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HHS Public Access
Author manuscript
Curr Opin Virol. Author manuscript; available in PMC 2023 February 01.

Published in final edited form as:
Curr Opin Virol. 2022 February ; 52: 148–160. doi:10.1016/j.coviro.2021.12.005.

Development of plant-made monoclonal antibodies against viral infections

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Abstract

Current plant-based systems offer multiple advantages for monoclonal antibody (mAb) development and production beyond the traditional benefits of low cost and high scalability. Novel expression vectors have allowed the production of mAbs at high levels with unprecedented speed to combat current and future pandemics. Host glycoengineering has enabled plants to produce mAbs that have unique mammalian glycoforms with a high degree of homogeneity. These mAb glycovariants exhibit differential binding to various Fc receptors, providing a new way to optimize antibody effector function for improving mAb potency or safety. This review will summarize the status of anti-viral mAb development with plant-based systems. The preclinical and clinical development of leading plant-made mAb candidates will be highlighted. In addition, the remaining challenges and potential applications of this technology will be discussed.

Keywords

plant-made antibodies; monoclonal antibody; mAbs; virus; viral diseases; plantmade biologic

Introduction

Monoclonal antibodies (mAb) are the predominant growing class of biopharmaceutical products in recent years. Since the first approval of Muromonab-CD3 in 1985 [1], more than one hundred mAb-based drugs have been approved by the US food and drug administration (FDA) as of March 2021 [2]. Largely due to their high specificity, mAb-based drugs have revolutionized the pharmaceutical industry and achieved great financial success, creating a global market valued at greater than $100 billion for cancer therapy alone [3].

Recently, the FDA has issued emergency use authorization (EUA) of several mAbs for severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) treatment, highlighting the

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significance of antibody drugs in fighting infectious diseases [4]. Currently, there have been four mAbs approved by the FDA for the prevention or treatment of virus-caused infectious diseases [2]. In 1998, palivizumab, the first mAb against infectious disease, was licensed for the prevention of respiratory syncytial virus (RSV) infection, the most common cause for severe bronchiolitis in young children [5]. Palivizumab prevents RSV entry into host cells through specific binding to the RSV envelop fusion protein and inhibiting membrane fusion [6]. In 2018, Ibalizumab, a humanized immunoglobulin G4, was approved for clinical management of human immunodeficiency virus (HIV)-1 infection with multidrug resistance [7]. As a CD4-directed post-attachment inhibitor, this mAb binds to CD4 T cells and blocks the conformation changes required for HIV-1 entry [7]. In 2020, two antibody drugs, Inmazeb and Ebanga, were licensed for the treatment of Ebola virus (EBOV) infections. Inmazeb is a combination of three mAbs: atoltivimab, maftivimab, and odesivimab; all three of which can bind to the EBOV glycoprotein simultaneously to prevent virus entry [8]. Ebanga contains one mAb called ansuvimab, which also binds the EBOV glycoprotein and blocks its interaction with the host cell receptor [9]. In addition to blocking virus attachment or membrane fusion to prevent virus entry, increasing evidence shows that antibodies can provide significant therapeutic effects against viral infections through fragment crystallizable region (Fc) mediated effector functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP) [10–12]. For example, two of the mAbs in the EBOV drug, Inmazeb, and ansuvimab, can induce ADCC in addition to blocking virus entry [13, 14], indicating the importance of effector functions in the treatment of EBOV infection.

Almost all approved mAb drugs are produced in mammalian cell culture, which requires high-tech facilities, sophisticated bioreactors, expensive downstream processing, cold storage and transportation, and sterile delivery methods [15–18]. As a result, mAb drugs produced by current technology platforms are prohibitively expensive, for example, with an average annual price of $142,844 for cancer treatment [19], making them unaffordable for the majority of ordinary citizens in the world [20]. The high cost of current mAb drugs calls for the development of alternative production systems based on non-mammalian cells.

Plants as production host for mAbs

Antibody production requires the expression of at least two types of polypeptides and their proper assembly into a multimeric structure, as well as complex-type glycan modifications. Despite this complexity, plants have been shown to have the ability to produce mAbs since the first mAb made in tobacco in 1989 [21]. Since then, numerous mAbs and their structural variants including IgGs, secretory IgAs, pentameric IgMs, camelid nanobodies, tetravalent mAbs, bifunctional mAbs, recombinant immune-complex (RIC), single-domain fragments, single-chain variable fragments (scFv), and diabodies have been produced, several of which have entered human clinical trials [17, 22–24].

Low cost, high scalability, and low risk of human pathogen contamination are the hallmarks of plant-based systems for producing mAbs [15, 25, 26]. Unlike mammalian cell culture systems, plant biomass can be generated in greenhouses with simple mineral solutions. This

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eliminates the need for capital-prohibitive bioreactors and expensive culture media, resulting in significant cost savings associated with upstream processing of mAb production [17, 27], Production of mAbs in mammalian cells carries the inherent risk of contaminating drugs with animal pathogens, especially those that are unknown or uncharacterized. As plants rarely carry pathogens that are infectious to humans, such risk is greatly reduced in plant-produced mAbs. The ease of producing multiple hetero-subunit proteins and the unique characteristics of the cell wall provides plant cells with another advantage for mAb production and potentially a new route of drug delivery. Functional and protease-resistant secretory IgAs and pentameric IgMs which require up to four hetero-subunits have been produced successfully in plants [22, 28]. Since these mAbs are encapsulated inside the plant cell wall, they are protected from acids and enzymes in the stomach, allowing them to enter the gut lumen where they are enzymatically released by gut commensal bacteria [29]. This opens the possibility of combating gastrointestinal tract (GI) viral diseases via oral delivery of edible plant materials that encapsulate mAbs against GI viruses. Since many viruses use mucosal surfaces as sites of entry, plant-cell delivered mAbs can be used as both prophylactics to block viral entry, and therapeutics to treat viral infection. Prophylactic applications of mAbs to preventive viral infections would enroll larger populations with repeated dosing, thereby, demand large amounts of mAbs. In this case, plant-based production platforms become more attractive due to their ease of scale-up production without demanding prohibitive capital investment or the time-consuming development of new production processes [17].

