered strains. A clear relationship emerged between the extent of truncating LPS and deep tissue colonization of mice, and it was determined that deletion of LPS into the core oligosaccharide region resulted in a severe drop in colonization while preservation of at least one sugar residue at the terminus of the O-antigen was sufficient to enable significant colonization of the Peyer's patches, liver, and spleen of orally and intranasally immunized mice. It was also conclusively demonstrated that strains which successfully colonized deep tissues induced excellent serum antibody responses against *S.* Typhimurium LPS [14].

Attenuation achieved by addition as well as subtraction. Up to this point, we have considered only the construction of attenuated vaccine strains by deletion of native endogenous functions, whether they be virulence determinants or metabolic factors. However, novel approaches have recently been reported in which fully functional factors from foreign bacteria have been engineered into a pathogenic strain with the intention of disrupting pathogenicity and

188 improving innate and adaptive immunity. This intriguing strategy has been applied to the
189 remodeling of the lipid A moiety of LPS which is responsible for the endotoxic activity of enteric
190 pathogens such as *Salmonella.* The lipid A moiety of LPS stimulates a strong innate immune
191 response via stimulation of the Toll-like receptor 4 (TLR4)-MD2 complex, and triggers a
192 vigorous inflammatory response that contributes to the reactogenicity of *Salmonella*. The
193 endotoxicity of lipid A is dependent on the number and length of hydrophobic acyl side chains
194 anchoring lipid A into the outer membrane [17], as well as the phosphorylation state of the
195 disaccharide backbone [18]. Lipid A is typically biphosphorylated in *Salmonella*, which enables
196 full induction of innate immunity through stimulation of TLR4-MD2. Kong et al. [19] reported that
197 insertion of foreign genes encoding non-homologous phosphatases into *S.* Typhimurium
198 resulted in attenuated strains with increased sensitivity to the bile salt deoxycholate and
199 reduced ability to colonize the deep tissues of mice after oral immunization. The LD50 for
200 engineered strains producing monophosphorylated lipid A increased 3-4 logs while strains with
201 totally non-phosphorylated lipid A were completely avirulent in mice. Despite the fact that
202 monophosphorylated strains still retained some virulence at high doses, this strategy may still
203 prove effective in reducing reactogenicity at high doses when combined with other attenuating
204 deletions in metabolic pathways.

205 LPS-remodeling strategies have also proven to be useful in the engineering of
206 attenuated strains of *Yersinia pestis*, the causative agent of plague. The lipid A membrane
207 anchor of *Y. pestis* LPS carries six hydrophobic side chains (hexa-acylated) when residing
208 outside its human host, but down-regulates acylation to a tetra-acylated form after infection in
209 response to the increase in temperature from ambient 26°C to 37°C. Hexa-acylated LPS is a
210 potent TLR4 agonist while tetra-acylated LPS binds poorly to TLR4-MD2, resulting in a
211 reduction in inflammatory responses. When *Y. pestis* was engineered for chromosomal
212 expression of a non-homologous acyl-transferase, forcing expression of hexa-acylated LPS at
213 37°C, the resulting strains were highly attenuated yet immunogenic when this mutation was

coupled with a metabolic deletion in a sugar utilization pathway. Mice immunized subcutaneously or intranasally with this construct were protected against both subcutaneous and intranasal challenge with fully virulent *Y. pestis* [20]. Interestingly, when attempts were made to further attenuate engineered plague vaccines by reducing the phosphorylation state of lipid A using a non-homologous phosphatase (an approach that proved successful when applied to *Salmonella*), this strategy was unsuccessful with *Y. pestis* [21].

Attenuation through replication. Until recently, all attenuating strategies developed to date had begun with a fully virulent pathogen and engineered insertion or deletion mutations into a wildtype strain with the goal of creating a vaccine candidate that was sufficiently attenuated to ensure safety but sufficiently robust to confer immunogenicity. A novel shift in this established paradigm was first reported by Curtiss et al. in 2009 [22], in which candidate vaccines were engineered to be fully virulent at the time of immunization, and to *become* attenuated as they replicated within the host. This attenuation strategy effectively avoids the over-attenuation of vaccines prior to reaching appropriate immune induction sites since the intricate balance between virulence factors and metabolic pathways of the engineered pathogen remain unchanged until limited replication within the host gradually manifests the attenuated state. This biological transition from a fully virulent organism to an attenuated vaccine is referred to as a regulated delayed attenuation phenotype (RDAP), and is engineered to be dependent on the intracellular concentration of the sugar arabinose at the time of immunization. To accomplish this requires the genetic targeting of several key metabolic chromosomal loci involved in the intracellular replication and survival of *Salmonella*, whose transcription levels are re-engineered to be controlled by the arabinose activator/repressor AraC [23]. In the presence of arabinose, AraC binds arabinose and subsequently activates transcription of any genes transcriptionally controlled by the arabinose promoter P_{BAD}; in the absence of arabinose, AraC is incapable of activating P_{BAD} and transcription ceases. Given that arabinose concentrations available to vaccine organisms after immunization will be insufficient to ensure synthesis of these critical

