2024

Harnessing innovative methods in antibody design and delivery for development of a novel nonhormonal contraceptive

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Dissertation

HARNESSING INNOVATIVE METHODS IN ANTIBODY DESIGN AND DELIVERY FOR DEVELOPMENT OF A NOVEL NONHORMONAL CONTRACEPTIVE

by

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B.A., Vassar College, 2018

Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

2024

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Nothing in life is to be feared, it is only to be understood.

-Marie Curie

DEDICATION

To my mom. My hero and blueprint. As a little girl, I watched in awe as you singlehandedly conquered a career in medicine while building a beautiful life for your daughters. Your constant love, support, and guidance have made me invincible. I could not have done this without you.

ACKNOWLEDGMENTS

Completing this degree has in no way been a solitary task and is a shared accomplishment among all who have helped me along the way. There are so many individuals who have provided immense support not only in an academic sense but also throughout my personal journey. Firstly, I'd like to thank my supervisor and mentor Deborah Anderson who graciously gave me the opportunity to be involved in such exciting and important research. Her unmatched insight and expertise in reproductive immunology have shaped my work into valuable contributions to the field. As a result, she has made me a better scientist.

Over the years, the members of the Anderson lab have become my second family. I'd especially like to thank Joe Politch and Jai Marathe for their ever-present friendship and perspective (both in science and life). You both have been crucial in preserving my sanity and getting me through those often harsh days of being a grad student. Thank you to all other current members of the lab, James Doud, Sarah Dohadwala, Matt Geib, Jess Barmine, and Samaa Rasheed, for all the ways you've supported my work. I must also give thanks to past members of the lab: to Jeff Pudney for his wisecracks and histology prowess, to Emilie Mausser who has been my partner in crime and an incredible role model, and to Gaby Baldeon Vaca who from the moment I joined the lab has been and continues to be an invaluable mentor.

I was lucky enough to work with some brilliant collaborators on different projects and as a result, I gained scientific insight on a diverse range of fields. I would like to thank Phil Santangelo and his lab at Emory University, especially Daryll Vanover and Jae Yeon

Joo, for bringing me into the exciting world of mRNA therapeutics and for so generously providing me with protocols, samples, resources, and wisdom in everything mRNA. Thank you to Cathy Costello and Chaoshuang Xia who agreed to embark on a glycobiology project with me and believed in my idea despite my inexperience. Your expertise in mass spectrometry and proteomics is nothing short of impressive.

I would like to thank my dissertation committee: my chair Andy Henderson, my second reader Cathy Costello, Darin Edwards, and PJ Maglione. Thank you for keeping my work focused and on track; your feedback has been indispensable these past few years. A special thanks to the co-chairs of the department of Molecular and Translational Medicine, Matt Jones and Valerie Gouon-Evans, for their help in guiding me towards the finish line.

I could not have survived this adventure called grad school without all of my peers within the PiBS program. It has been so special to be part of a support system in which we all have had one other's backs since orientation day. Our gripe sessions, group lunches, zoom calls, trivia teams, and theme parties have meant the world to me. Through this program, I have also found my partner Jimmy Conley. Jimmy, I cannot thank you enough for being so lovely and so patient with me through all the ups and downs. Your freshly brewed morning coffees, warm company, and unwavering partnership have made me feel at home in a foreign city.

Last but not least, I'd like to thank my family back home in New York and elsewhere. To my mom, dad, sisters, cousins, aunts, uncles, and grandparents- your unconditional love and support have been the greatest motivation to keep my head above

water. I want to especially thank my three sisters and soulmates, Juliana, Olivia, and Sofia, for being my best friends and strongest allies in my life. I know anything is possible with you three by my side. Though it has been difficult being apart and missing holidays, birthdays, etc., I have felt the whole family rooting for me from miles away. And for that, I will be forever grateful.

HARNESSING INNOVATIVE METHODS IN ANTIBODY DESIGN AND DELIVERY FOR DEVELOPMENT OF A NOVEL NONHORMONAL CONTRACEPTIVE

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ABSTRACT

The development of safer and more accessible contraceptive options is necessary to reduce the high number of unintended pregnancies worldwide. As monoclonal antibody engineering continues to revolutionize drug development, a variety of strategies are being harnessed to establish antibody-based contraceptives. Human Contraception Antibody (HCA), an immunoglobulin G1 (IgG1) monoclonal antibody that potently agglutinates human sperm, is a promising candidate for nonhormonal immunocontraception in women. Our group recently established the safety and efficacy of a topical IgG1 HCA-formulated dissolvable vaginal film. Though successful, we are currently working to further optimize and improve the HCA product. In this study, we characterized engineered variants of HCA. Bioactivities, specifically agglutination and effector functions, of multimeric and fragment crystallizable (Fc)-mutated variants were compared and inform further engineering of an optimal clinical profile. We then established an atomized mRNA mechanism for delivery of HCA to the female reproductive tract (FRT). The use of mRNA could provide several advantages including: efficiency, reversibility, safety, durability, and cost-effectiveness.

mRNA-encoded HCAs were expressed in several models of the FRT and were functional, sperm-specific, and safe. We also analyzed Fc *N*-glycans at the conserved glycosylation site on IgGs that regulate effector functions and compared the site-specific glycosylation on antibodies generated by two HCA expression platforms of interest, namely *Nicotiana benthamiana* and mRNA-transfected vaginal cells. Disparities in glycan site occupancy and glycoform populations between the two platforms were observed. Platform-specific HCA glycans resulted in differing levels of sperm phagocytosis, an Fc function. In summary, these studies provide a clearer understanding of engineered variants and delivery platforms to further advance the development of HCA as a novel, antibody-based female contraceptive.

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LIST OF ABBREVIATIONS

ADCC......Antibody-dependent cellular cytotoxicity

ADCP...... Antibody-dependent cellular phagocytosis

aHCA ......anchored HCA

ANOVA ......Analysis of variance

ASA......Antisperm antibody

BPE ...... Bovine pituitary extract

BV ......Bacterial vaginosis

CASA......Computer-assisted sperm analyzer

CDC ...... Complement-dependent cytotoxicity

CHO ......Chinese hampster ovary

CM ......Cervical mucus

CVL......Cervicovaginal lavage

DP......Drug product

dsRNA......Double-stranded RNA

E2 ......Estradiol

ELISA ...... Enzyme-linked immunosorbent assay

ER...... Endoplasmic reticulum

FBS......Fetal bovine serum

FcR...... Fc receptor

FDA...... Food and Drug Administration

Fig ......Figure

FRT .... Female reproductive tract

FucT .... Fucosyltransferase

GCRU.. General Clinical Research Unit

GMP.. Good manufacturing practices

GlcNAc .... N-acetylglucosamine

GPI .... Glycosylphosphatidylinositol

HC .... Heavy chain

HCA .... Human Contraception Antibody

HCD .... High energy collisional dissociation

HiC .... Heat-inactivated complement

HIV-1 .... Human immunodeficiency virus-1

HPF .... High power field

HSV-2 .... Herpes simplex virus-2

Ig .... Immunoglobulin

IUD.. Intrauterine device

IVT.. In vitro transcription

LARC.. Long-acting reversible contraceptive

LC.. Light chain

LC-MS .... Liquid chromatography-mass spectrometry

LNP .... Lipid nanoparticle

mAb.. Monoclonal antibody

MALDI-TOF.. Matrix-assisted laser desorption/ionization-time-of-flight

MOI..Multiplicity of infection

MHM..Multipurpose handling medium

MPT ....Multipurpose prevention technology

MPA ....Medroxyprogesterone acetate

mRNA .... Messenger RNA

MS..Mass spectrometry

MS/MS ....Tandem mass spectrometry

N9...Nonoxynol-9

NeuAc ....N-Acetylneuraminic acid

Nluc...Nanoluciferase

NK..Natural killer

NS..Nonsignificant

ORF...Open reading frame

P4 ....Progesterone

PBMC... Peripheral blood mononuclear cell

PBS..Phosphate-buffered saline

PCT ....Postcoital test

PLC ....Phospholipase C

PMA Phorbol-12-myristate-13-acetate

PMS..Progressively motile sperm

PTM ....Posttranslational modification

PVA.. Polyvinyl alcohol rEGF..Recombinant epidermal growth factor

Ref Std...Reference standard

RIG-I...Retinoic acid-inducible gene I

RSV...Respiratory syncytial virus

RT..Room temperature

sAc ....Soluble adenylyl cyclase

SEM ....Standard error of the mean

sHCA...Secreted HCA

STI...Sexually transmitted infection

TEER...Transepithelial electrical resistance

TLR .... Toll-like receptor

UTR...Untranslated region

VF...Vaginal fluid

WHO ....World Health Organization

XylT ....Xylosyltransferase

CHAPTER ONE: Introduction

A gap in contraceptive needs

Global rates and implications of unintended pregnancy

Almost half of all pregnancies worldwide are unintended[1,2]. An unintended pregnancy, whether wanted or unwanted, is a pregnancy that occurs in a woman[†] who was not planning to have any (or any more) children, was mistimed, or was earlier than desired. More specifically, there were 121 million unintended pregnancies annually between the years 2015-2019, accounting for around 48% of all pregnancies. Of these unintended pregnancies, 61% ended in abortion which was similar to the rate seen in 1990-94, even in settings where access is restricted. These are striking and alarming numbers for a host of reasons including socioeconomic and health implications and point to the need for better access, investment, and development of contraception.

Rates of unintended pregnancy vary by demographic, especially income group and region. Based on World Bank income groups, there is an inverse relationship between income and unintended pregnancy[1]. In general, people in high-income countries have better access to reproductive and sexual healthcare than those in low-income countries. Between 2015-2019, low-income countries had the highest rate of unintended pregnancy. Cultural

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[†]Not all individuals who become pregnant are women and girls; transgender and non-binary people also face barriers to sexual and reproductive health resources and can be at risk for unintended pregnancy. However, data in reports and surveys largely identify participants as women or girls. Therefore, to remain consistent and accurate with terminology in published data and to avoid incorrect assumptions[2], this work will generally refer to pregnancy risk and contraception use among women and girls.

and social stigma can also contribute to regional differences. Among women in low- and middle-income countries who had experienced an unintended pregnancy, 56% were not using any form of contraception[3]. Limited access to sexual and reproductive health services in these regions widens global disparities and economic inequalities. However, barriers to reproductive healthcare exist in settings with greater resources as well[4].