Technological advances in transient expression and vector engineering offer plant-based systems the robustness, speed, and versatility that are unmatchable by mammalian cell cultures for mAb production. The development of “deconstructed” viral vector systems such as magnICON, geminiviral, and pEAQ vectors has allowed for a high and consistent levels of mAb expression in short time [30–34]. For example, mAbs can be produced within 1-2 weeks with magnICON-based transient systems at levels of up to 5 mg mAb per gram of leaf fresh weight (LFW) [35]. Since the deconstructed vectors are delivered into plants via *Agrobacterium tumefaciens*, a bacterium that naturally transfers a portion of its plasmid into plant cells [36–38], the transient systems retain the flexibility of nuclear gene expression while increasing production speed and yield. As a result, this type of transient expression is the system of choice for obtaining gram levels of mAbs for pre-clinical studies. As mAb-based drug discovery involves an iterative process of successive modifications and functional evaluation, the speedy nature of the plant transient system in producing gram-levels of mAb variants will greatly accelerate this process. In addition, the “bridge” versions of deconstructed viral vectors have also been developed, so that scale-up production of mAbs can be done in stable transgenic plants with the same speed and high yield as in transient expression [39, 40]. Therefore, current plant expression platforms based on deconstructed viral vectors offer advantages in all stages of mAb-drug development, from early candidate screening to the final stage of large-scale manufacturing.

Glycoengineering of plant hosts provides another advantage over the mammalian cell-based system for mAb development. The mAbs are N-linked glycosylated and their sugar moieties have a significant impact on their folding, serum and shelf half-life, and functionality [41]. Thus, one approach to enhance the potency, safety, and stability of mAbs is to alter

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protein-associated sugars to achieve the desirable properties. The challenge is to develop biological systems that can consistently produce mAbs with specific and homogeneous glycans on demand. In mammalian cells, there is a large diverse population of Golgi-located glycoenzymes, giving rise to more than 2000 different *N*-glycans generated by a few hundred enzymes in the secretory pathway [42]. For example, Chinese hamster ovary (CHO) cell-derived mAbs exhibit substantial glycan heterogeneity, precluding the ability to generate distinct glycoforms that could be used to enhance their efficacy [43]. Glycoengineering of CHO cells has only achieved modest success, especially in producing defined N-glycoforms with high degrees of homogeneity. In contrast, plant cells have a much smaller repertoire of glycoenzymes, and plant glycoproteins usually bear a single dominant N-glycan structure [41]. The inherent lack of N-glycan heterogeneity of plant-produced glycoproteins has turned out to be an advantage for plant cells as a host for generating mAbs with homogeneous glycans [43]. Glycoengineering of host plants by deleting or suppressing the expression of plant-specific glycan genes or/and inserting mammalian glycosylation genes has achieved remarkable success [44]. Studies have shown that mAbs produced in glycoengineered plants do not carry any plant-specific glycans, eliminating the concern for immunogenicity and the potential risk of adverse effects for plant-produced mAbs [45, 46]. Furthermore, mAbs made in glycoengineered plants usually carry a homogenous (>90%) N-glycan structure compared to the mixture of multiple glycans exhibited by the same mAbs produced in CHO cells [47–50]. A portfolio of plant lines has since been developed, each producing mAbs with a unique mammalian N-glycoform [44] (Fig. 1). The availability of such glycoengineered plant lines provides the opportunity to develop mAbs with defined and uniform carbohydrate moieties to enhance their efficacy and/or safety.

Leading candidates for plant-derived mAbs

Multiple mAbs and mAb derivatives against viral pathogens have been developed in various plant species. The initial studies aimed to examine mAb expression with different plant-expression platforms and to test their equivalency in physiochemical structure and both *in vitro* and *in vivo* functionality with their mammalian cell-produced counterparts. The success of glycoengineering in plants has allowed the paradigm shift of using plants to develop “biobetters” to improve the efficacy and/or safety of mAb-based drugs. These plant-made mAbs are in various stages of development ranging from research and development (R&D), preclinical testing, compassionate use in human patients, to Phase I human clinical trials (Table 1). Representative plant-made mAbs will be discussed in the following sections based on their developmental stages and disease targets.

MAbs against Ebola virus—The Ebola outbreaks of 2014 attracted the attention of the public to plant-produced mAbs. Two critically ill American Ebola patients were given an experimental drug called ZMapp and quickly recovered [51]. ZMapp contains three chimeric mAbs produced in *N. benthamiana* plants. These mAbs can be produced quickly [52–54], and are highly efficacious in protecting mice and macaques against Ebola infection [55, 56]. Interestingly, a recombinant immune-complex vaccine based on these mAbs also protected mice from a lethal Ebola challenge [57, 58]. The potency of plant-derived mAbs is superior to that of their mammalian-produced counterparts, likely due to their homogenous GnGn mammalian glycan profile, which enhanced their binding to the Fc gamma receptor (FcγR)

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IIIA and possibly ADCC activity [55, 56]. ZMapp was used as an experimental drug in seven human patients during the 2014 Ebola outbreak. A subsequent study demonstrated that 100% of rhesus macaques can be successfully rescued by ZMapp even 5 days post a lethal Ebola challenge [59]. A phase I clinical trial was conducted in 2015 to assess the safety and efficacy of ZMapp. Although the trial did not reach its enrollment goal of 200 people due to the outbreak waning, 72 Ebola-infected subjects were enrolled, and, consequently, the results are not statistically significant. Nevertheless, ZMapp was found to be safe and well-tolerated, and patients who received ZMapp had a 40% lower risk of death [60]. During the 2018-19 Kivu Ebola outbreak in the Democratic Republic of the Congo, another randomized and controlled Phase I clinical trial was conducted for ZMapp along with three investigational agents. The results of this trial indicated that ZMapp lowered the mortality rate from ~ 70% to 49.7%, albeit the other candidates showed more superior efficacy [61]. Beyond the Ebola therapeutic target, the ZMapp clinical trials are also highly significant in several other aspects. It signifies the establishment of upstream and downstream processes for manufacturing plant-made mAbs that are compliant with current Good Manufacturing Practice (cGMP) regulations of the FDA. The clinical trial also helped drug approval agencies such as the FDA to navigate this new technology and pave a clearer regulatory pathway specific for plant-made mAbs. This should greatly streamline the approval of other plant-made mAbs.