regulatory proteins *in vivo*, loss of function will increase with every round of replication of vaccine organisms until maximum attenuation occurs. Using this transcriptional control system, gene cassettes encoding AraC linked to P_{BAD} were engineered by Curtiss et al. to replace the natural promoters controlling genes involved in iron regulation (*fur*), catabolite repression (*crp*), magnesium regulation of virulence factors (*phoPQ*), and stationary-phase protein expression (*rpoS*). All engineered strains were confirmed *in vitro* not to grow in the absence of arabinose on otherwise rich bacteriologic media. While strains carrying individually targeted regulatory genes proved to be insufficiently attenuated in the mouse model at elevated oral doses [22], later combinations of these mutations proved to yield highly immunogenic live vaccines in orally immunized mice [24]. This attenuating strategy was recently introduced into *S.* Typhi strains in anticipation of Phase 1 clinical trials [25] which have now been completed; candidate RDAP vaccine strains were proven to be safe and immunogenic in orally vaccinated volunteers, demonstrating that properly attenuated live *S.* Typhi vaccines such as RDAP vaccines can retain the ability to undergo limited replication within humans without eliciting clinical symptoms [26] .

FURTHER ENGINEERING of ATTENUATED STRAINS AS CARRIER VACCINES FOR DELIVERY of FOREIGN ANTIGENS.

Foreign gene expression and metabolic stress. It is clear from efforts to construct attenuated live vaccines that disruption of the metabolism of a pathogen results in attenuation and over-attenuation results in loss of immunogenicity. It therefore follows that attempts to further engineer additional expression technologies into a candidate vaccine, which may further impact already attenuated metabolic pathways, may destroy the efficacy of the resulting live vaccine without sufficient attention being paid to maintaining the fitness of the vaccine strain. This becomes a serious consideration in the development of live multivalent vaccines (hereafter referred to as carrier vaccines) designed for immunization against several unrelated pathogens

by delivering additional protective antigens to the immune system. The efficacy of any live carrier vaccine rests with its ability to present sufficient foreign antigen to the human immune system to elicit the desired protective immune responses. However, unregulated expression of foreign antigens diverts precious energy and metabolic resources away from the metabolism of the vaccine and into synthesis of proteins from which the vaccine derives no selective advantage either in growth or replication. As has been observed in clinical trials with RDAP vaccines, after immunization, live vaccines undergo a limited number of replications which allow limited colonization of lymphoid inductive sites to induce immune responses; further compromise of the metabolism of the live vaccine by unrestricted synthesis of foreign antigens will inevitably over-attenuate the vaccine and destroy immunogenicity.

The inevitable effect of foreign antigen synthesis on the metabolism of a live carrier vaccine could in principle be exploited to reduce the reactogenicity and improve the safety of a candidate vaccine with some residual reactogenicity (**Figure 1D**). While it is clear that over-expression of a foreign antigen will over-attenuate the resulting carrier vaccine (**Figure 1E**), careful attention to appropriate induction of foreign antigen synthesis, either by regulating the timing, level of synthesis, or processing of the antigen could in principle restore the balance between attenuation and immunogenicity to create a carrier vaccine with both excellent safety and immunogenicity (**Figure 1F**).

The concept of over-expression of foreign antigens leading to attenuation of otherwise metabolically fit organisms has been intentionally exploited recently to create carrier vaccines directly from wildtype pathogens, a strategy referred to as Attenuating Gene Expression (AGE) [27]. Support for this concept comes from the observation that over-expression of endogenous native proteins such as flagella severely attenuates wildtype *S.* Typhimurium by disrupting the bacterial outer membrane, resulting in elevated susceptibility to bile and an inability to replicate within murine macrophages. Although attenuated, these engineered strains remained immunogenic and conferred excellent protection against homologous challenge with fully

virulent *S.* Typhimurium [28]. Over-expression of native proteins was also confirmed to attenuate *Y*. *pestis*, wherein over-expression the *caf* operon, encoding the essential virulence capsule F1, was observed to dramatically reduce both intra-macrophage survival rates and the infectivity of otherwise fully virulent *Y. pestis* [29]. Over-expression of the *caf* operon was subsequently engineered as a foreign antigen gene cassette into wildtype *S*. Typhimurium, resulting in a carrier strain with severely reduced survival in murine macrophages and complete loss of virulence in mice. As expected, if expression of the *caf* operon was tightly regulated using the native *Y. pestis* temperature regulated promoter, the resulting carrier strain displayed excellent expression of the F1 capsule but also retained full virulence in mice [29]. Since such over-expression of foreign proteins clearly exerts metabolic pressure on the carrier strain, a selective advantage will arise for spontaneous deletion mutations arising that destroy foreign antigen synthesis. Therefore, reliance solely on the AGE strategy for engineering carrier vaccines will not be adequate and will require additional independently attenuating deletions to ensure safety.

