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Review

Recombinant immune complexes as vaccines against infectious diseases

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New vaccine technologies are needed to combat many existing infections and prepare better for those that may emerge in the future. The conventional technologies that rely on protein-based vaccines are still severely restricted by the sparsity and poor accessibility of available adjuvants. One possible solution to this problem is to enhance antigen immunogenicity by a more natural means by complexing it with antibodies in the form of immune complexes (ICs). However, natural ICs are impractical as vaccines, and significant research efforts have been made to generate them in recombinant form, with plant bioengineering being at the forefront of these efforts. Here, we describe the challenges and progress made to date to make recombinant IC vaccines applicable to humans.

Highlights

Immune complexes (ICs) between antigens and antibodies are naturally immunogenic by virtue of enhanced uptake by antigen-presenting cells.

Recombinant ICs have been generated by molecular engineering, which opens the path for scale-up manufacturing and consistent formulation.

Several iterations of recombinant ICs have been generated and tested as vaccine candidates for immunogenicity and protection against infectious diseases.

Plant biotechnology has emerged as an alternative and cheaper production system to mammalian cell platforms, raising the prospect for scalable, low-cost production of recombinant IC-based vaccines.

Recombinant IC technology is now poised to enter the clinical phase of testing and development.

Challenges in protein vaccine development and the adjuvant 'bottleneck'

To date, some of the most successful vaccines for infectious diseases, such as the triple-vaccine combination for diphtheria-tetanus-pertussis, hepatitis B virus (HBV), influenza, and pneumococcal pneumonia, have relied on using the alum **adjuvant** (see Glossary), which was licensed in the 1920s and remained the only adjuvant in use until the late 1990s, when the oil-in-water emulsion adjuvant MF59 was first included in a licensed product in Europe [1]. Since then, a very limited number of new adjuvants have been used in human vaccines, and they include the Adjuvant Systems AS0, CpG1018, and, most recently, the MatrixM adjuvant used in the malaria R21/MM [2] and Novavax COVID-19 vaccines [3]. This illustrates the issue of adjuvants often being seen as a major 'bottleneck' in vaccine development. Yet, protein antigens are poorly immunogenic on their own and fail to induce protective immunity, thus requiring adjuvants to elicit long-lasting immunity and protection from subsequent infections, as well as to help reduce the antigen dose. However, the downside of adjuvants in vaccine formulations is their potential for toxicity, which can range from mild irritation to the more serious adverse events, including the so-called autoimmune/inflammatory syndrome induced by adjuvants. This may manifest through a range of pathologic conditions, such as sarcoidosis, Sjögren syndrome, undifferentiated connective tissue disease, and various immune-related adverse events [4,5]. For these reasons, it would be highly desirable to reduce or altogether circumvent the need for exogenous adjuvants in protein vaccine formulations. One attractive possibility to do that is to enhance protein immunogenicity by a more natural means, such as complexing it with antibodies in the form of **immune complexes (ICs)**, for enhanced uptake by **antigen-presenting cells (APCs)**. Box 1 describes some of the initial successes with classical IC approaches in vaccinology but also highlights the challenges and limitations in terms of reproducible production and formulation, limiting their application. The need for producing IC in recombinant form therefore became an apparent prerequisite for translating the IC vaccine technology to application in humans. In this review, we describe the properties of ICs as a vaccine delivery platform against infectious diseases, with the main emphasis on **recombinant forms of ICs (RICs)**, and the progress made to date to achieve reproducible production and consistent formulation amenable for vaccine development and application in humans.

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Box 1. Classical IC vaccine approaches against infectious diseases

In our bodies, an external foreign substance stimulates both innate and adaptive immune responses via Ag-specific and nonspecific recognition mechanisms, with the adaptive responses leading to the formation of ICs. This occurs through the primary and especially secondary phases of the immune response, when induced antibodies are abundant and predominantly of the IgG isotype. ICs amplify an ongoing immune response, a property demonstrated long ago using model antigens in experimental models of immunization [56–58]. Since then, the early attempts at immunization with polyclonal sera-based ICs focused mostly on animal diseases, such as chicken bursal disease [59,60], Newcastle disease virus [41], equine herpesvirus 1 [61], and porcine parvovirus [62]. After the introduction of mAbs, the IC vaccine approach became more suited for human application, and mAb-based IC vaccine candidates, including, among others, for influenza [63], HIV [42,64] HBV [65–67], tick-borne encephalitis virus [68], *Francisella tularensis* [69], Ebola virus [17], and tuberculosis [70], have all been attempted. These studies have clearly demonstrated the potential of ICs as vaccine delivery systems but also highlighted certain limitations. Principally, the use of polyclonal sera as the source of antibodies for ICs is impractical, whereas mAbs rely on polymeric antigens or whole organisms or viruses to form ICs, as soluble monomeric antigen and mAb can only form single-molecular but not multimolecular immune complexes. Furthermore, preparation of ICs with soluble antigens requires the *in vitro* mixing of Ag and Ab at an optimum ratio, which is not easily reproducible, or the use of cocktails of mAbs to achieve complexing, which is expensive. Thus, the complexity of formulation does not lend itself well to pharmaceutical development. This greatly limits the potential of mAb-based ICs, and further molecular engineering was required to make them amenable for vaccine applications.