MAbs against human immunodeficiency virus (HIV)—Despite the global efforts in curbing the HIV epidemic in the last few decades, thousands of newly infected cases are still reported daily in sub-Saharan Africa with 1.5 million new cases in 2020 [62]. Broadly neutralizing antibodies (bNAb) that can neutralize multiple HIV isolates are promising candidates for treating and preventing HIV infection. However, the implementation of bNAb treatment programs in resource-poor regions, where HIV is the most prevalent, is greatly challenged by the cost and scale-up capability associated with the CHO-based mAb production system. As a result, several bNAbs have been produced in various plant-based systems [63–67]. For example, 2G12 has been produced both in maize and in *N. benthamiana* [63, 64]. In general, the antigen specificity, affinity, and neutralization potency of plant-made 2G12 and other bNAbs are comparable to those of their mammalian cell-derived counterparts. However, bNAbs produced in glycoengineered plants with specific and homogeneous glycoforms appear to have enhanced FcγRIIIA-binding, neutralization, and/or ADCC activity [63, 67, 68]. In addition to glycoengineering, engineering of other posttranslational modification (PTM) pathways in plants also overcome the challenge of producing functional bNAbs that require specific mammalian-type PTM. For example, the expression of human tyrosyl protein sulfotransferase 1 in *N. benthamiana* allowed the proper tyrosine sulfation in the CDR H3 region of two bNAbs, preserving their broad neutralization activity against HIV [66, 69]. bNAbs and their fragments have also been fused to antiviral lectins to enhance their potency. For example, cyanovirin-N (CV-N) was fused to the C-terminus of bNAb b12 heavy chain (HC) and expressed in transgenic tobacco. Compared to b12 or cyanovirin alone, the fusion protein exhibited increased anti-HIV potency [70]. Similarly, a bispecific bNAb-lectin fusion protein composed of the antigen-binding fragment (Fab) of bNAb VRC01 and Avaren was expressed in *N. benthamiana.* The VRC01 Fab-Avaren fusion protein demonstrated more potent neutralizing activity compared

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to a cocktail of the two parent molecules [71]. Pharmacokinetics and preliminary safety of plant-produced HIV bNAbs have been investigated in nonhuman primate models [65, 72]. For example, VRC01 produced in *N. benthamiana* was formulated in the form of intravaginal rings and tested as a topical microbicide for preventing sexual HIV infection in a macaque model. The rings were found to sustain VRC01 levels in the range of 100 -1000 μg per gram of vaginal fluid for up to 21 days without causing any adverse safety indications [65]. Excitingly, a first-in-human, double-blind, placebo-controlled, randomized, dose-escalation phase I clinical trial was conducted for tobacco-produced 2G12 as a vaginal microbicide in a single dose up to 28mg in healthy female volunteers [73]. 2G12 was found to be safe as no major adverse events were identified during the trial and no specific anti-2G12 immunological changes were detected in serum or vaginal fluid after intravaginal administration of this mAb at any dosage [73]. Importantly, the clinical trial has allowed the identification of key regulatory issues specific to plant-made mAbs. Furthermore, cGMP-compliant procedures from seed bank establishment, to plant cultivation, to downstream processing have all been developed to address the key regulatory issues identified above [73]. The trial also helped the European regulatory agencies to establish specifications in identity, purity, and potency for plant-made mAbs acceptable for human applications [73]. Therefore, the successful completion of this clinical trial marks a significant milestone towards the eventual approval of the first plant-made mAb commercial product. Overall, the successful production and efficacy optimization of HIV bNAbs in plants supports the prospect of providing affordable mAb-based prophylactics and therapeutics to control HIV in resource-poor parts of the world.

MAbs against flaviviruses—The ease of producing mAbs that carry homogeneous and distinct mammalian glycans in glycoengineered plants offers opportunities to improve the efficacy and safety of mAb-based therapeutics against flaviviruses. West Nile virus (WNV) is a neurotropic virus that causes life-threatening WNV neuroinvasive diseases including encephalitis, meningitis, and acute flaccid paralysis [74, 75]. Our laboratory has developed a plant-derived mAb E16 that recognizes an epitope in domain III of WNV envelope protein [76]. A single dose of plant-made E16 protected mice from lethal infection of WNV, even given 4 days post-infection, when the virus has entered the brain [47–50]. Since most mAb therapeutics would have very limited efficacy in treating neuroinvasive diseases due to the incapability to cross the blood-brain barrier (BBB), we explored the possibility of using plant systems to produce more complexed antibody variants to facilitate their crossing of the BBB. Specifically, we designed a tetravalent E16 variant (Tetra E16) with two sets of Fabs [77]. Tetra E16 exhibits differential binding affinity to C1q and various FcγRs. Furthermore, Tetra E16 neutralizes WNV infection and protects mice from WNV lethal challenge equivalently as the parent E16 [77] (Fig. 2). These studies indicate plants are capable of producing complexed antibody variants, establishing the foundation to develop bispecific antibodies that can have BBB permeability [77] For example, bifunctional mAbs with a similar structure as the Tetra E16 but with one of the two sets of Fabs binding to a specific receptor (e.g., insulin receptor [78]) on the BBB may have the desired bi-functionality. That one set of Fabs would facilitate their transport into the brain and the other set of Fabs would retain its therapeutic activity against WNV in the brain (Fig. 3).

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Recently, we have demonstrated that such bifunctional mAb can be successfully expressed and assembled in plants with its specificity to each antigen preserved [79].