Metabolic stress and instability of expression plasmids. Expression and delivery of foreign antigens by attenuated carrier vaccines can be accomplished either by plasmid-mediated expression or by integration of foreign genes into the vaccine chromosome. When using multicopy expression plasmids, induction of antigen expression can introduce sufficient metabolic stress upon the carrier vaccine to result in a selective advantage for plasmid loss, which eliminates this metabolic stress and allows a restoration of fitness. If rapid plasmid loss occurs *in vivo* following immunization, antigen-specific immunity will be lost as well. Since the use of antibiotics for plasmid maintenance (a practice commonly used under laboratory conditions) is of little use *in vivo* and is currently discouraged by the Food and Drug Administration for use with human oral vaccines, non-antibiotic strategies are needed for ensuring plasmid maintenance *in vivo* and enhancing antigen-specific immunity. An effective yet simple solution to this dilemma was devised in which a gene encoding an essential function

within the carrier vaccine was deleted from the chromosome and placed instead on a multicopy expression plasmid. Loss of the plasmid would then result in a non-viable carrier vaccine, thereby ensuring plasmid maintenance *in vivo* during limited replication and colonization of inductive lymphoid tissues. When chromosomal targets for such deletions involve enzymatic functions whose metabolic products can be added to the growth medium during *in vitro* cultivation, construction of multivalent carrier vaccines becomes reasonably straightforward. This strategy was exploited by Galán et al. [30] more than two decades ago by targeting a metabolic pathway involved in the synthesis of the *S.* Typhimurium cell wall. The enzyme aspartate β-semialdehyde dehydrogenase (Asd) is essential for the proper synthesis of the bacterial cell wall and several amino acids [31], and loss of Asd activity results in lysis of the bacterium resulting from an inability to correctly assemble the peptidoglycan layer of the cell wall. Attenuated strains in which Asd-encoding plasmids have not yet been introduced can be efficiently propagated by adding the metabolite diaminopimelic acid (DAP) to the growth medium until Asd-stabilized plasmids have been introduced. Using this plasmid stabilization strategy, attenuated strains of *S.* Typhimurium were constructed in which *asd* was encoded by high copy number plasmids, and the resulting carrier strains were evaluated for plasmid retention in orally immunized mice. For carrier strains recovered from deep tissues of immunized mice, 99% of recovered vaccine organisms retained Asd-stabilized expression plasmids *in vivo* (despite the very high copy number of these plasmids), compared to only 10% of vaccine organisms retaining unstabilized lower copy number conventional plasmids after recovery from mice [32].

This remarkably versatile strategy for plasmid maintenance was later expanded to include non-catalytic proteins such as the single-stranded binding protein (SSB) which is essential for DNA replication, recombination, and repair [33;34]. Since SSB produces no metabolic products that can be added to cultures *in vitro*, chromosomal deletion of *ssb* required the sequential use of temperature-sensitive suicide plasmids to establish this maintenance

system [35]. Use of SSB-stabilized plasmids in attenuated *S.* Typhi carrier vaccines ultimately showed that antibody responses elicited in mice against foreign antigens delivered by *S*. Typhi carrier vaccines were inversely related to the metabolic burden imposed by expression of the foreign antigen, and that these responses were improved when antigens were expressed from low-copy-number SSB-stabilized plasmids carried by less attenuated carrier vaccines [35].

Minimizing metabolic stress by synchronizing antigen expression with metabolism. Success with plasmid maintenance systems that essentially guarantee plasmid stability *in vivo* can quickly lead to additional problems with over-attenuation frequently associated with plasmid-based delivery of foreign proteins. Due to the multicopy aspect of plasmids, when foreign genes are induced there is a rapid rise in antigen synthesis (**Figure 2A**) [36] which tends to slow growth rate and reduce the colonization capacity of the carrier vaccine due to the severe diversion of metabolic resources [37]. Over the years, vaccine developers have created a number of tools for reducing the effect of this antigen burst through tightly regulating control of the transcription, translation, and export of foreign antigens. These techniques have been reviewed elsewhere in considerable detail by ourselves [38-40] and other groups [41;42], and will not be recapitulated here. Slightly more unconventional efforts to minimize the effects of antigen burst have recently been reported in which an attempt is made to link the timing of antigen synthesis directly to the physiology of the carrier vaccine.