RIC technologies

Fc fusion proteins have been used extensively as an important backbone for Ab derivatives (e.g., Ab–drug conjugates and immune cytokines) and have shown good efficacy and no adverse side effects in humans [6,7]. Vaccines have also been developed using Fc fusion technology because there are many advantages, including (i) easy purification using protein A/G affinity chromatography; (ii) the fact that Fc promotes the correct folding of the fusion protein [8] and enhances binding to APCs [9], resulting in increased (in the context of infectious diseases) or suppressed (in the context of autoimmune and chronic inflammatory diseases) Ag immunogenicity [10]; and (iii) the fact that Fc fusions can improve recombinant immunogen solubility and stability and thus prolong half-life [8,11–13].

However, using the monomeric antibodies or Fc component [constant domain of the antibody heavy chain (HC)] alone to deliver Ag to immune cells has one major limitation in that, being monomeric, they can bind only to high-affinity FcγRI but are unable to crosslink multiple FcγRs or bind to low-affinity FcγRs, including FcγRIIA and FcγRIII, which are important for amplifying immune responses by ICs. In contrast, classical ICs that contain multiple Fc domains can enhance Ag uptake through the increasing binding activity to all FcγRs, including those with high and low affinity [14]. This is possible because of their multimeric nature, with polyclonal IgGs bound to the same antigen or organism, as depicted in Figure 1, enabling uptake of ICs by APCs and subsequent antigen presentation to T cells, as explained in greater detail in Box 2. Monoclonal antibodies (mAbs) can partly replicate this, but only when bound to whole organisms (Figure 1) and only if the surface antigen is of sufficiently high density to allow FcγR crosslinking; they cannot do so with soluble antigens used in conventional subunit vaccines. Similarly, Fc fusion proteins also fail to form multimeric complexes and consequently are ineffective as vaccines (Figure 1). Therefore, despite the many advantages of Fc fusion proteins listed in the preceding text, their inability to form ICs remained a major obstacle for vaccine design, an obstacle that has now been overcome by means of advanced molecular engineering, as described in the following section.

RICs based on full-length mAb

The pioneering attempts to generate ICs in recombinant form date back to 2006, when, using transgenic tobacco plants as the expression system, Ma and colleagues first generated fully RICs using a tetanus [15] and then the Ag85B and Acr (HspX) tuberculosis antigens [16] by expressing antigen fused to the C-terminus of the antibody HC of a cognate mAb. The design of the RIC fusion molecule resulted in the expression of Ab-Ag at a 1:2 ratio (Ag was fused to

Glossary

Adjuvants: vaccine ingredients that help induce a stronger immune response to the vaccine antigens. They work by a variety of different mechanisms, including antigen deposition, microbial mimicry, engagement of cell surface receptors on APCs, induction of localized inflammatory response, and influx of immune cells. Because of potential for toxicity, adjuvants need to meet stringent safety requirements before use in humans.

Antigen-presenting cells (APCs): specialized cells of the immune system that take up antigens or microbes, process them by one or both antigen presentation pathways (endogenous, via MHC-I; exogenous, via MHC-II), and present the resulting MHC-peptide complexes to antigen-specific T lymphocytes. MHC-I peptides are predominantly presented to CD8 T cells, whereas MHC-II peptides are presented to CD4 T cells. The co-called professional APCs primarily include dendritic cells and macrophages, with B cells also participating in antigen presentation under specific conditions.

Good Manufacturing Practice (GMP): a set of protocols and processes which ensure that products are consistently produced and controlled according to quality standards. This is a prerequisite for all medicinal products and ensures that they are of consistent high quality, appropriate for their intended use, and meet the requirements of the marketing authorization or clinical trial authorization.

Immune complexes (ICs): formed between antigens or whole organisms (i.e., bacteria or viruses) and polyclonal antibodies. The minimal requirement for an IC is complexing of antigen with at least two different antibody molecules. ICs are formed during infection to either amplify the immune response [by targeting complexed antigens to antibody heavy chain Fc receptors (FcR) on APCs or by activating complement] or sequester the excess antigens from circulation, usually to the liver or spleen, where they are destroyed.

Plant biotechnology: production of therapeutic molecules in plant cells using stable nuclear transformation or transient expression approaches. The advantages of this technology are the low initial investment cost, ease of scalability (greenhouses), and lack of risk of human pathogen transmission.

Recombinant immune complexes: ICs that are made by recombinant

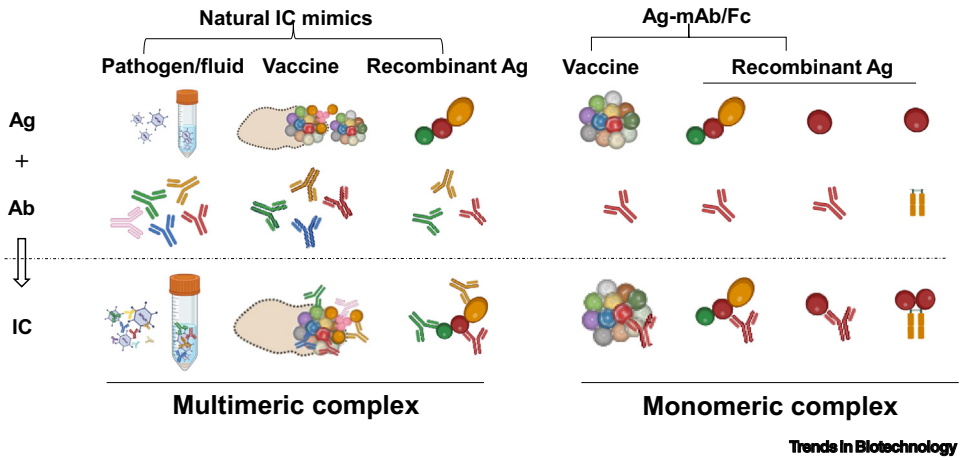

Figure 1. Formation of classical immune complex (IC) with polyclonal antibodies versus monomeric complex formation with monoclonal antibodies (mAbs) or Fc component alone. Shown are different ways of generating ICs as potential vaccines, with either polyclonal antibodies (indicated in different colors) or mAbs; presence of multiple or a single target epitope is indicated with differently colored circles. Note: The monomeric complex on the far right is based on Fc-antigen (Ag) fusion protein, with no antibody light chain present.