The four serotypes of dengue virus (DENV1-4) represent one of the largest global disease burdens with over 3 billion people at risk for infection [80]. DENV infections are prone to the development of antibody-dependent enhancement of infection (ADE). ADE occurs through the mechanism of immune-complex formation between the infecting flavivirus and the preexisting antibodies, including therapeutic mAbs, that bind to the virus but are non-neutralizing or at sub-neutralizing concentrations. Such immune complexes bind to FcγR-bearing cells, resulting in increased virus uptake and infection [81–83]. Therefore, ADE may render anti-flavivirus mAb treated subjects more at risk of developing severe disease, including life-threatening dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) [84, 85]. To address this major impediment, our laboratory used E60 mAb as a model to investigate if plants can help to overcome ADE by modulating mAb glycosylation. Various E60 glycoforms were produced in *N. benthamiana* and each was shown to carry a single expected N-glycoform with a high degree of homogeneity [86]. Furthermore, E60 glycovariants retain neutralizing potency against multiple DENV serotypes similar to the mammalian cell-made E60 [86]. Strikingly, plant-produced E60 glycovariants forego ADE activity, in contrast to mammalian E60 that exhibits strong ADE activity (Fig. 4) [86]. Our *in vivo* studies suggest that in contrast to CHO cell-produced E60 that has no therapeutic efficacy, plant-made E60 has gained potent post-exposure therapeutic activities in both lethal infection only and ADE mouse models [87]. These results indicate that by modulating N-glycans via plant glycoengineering, mAbs can be transformed from inactive binding antibodies into protective mAbs with potent *in vivo* efficacy even after the occurrence of ADE. This strategy is far more superior to the current approach of mutating the N-glycosylation recognition sequence used in CHO cells, as the latter method would completely remove all N-linked glycans from a mAb, causing total abolishment of mAb binding to all FcγRs and loss of effector function that may be required for the full therapeutic efficacy of a mAb [88–90]. Similarly, glycovariants of anti-Zika virus mAbs produced in plants also showed greatly reduced ADE activity in enhancing DENV infection [12]. Thus, N-glycan modulation by using glycoengineered plants has the potential to overcome ADE and increase the efficacy for mAb-based therapeutics against other ADE-prone viruses such as coronaviruses, paramyxoviruses, and lentiviruses [91].

MAbs against SARS-CoV-2—SARS-CoV-2 is the causal agent of coronavirus disease 2019 (COVID-19) and has infected over 255 million people, causing more than 5.1 million deaths globally since its emergence [92]. While vaccines remain the strategy of choice to curb the current COVID-19 pandemic, post-exposure therapeutics are also in critical demand to treat seriously ill COVID-19 patients and to prevent infection in certain vulnerable populations such as the unvaccinated or recently vaccinated high-risk patients. Neutralizing mAbs, especially those that target the spike (S) protein of SARS-CoV-2, have shown promising therapeutic potential in animal models and human patients [4]. The S protein of SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the target cell surface, thereby, facilitating cell binding and fusion [93]. MAbs targeting the S protein, therefore, can neutralize the virus by interfering in its ability to bind

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and fuse with the target host cell. Currently, there are more than 20 mAbs directed to the S protein that have entered clinical trials, and three mAb-based therapies have been granted EUA by the FDA for treating COVID-19 patients [4]. These therapies include two combinational therapies of bamlanivimab/etesevimab and casirivimab/imdevimab, as well as a monotherapy of sotrovimab [94–96]. Since the robust and rapid nature of plant transient expression allows for the production of mAbs at unprecedented speed, plants have been explored to quickly produce several types of mAbs against SARS-CoV-2 aiming to contribute to developing efficacious and affordable therapeutics to control the current COVID-19 pandemic. For example, two neutralizing mAbs against epitopes on the receptor-binding domain (RBD) of the S protein were expressed in *N. benthamiana* plants [97]. The mAbs were expressed in leaves within 4 days after the introduction of mAb-coding sequences, although the expression levels are relatively low [97]. The plant-made mAbs retain their RBD-binding specificity and neutralization activity. Similarly, expression of ScFv-Fc and camelid nanobodies has also been attempted in *N. benthamiana* [98]. A ground-breaking study came from Dr. Steinkellner's laboratory, in which they examined and compared the neutralization potency of two *N. benthamiana*-produced anti-RBD mAbs in three formats: IgG1, IgA1 monomer, and IgA2 dimers [99]. While the three mAb variants showed similar binding to RBD, their neutralization potency against SARS-CoV-2 was significantly different, specifically in the order of IgG1< IgA1 monomer < IgA1 dimer, with IgA1 dimer having an up to 240-fold increased potency over the monomers [99]. In collaboration with our laboratory, Dr. Steinkellner's group also compared the neutralization potency of the two anti-RBD mAbs in the four IgG subclasses [100]. IgG3 subtype was found to exhibit an up to 50-fold superior potency compared to the other subclasses [100]. These studies have revealed the importance of antibody multivalency and S protein cross-linking on the viral surface for neutralization potency and will have significant implications for COVID-19 vaccine and therapeutic development.

A characteristic seen in severe cases of COVID-19 appears to be a dysregulated immune response, resulting in cytokine storm and immunopathology [101]. Specifically, increased proinflammatory cytokine levels have a strong correlation with severe symptoms, and higher interleukin 6 (IL-6) levels are associated with non-survivors and patients needing mechanical ventilation [101–103]. One approach to curb the hyperinflammatory response is the inhibition of IL-6 signaling, as a large clinical trial using anti-IL-6 receptor (IL-6R) mAbs along with the use of corticosteroids on COVID-19 patients showed statistically significant benefit of the mAb treatment in improving patients' clinical outcomes and survival [104]. To this end, our laboratory explored the feasibility of using plants to produce an anti-IL-6R mAb and examined its utility in reducing IL-6 signaling in a model, which simulates IL-6 induction during SARS-CoV-2 infection. *N. benthamiana*-produced anti-IL-6R mAb (pIL6RmAb) was shown to effectively inhibit IL-6 signaling in a cell-based model system. Furthermore, pIL6RmAb also suppressed IL-6 signaling that was induced by the exposure of human peripheral blood mononuclear cells to the spike protein of SARS-CoV-2 [105]. This study demonstrates the capacity of plants for producing functionally active mAbs that block cytokine signaling and implies their potential efficacy to curb cytokine storm in COVID-19 patients.

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MAbs against other viruses—Plant-based mAb therapies against other viruses including RSV, chikungunya virus (CHIKV), rabies virus, and Herpes simplex virus-2 (HSV-2) have also been developed. Palivizumab is a neutralizing mAb targeted to the fusion protein of RSV that has been approved as prophylaxis for severe RSV infection in high-risk infants [106]. However, CHO cell-produced palivizumab is cost-prohibitive, which limits its application to the elderly who are also suffered from RSV-causing morbidity and mortality [107]. Encouragingly, plant-produced palivizumab was found to be equivalent in its efficacy both *in vitro* and in animal models [108]. Thus, plant-made palivizumab may provide an economical alternative to treat infants, as well as open up new markets for palivizumab.