Health and wellbeing outcomes for both mother and child can be influenced by the intention status of the pregnancy. For example, there is an increased risk of psychological distress at 9 months postpartum following an unplanned pregnancy among partnered women[5]. Distress can be contributed to and exacerbated by socioeconomic position and readiness for motherhood. Birth outcomes can also be affected by pregnancy intent. Though there are gaps in literature and studies show mixed effects, there is evidence to suggest that in some countries, there is a positive association between unintended pregnancy and maternal risk behaviors like substance abuse, smoking, and caffeine intake[6]. In turn, this can lead to increased risk of congenital anomalies, spontaneous abortion, low birth weight, and premature delivery.

Population growth is one other factor of concern associated with unintended pregnancies. The current world population is 7.6 billion, and this number is predicted to increase to 9 billion by 2050[7]. Human activity contributes to pollution, depletion of natural resources, a decline in wildlife, and overall climate disruption. The development and widespread use of new contraceptives could potentially help to stabilize human population growth and health of the planet for future generations[8].

Current state of the contraception market

There are several available methods of contraception on the market today (Fig 1.1). In order of greatest efficacy, they include: permanent, surgical methods (tubal ligation and vasectomy), long-acting reversible methods (intrauterine devices (IUDs), rings, patches, and implants), short-acting reversible methods (injectables and oral pills), barrier methods (condoms and diaphragms), and natural planning (fertility awareness and coital withdrawal). Despite these available methods, contraceptive satisfaction and continuation rates among contraceptive users in the US are low[9]. In fact, one study reported that 46% of women using reversible contraceptives discontinued at least one method in their lifetime because they were unsatisfied[10]. One of the most reported reasons for dissatisfaction is side effects which is most often seen with use of hormonal methods. Hormonal contraceptive methods such as estradiol and progestin products dominate the contraception market, although there are certain risks associated with these methods. Besides frequency of undesired side effects like mood changes and irregular periods, other health risks include venous thrombosis or blood clots[11]. Additionally, one study found that the risk of breast cancer was slightly higher among women who currently or recently used contemporary hormonal contraceptives compared to women who had never used hormonal contraceptives, and this risk increased with longer durations of use[12].

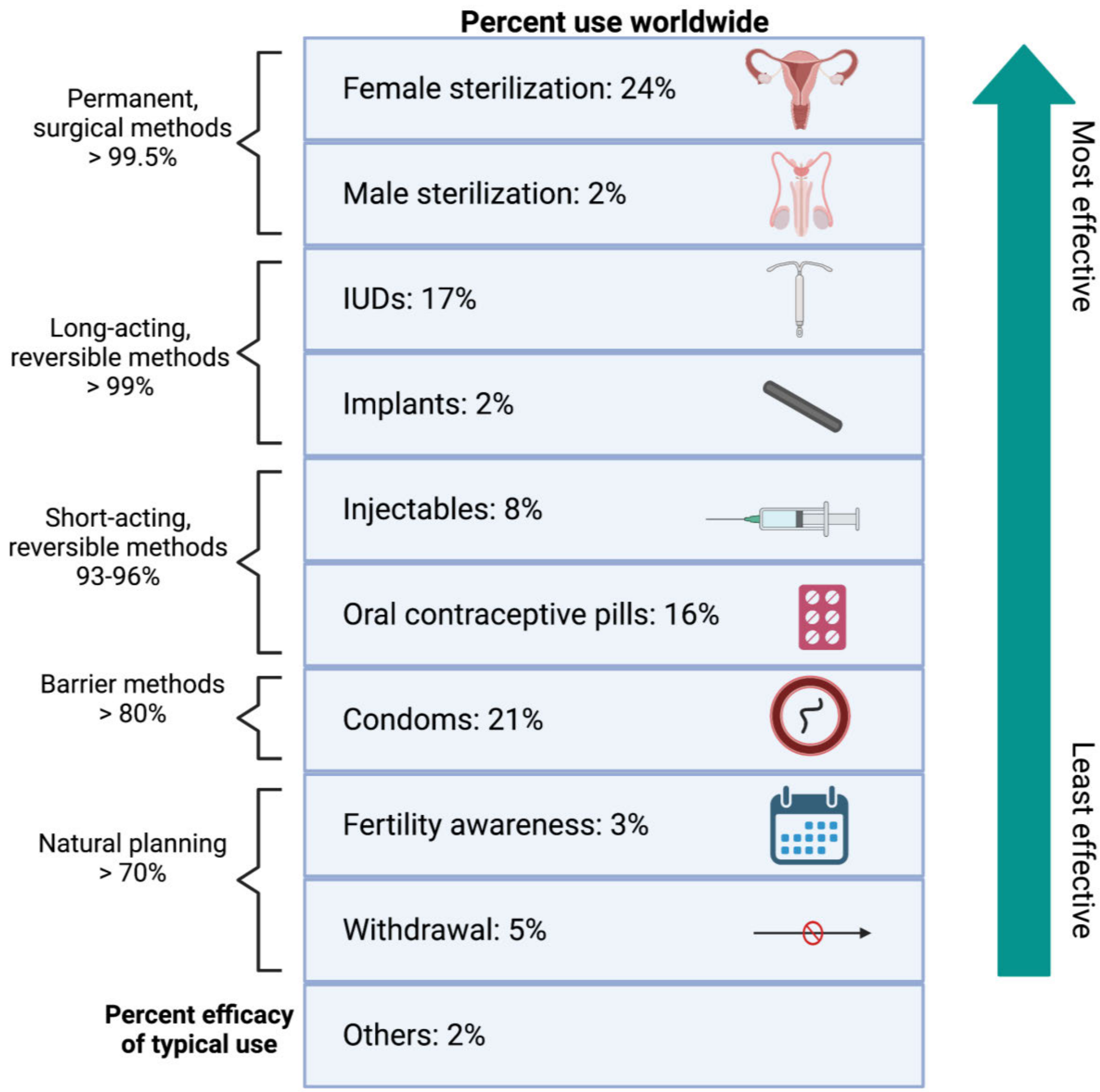

Figure 1.1- Modern contraceptive methods. Created using data from Trussell et al. (2018) and United Nations data booklet (Contraceptive Use by Method 2019). Figure generated in Biorender.com.

Long-acting reversible contraceptives (LARCs) such as intrauterine devices and implants have been shown to be superior in efficacy and have higher rates of satisfaction and continuation among users compared to other methods[13]. But despite their effectiveness, LARCs are not widely used in the United States due to lack of accessibility, misconceptions, and initial costs. Concerted efforts in the US to increase LARC use have led to several regional initiatives including the CHOICE project in St. Louis, Missouri. After removing barriers related to cost and knowledge, this initiative resulted in a decrease in unintended pregnancy, especially among teens, and a decrease in abortions[14]. Removing barriers and improving upon current options of reversible contraceptive products may help decrease the rate of unplanned pregnancies.

Clear demand for the development of novel contraceptives

High rates of unintended pregnancy and contraceptive discontinuation demonstrate that the currently available contraceptives are insufficient and do not meet the needs of all women. Thus, development of new contraceptive products to create more options and autonomy for women is crucial to address this unmet need. Substantial disparities in the rates of unintended pregnancies with respect to race, ethnicity, and income level indicate the need for more accessible and cost-effective options. In creating new methods, it is imperative to consider and incorporate user preferences to enable adoption and continuation of contraceptives. These preferences include nonhormonal options, milder or no side effects, self-administered topical methods, and cost-effectiveness[15].

It is important to note that promising approaches to male contraception are currently under development. These include both hormonal and nonhormonal molecularly targeted male contraceptives. One male hormonal method currently in clinical trials is a nestorone

+ testosterone combination gel which reduces sperm counts with minimal adverse effects[16]. A leading nonhormonal small molecule contraception candidate targets soluble adenylyl cyclase (sAC) which is essential for sperm motility and maturation[17]. The use of an acutely-acting sAC inhibitor demonstrated temporary infertility in mice. Development of male contraceptives would not only help prevent unintended pregnancies but would also lessen the contraceptive burden on women and contribute to equity between sexes.

Monoclonal antibodies as a promising frontier

The synthetic production of monoclonal antibodies (mAbs) from hybridomas was first described by Kohler and Milstein in 1975[18] and has led to use of the antibody as a powerful human therapeutic. The commercialization of the first therapeutic monoclonal antibody, OKT3, in 1986 marked the beginning of antibody-based drug products dominating the biopharmaceutical industry. There are about 175 mAbs in regulatory review or approved[19], the majority targeting cancers, with 12 new antibody-based therapeutics approved just in 2022. The global mAb market size was valued at USD 210.06 billion in 2022 and is projected to grow 11% from 2023 to 2030[20]. Some factors contributing to the increasing popularity of mAbs are high specificity and low risk of safety concerns due to antibody humanization. Recent advances in mAb technology have also led to high product yields and reduced costs[21]. Thus, monoclonal antibodies have great potential to address unmet medical needs such as unintended pregnancy and to make products more accessible to a wider demographic.

Human Contraception Antibody (HCA)

Antisperm antibodies

Spermatozoa were discovered to be immunogenic over a century ago[22]. Antisperm antibodies (ASAs) were detected in the blood of animals and women that had been immunized with sperm. They can also occur naturally in both men and women and are associated with infertility[23]. ASAs are highly diverse in terms of cause, immunoglobulin class, antigen-reactive site, mechanism of action, and degree of fertility impairment[22]. ASAs can affect fertility on several levels: 1) gamete interactions such as sperm penetration of the zona pellucida, 2) impaired transport through the female reproductive tract (FRT) via agglutination or cervical mucus trapping, or 3) complement-mediated damage or immobilization of sperm. Though the cause of infertility is not always apparent in infertile couples, previous studies have documented several etiologies of ASAs[22]. In men, breakdown of the blood-testis barrier can expose the immune system to auto-antigens on sperm. Vasectomies can also induce ASA production. Etiologies are not as clear in women, since immuno-suppressive agents are naturally present in seminal plasma to reduce immune function of T cells, B cells, macrophages, and NK cells and prevent immunity of sperm in women after coitus. Lack of immunosuppressive activity or immune activation as a consequence of FRT infection may cause ASAs.

Upon deducing that ASAs can cause infertility, the WHO convened two workshops in the 1980s to characterize monoclonal antibodies against sperm-specific and trophoblast-specific antigens to identify appropriate candidates for the development of contraceptive vaccines[24]. The ASAs were characterized by assays to detect agglutination activity, complement-dependent sperm cytotoxicity, immunobead binding, and specificity[22]. Of the 111 antibodies evaluated, two anti-trophoblast and three anti-sperm monoclonal antibodies demonstrated high specificity with no cross-reactivity in non-reproductive tissues. However, interest in contraceptive vaccines declined shortly thereafter due to concerns for safety and contraceptive irreversibility.