each Ab HC). This allowed the antibody to bind to an adjacent same molecule and effectively multimerize, forming ICs (Figure 2, Key figure). Moreover, it was demonstrated in mouse immunization studies that serum Ab responses [15] and protective cellular immunity [16] were induced without additional adjuvants. In a similar fashion, Phoolcharoen and colleagues [17] transiently expressed Ebola vaccine in plants by genetically fusing the Ebola GP1 glycoprotein subunit to a specific Ab HC and demonstrated high titers of Ebola-specific IgG Abs in immunized mice. Thus, these studies have shown that RICs represented an attractive immunization strategy if they can be manufactured reproducibly at large scale, a prerequisite for **Good Manufacturing Practice (GMP)** production. However, some limitations of the RIC technology became apparent. The molecular design of the RIC did not allow control over the size of the IC formed and suffered

technologies and based on a single mAb. Multiple antibody molecules within the complex are enabled by molecular engineering rather than polyclonal antibodies binding to the same antigen. The assembled complexes are functionally representative of natural ICs but are more amenable for reproducible production and consistent formulation.

Box 2. IC mechanism of immunogenicity

Dendritic cells (DCs) are the most important APCs in the context of priming immune responses and vaccination. It is well known that they not only can present exogenous antigens to CD4 T cells but also are capable of cross-presentation of endogenous antigens to CD8 T cells, which can be potentiated by adjuvants [71,72]. In this context, an IC-based vaccine that contains no exogenous adjuvants can still compensate by virtue of greatly enhanced uptake through FcγRs on the surface of APCs, because uptake of soluble antigen alone and subsequent cross-priming are generally very inefficient and require very high Ag concentrations [73]. This contrasts with very efficient uptake and internalization by phagocytosis of Ag coupled to latex beads, which leads to MHC classes I and II–restricted Ag presentation in both macrophages and DCs [74,75]. Thus, the pathway by which antigens are internalized appears to impact the efficiency of presentation by both MHC classes I and II molecules, and ICs can both engage these pathways effectively and prime both $CD4^+$ and $CD8^+$ T-cell responses [76]. This can be further potentiated by IC-mediated activation of the complement cascade and retargeting of C3-decorated ICs to complement receptor CR2 on APCs [77]. Ab binding to Ag also leads to protection of the Ag from proteolysis extracellularly [78], and this can lead to modulation of Ag processing as well as Ag presentation [79].

IC-mediated cellular activation triggers transcriptional programs inside the APC, such as enhanced expression of proinflammatory cytokines as well as upregulation of membrane-expressed coactivating molecules, including CD80 and CD86, and MHC classes I and II–driven presentation of Ag-derived peptides to T cells [80]. However, apart from the activating Fc receptors, the APCs also express inhibitory receptors, and their conserved expression among species suggests that immune modulation by ICs is tightly regulated [81]. Furthermore, the expression of various FcRs is strongly influenced by the cellular and cytokine environment [82]. For example, TGF-β1 downregulates surface expression of FcγRI and FcγRIII on monocytes, whereas IL-4 increases the expression of the inhibitory FcγRIIB [83]. In contrast, IFN-γ alone or in combination with TNF-α increases expression of the activating Fc receptors on monocytes [82,84]. Therefore, IC vaccine approaches need to consider which activating and inhibitory Fcγ receptors are expressed by APC subsets targeted by the vaccine and define how their expression patterns are regulated after IC-mediated activation *in vivo*.

Key figure

Schematic representation of recombinant immune complex (RIC)-based vaccine constructs