One way to link the synthesis of foreign antigens to vaccine physiology is an extension of the technique of regulated delayed attenuation, in which the timing of antigen synthesis is intimately linked to replication of the vaccine *in vivo*. This strategy is referred to as regulated delayed antigen synthesis (RDAS, **Figure 2B**) [43], and was created by engineering modifications to the well characterized lactose repressor (LacI) transcriptional control system frequently employed to control foreign antigen synthesis [44]. In the absence of lactose or other synthetic sugar analogs, LacI binds to its cognate promoter and prevents transcription of the downstream open reading frame; the binding of LacI to lactose or synthetic analogs causes an

allosteric shift in LacI conformation which derepresses transcription, with subsequent commencement of foreign antigen synthesis. By replacing the natural *lacI* promoter with an arabinose-controlled *araC* promoter, antigen synthesis is then linked to intracellular concentrations of arabinose, in a strategy similar to the previously discussed delayed attenuation system. Antigen synthesis is then ultimately linked to the replication and growth rate of the bacterial strain, with diminishing intracellular concentrations of arabinose leading to a shift in protein synthesis away from LacI expression and towards an increase in foreign antigen synthesis (**Figure 2B**). Such an approach would theoretically allow remarkable diversity in the timing of antigen delivery by a carrier vaccine. Antigen production in vaccine organisms prepared in the presence of arabinose would be tightly repressed prior to immunization; after oral administration, transient exposure to lactose after immunization could enable a quick but temporary burst of foreign antigen production, with full and sustained induction occurring as the vaccine grows and colonizes lymphoid tissues and intracellular LacI concentrations drop.

A thorough characterization of plasmid-based regulated delayed antigen synthesis in attenuated *S.* Typhimurium carrier vaccines was described by Wang et al. [43], and this work clearly demonstrated that the over-attenuating effects of poorly regulated antigen expression from multicopy plasmids could be effectively overcome by an arabinose-controlled LacI-mediated antigen gene expression system. When mice were orally immunized with RDAS carrier vaccines delivering a protective pneumococcal surface protein (PspA) antigen from *Streptococcus pneumoniae*, 52% of vaccinated animals were protected against challenge with fully virulent *S. pneumoniae*, while only 21% of mice receiving vaccines constitutively over-expressing the foreign antigen were protected [43].

In a related approach linking the timing of antigen synthesis directly to the physiology of the carrier vaccine, initial studies have demonstrated the feasibility of controlling the timing of foreign antigen expression encoded by chromosomally engineered expression cassettes, without the need for plasmids. Although expression of foreign antigens exclusively from

chromosomally integrated gene cassettes offers the substantial advantage of minimizing any metabolic burden associated either with multicopy expression plasmids or the foreign antigens they encode, the challenge with this approach has been synthesizing sufficient levels of foreign antigen capable of eliciting relevant immune responses despite the significant drop in copy number of the cassettes encoding these antigens. Using a cassette encoding the model foreign antigen green fluorescent protein (GFP), Wang et al. [36] described a novel chromosomal expression strategy designed to compensate for the inherent disadvantage of lower gene dosage (versus plasmid-based expression) by integrating a single GFP-encoding gene cassette into multiple chromosomal sites already inactivated in an attenuated *S.* Typhi vaccine candidate. Using GFP-encoding cassettes integrated into both *guaBA* (which displays growth-regulated transcriptional control of antigen synthesis [45]) and *htrA* (which displays transcriptional control of antigen synthesis in response to metabolic stress during growth [46]), cumulative synthesis of GFP from these two integration sites was observed to be superior to single integrations. Most importantly, it was demonstrated that GFP expression increased in a growth phase-dependent manner, suggesting that foreign antigen synthesis could be "tuned" to the physiology of the carrier vaccine [36]. This promising chromosomal expression technology is currently being combined with plasmid-based expression from stabilized plasmids for delivery of several protective antigens from *Y. pestis*, delivered by a single multivalent *S.* Typhi carrier vaccine.

Site of antigen delivery and subsequent immune response. In addition to tightly regulated expression of foreign antigens, it is now clear that the manner in which these antigens are delivered to the immune system can have a profound impact on the resulting immune responses and ultimate success of a carrier vaccine. The induction and extent of mucosal, humoral, or cellular immunity can be significantly influenced by whether foreign antigens are expressed within the carrier vaccine or exported out of the live vaccine, as well as whether antigens are expressed prior to host cell invasion or delivered by intracellular carriers. It is now reasonably well established that antigen-specific humoral immunity can increase significantly

when antigens are exported either to the carrier surface or extracellularly into the surrounding milieu, rather than remaining in the cytoplasm [47-49]. It has also been reported that cellular immunity to surface antigens delivered by intracellular carriers is superior to immunity targeting cytoplasmic antigens [50].

Cellular responses can also be improved by injection of foreign proteins from intracellular carrier vaccines into the cytoplasm of antigen-presenting cells via *Salmonella* type III secretion systems, a technique first described over a decade ago by Russmann et al [51]. Hegazy et al. [52] have further developed this approach by conducting a methodical analysis of the translocation efficiency of a panel of SPI-2 effector proteins when used as carriers for antigenic passenger domains fused to the carboxyl terminus of the SPI-2 carrier. They observed that for *S.* Typhimurium carrier vaccines delivering passenger domains of listeriolysin O (a protective antigen from *Listeria monocytogenes*) fused to SPI-2 effectors, translocation of fusions into murine bone marrow-derived dendritic cells displayed varying efficiencies *in vitro,* depending on the specific effector fusion involved. However, in mice orally immunized with these carrier strains, stimulation of *L. monocytogenes* antigen-specific cytotoxic T-cells did not strictly correlate with *in vitro* translocation efficiencies. This disparity may be a reflection of artificial induction conditions used for *in vitro* expression, which may have little relevance to *in vivo* microenvironmental induction conditions. However, it was clear from these studies that choosing the right SPI-2 effector for translocation of a vaccine antigen can elicit robust levels of cytotoxic immunity against intracellular pathogens.