One of the sperm antigens of particular interest in these workshops was CD52g. A Japanese lab headed by Shinzo Isojima first isolated the anti-CD52g IgM monoclonal antibody, H6-3C4, from blood cells of an infertile woman whose serum had demonstrated sperm agglutinating and immobilizing activity[25]. John Herr independently developed a mouse anti-CD52g IgG monoclonal antibody designated S19 that also exhibited sperm agglutination[23]. CD52g is a male reproductive tract-specific glycoprotein that is produced and secreted by epididymal epithelial cells. A unique *N*-linked glycan is present on sperm CD52, a GPI-anchored protein[26] (Fig 1.2). Mature sperm acquire CD52g via GPI membrane insertion during epididymal transport. The epitope is localized to the entirety of the sperm surface and is also abundant in its soluble form in seminal plasma. The function of CD52g is currently unknown.

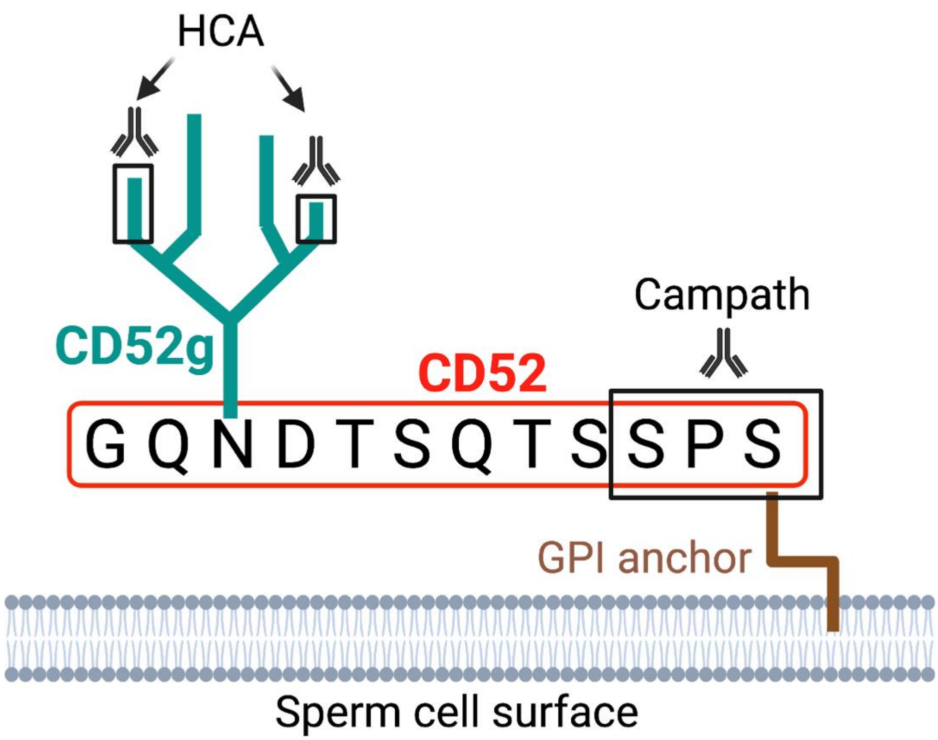

Figure 1.2- CD52g- A male reproductive tract-specific epitope. Figure adapted from Diekman et al. (1999) and generated in Biorender.com.

Development and characterization of HCA

Immunocontraception has recently regained traction as a means of nonhormonal contraceptive development. Using the variable region nucleotide sequence of H6-3C4, our research group, the Boston University Contraceptive Development Research Center, engineered a human IgG1 isotype of the anti-CD52g monoclonal antibody called the Human Contraception Antibody (HCA)[27]. The main mechanism of action of this IgG mAb is potent, dose-dependent agglutination of sperm. HCA prevents sperm from traversing cervical mucus, consequently blocking fertilization. HCA is also capable of trapping sperm in cervical mucus. Mucus trapping occurs when gel-forming mucins form weak bonds with sperm and crosslink them to cervical mucus[28]. The sperm are thus able to move in place but unable to make forward progression. This is known as the shaking phenomenon. Preclinical research performed in our lab showed that HCA agglutinates human sperm under a variety of physiologically relevant conditions including: low pH, in the presence of BV-associated microbiota, in the presence of seminal plasma, and varying sperm concentrations[29]. In addition, in vitro safety tests showed that the mAb did not induce inflammation in a vaginal tissue model. The noninflammatory, reversible, and potent nature of HCA solidifies it as a candidate for further contraceptive product development.

Topical delivery to the female reproductive tract

The efficacy of new forms of contraception is largely dependent on the mode of drug administration and delivery. Topical drug administration to the FRT has several advantages over oral or systemic administration including avoidance of the harsh gastrointestinal environment, avoidance of the hepatic first-pass effect, ability to utilize smaller drug doses, and reduced off-target and side effects[30]. Despite these advantages, however, the unique anatomy and physiology of the FRT (Fig 1.3) present certain obstacles with respect to topical drug delivery. Firstly, vaginal rugae substantially increase the internal surface area making adequate drug distribution challenging. Highly viscous cervicovaginal mucus can also serve as a physical barrier for drug penetration. Other physiological aspects of the vagina that may pose challenges include low vaginal pH, dynamic hormone levels which affect mucus composition, and the presence of diverse microbiota including *Lactobacilli* and species associated with bacterial vaginosis.

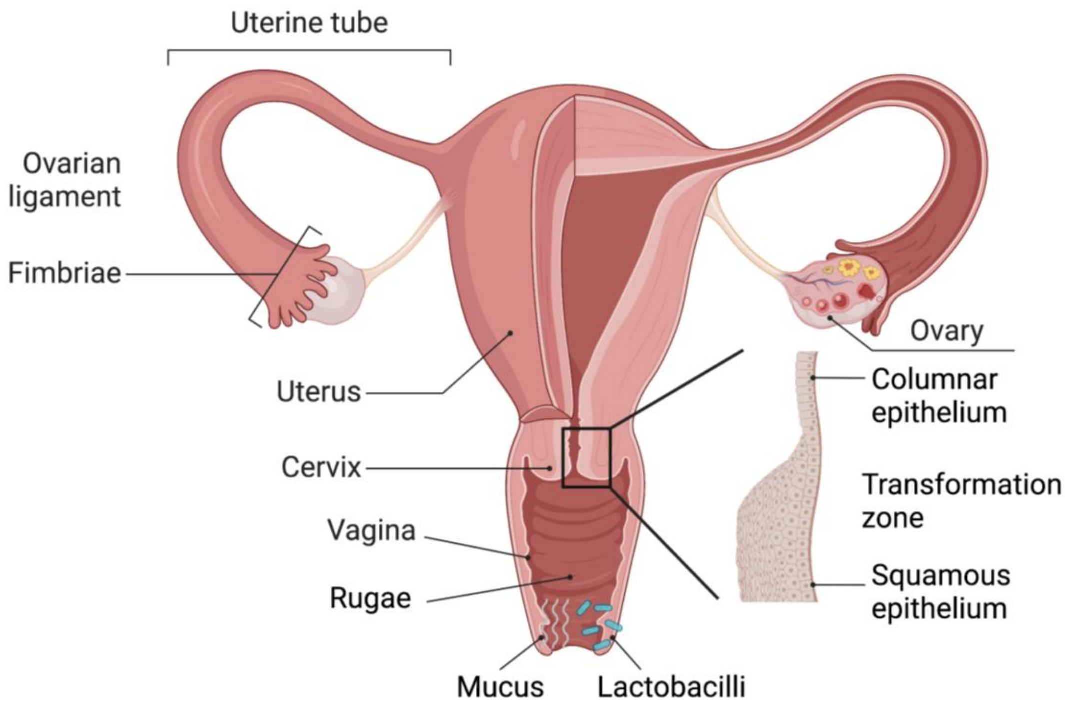

Figure 1.3- Anatomy of the female reproductive tract. Figure generated in Biorender.com

Many research groups, including our own, are increasingly interested in the development of topical products such as intravaginal rings and polymer films for the delivery of contraceptives and microbicides including mAbs such as HCA[31]. In addition, approaches using synthetic messenger RNA (mRNA), which have seen recent breakthroughs in the field of vaccinology[32], are currently being harnessed for vaginal delivery of monoclonal antibodies. This mRNA approach will be further discussed in later sections.

The future of an HCA product

Our group has seen recent success in the topical delivery of mAbs expressed in *Nicotiana benthamiana*. *N. benthamiana* is a relative of the tobacco plant and has gained popularity as an antibody production platform due to its scalability, cost-effectiveness, versatility, and rapid, customizable expression[33]. In brief, mature *Nicotiana* plants are transfected with *Agrobacteria* containing viral replicons that encode for HCA genes and are harvested 8 days later after expression of full-length, assembled mAbs. A transgenic strain of *Nicotiana* is used to eliminate non-mammalian glycans. The purified antibodies are then formulated into a polyvinyl alcohol (PVA)-based polymer to produce homogeneous, square pieces of dissolvable film within the milligram concentration range under good manufacturing practice (GMP) standards.

The *Nicotiana* mAb topical delivery approach was first used by our group to topically administer MB66, a film containing mAbs against HIV-1 (VRC01) and HSV-1,2 (HSV8). A phase 1 clinical trial to assess pharmacokinetics, safety, and efficacy of MB66 demonstrated that effective levels of mAbs persisted in the vaginal secretions through 24 hours post-treatment, and that the product was safe, well tolerated, and acceptable among participants[34]. Acceptability of the film was attributed to its on-demand format, rapid effectiveness, compactness/discreetness, and lower degree of discharge compared to gel products[27].

In a similar manner, a vaginal film containing HCA also demonstrated safety and efficacy in a phase 1 clinical trial[35] (see Appendix 1 for select data). Postcoital testing revealed high efficacy after a single use of the product, with virtually no progressive sperm in cervical mucus. Follow-up visits demonstrated contraceptive reversibility. The product was safe and found acceptable by both female participants and their male partners. Thus, the HCA film is a viable candidate for a new, nonhormonal contraceptive and will undergo further testing.

The long-term goal for HCA is to incorporate it into a multipurpose prevention technology (MPT) product that addresses multiple reproductive health indications such as pregnancy, HIV, HSV, and other STIs[36]. Due to the wide prevalence of sexually transmitted infections, many consumers prefer products with combined protection. To date, the condom is the only available MPT product, though current efforts in the field are directed towards the development of new classes of FDA-approved MPTs such as MB66 and the dapivirine ring[37]. Combining HCA with anti-STI antibodies in a vaginal film or intravaginal ring is a promising approach for an MPT that is currently being pursued by our group.