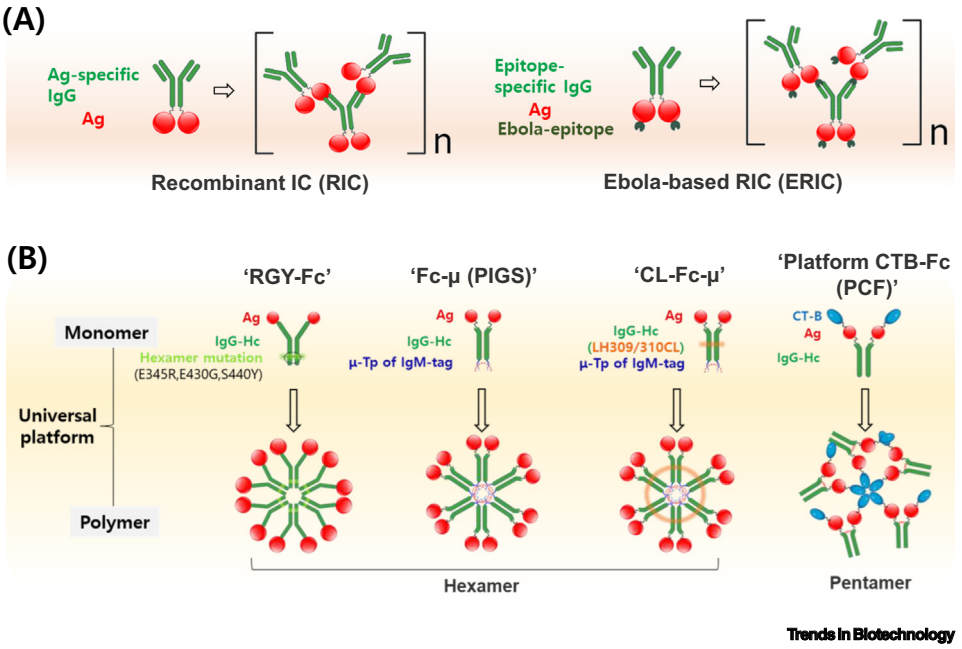

Figure 2. (A) RIC and ERIC require antibody binding domains (including light chain) for multimerization and self-assembly through antigen (Ag)-antibody (Ab) binding. The left panel represents classical RIC [15–17], and the right panel represents the universal version of RIC, which incorporates an anti-Ebola monoclonal antibody (mAb), the antigen of interest and an Ebola epitope tagged at the C-terminus of the construct (ERIC [18,19]). (B) Universal and size-predefined polymeric IC platforms that do not require antibody binding function (no light chain) but are based on the IgG heavy chain (HC) polymeric scaffold (PIGS, first three panels), or subunit B of the cholera toxin (CTB) (PCF, right panel). Multimerization of PIGS can be achieved by either a three–amino acid mutation in the Fc region of IgG ('RGY-Fc,' first panel [20]) or insertion of an IgM tail piece (Fc-m, second panel [29,30,32]) or insertion of an IgM tail piece and replacement of lysine to cysteine in CH2 domain ('C-Fc-μ,' third panel [28]). Multimerization of PCF occurs through CTB.

from an antibody 'epitope masking' effect. Although this effect could impact the B but not T cell responses, it could potentially be a serious limitation if the chosen epitope is critical to protection against infection. Furthermore, a specific antigen and mAb would be required for every vaccine candidate. To overcome the latter, Kim and colleagues [18] and Diamos and colleagues [19,20] modified RICs by inclusion of the Ebola 6D8 epitope to the Ab HC-Ag fusion polypeptide, so that the molecule could still assemble into RICs (because the antibody is specific for the 6D8 epitope) but now carries an additional antigen of choice. This universal platform, termed 'ERIC' (Figure 2) was successfully applied to generate vaccine candidates for dengue [18], human papillomavirus [19], and Zika virus [20]. In each case, high Ag-specific IgG titers could be induced without exogenous adjuvants, thus making RIC and ERIC effectively self-adjuvanting vaccines [21].

Although promising in terms of their immunogenic potential, RIC/ERIC technology still had the shortcoming that molecules could assemble freely into multimeric complexes of various sizes that cannot be easily controlled. The major factors driving the self-polymerization process are

the affinity of Ab-Ag binding, protein concentration, and incubation time, with all impacting the size of the formed RIC. Thus, Diamos and colleagues [22] showed that reducing the antibody affinity within ERIC by shortening the epitope tag resulted in more soluble and smaller complexes while retaining C1q binding and immunogenicity. The difficulty in controlling the size of RICs (a prerequisite for GMP production) is that if either concentration or the incubation time is not optimal, the RIC might not form or may grow too large and eventually precipitate, as can happen with natural ICs. Therefore, although these RIC strategies are an important proof of concept, they still required significant improvements to achieve consistency of characteristics and production to make ICs translatable into vaccine candidates for humans.

Polyimmunoglobulin scaffold (PIGS)-based RICs

The capacity for IgG to assemble into multimeric forms that enable multivalent attachment to antigen has also been explored. For example, anti–HIV-1 IgG-2G12 formed a dimer/trimer through intramolecular domain exchange, which increased neutralization potency compared with monomeric 2G12 [23]. Another example is the anti–HIV-1 gp120 antibody IgG1-b12 (1HZH) clustering into hexamers through specific noncovalent Fc interactions [24]. Furthermore, Diebolder and colleagues [25] reported that hexamers of anti–HIV-1 IgGs formed through a triple mutation (IgG1-005-RGYor IgG1-7D8-RGY: E345R, E430G, S440Y) were readily assembled in the HEK293 cells, which was demonstrated by a range of size measurement methods. Taking advantage of the latter approach, Diamos and colleagues [22] modified the anti-Ebola 6D8 IgG HC by introducing a triple mutation (E345R, E430G, S440Y) and fused it to Zika EDIII antigen at the N-terminal side of IgG HC (ZHx). Soluble extracts of ZHx contained both low- and high-density material, suggesting that the combination of light chain removal and RGY mutations contributes to the formation of larger aggregates, resulting in low expression yield in plants (<0.1 mg/kg fresh leaf weight) compared with other RIC-based Zika EDIII fusion multimers. Even so, ZHx induced potent neutralizing antibodies against the Zika virus in mice.

Apart from IgG, other antibody isotypes can also form ICs, and the hexameric IgM is particularly effective because of its multimeric structure. However, unlike the polyclonal nature of classical IgG ICs, IgM forms monoclonal ICs, a property that lends itself well to recombinant technology. This property of IgM was first explored for generation of therapeutic IgG antibodies with increased avidity more than three decades ago by Smith and Morisson [26,27] and more recently as an Fc-fusion protein vaccine strategy by Mekhaiel and colleagues [28] in the context of malaria (see also review by Czajkowsky and colleagues [11]). This approach relies on insertion of the 18–amino acid IgM C-terminal ‘tail piece’ sequence to the C-terminus of IgG HC, which also includes insertion of Cys414 of hIgM to allow crosslinking of monomers, enabling it to form hexamers much like IgM does (Figure 2). Furthermore, if coexpressed with the J chain, fusion protein forms pentamers, as indeed does pentameric IgM. The advantage of this approach over RIC/ERIC is that it dispenses altogether with the antibody light chain and the antibody binding domains, because polymerization is achieved through the C-terminally linked IgM tail piece, with the antigen being fused to the N-terminus of the IgG HC. The resulting vaccine platform thus not only is universal (PIGS) but also has well-defined size, making it more amenable than the RIC/ERIC for consistent and reproducible production.