Infection of CHIKV in humans can cause debilitating polyarthralgia that may persist for months to years and affects multiple joints [109]. The reemergence of CHIKV has resulted in millions of cases of severe, often chronic, arthralgia, and no therapeutics have been approved for human use [110]. MAbs against CHIKV envelope 1 and 2 protein (E1 and E2) have been shown to be protective against CHIKV infection in mouse models and Fc effector function has been implicated in their efficacy [111]. Our laboratory took the initiative to investigate if the efficacy of these mAbs can be improved by glycan modulation via using glycoengineered plants. Our results showed that an anti-CHIKV E1 mAb expressed in wild-type and glycoengineered *N. benthamiana* plants carried the unique N-glycans expected from those plant lines [24]. While both of the mAb glycovariants showed potent neutralization activity *in vitro* and efficacy in mice against CHIKV infection, their *in vivo* potency differed significantly, with the GnGn glycoform exhibiting superior potency in reducing viral load and in improving clinical symptoms [24, 112]. As CHIKV and DENV are co-circulating in the same parts of the world and co-infection with both viruses is likely, we also developed a bifunctional mAb in plants to meet the need for an effective therapeutic for patients in endemic areas. The plant-made bifunctional mAb showed the specific binding for both viruses and exhibited potent neutralization activity against both CHIKV and DENV [79]. These studies provide further examples of using glycoengineered plants to improve the efficacy of anti-viral mAbs via glycan modulation.

The current supply of prophylaxis for rabies virus is in the form of immunoglobulins from horses or humans immunized with rabies antigens [113]. The limited supply of such immunoglobulins and biosafety concerns for equine and human-derived products promoted the development of anti-rabies mAbs in plants. A mAb produced in transgenic tobacco leaves showed similar neutralizing potency and post-exposure prophylactic efficacy in hamsters compared to the equivalent mAb produced in hybridomas or anti-rabies human immunoglobulin [114]. This indicates that plant-derived mAbs are promising alternatives to address the supply and biosafety challenges of current post-exposure prophylaxis for treating rabies infection.

Genital herpes caused by HSV-2 is a global problem associated with significant morbidity and more than 491 million people worldwide are living with this incurable disease [115]. Furthermore, HSV-2 infection often the sexual transmission of HIV [115]. To explore the feasibility of developing inexpensive topical prophylaxis against HSV-2 infection, a transgenic soybean-produced anti-HSV glycoprotein B mAb was compared to the same mAb produced in mammalian cell culture for their *in vitro* properties as well as *in*

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vivo activity in a mouse model [116]. It was shown that the plant-produced mAb and its mammalian cell-derived counterpart had similar stability and diffusion rate in human cervical mucus and prevented vaginal HSV-2 infection similarly in a mouse model [116]. Notably, a *N. benthamiana*-produced mAb against HSV-2 (HSV8-N) was combined with an anti-HIV-1 mAb (VRC01-N), formulated into a vaginal film (MB66), and recently tested in a Phase I clinical trial to assess their safety, pharmacokinetics, and *ex vivo* efficacy in preventing HSV-2/HIV-1 infection [117]. The results from this trial indicated that repeated intravaginal applications of MB66 were safe and well-tolerated, and vaginal samples collected 24 hours post MB66 insertion significantly neutralized both HSV-2 and HIV-1 *ex vivo* [117] This clinical trial set a new milestone in the journey of plant-made mAbs to become approved anti-viral drugs and testified the feasibility of using plant-derived mAbs for topical applications.

Challenges and perspective

The successful production of full mAb and their variants in multiple defined human glycoforms with high yield, speed, enhanced scalability, and cost-effectiveness demonstrates plants' potential as an attractive alternative platform for mAb development and production. However, there are remaining challenges that must be overcome to realize the full potential of plant-made mAbs. For example, despite the successes of large-scale production of several mAbs, large-scale downstream processing of mAbs from plant biomass remains challenging. Unlike mammalian cell-based platforms in which mAbs are usually secreted by cells into the cell culture media, full-length mAbs secreted by plant cells are retained in the extracellular space between the plasma membrane and the cell wall, a compartment known as apoplast [15]. Consequently, downstream processing of mAbs from plants usually requires tissue and cell disruption, which releases plant host proteins and proteases into the feed stream. Furthermore, plants usually produce abundant fibers and other solid debris and some *Nicotiana* species contain high levels of phenolics and alkaloids [118]. As a result, downstream processing is far more complex for plant-made mAbs. For example, clarification of plant extracts cannot be achieved simply by filtration as in the case of mammalian cells, and direct loading of plant extracts onto protein A column may cause resin fouling [119]. To overcome these challenges, several new strategies have been explored. An integrated method that combines two or more operations of extraction, bulk enrichment, purification, and concentration is one of these strategies. For example, our research has demonstrated that aqueous two-phase separation (ATPS) can combine separation, purification, and concentration in one operation. By using a plant-produced and unpurified hydrophobin-protein A fusion protein, we extracted and enriched plant-derived mAbs in one step to a comparable purity as those extracted and purified by conventional protein A chromatography [54, 120]. Another strategy is to avoid cell disruption and isolate mAbs directly from the apoplastic fluid by centrifugation [98]. While this method works for smaller mAb variants such as nanobodies and ScFvs, the sizes of full-length human mAbs are too large to pass the cell wall [15]. Nevertheless, attempts are being made to loosen the cell wall by treatment with a combination of chelators, pectinase, and cellulase aiming to allow extraction of full mAbs directly from apoplastic fluid [121]. An alternative approach from a different perspective is to further increase the expression levels of mAbs in plants. While transient expression with deconstructed viral vectors has allowed competitive levels

of expression up to 5 g mAb per kilogram LFW [35], higher mAb expression levels will reduce the extraction volume and in turn, reduce consumable expenditure and the overall cost of downstream processing. The combination of these strategies is more than likely to resolve some of the challenges of downstream processing. However, further optimization of the explored technologies and/or the introduction of new processing methods are required to fully overcome this major impediment.