Room for improvement: Expanding on IgG HCA through antibody engineering

Monoclonal antibody design and engineering

Despite recent success in the HCA vaginal film clinical trial, there is still room to improve upon an HCA product given fast-paced, innovative progress in antibody engineering. Advances in mAb production techniques have enabled optimization of certain antibody properties such as potency, half-life, effector function, and stability through tailoring of both the Fc and Fab regions[38]. The majority of mAbs on the market to date are of the IgG1 subclass. IgG antibodies are composed of two identical heavy chains and two identical light chains. The Fab portion, which contains the variable sequences, is responsible for highly specific epitope binding and the Fc portion elicits effector functions through immune receptor binding. Disulfide bonds link heavy chains to each other within the hinge region and link heavy chains to their light chains. IgGs are also heavily modified with posttranslational modifications, especially glycosylation, that influence Fc receptor (FcR) interactions. When designing and manufacturing new mAb-based therapeutics, certain factors should be taken into consideration to generate an antibody with a certain desired clinical profile (Fig 1.4). Different antibody isotypes (e.g. IgG, IgA, IgM) convey distinct functions, as do antibody allotypes related to the species of origin and expression system. These factors can influence effector FcR and complement binding as well as immunogenicity. Therefore, design strategies and engineering options can be harnessed to fine-tune mAbs for improved safety and efficacy.

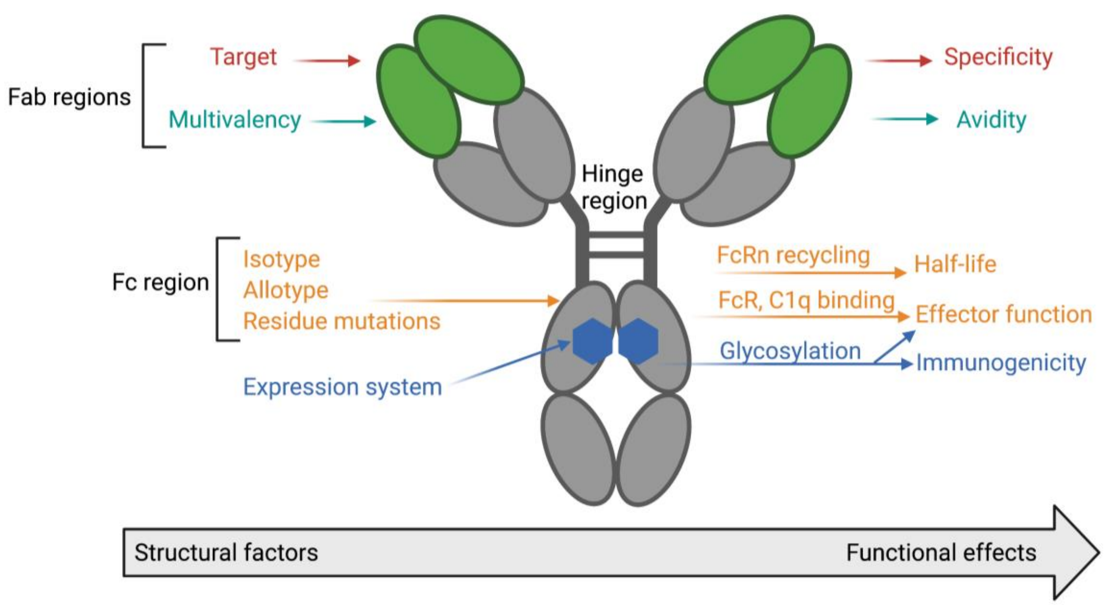

Figure 1.4- Structural considerations for antibody design and engineering. Figure adapted from Goulet & Atkins (2020)[39] and generated in Biorender.com.

Increasing Fab valency

The antigen-binding regions of antibodies can be recombinantly engineered to increase valency (Fig 1.5). Multivalent mAb constructs have multiple binding sites, usually through the addition of extra Fab arms, resulting in higher avidity or total binding strength at a single epitope site. For example, recombinant trivalent cancer antibodies maximize tumor targeting through enhanced biodistribution and functional affinity compared to bivalent counterparts[40]. Multivalent HCA constructs are also currently being explored to increase sperm agglutination potency. A hexavalent version of HCA possessing 6 Fab arms per molecule called “Fab-IgG-Fab” (FIF) demonstrated a 10-fold greater sperm agglutination than parent IgG[41,42].

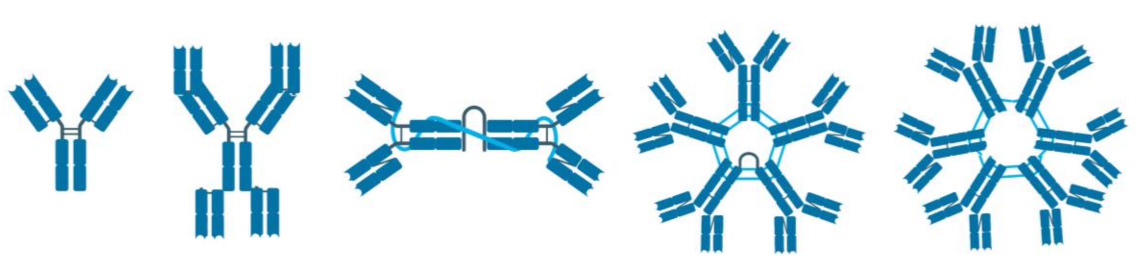

Figure 1.5- Structure of multivalent antibody variants. From left to right: Parent IgG, Fab-IgG-Fab (FIF), dimeric IgA, IgM, IgG/t (hexamer engineered using IgM tail). Figure generated in Biorender.com.

Another way to increase avidity is through multimerization, which requires polymerization of multiple IgG molecules to not only increase Fab sites but also Fc regions. One naturally occurring example of this is the isotype IgM, which polymerizes to form a pentamer with the J-chain or a hexamer without the J-chain[43]. However, recombinant IgM expression has historically been technically challenging; its complex structure and protein folding result in poor purification and oligomerization quality, poor stability and solubility, and low titers[44]. Advances in biotechnology have made it possible to circumvent these issues by directly fusing IgGs at their C-terminal lysine residues to the 18-amino acid C-terminal tailpiece of IgM resulting in a fully human hexamer with no mutations[45,46]. Not only is epitope binding more potent in these constructs, but the multimerization of Fc regions can also augment FcR binding functions like complement activation[47]. Our group has successfully constructed an HCA hexamer using this method which we call "IgG/t" ("t" for tailpiece). We have also successfully manufactured dimeric IgA HCA.

The Fc region and immune cell interactions within the FRT

The female reproductive tract is a unique and complex immune environment that is highly regulated by sex hormones throughout the menstrual cycle[48]. The lower reproductive tract (i.e. vagina and ectocervix) is composed of stratified squamous epithelium, which contains differentiating basal epithelial cells, intermediate epithelial cells, and the apical stratum corneum[49]. The upper reproductive tract (i.e. endocervix, endometrium, and uterus) is composed of a single layer of columnar epithelium. Immune cells such as T cells, B cells, macrophages, neutrophils, NK cells, and dendritic cells can be found underlying the vaginal epithelium and can be recruited through production of cytokines and chemokines[50]. The transformation zone, where the upper and lower epithelia meet, contains the highest number of immune cells[51], however immune cells fluctuate with hormone levels in order to meet the specific needs of the FRT such as defense against sexually transmitted pathogens and protection of a fetus or sperm from immune attack.

The predominant immunoglobulin isotype in the FRT is IgG, followed by IgA. Basal cells within the epithelia express the neonatal receptor FcRn which transports IgG from the basal layers to the lumen[52] where immunoglobulins are retained by the stratum corneum and eventually released. IgGs and IgAs execute protective functions through Fc interactions with Fcγ and Fcα receptors on immune cells, respectively. For example, macrophages expressing FcRs bind antibodies via the Fc region to enable recognition and phagocytosis of pathogens or foreign cells. The IgG Fc region can also interact with other immune components within the FRT such as complement and mucins to mediate cytotoxicity and mucus trapping[53]. Our research is beginning to elucidate HCA Fc-mediated effector functions.

Mutations of selected residues within the Fc region can affect FcR affinity and effector function. The established LALAPG amino acid substitution series (L234A/L235A/P329G) on the Fc region abolishes immune effector functions through reduced binding to Fc receptors and C1q[54]. These Fc-engineered variants are useful in scenarios where inflammation and immune responses are unwanted, like in the FRT. Several LALAPG antibodies have entered clinical trials. Other combinations of Fc residue mutations identified as the point of contact for Fc receptors can be engineered to alter Fc function, either improving or reducing receptor binding, to produce a desired clinical profile[55]. Applying these antibody engineering approaches to HCA could help further optimize a final HCA product.

The conserved IgG Fc N-glycan

Fc function is also highly regulated by *N*-glycosylation. Glycosylation is the most common posttranslational modification (PTM) on monoclonal antibodies; *N*-glycosylation occurs when an oligosaccharide is attached to the nitrogen atom of an asparagine (Asn) side chain. Monoclonal antibodies contain a conserved *N*-linked glycan at Asn 297 on the heavy chain constant domain (CH2) of the Fc region. This glycan profoundly influences a number of biological properties of an antibody such as pharmacokinetics, stability, safety, and most notably effector function[56]. *N*-glycans are required for activation of Fc receptors and complement to clear antigens through classic mechanisms such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC) as it is integral to the Fc structure. Depending on which specific features are present in the sugar moieties, certain glycan compositions can dictate receptor binding capacity. For example, a core glycan fucose dampens ADCC activity[57].

Because of the role glycosylation plays in a therapeutic antibody's functional profile, it is important to consider this feature as a critical quality attribute during engineering, production, and development[58]. However there is little published guidance on when and to what extent to implement glycosylation monitoring[59]. Choice of production platform, fermentation conditions, and expression system can affect glycan patterns; plant and mammalian host cells can often generate glycoforms that are immunogenic in humans[60]. Analytical methods such as mass spectrometry (MS) can be utilized to characterize the glycan pattern on antibodies prior to commercialization for quality control.

Current efforts in glycoengineering may facilitate the customization of glycans to generate a more homogenous and functionally desired profile.