There are several examples of application of PIGS-based vaccine constructs. Although in the original Mekhaiel study [28] the polymeric constructs carrying a malaria antigen appeared less immunogenic than the monomeric fusion protein in mice, in our own studies, they were superior to monomers when applied in the context of a dengue antigen, based on either mouse [29] or human IgG [30], with the latter construct being tested in human FcγR1 transgenic mice [31]. The induced antibodies displayed strong neutralizing potential against the four serotypes of the

dengue virus [30]. Similarly, the PIGS vaccine platform was also applied for *Mycobacterium tuberculosis* [32] and porcine epidemic diarrhea virus (PEDV) infection. In the latter study, PEDV-PIGS were administered subcutaneously or orally to mice, but only oral administration led to mucosal Ag-specific immune responses [33]. Therefore, the immunogenicity of antigens within the PIGS constructs may in part at least be dependent on their structural/conformational requirements.

Platform CTB-Fc (PCF)-based RIC

A further iteration of an RIC is represented by the 'PCF' vaccine platform, or platform CTB-Fc. The advantage of this vaccine platform over PIGS is that it also includes the nontoxic subunit B (CTB) of the cholera toxin, a potent mucosal molecular adjuvant, a modification brought about to suit mucosal as well as systemic immunization. Numerous studies have demonstrated that targeting Ag to FcRs enhances both humoral and cellular immune responses systemically (reviewed in [21]), but little is known about whether this same strategy can be used to enhance protection against mucosal pathogens. Most recently, we demonstrated that the inclusion of CTB to ICs markedly enhanced mucosal immune responses in mice when compared with PIGS. Thus, using a dengue antigen, we demonstrated that both mucosal and systemic responses were enhanced following intranasal boosting of systemically primed mice compared with systemic immunization alone or systemic prime intranasal boost with PIGS incorporating the same dengue antigen [34]. The mucosal response was characterized by high titers of both IgA and IgG in the bronchoalveolar lavage, which could not be induced by PIGS. This clearly demonstrated the value of CTB as a potent mucosal adjuvant, but, considering that all the three vaccine components (CTB, Fc, and antigen) were expressed within a single polypeptide, this vaccine platform has a significant advantage over conventional protein subunit vaccines because it does not require exogenous or chemical adjuvants to induce robust immunity and can be therefore considered fully self-adjuvanting.

An essential feature of PCF is that unlike other forms of Fc-fusion protein–based ICs that polymerize through Fc, it does so through pentamerization of CTB [35], giving the IC a defined size and delivering five copies of antigen to APCs with each polypeptide, which further increases due to the dimerization of IgG-Fc and immune complex formation (Figure 2). CTB can engage gangliosides on mucosal epithelial cells, but without the prohibitive inflammatory response of the holotoxin A (CTA) and the risk of binding to olfactory sensory neurons located in the nasal epithelium. The main safety concern of full CT or indeed all AB_5 enterotoxins in mucosal vaccines is that they can potentially induce adverse effects, as observed in a clinical trial of a nasal HIV vaccine formulated with the heat-labile *Escherichia coli* toxin, LT-I (Adjuvant LTK63) [36]. This risk is greatly minimized by removal of the A subunit (enzyme ADP-ribosyl transferase) while retaining the immunomodulatory activity of the B subunit. Although CTB is used in the oral Dukoral cholera vaccine (SBL Vaccines), no human studies have been reported for aerosolized delivery of vaccines containing CTB, although a very recent study using a SARS-CoV-2 antigen complexed with CTB showed no side effects in nonhuman primates following aerosolized delivery [37]. Furthermore, our preferred route of aerosolized delivery is through the mouth rather than the nose, minimizing further any risk of binding to olfactory neurons. Thus, using a mash nebulizer, we demonstrated the PCF can be aerosolized without a loss of protein or biological activity of its components [34], making it suitable for aerosolized mucosal delivery through a mouthpiece. However, the construct could also be used as an injectable vaccine, because CTB is well known to induce robust systemic immunity when coadministered with antigens [38], or indeed by a combination of systemic prime and mucosal boost, as demonstrated in our study [34]. Furthermore, there is also a scope to generate PCF vaccine constructs in the form of mRNA, which could further simplify production and reduce cost. mRNA vaccine technology achieved tremendous success during the COVID-19 pandemic and is likely to play a major role in future outbreaks.