Other challenges for plant-made mAbs includes skepticism and lack of interest by industry experts and large pharmaceutical companies. ELEYSO™ is the only plant cell-made biologic approved by the FDA to date, but there are still no plant-made mAbs that have been licensed for human use [122]. The lack of a clear regulatory pathway in the past is partially responsible for the lingering skepticism and the inertia by large pharmaceutical companies, as regulatory agencies like FDA were uncertain about how to fit biologics made by this novel yet complex technology into their approval framework established mostly for biologics produced in mammalian cells. The approval of ELEYSO™ by the FDA along with the clinical development of ZMapp and 2G12 has cleared the regulatory pathway and slowly warmed up the interest of large pharmaceutical companies towards plant-made biologics including mAbs [122–124]. For example, Pfizer entered into an agreement to license the worldwide rights for commercializing ELEYSO™, and GlaxoSmithKline (GSK) is co-developing a plant-made COVID-19 vaccine with a Canadian plant-based biotech company [125]. These progresses should facilitate the commercial development of plant-made mAbs and streamline the approval of those that have shown safety in human clinical trials.

Since mAb titers 5.0 g per liter can be readily achieved in mammalian cell cultures [126], expression levels 5.0 g per kilogram LFW may be required for plant-made mAbs to compete with those derived from mammalian cells [119]. Thus, it is unlikely that plants will become the primary platform for mAb production in the foreseeable future. Instead, several special niches are waiting to be explored by this technology. For example, plants would be optimal to produce mAb biosimilars because they can produce large amounts of mAbs rapidly at a low cost. Similarly, plants should be a system of choice for producing mAbs with an extraordinarily large-quantity demand, such as prophylactic mAbs that are needed to cover a large population with repeated dosing requirements. Glycoengineered plants should be employed to develop and produce safer and more efficacious mAb biobetters due to their flexibility in producing mAbs with specific and homogeneous mammalian glycans and other PTMs that preferentially bind to various FcγRs, thereby, modulating mAb effector functions. As demonstrated by the development of several anti-SARS-CoV-2 mAbs in plants, the rapid nature of plant transient expression systems should be used more extensively to quickly produce various mAb glycovariants, isotype variants, and subtype variants to improve the efficacy and safety of prophylactic and therapeutic candidates in fighting the current COVID-19 and future potential pandemics [97, 127, 128]. The current priorities for the field of plant-made mAbs should be the focus on overcoming the challenges in downstream processing, actively engaging large pharmaceutical companies in co-developing new mAb drugs, and vastly expanding the approval pipeline of plant-made mAbs. We speculate that the first plant-made mAb-based antiviral drug should be approved within this decade.

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Acknowledgements

The authors thank Josh Lesio for the critical reading of the manuscript. Relevant research in the author's laboratory was supported in part by NIH-NIAID grants U01 AI075549 and R21/R33AI101329 to Qiang Chen

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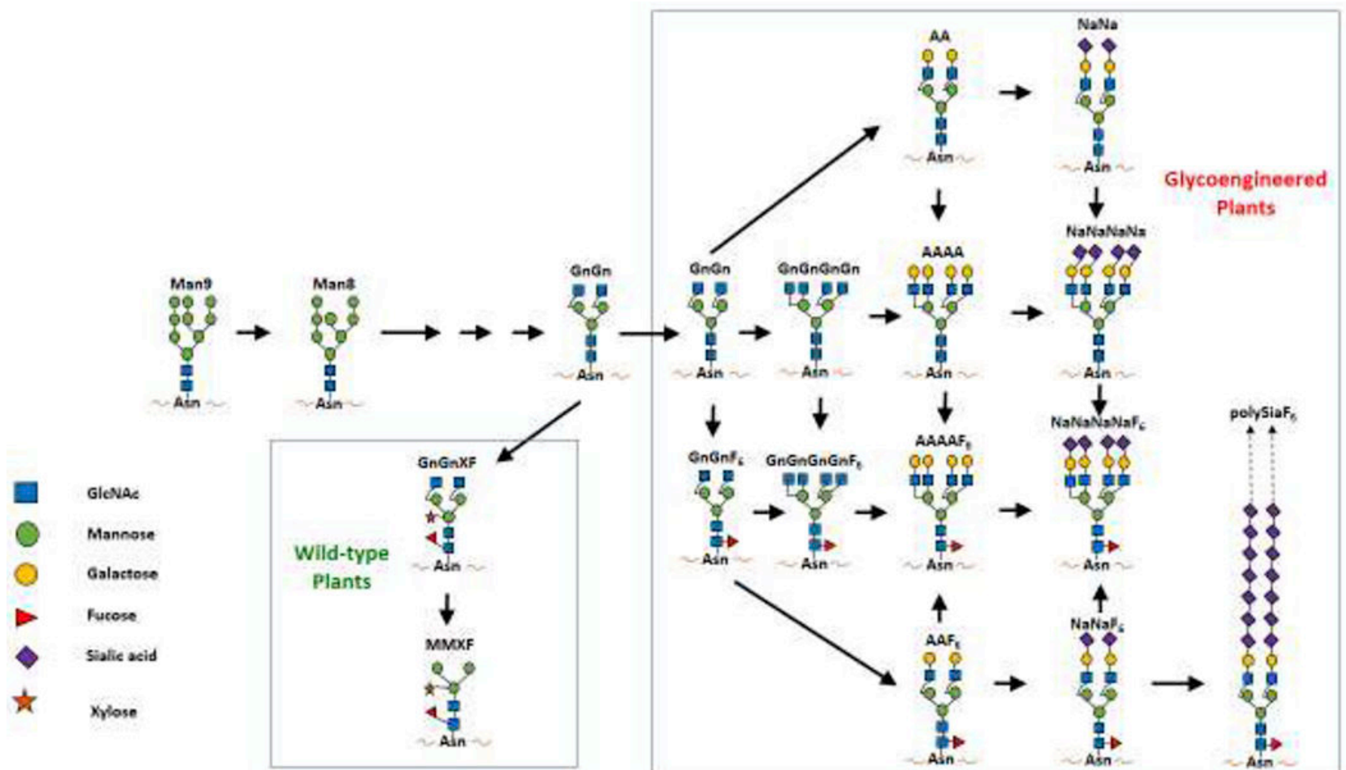

Figure. 1. Major N-glycan structures in wild-type and available glycoengineered *N. benthamiana* plant lines.