A novel mRNA delivery system of HCA

Advantages of mRNA delivery

The delivery of nucleotide sequences encoding mAbs is a promising alternative to traditional methods of protein production[61]. Rather than administering a mAb protein generated in a foreign host, mRNA therapeutics rely on delivering genetic information directly to the host for in situ production. This has several advantages over both protein and plasmid DNA delivery: there is no risk of genome integration and expression is transient, both contributing to a favorable safety profile, the mRNA production system is cost-effective and does not rely on mammalian cells, and PTMs are native to the host[62]. This mRNA approach has a wide breadth of applications since scalable quantities of mRNA can be designed and produced rapidly using good manufacturing practice without specific optimization given the mRNA sequence is available. This is especially useful in the context of disease outbreaks, as recently seen with the SARS-CoV-2 pandemic wherein pharmaceutical companies like Pfizer-BioNTech and Moderna saw success in the rapid production and efficacy of an mRNA vaccine[63]. These vaccines generate potent neutralizing antibody responses in humans with one or two immunizations.

Modifying synthetic mRNA

Several modifications are commonly made to synthetic mRNA to optimize its efficiency in protein expression for therapeutic applications (Fig 1.6). One widely used modification is the substitution of wild-type uridine by N1-methyl-pseudouridine (N1**Ψ**). Incorporation of this modified nucleotide reduces immune stimulatory activity and thus degradation since pseudouridine fails to activate innate immune sensors like Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)[64]. Protein yield is dependent on translation rate and ribosome density. Pseudouridine increases translation efficiency by favoring ribosome loading and recycling[65]. Prior to application, synthetic mRNA is purified for the removal of double-stranded RNA (dsRNA) byproducts of in vitro transcribed (IVT) mRNA synthesis. Synthetic mRNAs also include a 5′ cap for stability and promotion of ribosome recruitment via binding initiation factor 4E. The 3′ UTR and poly(A) tail length can be modified to regulate half-life, which is based on the processivity of poly(A) polymerase[66]. Taken together, these modifications provide an mRNA product that resembles mature, naturally occurring mRNA in eukaryotic cells.

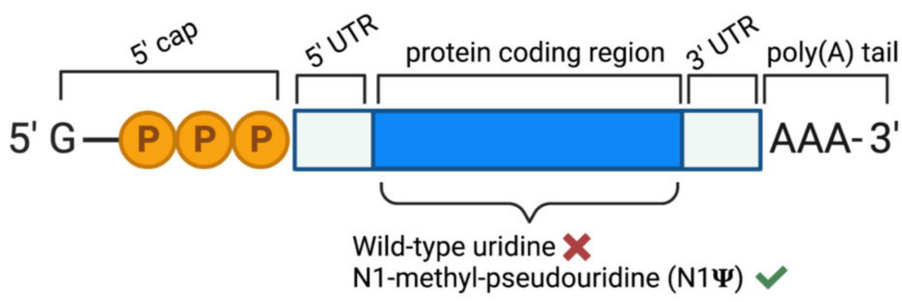

Figure 1.6- Common modifications on synthetic mRNA for therapeutic use. Within the protein coding region, N1-methyl-pseudouridine is used instead of wild-type uridine. UTR, untranslated region. Figure generated in Biorender.com.

There are several modes of mRNA delivery. The simplest method is administration of naked mRNA, though various classes of nanoparticles (NPs) are being explored to enhance delivery. NPs are self-assembled particles that can be used to load mRNA for in vivo delivery. The most popular delivery vehicle in use is the lipid nanoparticle (LNP) which allows endosomal release of mRNA into the cytoplasm. LNPs contain four main components: an ionizable cationic lipid for self-assembly (~100 nm), polyethylene glycol (PEG) to increase half-life, stabilizing cholesterol, and phospholipids to support the lipid bilayer[32]. NPs can also be formulated to target a specific location within the body either by net charge or with the inclusion of a target ligand. For example, positively charged LNPs target the lung and negatively charged LNPs target dendritic cells in lymphoid tissue and bone marrow[32].

Current therapeutic uses of mRNAs encoding monoclonal antibodies

Though monoclonal antibodies have become one of the most rapidly progressing and promising therapeutic approaches in biopharmaceutics, their success is limited by production costs and scalability[67]. mRNA-encoded mAbs may provide a more efficient alternative to traditional mAb production platforms. The first indication of mRNA as a platform for antibody production was in 2017 by Pardi et al. who demonstrated robust expression of an anti-HIV-1 monoclonal antibody following systemic delivery of modified mRNAs formulated into lipid nanoparticles (LNPs)[68]. Since then, mRNA has been used to successfully encode mAbs against infections and tumors in animal models. Infectious disease applications include respiratory syncytial virus (RSV), chikungunya virus, and

Zika virus[67]. For treatment of tumors, delivery of mRNAs encoding antibodies against non-Hodgkin's lymphoma tumor and HER2 induced antibody titers in vivo. mRNA-expressed bispecific T cell-engaging antibodies are also being explored to treat malignancy[67].

Topical delivery of mRNA to the FRT for HCA expression

The use of aerosolized (i.e. atomized) mRNA to transfect mucosal interfaces in vivo for monoclonal antibody expression was established by the Santangelo group, our collaborators at Emory University. The principle behind this delivery method is that hypotonic solutions such as water, when delivered via aerosol, alter the pressure at the host membrane facilitating pore formation and direct access of mRNA to the cytosol. Once in the cytosol, the heavy and light chain mRNAs are translated by the transfected cell's ribosomal machinery and assembled antibody is either secreted or, with the inclusion of a GPI-linker, anchored to the plasma membrane.

The Santangelo group previously demonstrated this paradigm in two different disease applications. Firstly, they showed that GPI-anchored mRNA-expressed anti-RSV antibodies, delivered via aerosol, were retained on the plasma membrane of transfected murine lung epithelial cells and prevented RSV infections in vivo[69]. Secondly, they showed that the entire lower female reproductive tract in sheep and macaques was conducive to transfection by aerosolized, naked, synthetic mRNA encoding a broadly neutralizing HIV-1 antibody[70]. After 28 days post-transfection, the GPI-anchored antibodies were still detectable in genital secretions and tissues at concentrations that effectively neutralized HIV.

An aim of this dissertation research was to apply this concept to HCA delivery by expressing mRNA-encoded HCA in the vagina (Fig 1.7). The mRNA-encoded HCA can either be secreted into the vaginal environment or anchored onto epithelial cells to prolong retention time and thus provide more long-term contraception. The mRNA can be delivered either complexed with a nanoparticle or naked in water to prevent immunostimulation. Aerosol-delivered HCA would be self-administered using an easy-to-use spray applicator currently undergoing design optimization. Overall, this HCA delivery system could provide a method of contraception that is safe, topical, reversible, nonhormonal, cost-effective, and controllable for retention time.

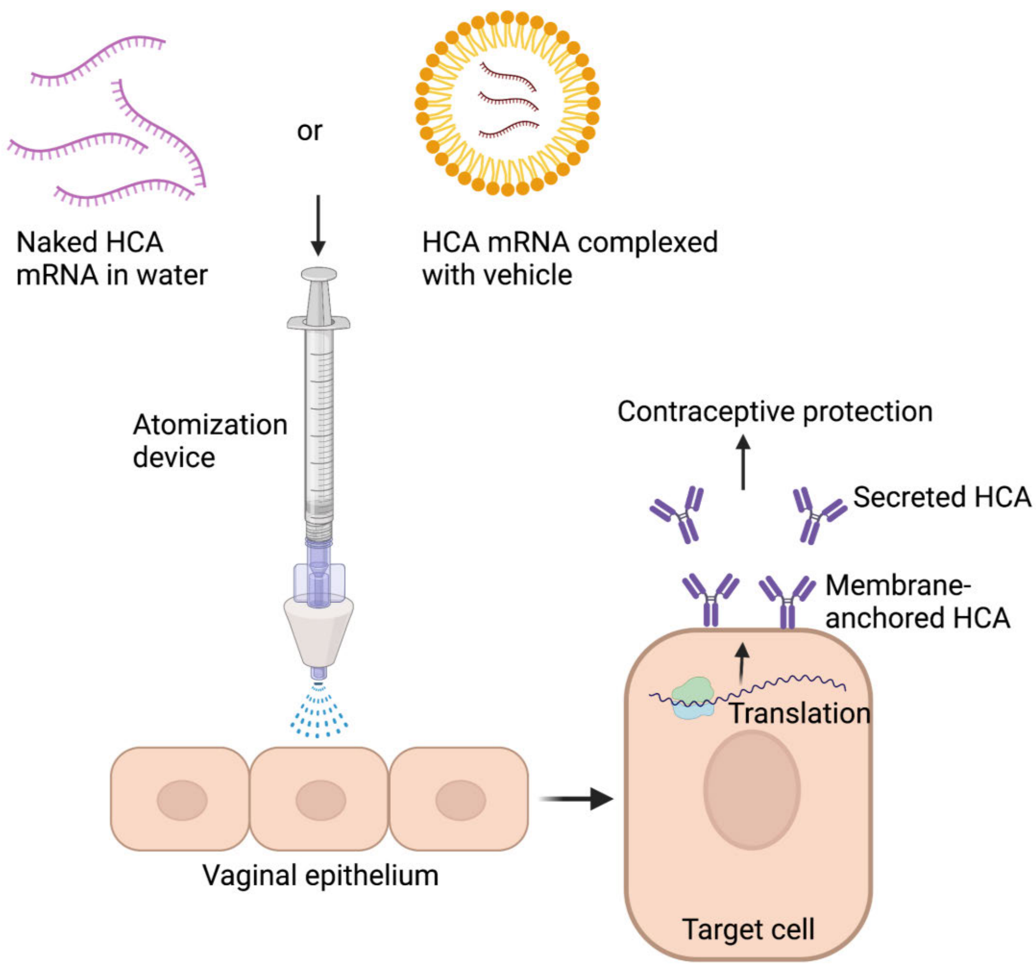

Figure 1.7- Schematic of atomized HCA mRNA delivery and antibody expression in the female reproductive tract. Either naked mRNAs in water or mRNAs complexed in a nanoparticle are loaded into an atomization device. mRNA is then sprayed directly onto vaginal tissue. Target cells uptake the mRNA and use ribosomal machinery within the cytosol to translate and express HCA. Depending on the mRNA construct, the HCA will either be secreted into the vaginal environment or anchored to the plasma membrane. Figure generated in Biorender.com.

Dissertation objectives

Rationale

There is an unmet need for the development of safer and more accessible contraceptive options to reduce high rates of unintended pregnancies worldwide. Development of antibody-based methods may represent a favorable approach in bringing more nonhormonal options to the market. Human Contraception Antibody (HCA) is an IgG1 monoclonal antibody that potently agglutinates and immobilizes human sperm, and is a promising candidate for nonhormonal immunocontraception in women. The studies below utilized antibody engineering and synthetic mRNA as innovative methods to improve the design and delivery of HCA. Overall, this body of work has the potential to provide a new mode of contraception for women seeking a nonhormonal, efficient, reversible, and self-administered product.