Table 1. Pathogenic targets and expression systems for recombinant IC vaccines

Diseases	Type of Ig	Antigen	IC form	Expression	Immunization	Result	Refs
Tetanus	mIgG2a HC + κ-C	47 kDa tetanus toxin fragment C (TTFC)	IgG-TTFC: Ag fused to C-terminus of α-tetanus IgG (278-02 mAb) HC	Transgenic plants (*N. tabacum*)	s.c. without adjuvant	IgG-TTFC RIC elicited Ab titers that were more than 10 000 times higher than those observed in animals immunized with the antigen alone. Animals fully protected against lethal challenge.	[15]
Ebola (EBOV)	hIgG1 HC + λ-C	GP1	ERIC: Ag fused to C-terminus of α-Ebola IgG (6D8 Mab)	Transient plants (*N. benthamiana*)	s.c. with adjuvant	Codelivery of the ERIC with Toll-like receptor agonists (PIC) elicited higher Ab response and protected against a lethal challenge by Zaire EBOV in mice.	[17]
HSV	hIgG1 HC + k-C	gD	ERIC: gD fused to C-terminus of α-Ebola IgG (6D8 mAb) by adding a self-binding epitope	Transient plants (*N. benthamiana*)	s.c. without adjuvant	ERIC produced high-titer anti-gD Ab responses.	[85]
TB (MTB)	mIgG1	Acr-Ag85B fusion	TB-ICM: Ab:Ag mixture at 1:20 molar ratio	*E. coli* for Ag fusion protein and hybridoma cell line for Ab	s.c. with/without adjuvant	TB-ICM induced a strong Th1/Th2 mixed type antibody response, which was comparable to cholera toxin adjuvanted antigen.	[70]
TB (MTB)	mIgG1 HC + k-C	Acr-Ag85B	TB-RICs: Ag fused to C-terminus of α-Acr IgG (TBG65 mAb)	Transgenic plants (*N. tabacum*)	i.n. boosting of BCG without adjuvant	The mucosal TB-RICs induced both anti-Acr and anti-Ag85B Ab and cellular responses and resulted in reduced *M. tuberculosis* infection in their lungs.	[16]
Dengue (DV)	mIgG2a HC + k-C	cEDIII	D-ERIC: Ag fused to C-terminus of α-Ebola IgG (6D8 Mab) adding a self-binding epitope	Transient plants (*N. benthamiana*)	s.c. without adjuvant	The sera of animals immunized with D-ERIC showed high specific IgG titers and induced a potent, virus-neutralizing anti-cEDIII humoral immune response without exogenous adjuvants.	[18]
Influenza virus	hIgG1	Hemagglutinin (HA)	Trimeric IC: HA complex with mAb (PY102)	HA proteins in a baculovirus system and anti-HA IgG in 293T cells	i.v.	Immunization with sialylated Fc-HA ICs elicited protective, high-affinity IgGs against the conserved stalk of the HA.	[63]
HPV	hIgG1 HC + κ-C	14–122 aa of HPV16 L2	HPV-ERIC: Ag fused to C-terminus of α-Ebola IgG (6D8 mAb) by adding a self-binding epitope	Transient plants (*N. benthamiana*)	s.c. without adjuvant	Codelivery of VLP and RIC together resulted in a strong synergistic enhancement of anti-L2 antibody (representing a 700-fold increase over immunization with Ag) after two doses and a 2-fold increase over the RIC-alone group after third dose.	[19]
Zika (ZIKV)	hIgG1 HC + κ-C	E protein domain 3 of Zika virus (ZE3)	Zika-ERIC: Ag fused to C-terminus of α-Ebola IgG (6D8 Mab) by adding a self-binding epitope	Transient plants (*N. benthamiana*)	s.c. without adjuvant	Up to 150-fold higher Ab titers than ZE3 alone; neutralized ZIKV	[20]
Malaria (*Pf*)	hIgG1 HC or mIgG2a HC	19 and 42 kDa subunit of MSP1	Monomeric *Pf*MSP1$_{19}$-hIgG1-Fc-LH309/310CL and polymeric *Pf*MSP1$_{19}$-hIgG1-Fc-LH309/310CL-TP	CHO-K1 or HEK cell	s.c. with/without adjuvant	The monomeric Fc fusions induced Ag-specific IgG1 responses, but the Ab titer was not improved with additional adjuvant, and this effect was maintained even after challenge with parasites. Significantly lower *Pf*MSP1$_{19}$-specific and a complete absence of anti-hIgG1-Fc responses observed from the hexamer. No improvement in Ag-specific Ab titers, or resolution of malaria, were seen when	[28]

(continued on next page)

Table 1. (continued)

Diseases	Type of Ig	Antigen	IC form	Expression	Immunization	Result	Refs
						immunizations with hexamer were repeated in the presence of alum.	
Dengue (DV)	Truncated HC of hIgG1 or mIgG2a	cEDIII	D-PIGS: Ag fused to N-terminus of an extended Fc to form multimer of Y-shape	Transient plants (*N. benthamiana*) and transgenic CHO	s.c. with/without adjuvant	D-PIGS induced Ag-specific neutralizing antibodies and cell-mediated immune responses in both wild-type mice and CD64 transgenic mice. D-PIGS were able to prime immune cells from human adenoid/tonsillar tissue *ex vivo* as evidenced by antigen-specific $CD4^+$ and $CD8^+$ T-cell proliferation, IFN-γ and antibody production.	[29,30]
TB (MTB)	Truncated HC of hIgG1 or mIgG2a	Ag85B	TB-PIGS: Ag fused to N-terminus of an extended Fc to form multimer of Y-shape	Transient plants (*N. benthamiana*) and transgenic CHO	s.c. or i.n.	TB-PIGS were highly immunogenic with and without adjuvant, but no protection against *M. tuberculosis* challenge.	[32]
Zika (ZIKV)	hIgG1 Fc/HC	ED3	ZED3 fused to hFc	Transient plants (*N. benthamiana*)	s.c. without adjuvant	Zika-specific antibodies elicited with high titers and neutralization Ab titers reached up to 150-fold higher than Ag alone after two doses without adjuvant.	[22]
PED (PEDV)	Truncated HC of hIgG1 or mIgG2a	COE	PEDV-PIGS: Ag fused to N-terminus of an extended Fc to form multimer of Y-shape	Transient plants (*N. benthamiana*)	s.c. and oral administration with/without adjuvant	PEDV-PIGS induced both systemic and mucosal antigen-specific immune responses when combining routes.	[33]
Dengue (DV)	Truncated HC of hIgG1 or mIgG2a	cEDIII	D-PCF: Dengue Ag fused between Platform CTB and Fc (PCF)	Transient plants (*N. benthamiana*)	s.c. prime i.n. boost regimen without adjuvant	D-PCF, vaccination by a combination of systemic and mucosal routes, raised robust systemic and mucosal immune responses, as well as systemic T cell responses, significantly higher than those induced by a related Fc fusion protein but without CTB.	[34]