In wild-type plants, glycoproteins usually carry a single dominant N-glycan structure, GnGnXF or MMXF which contain plant-specific glycans of β1,2-xylose (X) and α1,3-fucose (F). By suppressing gene expression for xylosyl- and fucosyltransferases, plant-specific N-glycans can be eliminated from mAbs made in plants. Moreover, glycoengineered plants generated by introducing mammalian glycoenzymes to specific compartment of Golgi complex can overcome CHO cells' inability to synthesize multi-antennary N-glycans, and can now produce mAbs with defined and homogeneous N-glycans on demand, including α1,6-fucosylated, bisected, tetra-antennary, bi-galactosylated, and bi-antennary sialylated complex N-glycoforms. (Glycan nomenclature according to Consortium of functional glycomics, see http://www.proglycan.com/upload/nomen_2007.pdf)

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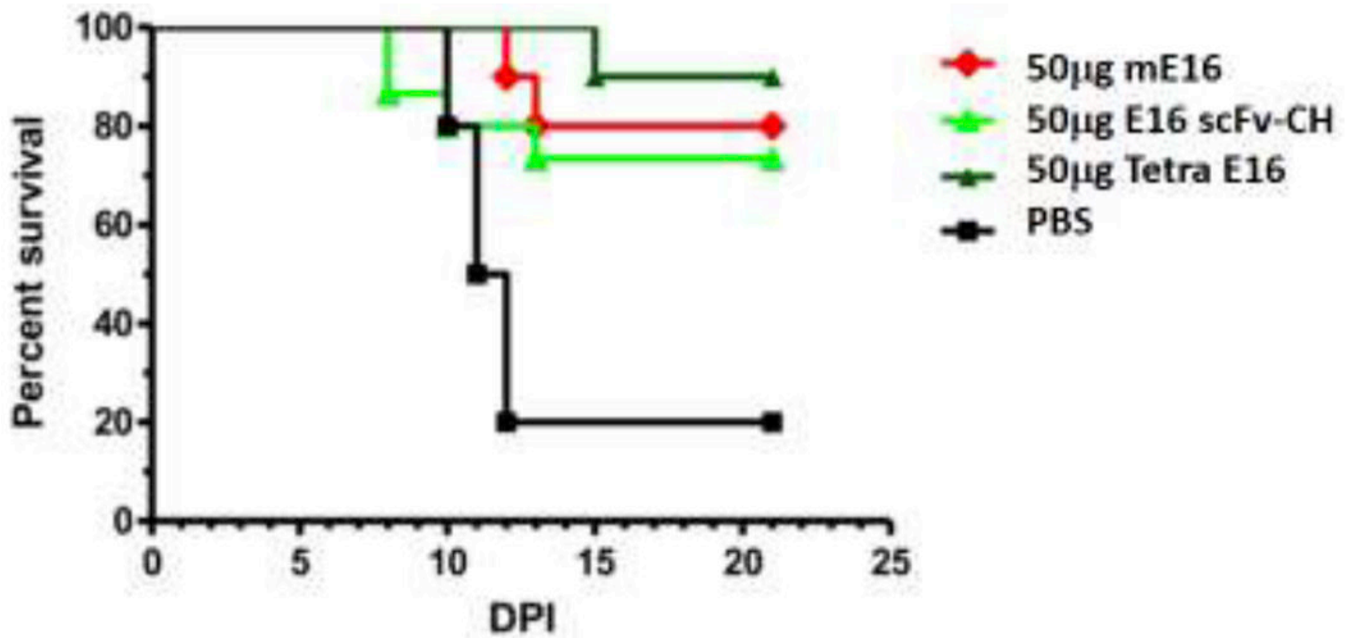

Figure 2. Plant-made E16 mAb variants protected mice against a lethal challenge of WNV infection.

Wild type C57BL/6 mice were infected with 10^2 PFU of WNV. A single 50 μg dose of single-chain E16 (E16 scFv-C_H or Tetra E16 was given to mice via an intraperitoneal route 4 days after infection. Mammalian cell-made E16 (mE16) was used as a positive control. Survival data was pooled from several independent experiments ($n > 10$ mice per dose) and analyzed by the log-rank test. (Adapted from reference [47] with permission)

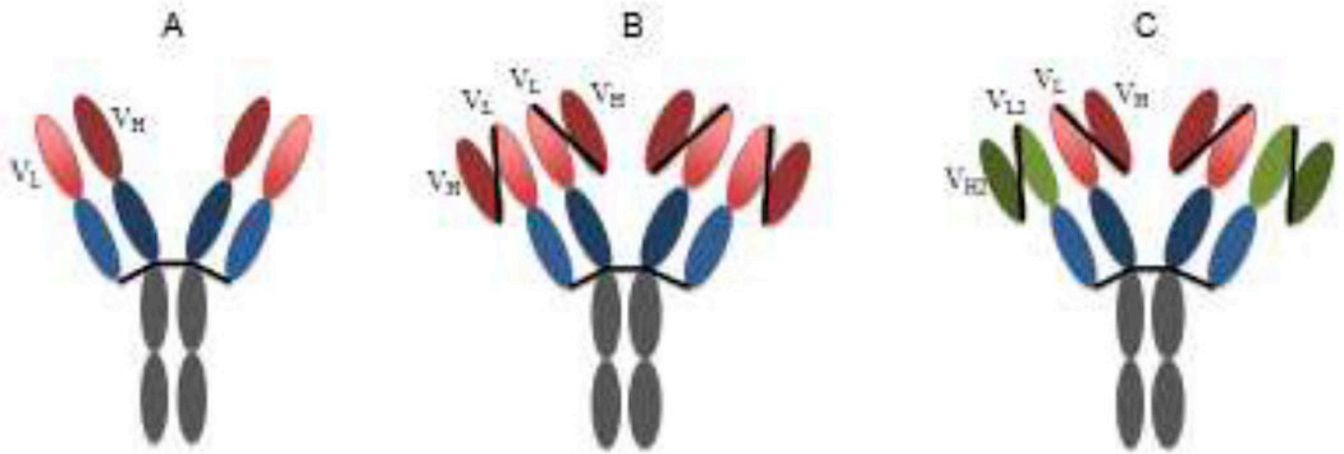

Figure 3. Tetravalent E16 and bifunctional E16 design.