Project aims

Aim 1: Delineate Fab and Fc-mediated functions of HCA using an Fc-silenced mutant HCA-LALAPG to further evaluate HCA as a product.

We were interested in an Fc-mutated variant of HCA for two reasons: 1) to use as a tool to assess HCA Fc effector functions and 2) to determine if it could serve as a less inflammatory alternative to HCA. Sperm agglutination was compared between HCA and HCA-LALAPG to ensure Fab function was conserved. For Fc function, we specifically evaluated antibody-dependent cellular phagocytosis, mucus trapping, and complement-dependent sperm immobilization. Parent HCA was able to mediate all Fc functions evaluated while HCA-LALAPG was not.

Aim 2: Establish a topical mRNA delivery system of HCA and engineered variants using in vitro models of the female reproductive tract (FRT).

mRNA delivery by both atomization and traditional transfection methods were utilized to achieve expression of HCA in culture supernatant from a vaginal epithelial cell line and a 3D vaginal tissue model. Antibody retention and pharmacokinetics post-mRNA delivery were determined and were prolonged by anchoring the antibody to the membrane of vaginal cells with a GPI-linker. Bioactivity levels and sperm specificity of the mRNA-produced antibodies were assessed using agglutination assays and immunofluorescence, respectively. Vaginal tissue health following mRNA delivery and exposure was evaluated to ensure the safety of our method. mRNA-expressed HCA multimers were also explored to enhance agglutination potency.

Aim 3: Compare Fc *N*-glycan profiles of relevant production methods of HCA and determine their implications on quality control.

We characterized the conserved IgG Fc *N*-glycan on HCA, the glycosylation site necessary for effector function. IgG Fc *N*-glycan profiles and site occupancy of HCA produced in its current commercial platform, *Nicotiana benthamiana*, and HCA expressed via mRNA in vaginal cells, our new platform of interest, were compared to inform further production and development. We determined that differences in glycan profiles between platform-specific HCAs regulated antibody-dependent cellular phagocytosis (ADCP), an Fc function.

CHAPTER TWO: LALAPG variant of the human contraception antibody (HCA) reduces Fc-mediated effector functions while maintaining sperm agglutination activity

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Mausser E, Nador E, Politch JA, Pauly MR, Marathe JG, Moench TR, et al. (2023) LALAPG variant of the Human Contraception Antibody (HCA) reduces Fc-mediated effector functions while maintaining sperm agglutination activity. PLoS ONE 18(3): e0282147. https://doi.org/10.1371/journal.pone.0282147

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Contributions: EM performed the sperm immobilization and cervical mucus penetration assays. EN performed the agglutination and phagocytosis assays. JGM organized cervical mucus sampling. JAP organized semen sampling and assisted in statistical analysis. DJA led conceptualization of the study. MRP, TRM, LZ, and KJW provided reagents. All authors contributed to the writing, reviewing, and editing of this manuscript.

Abstract

High rates of unintended pregnancies worldwide indicate a need for more accessible and acceptable methods of contraception. We have developed a monoclonal antibody, the Human Contraception Antibody (HCA), for use by women in vaginal films and rings for contraception. The divalent F(ab')2 region of HCA binds to an abundant male reproductive tract-specific antigen, CD52g, and potently agglutinates sperm. Certain other antibody activities mediated by the Fc region such as mucus trapping, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular phagocytosis (ADCP) could have beneficial or negative effects. The purpose of this study was to document HCA Fc effector functions and determine whether an engineered variant of HCA with a modified Fc region, HCA-LALAPG, retains desirable contraceptive activity while minimizing Fc-mediated effects. Fab and Fc functions were compared between HCA and HCA-LALAPG. Fab activity was assessed using sperm agglutination and modified swim-up ("sperm escape") assays. Fc functions were assessed by CDC (sperm immobilization), ADCP, and cervical mucus penetration assays. HCA and HCA-LALAPG showed equivalent activity in assays of Fab function. In the assays of Fc function, HCA supported strong CDC, ADCP, and sperm trapping in cervical mucus whereas HCA-LALAPG demonstrated little to no activity. HCA and the HCA-LALAPG variant were both highly effective in the sperm agglutination assays but differed in Fc mediated functions. Use of the HCA-LALAPG variant for contraception in women could reduce antibody-mediated inflammation and antigen presentation but may have reduced contraceptive efficacy due to much weaker sperm trapping in mucus and complement-dependent sperm immobilization activity.

Introduction

The United Nations reports that almost half of all pregnancies are unintended due to lack of or incorrect use of effective contraception[2]. This points to the need for better contraception education and services, and access to more acceptable, easy to use contraception methods. Male contraception and new nonhormonal products for women may fill this gap and allow greater control of reproduction. A new contraceptive product that is low-cost, easily accessible, user-controlled, non-invasive, reversible, safe, and unobtrusive during intercourse might be popular among women who presently do not consistently use effective contraception methods due to their high cost, limited access and undesirable side effects[15,71]. A promising new contraceptive candidate is the Human Contraception Antibody (HCA), an antisperm antibody currently in clinical development as a topical nonhormonal contraceptive for women[29]. HCA binds to a glycoprotein, CD52g, on the surface of sperm, and has been shown to rapidly agglutinate sperm and prevent their progression through the female reproductive tract.

Over 100 antibody-based drugs have been approved by the US Food and Drug Administration (FDA) with many more currently in clinical trials[72]. While a majority of these are for treatment of cancer and autoimmune disorders, there is growing interest in using antibodies to prevent and treat a wider range of indications, including infectious diseases and unintended pregnancy. We are currently using a transgenic *Nicotiana*-based production platform to produce monoclonal antibodies (mAbs) for reproductive health. Our group recently tested a multipurpose prevention technology (MPT) product, a vaginal film containing *Nicotiana*-based mAbs against human immunodeficiency virus 1 (HIV-1) and herpes simplex virus type 2 (HSV-2), in a phase 1 clinical trial. The film, MB66, was safe and maintained antiviral activity in the vaginal mucosa for up to 24 hours[34]. The contraceptive mAb, HCA formulated into a vaginal film was recently tested in a phase 1 clinical trial where it demonstrated safety and contraceptive efficacy in postcoital tests (Clinical Trial Registration: NCT04731818). The HCA vaginal films used in the phase I clinical trials are manufactured at 20 mg per film. The expected concentration of HCA in the FRT following film application may be lower than 20 mg due to user error and/or differences in cervicovaginal secretion volume, but the exact concentration is not yet known and will be answered by ongoing clinical trials.

The majority of mAbs produced for clinical applications, including our lead HIV-1, HSV-2, and HCA mAbs, belong to the IgG1 antibody subclass[73]. IgG1 mAbs bind to target antigens through the variable (Fab) region and can participate in other effector functions through the constant (Fc) region. Many immune cells express receptors that specifically bind to the Fc region of IgGs (FcγRs). FcγRs recognize IgG-coated targets such as opsonized pathogens or immune complexes; cross-linking leads to internalization and activation of downstream signaling cascades. Two effector cell activities elicited by FcγR activation include antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cellular cytotoxicity (ADCC). The IgG1 Fc region also contains binding sites for the complement component C1q which can initiate the complement cascade leading to complement-dependent cytotoxicity (CDC)[74]. IgG Fc can also bind to the neonatal Fc receptor, FcRn, that regulates antibody recycling[75]. The Fc region may also play a role in trapping pathogens and sperm in mucus. Jager et al. were the first to report that sperm coated with antisperm antibodies become trapped in cervical mucus and that the Fc region may play a role[76]. A number of recent studies provide further evidence that multiple weak bonds between IgG-Fc and mucins trap IgG-coated pathogens and sperm in mucus[53].

Fc-mediated antibody functions are desirable for certain applications such as mAb-mediated pathogen clearance, but they may be undesirable in other situations. ADCC and CDC can elicit inflammation[77] and ADCP may stimulate downstream adaptive immune responses by facilitating antigen presentation[78]. In the case of HCA vaginal film, an Fc-mediated inflammatory response could enhance HIV transmission[79], and presentation of sperm antigens by immune cells in the female reproductive tract following ADCP could potentially lead to antisperm immunity and infertility[80]. Fc interactions and consequent effector cell functions can be tailored through Fc engineering[81], and a number of genetically engineered Fc variants have been introduced. One such variant, LS, contains a mutation associated with improved binding to the FcRn receptor and increased antibody serum half-life which would require less frequent dosing of an antibody-based drug[82]. Fc engineering can also be used to reduce effector function to minimize unwanted inflammation and acquired immunity induced by antibody-based drugs. The most common of these Fc designs, known as LALAPG, includes three point mutations: L234A, L235A, and P329G[83] (Fig 2.1). LALAPG variants inhibit binding to FcγRs and C1q while FcRn binding and Fc stability remain unaffected[84]. Effects of LALAPG mutations on Fc-mucus interactions have not been described. Several antibodies with LALAPG mutations are currently undergoing testing in clinical trials[54]. Engineering the Fc region of antibody-based drugs can help tailor the drug for a specific desired effect.

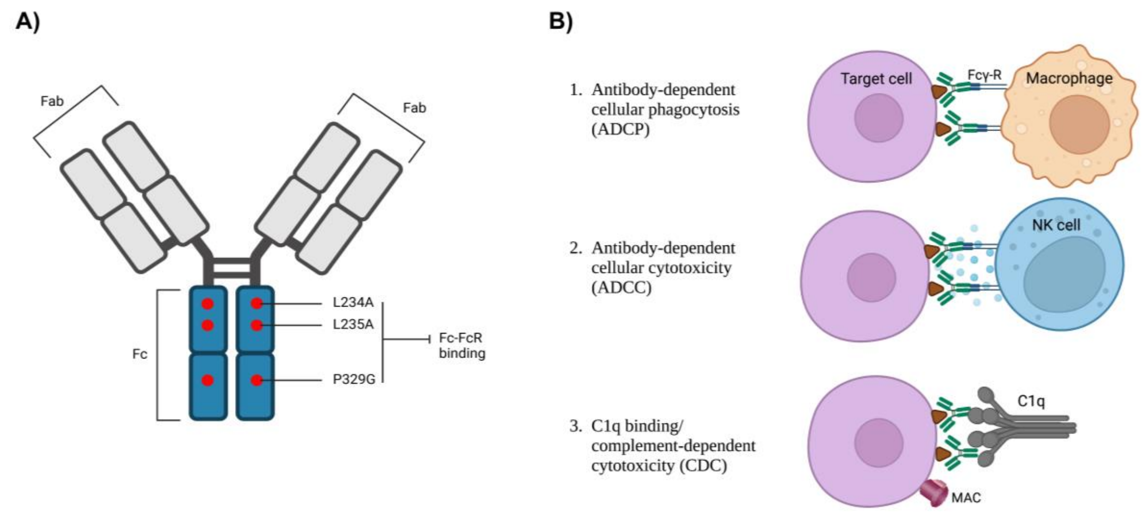

Fig 2.1- LALA-PG mutation and Fc functions of IgG. A) Diagram of Fc-engineered HCA variant. Red dots on the Fc region indicate approximate locations of the three amino acid mutations and their respective residue substitutions. B) Fc functions inhibited by the Fc LALA-PG mutation. Created with BioRender.com.