Abbreviations: cEDIII, consensus EDIII from serotype 1–4; DV, Dengue virus; EBOV, Ebola virus; hIgG, human IgG; HPV, human papillomavirus; HSV, herpes simplex virus; i.n. intranasal; i.v., intravenous; mIgG, murine IgG; MSP1, merozoite surface protein 1; MTB, *Mycobacterium tuberculosis*; PEDV, porcine epidemic diarrhea virus; *Pf*, *Plasmodium falciparum*; s.c., subcutaneous; TB, tuberculosis; ZIKV, Zika virus.

Expression systems for RICs

RICs are complex molecules that require appropriate expression systems for assembly. Because of the presence of an IgG-Fc component that requires dimerization via a disulfide linkage, the *E. coli* expression system is not suitable or is inefficient at best. Higher expression systems are required, and mammalian cell culture is ordinarily used because of its widespread use for production of protein-based therapeutics and vaccines. Indeed, many Fc-fusion therapeutics are made in mammalian cells, finding applications in a range of human conditions, including autoimmune diseases [39] and particularly cancer [40], whereas progress in bringing ICs as vaccines to the clinic has been comparatively much slower [41–43]. This is partly because to be immunogenic, the Fc-fusion proteins in vaccines ideally need to be in the form of ICs rather than just monomeric Fc fused to a partner antigen. Assembly of RICs requires an expression system that is capable of correct folding, structural assembly, and post-translational modifications (including glycosylation) to allow secretion (extracellular) or accumulation in cellular organelles (i.e., endoplasmic reticulum). The advantage of mammalian expression systems over *E. coli* is that they possess the required machinery for expression, assembly, and secretion of such complex structures. However, this comes at the cost of an expensive technical setup for production, sometimes

nonhomogeneous product due to multiple glycoforms, and the risk of contamination with human and animal pathogens associated with the use of animal products. Several RIC vaccine candidates were produced in this expression system (some listed in Table 1).

An alternative expression system is plants, which, similar to mammalian cells, can express and correctly assemble complex protein structures such as antibodies [44], including the even more complex secreted variants [45,46]. Thus, we and others have used *Nicotiana tabacum* and *Nicotiana benthamiana* plants to express several iterations of RICs as vaccines, including RIC [15–17], ERIC [18], PIGS [29,30,32], and PCF [34] (Table 1). The advantages of plant expression systems include relatively inexpensive technical setup (greenhouses), ease of scalability, and the absence of animal or human pathogens. Thus, we and others have used this expression platform for expression of several therapeutic proteins and vaccines against a range of human conditions, including infectious diseases and cancer (recently reviewed in [47]). One post-translational modification is particularly important when using plants as the expression system, which is glycosylation. Plant glycans differ from those present in mammalian proteins, and there is a concern of potential immunogenicity when applied to humans, although little evidence of concern has been raised from preclinical studies. Thus, Chargelegue and colleagues [48] showed that a murine mAb produced in transgenic plants with plant-specific glycans was not immunogenic in mice. However, to minimize any such risks, plants can be genetically engineered to produce glycans that are more like those in mammalian proteins. This includes removal of fucose and xylose residues [49] and retention of expressed proteins within the endoplasmic reticulum, which ensures only homogeneously mannosylated protein product, but without the complex glycan structures found in mammalian cells [50]. Although a relatively young biotechnology, plant production systems have made major strides in the last two decades, and this technology is now emerging as a viable and cheaper alternative to the conventional animal cell bioreactors for large-scale production of therapeutic proteins. Some of the recent demonstrations of this progress are represented by the successful application of a cocktail of three plant-expressed antibodies for treatment of Ebola infection in the early stages of the 2013 Ebola outbreak in Africa and subsequent demonstration of the efficacy in clinical trials [51], most recently by the licensure of a plant-expressed COVID-19 vaccine by the Medicago company [52]. Although neither has led to a sold commercial product (for reasons that are beyond the scope of this review), these successes underline the huge but untapped potential of this protein manufacturing platform. We believe that plant expression systems could play a major role in the future as a low-tech, low-cost production platform for vaccines in low- and middle-income countries, including the RIC described in this review.

Conclusions and future directions

Classical ICs possess significant immunogenic potential, but their use as vaccines has been restricted by several impractical features that do not lend themselves well to vaccine design and manufacture. Only with the availability of advanced molecular engineering techniques over the past decade or so has it been possible to produce ICs in recombinant form, a prerequisite for GMP production. Remarkably, **plant biotechnology** was at the cutting edge of advancing the field of RIC production and proof-of-concept testing. A variety of engineering techniques have been used to address the issues of IC size, structure display, and monoclonality, as well as universal applicability. Some of the key advantages of RICs as immunogens are that they can target APCs directly, can activate complement to further amplify the response, can deliver multiple copies of antigen to APCs, present multiple vaccine components within a single molecular platform, protect the antigen from proteolytic degradation (especially at mucosal sites), and, importantly, they can induce both humoral and cellular (including cross-presentation to CD8 T cells) immune responses. However, despite these advantages and the significant progress

Outstanding questions

How critical will be the homogeneity of recombinant ICs as a manufactured vaccine product? Although conventional proteins (antigens) are relatively easily produced in homogeneous form, recombinant ICs are complex structures that require multimerization, a process that may yield less homogeneous product that will likely require additional purification steps, reduce yields, and add to the cost of production. Can we improve the control of IC multimerization and IC homogeneity by further genetic manipulations?