Antigen delivery by regulated lysis. One rather extraordinary method for delivery of intracellular antigens to lymphoid inductive sites is through outright lysis of the carrier vaccine to release cytoplasmic contents including foreign proteins (depicted schematically in **Figure 2C**). Success with this approach will depend on the timing of lysis, which must occur as vaccine organisms are reaching inductive sites. Since delayed attenuation technologies proved that it was possible to control the timing of induced attenuation to coincide with deep tissue

448 colonization and elicitation of protective immunity, it was considered plausible to adapt the
449 delayed phenotype strategy to achieve delayed lysis of a carrier vaccine and test the
450 immunogenicity of these novel constructs. Successful testing of this delayed lysis strategy was
451 first reported by Kong et al. [53] who genetically engineered a programmed lysis system based
452 on arabinose-controlled production of two key enzymes involved in the synthesis and
453 mechanical stability of the carrier vaccine cell wall. Diaminopimelic acid and muramic acid are
454 explicitly required to ensure the integrity of the peptidoglycan layer of the carrier vaccine cell
455 wall, and synthesis of these two components requires enzymes encoded by chromosomal *asd*
456 and *murA* genes respectively. The requirement for synthesis of aspartate β-semialdehyde
457 dehydrogenase (Asd) to ensure the integrity of the cell wall was first exploited for maintenance
458 of plasmids delivering foreign antigens (described above), with lysis of the vaccine resulting
459 from plasmid loss and cessation of Asd synthesis. To ensure complete lysis of vaccine
460 organisms and full release of cytoplasmic contents, arabinose-controlled synthesis of Asd was
461 coupled with additional arabinose-controlled synthesis of MurA. To establish exquisitely
462 stringent regulation of Asd and MurA synthesis, an arabinose-regulated anti-sense RNA system
463 was also engineered onto the antigen-expressing multicopy plasmid. In the absence of
464 arabinose, chromosomal transcription of both *asd* and *murA* ceased, as did transcription of an
465 additional chromosomally encoded repressor of the anti-sense-RNA system. Therefore,
466 cessation of both *asd* and *murA* transcription was simultaneously accompanied by induction of
467 high levels of antisense RNA synthesized from the expression plasmid, effectively blocking
468 residual translation from any lingering *asd* or *murA* transcripts. These concerted activities lead
469 to cell lysis and release of vaccine antigens [53]. In mice orally immunized with carrier vaccines
470 engineered for constitutive periplasmic synthesis of the *S. pneumoniae* protective PspA antigen,
471 coupled with delayed lysis to release PspA, excellent serum antibody responses against both
472 the foreign antigen and carrier-specific outer membrane proteins were reported. No viable
473 vaccine organisms were detected in host tissues after three weeks, demonstrating that these

474 engineered strains were able to proliferate long enough to stimulate humoral immunity but were
475 eventually completely cleared from the vaccinated host [53]. This last point may have relevance
476 to previous clinical trials in which orally administered *S.* Typhimurium WT05 vaccine strains
477 proved unacceptably attenuated and were shed for greater than three weeks from vaccinees [7].
478 The development of the delayed lysis strategy for carrier vaccines paves the way for
479 significant improvements in the use of carriers for delivery of DNA vaccines. Although several
480 early reports supported the feasibility of delivering DNA vaccines using attenuated carrier
481 strains to elicit immune responses [54-56], attempts to expand this vaccination strategy have
482 proven frustrating. The use of delayed lysis to improve DNA vaccine delivery and ensuing
483 immune responses has now been reported by Kong et al. [57]. In addition to the delayed lysis
484 strategy, additional vaccine modifications required for successful delivery of the DNA vaccine
485 involved: 1] increased expression of the SPI-1 activator HilA protein, resulting in a hyperinvasive
486 phenotype for the carrier strain to improve intracellular delivery of the DNA vaccine, 2]
487 inactivation of the SPI-2 effector protein SifA allowing *Salmonella* to escape from intracellular
488 vacuoles into the target cell cytoplasm, 3] inactivation of additional SPI-2 effectors which
489 normally induce apoptosis of eukaryotic cells by intracellular *Salmonella*, thereby allowing
490 sufficient time for DNA vaccines to traffic to the nucleus, and 4] insertion of multiple DNA
491 vaccine nuclear-targeting sequences to facilitate efficient delivery of the DNA vaccine to the
492 target cell nucleus after cytoplasmic lysis of the carrier. An optimized DNA vaccine encoding
493 the influenza hemagglutinin (HA) protective antigen, delivered by an optimized delayed lysis *S.*
494 Typhimurium carrier vaccine, induced complete protection in orally immunized mice against a
495 lethal intranasal challenge with 100 LD50s of fully virulent influenza virus [57]. Given that DNA
496 vaccines are virtually silent in carrier strains, plasmid-mediated metabolic attenuation of the
497 carrier is significantly reduced, and such vaccines have great potential in applications for which
498 conventional vaccination strategies have proven unsatisfactory.
499

NOVEL APPLICATIONS of ENGINEERED VACCINES.