As part of clinical development, various engineered versions of HCA are being tested to determine whether they enhance antibody potency and reduce potential side effects. HCA variants have been engineered with increased valency to enhance agglutination potency[42]. A LALAPG HCA variant has also been developed and is being explored as a means to reduce the potential for undesirable Fc-mediated side effects such as inflammation and acquired immunity[55,85]. In this study, we compared Fab and Fc functions of HCA and HCA-LALAPG to more fully elucidate the mechanisms of action of HCA, and to inform the optimal design of this antisperm antibody for contraceptive use.

Methods

Antibodies

Human Contraception Antibody (HCA)/HCA-LALAPG:

HCA, a monoclonal human anti-CD52g mAb, was produced in *Nicotiana benthamiana* from the variable region sequence of H6-3C4[86] and an optimized human IgG1 constant region as previously described[29]. This IgG1 form of HCA is also referred to as HC4-N. Transgenic *Nicotiana* plants with knockdown of fucosyl- and xylosyl-transferases allowed for the insertion of humanized glycans as previously described[33]. HCA-LALAPG was produced in the *Nicotiana* mAb platform from a DNA template with three amino acid substitutions in the Fc region: L234A, L235A, and P329G.

Campath-1:

Campath-1, or alemtuzumab, is a humanized IgG monoclonal antibody used for cancer immunotherapy (ThermoFisher Scientific Cat# MA5-16999, Waltham, MA, USA, RRID: AB_2538471). Campath-1 binds to a peptide epitope present on CD52, an antigen found on normal and malignant hematopoietic cells, and CD52g, a heavily glycosylated male reproductive tract-specific form of CD52. HCA binds to a carbohydrate epitope only found on CD52g. Campath-1 was used as a positive control for the sperm phagocytosis assay as it has been shown to bind to sperm[4] and activate ADCP by macrophages[87].

VRC01-N:

VRC01-N, a broadly neutralizing anti-HIV-1 IgG1 antibody[88], was also produced in *Nicotiana*. It was used as an isotype control in the sperm phagocytosis assay.

Sample collection and processing

This study was approved by the Institutional Review Board at Boston University Medical Campus (Human Subjects Protocols H36843 and H41454). All participants provided written informed consent prior to participation.

Semen:

Human semen samples were obtained from healthy men aged 18-45 after at least two days of sexual abstinence. All samples met the WHO criteria for fertility[89]. Samples were processed within one hour of collection and liquefaction at 32 °C. All sperm incubations were conducted at 32 °C as this is approximately the temperature within the testes, which is lower than normal body temperature. Whole semen was overlaid on an equal volume of 90% ISolate density gradient (FUJIFILM Irvine Scientific; Santa Ana, CA, USA) and centrifuged at 300 x g for 20 minutes. The sperm pellet containing progressively motile sperm was re-suspended in Multipurpose Handling Medium (MHM; FUJIFILM Irvine Scientific; Santa Ana, CA, USA). Sperm concentration and motility parameters were assessed using a Computer-Assisted Sperm Analysis system (CASA; Human Motility II software, CEROS II, Hamilton Thorne, Beverly, MA, USA). Sperm concentrations were adjusted to 25-40 x 10^6/mL for use in the assays.

Cervical mucus:

Midcycle cervical mucus was collected by a trained physician from reproductive-aged women within 48 hours of a positive ovulation test (Digital Ovulation Predictor Kit, Clearblue). An endocervical pipelle (Aspirette Endocervical Pipelle, Cooper Surgical, Trumbull, CT, USA) was used to aspirate pure cervical mucus directly from the endocervical canal. None of the women were on hormonal contraception. Ovulation was confirmed for all women by evaluation of the mucus (clear with spinnbarkeit characteristics), and by estrogen and progesterone levels in blood. Cervical mucus was diluted 1:3 in PBS and aspirated into flat capillary tubes (Borosilicate Capillary Glass Slide, 0.30 x 3.0 mm, 50 mm, Electron Microscopy Sciences, Hatfield, PA, USA). Both ends of the tube were sealed with parafilm, and samples were stored at 4°C for up to 5 days. Cervical mucus samples from four different donors were used for this study.

Agglutination kinetics assay

The effects of HCA and HCA-LALAPG on sperm agglutination were determined using the sperm agglutination kinetic assay as described previously[4]. A 2 µL sample of sperm cell suspension in MHM was added to a multi-Spot microscope slide (ThermoScientific, Waltham, MA, USA). An equal volume of antibody diluted in MHM was mixed into the well containing sperm. Agglutination of sperm was observed in real time on an Olympus inverted microscope at 10x. The time elapsed to achieve complete agglutination (i.e., 100% agglutinated sperm), was recorded. Since it has been reported that sperm can reach the endocervical canal in 3-5 minutes, a cutoff time of 2.5 min was used for the assay[29,90,91]. Data points were acquired in triplicate and the experiment was repeated three times using semen from different donors.

Sperm escape assay

The percentage of progressively-motile sperm that escaped sperm agglutinates after antibody exposure was quantified as described previously[29]. Sperm were added to PCR tubes containing HCA, HCA-LALAPG or medium alone (control) and incubated at room temperature at a 45-degree angle for 5 minutes. A 2.5 µL sample was taken from the top millimeter of the tube and placed in a 4-chamber slide (Microcell 15424, Vitrolife, San Diego, CA, USA) for counting on the CASA. Data points were acquired in triplicate and the experiment was repeated using semen samples from three different donors. Data were presented as percent of control (medium alone).

Complement-dependent sperm immobilization test

The sperm immobilization test was performed as described previously[29,92,93]. Freshly collected serum, used as a complement source, was stored at -80 °C and thawed on ice before initiating the assay. Briefly, 2.5 µL of washed sperm were sensitized with 5 µL of human serum for 5 minutes, then 25 µL of diluted antibody was added and the samples were incubated in 4-chambered slides inside a wet box for 60 minutes at 32 °C. Resulting sperm motility was measured using the CASA. Heat-inactivated complement (HiC), used as a negative control, was prepared by heating serum at 56 °C for 30 min. Experiments were performed three times with samples from different semen donors.

Cervical mucus penetration assay

Capillary tubes containing cervical mucus were warmed to 32 °C and each 1 cm interval on the capillary tube was marked. Motile sperm were prepared as described above but re-suspended at a concentration of 25-40 x 10^6/mL in seminal plasma instead of MHM. Antibodies were diluted to 12.5 μg/mL in MHM. Ten μL of diluted antibody or MHM alone (control) was added to one end of the capillary tube, and the opposite end was then sealed with parafilm. The open end of the capillary tube was inserted horizontally into a tube containing 100 μL of sperm so that the sperm would swim through the antibody or medium (control) before entering the column of cervical mucus. Tubes were incubated in a humidified chamber at 32 °C. At 30-, 60- and 90-minute timepoints, sperm were assessed at 1, 2, 3 and 4 cm depths using the CASA. Progressive sperm in each section were scored by manual review of the CASA output by a trained researcher.

Antibody-dependent sperm phagocytosis assay

U937 (CRL-1593.2, obtained directly from ATCC) pro-monocytes were seeded onto sterile glass coverslips in 6-well plates at a density of 0.5 x 10^6 cells/ml in 3 mL of RPMI 1640 complete medium (Gibco, ThermoScientific, Waltham, MA, USA; supplemented with +L-glutamine, 10%FBS, 1% Penicillin-Streptomycin). Cells were treated with 100 ng/mL phorbol-12-myristate-13-acetate (PMA) to induce differentiation into macrophages[94] and were incubated under an atmosphere of 5% CO_2 at 37 °C for 48-72 hours, after which they became adherent to the coverslips.

The sperm phagocytosis assay was performed as described by Oren-Benatoya et al. with modifications[95]. In brief, the growth medium in the macrophage cultures was discarded and 1 x 10^6 sperm in either MHM or in MHM supplemented with 50 µg/mL antibody were added to each well. Following a 30-minute incubation at 37 °C and 5% CO_2, the macrophage cultures were washed 3X with PBS. After another 30-minute incubation at 37 °C in PBS, the macrophage cultures were incubated in trypsin for 5 minutes to remove non-specifically bound spermatozoa. The coverslips were treated with Differential Quik III fixative solution (Polysciences, Warrington, PA, USA), stained with Differential Quik III stain solution I and II, and washed in tap water before being mounted to glass slides. Macrophages were observed under a light microscope at X200 magnification, and the number of spermatozoa associated with 100-300 macrophages was counted for each treatment group. Number of internalized sperm per macrophage was also assessed and characterized as sperm partially engulfed, either head or tail, or wholly engulfed by a macrophage. The experiment was repeated three times using semen samples from different donors.

Statistical analyses

Data were analyzed by either repeated measures analysis of variance (ANOVA) or mixed-effects analysis. A significant analysis was followed by either post hoc Tukey or Sidak multiple comparison tests as indicated by the type of analysis. If continuous variables were not normally distributed, they were log (natural) transformed prior to analysis. The equality of variances of the pairwise differences of within subject conditions was assessed through a test of sphericity (Geisser-Greenhouse's epsilon). GraphPad Prism (Version 9.4.1; GraphPad Software Inc.; San Diego, CA, USA) and JMP Pro (Version 15.2.0; SAS Institute Inc., Cary, NC, USA) were used for statistical analysis and graph creation. Differences were considered to be statistically significant when $p<0.05$.

Results

Both HCA and HCA-LALAPG robustly agglutinate sperm

Because agglutination of sperm relies on the Fab portion of HCA, Fc mutations would not be expected to impact agglutination potency. We compared the agglutination function of parent HCA and HCA-LALAPG using agglutination kinetics and sperm escape assays. As expected, the two antisperm antibodies did not differ in time to 100% agglutination over a wide concentration range ($p > 0.6$, Fig 2.2A). In fact, the two concentration curves appeared to be superimposed. At 100 µg/mL, both antibodies agglutinated 100% of sperm within 30 seconds. At the lowest concentration, 6.25 µg/mL, the antibodies agglutinated 100% of sperm within 60 seconds, well below the 150 second time cutoff. Likewise, HCA and HCA-LALAPG did not differ significantly in percent of progressively motile sperm cells in the sperm escape assay (sperm that "escaped" agglutination), following a 5-minute incubation of sperm and antibody ($p > 0.7$, Fig 2.2B). At an antibody concentration of 100 µg/mL, the number of progressively motile sperm for both the HCA and HCA-LALAPG antibodies was <1% of the medium-only control; the percent increased to 12-15% at an antibody concentration of 6.25 µg/mL.