How important is the choice of antibody and/or isotype when generating recombinant IC? This may be relevant not only for affinity of binding to Fc receptors on APCs but also for proteolytic stability, half-life, and bioavailability of the vaccine within tissues, and further comparative studies should be undertaken to address these issues.

Should recombinant IC-based vaccines predominantly target viral infections because the protective antigens and correlates of protection against them are better defined than for the more complex organisms? Recombinant ICs may not be the most suitable vaccine platform for screening of many antigens, because each would require its own genetic construct, whereas conventional protein plus adjuvant strategies can do that much more easily. Choice of the initial target disease using recombinant IC vaccine technology is therefore likely to be important for the success of this vaccine approach.

Although absence of exogenous adjuvants in recombinant IC vaccines is an attractive prospect, it remains to be fully determined if the induced immune responses are as robust and protective as in adjuvanted vaccines. Will there be an acceptance for a 'middle ground' where, for example, in some instances, adjuvant still may be required but at a lower (and therefore less toxic) dose?

Given that plant biotechnology has made the most advances in recombinant IC engineering, has this protein production system reached the required level of maturity to compete with the conventional mammalian expression systems for manufacture of such biologics? Are

made, much further optimization is required (see Outstanding questions). ICs can be both activating and inhibitory, and this is likely influenced not only by their overall size and the class of antibody used but also by the type of APCs they encounter and the relative density of the FcγRs on them. These parameters are more challenging to control and are primarily dependent on the route of immunization. Furthermore, the presence of glycans on the Fc antibody domain and their type (depending on whether expressed in plants or mammalian cells) can further influence the nature of the interaction of ICs with cells *in vivo*. Both the Fc component and the antigen itself may be glycosylated, and this means that ICs could bind to a variety of lectins present on the APCs, which could compete with FcγR binding, with unknown consequences. Therefore, further work is required to fully harness the potential of RIC technology, including glycouniformity, better stability and half-life *in vivo*, enhanced immunogenicity, and fully defined size and structure, as well as the scalability of production. Nevertheless, the studies described in the preceding text have demonstrated that this is an attainable target and that the incremental successes achieved through repeated iterations of RICs could eventually yield a vaccine product that could be used in humans for prevention of infectious diseases. In that regard, we believe that RIC-based vaccines would be particularly suitable for mucosal applications, because this is a more challenging route of immunization for protein vaccines than the systemic routes due to the tolerogenic immune mechanisms at the mucosa and higher stringency safety requirements, especially for the respiratory route, due to proximity of the brain (nasal spray) or pulmonary discomfort (inhalation). The mucosa, including the respiratory tract, covers a large surface area and continuously interacts with the environment (exposome) and nonpathogenic microbiome. This high antigenic load and constant exposure to microbes necessitates mucosal immunoregulatory mechanisms that are vital to maintain homeostasis and prevent damaging chronic inflammatory responses [53]. This has significant implications for vaccine development; for example, many Toll-like receptor agonists that are effective adjuvants when injected have poor efficacy when administered mucosally and require complex formulations with other compounds [54]. Consequently, only vaccines based on killed or attenuated whole organisms have been successful, whereas no protein-based adjuvanted vaccine for mucosal application has ever reached licensure [55]. Being protein-only and self-adjuvanting vaccines by virtue of their specific targeting of the FcγRs on APCs, the RIC could circumvent the adjuvant issue altogether and, in that way, minimize safety concerns as well as streamline the protocols for formulation, storage, and clinical testing.

The important next step for RIC vaccine technology will be generating the necessary clinical profile documentation to meet the regulatory requirements. The vaccine product will require stringent manufacturing quality measures that include sourcing and quality of raw materials, absence of contaminating agents, GMP compliance, product homogeneity, consistency of batches, in-process controls and specifications, and potency testing of the final lyophilized formulation. In this context, it is important to bear in mind that in contrast to chemically defined medicines, RICs are highly complex biological products that cannot be sufficiently characterized solely on the basis of physicochemical properties, and additional functional assays will need to be performed for each fusion polypeptide component during the manufacturing process and storage. As with any other new vaccine technology, the first application of RICs will require extensive quality control and clinical profiling, but the key characteristics and manufacturing experience will subsequently be useful for other IC vaccines to accelerate their clinical development and approval. The clinical development pathway for an RIC vaccine will depend on its indication and target population, and thus clinical evaluation, trial design, and endpoints will be specifically tailored to the product. In general, the clinical immunogenicity, safety, and effectiveness will need to be evaluated in phases I–III clinical trials, and the necessary documentation will need to be generated regarding the vaccine's effectiveness and safety, before licensure. This will require concerted and sustained effort over the next 5 or so years to progress the RIC vaccine technology to application in humans.

regulatory approvals of plant-based biologics likely to be more difficult than for the other production systems, and what can be done to expedite them?

How can clinical development of recombinant IC be accelerated? Despite reaching a high level of maturity, the technology is yet to enter the clinical phase of development and testing, and it would be important to identify and overcome the barriers to progress, whether they be related to manufacturing, regulatory issues, or insufficient preclinical data, to attract industrial sector involvement.

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Declaration of interests

R.R. and M.-Y.K. are coinventors on patent application PCT/GB2022/052418 on PCF vaccine technology. The authors of this review article declare no other conflicts of interest.

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