Engineered vaccines as "reagent strains". Aside from the conventional deployment of engineered strains as live and carrier vaccines, several additional applications of these strains have arisen which bear mention, including use in conjugate vaccine development and novel approaches to cancer treatment and prevention. Conjugate vaccines represent a versatile subunit vaccine strategy in which protective immunity can be targeted against capsular and outer membrane polysaccharides of a variety of Gram-negative bacteria. Along with the burgeoning interest in conjugate vaccines against pathogenic bacteria comes the problem of economic and safe purification of the polysaccharide haptens and the carrier proteins from which the conjugate vaccines are manufactured. Tennant et al. [58] have recently reported the development of "reagent strains" which have been specifically engineered for efficient purification of conjugate components from a single attenuated strain. These reagent strains can then be used to develop homologous conjugate vaccines comprised of purified endotoxin-free core-O-polysaccharides (COPS) chemically conjugated to purified flagellin monomers. Given the simplicity of the approach, this technique was applied to the engineering of reagent strains derived from both *S.* Typhimurium and *S*. Enteritidis pathogens. The engineering of these reagent strains was accomplished with three key steps in which fully virulent pathogens were first rendered auxotrophic for guanine by deletion of the chromosomal *guaBA* locus, followed by a deletion of a master *clpP* regulatory locus which resulted in hyperflagellation of the reagent strain. The final critical engineering step involved deletion of a FliD capping protein involved in the polymerization of flagellin monomers into fully functional flagella; in the absence of FliD, flagellin monomers were exported into the surrounding medium and could be efficiently purified away from intact bacteria. When evaluated in mice, Simon et al. [59] observed that conjugate vaccines developed against *S.* Enteritidis generated robust flagellin-specific antibody responses and higher anti-LPS IgG responses than observed in mice immunized only with unconjugated COPS. Most importantly, in mice challenged with fully virulent *S.* Enteritidis, conjugate vaccines

conferred 100% protection in vaccinated mice receiving fractional doses down to 0.25 μg, and 90% efficacy in mice immunized with as little as 25 ng of conjugate vaccine [60]. Genetically engineered reagent strains therefore represent a remarkably straightforward method from which highly immunogenic homologous flagellin-based conjugate vaccines can be safely and economically manufactured from a single attenuated strain.

Engineered vaccines as interventions against cancer. Another intriguing application of engineered strains targets development of therapeutic interventions against metastatic cancer. Vendrell et al. [61;62] have described in two recent studies, encouraging results using an engineered *S.* Typhi attenuated vaccine candidate as a therapeutic intervention to promote tumor reduction when injected directly into the tumor and surrounding draining lymph nodes. This approach was used in mouse models of breast cancer [61] and T-cell lymphoma [62], and in both cases resulted in significant infiltration of activated neutrophils not observed with untreated tumors. A reduction in invasion of tumor cells into nearby tissue, along with a reduction of potentially immunosuppressive T_{reg} cells into the draining lymph nodes, accompanied delayed development of metastases and increased survival times in both studies as well. In the case of immunotherapy against the mammary adenocarcinoma, significant infiltration of neutrophils was observed in necrotic areas that formed micro-abscesses [61]. Remarkably, immunotherapy with the metastatic T-cell lymphoma resulted in complete tumor regression in 10% of treated animals (3 out of 30 treated), which engendered tumor-specific immunity when rechallenged with the homologous tumor (in 2 of the 3 re-challenged animals) [62]. Interestingly, although the attenuated vaccine strain was engineered to be dependent on guanine for growth, viable organisms were recovered from tumor tissues for up to 7 days after injection in both studies; it was hypothesized that a nutrient-rich environment required by a rapidly growing tumor might provide just enough benefit to vaccine organisms to enhance persistence, which could theoretically enhance antitumor inflammatory responses and promote tumor-specific cytotoxicity either directly or indirectly.