A. E16. **B.** Tetravalent E16. C. Bifunctional E16 that are designed to enhance its ability to cross the blood brain barrier. V_L: variable region of light chain; V_H: variable region of heavy chain; V_L/V_H: the first pair of antigen binding sites that bind and neutralize WNV. V_{L2}/V_{H2}: a second pair of antigen binding sites that bind to surface receptors on the endothelial cells of the BBB. This may enhance the permeability of the bifunctional antibody across the BBB via receptor-mediated transcytosis.

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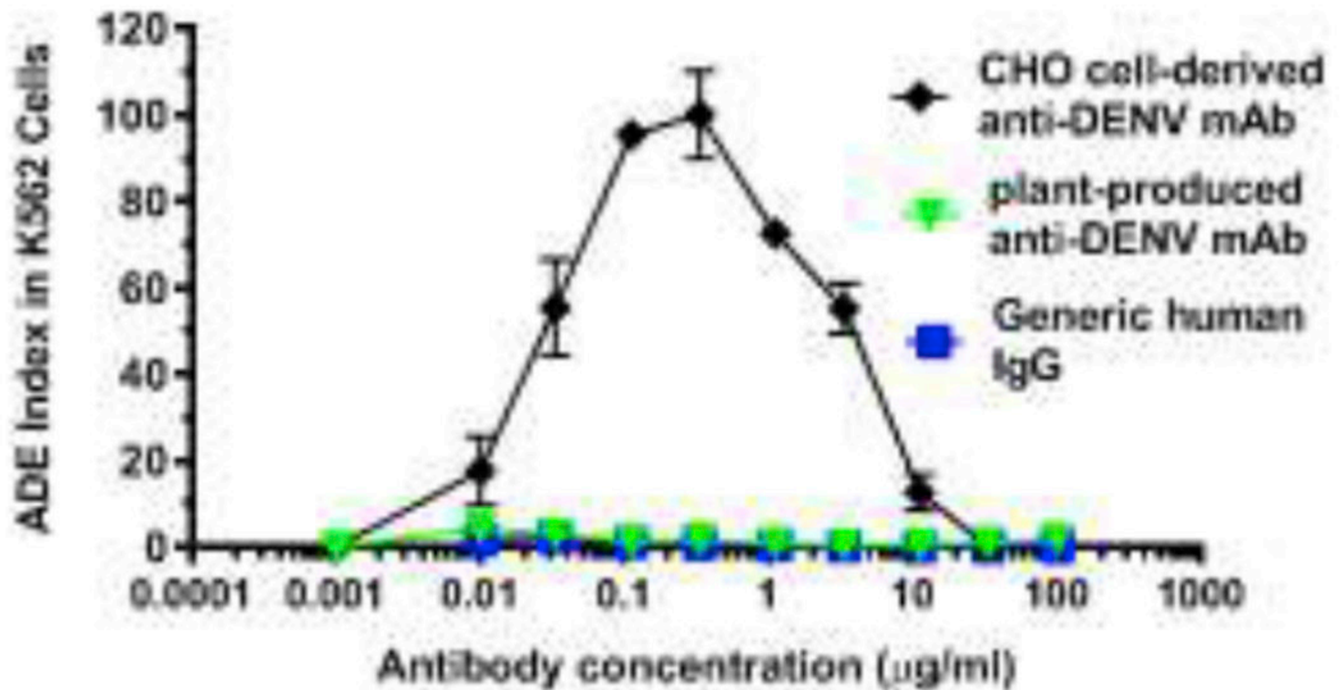

Figure 4. Antibody-dependent enhancement of pE60 variants.

MAb glycovariants were serially diluted and mixed with DENV-2. The mAb and virus mixture was then added to FcγR-expressing K562 cells. Cells were fixed, permeabilized and stained with anti-DENV E antibody 4G2 48 hr later, and analyzed by flow cytometry for DENV infection of cells. (Adapted from reference [86] with permission)

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Table 1:

Representative anti-viral mAbs made in plants at various developmental and clinical stages

Product	Disease application	Plant host	In vivo efficacy	Development stage	References
IgG1 (2G12)	HIV prophylactic	Transgenic tobacco, corn endosperm, and *N. benthamiana* (transient expression)	Protection in mice, safety in humans	Phase I	[63, 64, 73]
IgG1 (ZMApp)	Ebola virus therapeutic	*N. benthamiana* (transient expression)	Protection in mice and rhesus macaques, safety and efficacy in humans	Phase I	[59, 60, 61]
IgG1	Genital herpes (HSV-2)/HIV prevention	Transgenic Soybean *N. benthamiana* (transient expression)	Prophylactic protection in mice Safety and *ex vivo* activity in humans	Phase I	[116, 117]
IgG1	West Nile virus prophylactic and therapeutic	*N. benthamiana*/Lettuce (transient expression)	Protection in mice	Pre-clinical	[47–50, 77]
IgG1	Dengue virus therapeutic	*N. benthamiana* (transient expression)	Protection in mice	Pre-clinical	[86, 87]
IgG1	Zika virus therapeutic	*N. benthamiana* (transient expression)	Protection in mice	Pre-clinical	[12]
IgG1	Chikungunya virus therapeutic	*N. benthamiana* (transient expression)	Protection and reduced ankle inflammation in mice	Pre-clinical	[24, 79, 112]
IgG1	Rabies virus prophylactic	Transgenic tobacco	Prophylactic protection in Hamster	Pre-clinical	[114]
IgG1	Respiratory syncytial virus prophylactic and therapeutic	*N. benthamiana* (transient expression)	Prophylactic and therapeutic protection in cotton rats	Pre-clinical	[108]
RIC	Ebola virus vaccine	*N. benthamiana* (transient expression)	Protection in mice	Pre-clinical	[57, 58]]
IgG1	SARS-CoV-2 cytokine storm prevention	*N. benthamiana* (transient expression)	Not reported	Research	[105]
IgG1	SARS-CoV-2 therapeutic	*N. benthamiana* (transient expression)	Not reported	Research	[97, 99, 100]
IgG3	SARS-CoV-2 therapeutic	*N. benthamiana* (transient expression)	Not reported	Research	[100]
IgA1 dimer	SARS-CoV-2 therapeutic	*N. benthamiana* (transient expression)	Not reported	Research	[99]

RIC: recombinant immune complex, Pre-clinical: Efficacy demonstrated in animal models, Research: Efficacy only tested *in vitro*.