552 In addition to therapeutic interventions, work has also progressed in the development of
553 attenuated *Salmonella* carrier strains as therapeutic vaccines that target adaptive immunity in
554 addition to innate immunity for resolution of tumors. Xiong et al. [63] have reported the
555 construction of attenuated *S.* Typhimurium carrier vaccines for intracellular delivery of an
556 important tumor associated antigen, survivin, which is involved in tumor persistence,
557 proliferation, and invasion [64]. In these carrier vaccines, delivery of survivin is accomplished by
558 fusion to the T3SS SPI-2 effector protein SseF, which is translocated into host antigen
559 presenting cells to elicit antitumor activity. In this study, comparison of several strains
560 combining various deletion mutations to achieve attenuation demonstrated that properly
561 attenuated carrier vaccines were able to inhibit tumor growth in orally immunized mice
562 subcutaneously challenged with colon carcinoma cells or challenged by intracranial injection of
563 glioblastoma cells. The antitumor efficacy of these carrier vaccines was further improved in this
564 work by enhancing innate immunity through intraperitoneal co-administration of a ligand
565 adjuvant to stimulate natural killer T-cells [63].
566 Live attenuated cancer vaccines have also shown promise in a Phase 1 clinical trial in
567 which an attenuated strain of *Listeria monocytogenes* was evaluated in patients with late stage
568 metastatic cervical cancer. In this study [65], a streptomycin-resistant strain of *L.*
569 *monocytogenes* was attenuated by chromosomal deletion of an essential gene encoding a
570 master virulence regulator (PrfA), which was then placed on a multicopy plasmid to enhance
571 plasmid retention *in vivo*. Incomplete plasmid-based complementation of the chromosomal *prfA*
572 deletion mutation was reported to attenuate pathogenicity by 4-5 logs [66]. This expression
573 plasmid further encoded export of the human papillomavirus oncoprotein E7 (HPV-16 E7) fused
574 to listeriolysin O [67]. The carrier vaccine proved somewhat reactogenic in all patients receiving
575 intravenous infusions of two doses ranging from 1×10^9 to 1×10^{10} CFU and spaced three
576 weeks apart. However, 30% of patients experienced a reduction in tumor size, and overall

577 median survival was 347 days, versus a median survival time of 6-7 months for patients treated
578 by more conventional methods.
579
580 **CONCLUSIONS**
581 In this review, we have not attempted to provide an exhaustive recapitulation of
582 engineering techniques and strategies currently available for constructing attenuated live strains
583 and carrier vaccines. Rather, we have been intentionally selective in citing examples that
584 effectively illustrate the fundamental and central theme of this review in which we stress the
585 importance of metabolic fitness and its impact on the immunogenicity and protective efficacy of
586 live vaccines. We have highlighted a wide array of engineering techniques and strategies which
587 when properly and carefully applied can achieve the critical balance between safety and
588 immunogenicity that ultimately determines the success or failure of live vaccines in clinical trials.
589 We believe that with the availability of sufficient genomic data and armed with relevant data on
590 mechanisms of pathogenicity, today's vaccine developers may be constrained only by
591 imagination and persistence in creating highly immunogenic live attenuated vaccines against an
592 ever-increasing variety of emerging diseases of importance to public health.
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FIGURE LEGENDS

Figure 1. Balancing reactogenicity and immunogenicity in the development of live vaccines. Both the safety and immunogenicity of a live bacterial strain are schematically represented in the left and right sides of each panel as a gradient of values. For safety, the gradient extends from a region of unacceptable reactogenicity (red zone denoted by "R") to a region of acceptable non-reactogenicity (green zone denoted by "NR"); similarly, for immunogenicity, the gradient extends from a region of minimal immunogenicity (red zone designated as non-immunogenic, "NI") to the desired region of immunogenicity (green zone designated as immunogenic, "IM"). **Panels A-C** schematically represent construction of a properly attenuated vaccine strain; and **Panels D-F** represent construction of a carrier vaccine expressing additional protective antigens from unrelated pathogens. In this graphic, pathogenic organisms are represented by **Panel A**, over-attenuated vaccine strains engineered from pathogens are represented by **Panel B**, and properly engineered attenuated vaccines are represented by **Panel C**. Given that expression of a foreign antigen can elevate metabolic burden and increase attenuation, starting with a slightly reactogenic vaccine candidate (**Panel D**) and over-expressing a foreign antigen (purple filled circle labeled "Ag") will tip the balance and result in a safe but non-immunogenic live vaccine (**Panel E**), while expression of sufficient

628 levels of foreign antigen to elicit immunogenicity without over-attenuating the carrier strain will
629 yield both a safe and immunogenic carrier vaccine (**Panel F**).
630
631 **Figure 2. Strategies for developing safe and immunogenic carrier vaccines.** Candidate
632 live vaccines are depicted as elongated circles, and the degree of attenuation is represented by
633 shades of blue in which deep shades represent non-attenuated strains with shades becoming
634 paler with increasing attenuation. Production of foreign antigen is depicted as red dots.
635 Immunization is represented as a black arrow extending across a vertical black line labeled
636 “host barriers". **Panel A** depicts conventional strategies in which an attenuated strain is
637 engineered for tightly regulated expression of genes encoding foreign antigens after reaching
638 inductive sites within the host, resulting in a burst of antigen synthesis and an increase in
639 attenuation of the carrier vaccine. **Panel B** depicts the combined strategies of Regulated
640 Delayed Attenuation Phenotype (RDAP) and Regulated Delayed Antigen Synthesis (RDAS).
641 Fully invasive carrier vaccines are used for immunization, which undergo limited replication and
642 become increasingly attenuated with each round of replication (RDAP). As replication proceeds
643 and attenuation increases, synthesis of foreign antigen also begins to increase as vaccine
644 organisms reach lymphoid inductive sites (RDAS). Progression of both attenuation and antigen
645 synthesis is inextricably linked *in vivo* to depletion of intracellular levels of arabinose as limited
646 replication proceeds (see text for further details). **Panel C** depicts the combined strategies of
647 RDAP and regulated delayed lysis to improve delivery of foreign antigens (or DNA vaccines) to
648 immune inductive sites. As replication proceeds and attenuation increases (RDAP), regulated
649 induction of foreign antigen synthesis begins, accompanied by complete lysis and release of
650 cytoplasmic contents into antigen presenting cells as vaccine organisms reach lymphoid
651 inductive sites (see text for more details).

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Figure 1-revised

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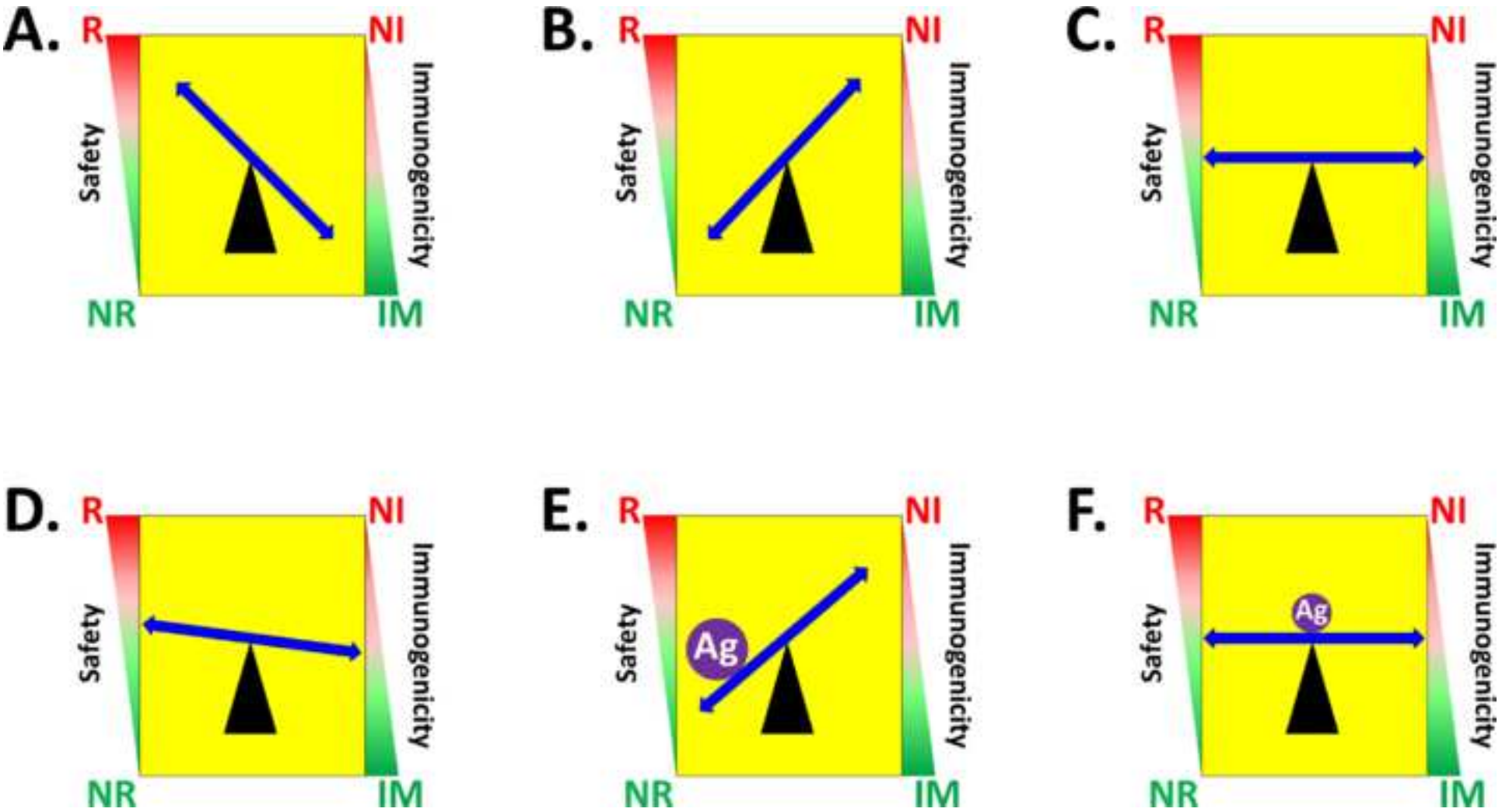

Figure 2

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