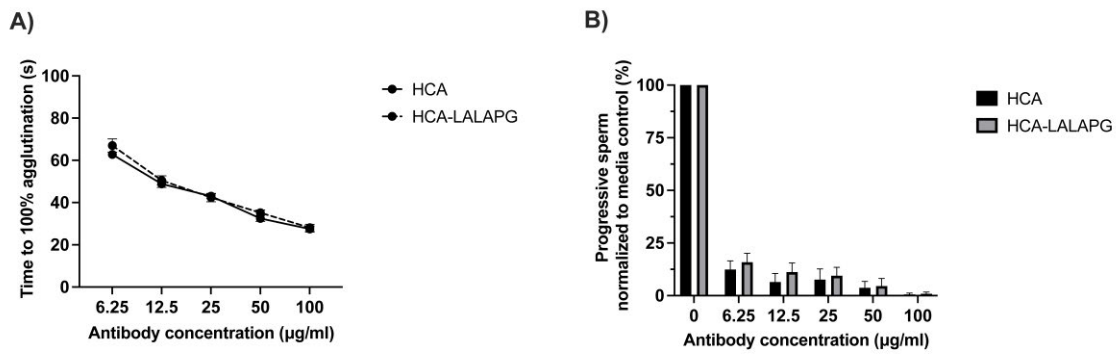

Fig 2.2- Sperm agglutination by HCA and HCA-LALAPG. A) Agglutination kinetics assay shows similar dose response curves for HCA and HCA-LALAPG. B) Sperm escape assay shows similar percent of progressive sperm relative to the medium-only control for HCA and HCA-LALAPG at all observed antibody concentrations. Data are expressed as means ± SEM of three independently performed experiments. Statistical analyses were conducted using repeated measures two-way ANOVA of log-transformed data followed by Sidak multiple comparisons test. No significant differences were found between the two groups for either assay ($p > 0.6$). SEM, standard error of the mean

HCA immobilizes sperm in the presence of complement whereas HCA-LALAPG does not

HCA significantly immobilized sperm in the presence of complement; sperm motility was significantly reduced at HCA concentrations ranging from 12.5 to 3.12 µg/mL (Fig 2.3, $p < 0.001$). At these concentrations, HCA agglutinated sperm but there were sufficient numbers of non-agglutinated sperm to enable motility assessment. Agglutinated sperm clump together but still retain movement though they do not show forward progression ("wiggling in place"). In complement-mediated immobilization, sperm still clump together in agglutinates, but the sperm are static and show no movement. Sperm motility was not affected by HCA in the presence of HiC. HCA-LALAPG, which is unable to bind the C1q complement protein, did not immobilize sperm at any antibody concentration tested ($p = 0.9105$).

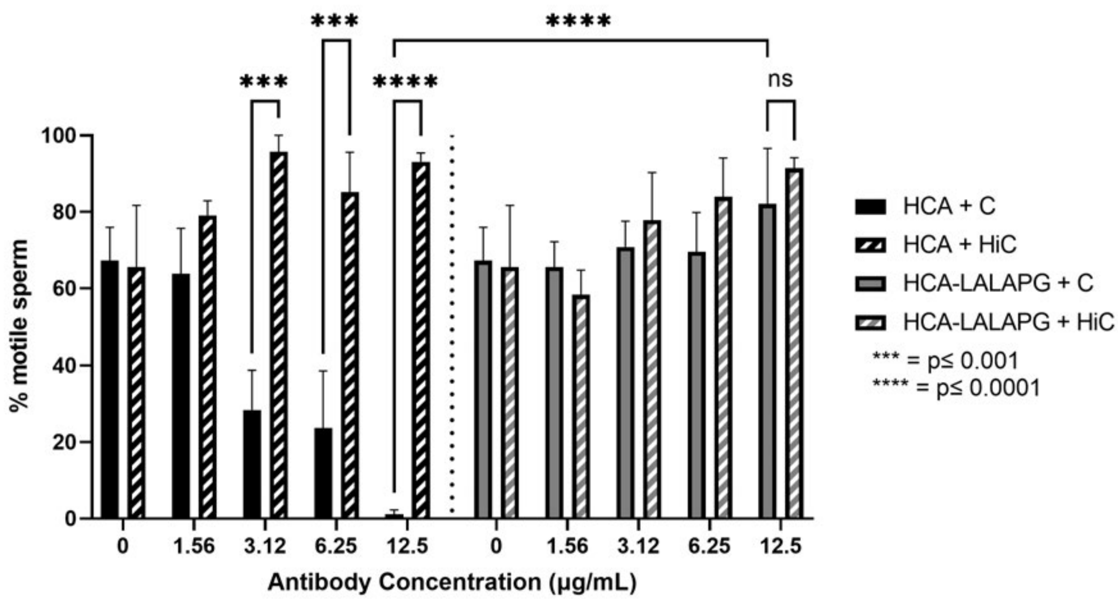

Fig 2.3- Sperm immobilization test with HCA and HCA-LALAPG. HCA significantly immobilized sperm in the presence of complement (C) in a concentration-dependent manner ($p = <0.0001, 0.0005, 0.0001$ for 12.5, 6.25, and 3.12 µg/mL, respectively). Heat-inactivated complement (HiC) was used as a negative control and did not cause immobilization. HCA-LALAPG did not significantly affect sperm motility with either C or HiC ($p = 0.9105$). Data are shown as mean ± SEM and is representative of three independently performed experiments. Statistical analyses were conducted using a two-way ANOVA followed by a Tukey multiple comparisons test. Results were significant when $p \leq 0.05$. (*** = $p \leq 0.001$, **** = $p \leq 0.0001$)

HCA traps sperm in cervical mucus whereas HCA-LALAPG does not

HCA significantly reduced the penetration of progressive sperm through midcycle cervical mucus at the 30-, 60-, and 90-minute timepoints compared to the no-antibody control and HCA-LALAPG. (Fig 2.S1 and Table 2.S1). By the 90-minute timepoint, the number of progressively motile sperm in cervical mucus was dramatically lower for HCA-treated sperm than for medium- and HCA-LALAPG-treated sperm at all four penetration depths (Table 2.1A). The number of progressively motile sperm in cervical mucus after treatment with HCA-LALAPG did not differ significantly from the medium-only control condition and was significantly higher than the number of progressively motile sperm treated with HCA (Table 2.1B and Fig 2.4 for specific pairwise comparisons).

Table 2.1- Cervical mucus penetration at 90 minutes with HCA and HCA-LALAPG.

A.

Condition	90 minutes			
	1cm	2cm	3cm	4cm
Control (no antibody)	65.00 ±34.59	75.00 ±43.16	31.5 ±21.74	10.75 ±8.43
+HCA	2.5 ±1.89	5.75 ±3.33	1.75 ±0.85	1.00 ±0.71
+HCA-LALAPG	33.75 ±12.99	34.5 ±12.48	58.75 ±36.54	22.5 ±21.17

B.

Comparison	90 minutes			
	1cm	2cm	3cm	4cm
Control vs. HCA	p<0.0001	p=0.0001	p=0.002	p=0.02
Control vs. HCA-LALAPG	p=0.38	p=0.75	p=0.35	p=0.10
HCA vs. HCA-LALAPG	p<0.0001	p=0.0007	p<0.0001	p=0.03

A) Number of progressively motile sperm in the cervical mucus penetration test after 90 minutes (shown as mean ± SEM). B) Statistical differences between treatment groups at each distance after 90 minutes.

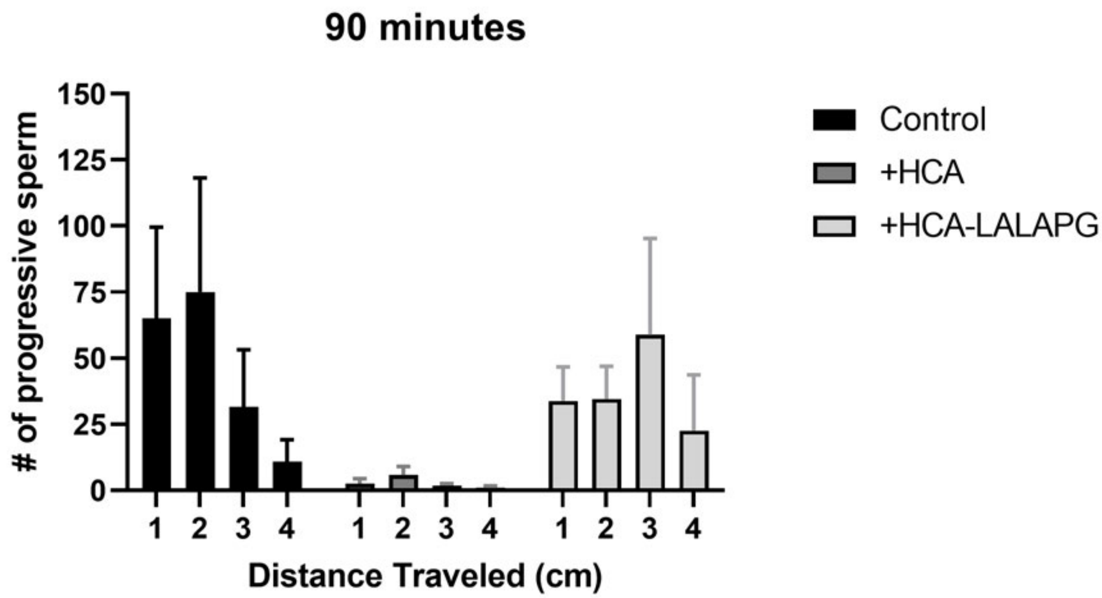

Fig 2.4- Cervical mucus penetration test with HCA and HCA-LALAPG. The number of progressively motile sperm at 90 minutes in ovulatory cervical mucus was significantly lower in the HCA treatment group than with HCA-LALAPG at all capillary tube depths (1-4 cm). Both antibodies were used at (12.5 μg/mL). Data are shown as mean ± SEM and is representative of four independent experiments, each with a different cervical mucus donor. Statistical analyses were conducted using a repeated measures ANOVA of log transformed data followed by a Tukey multiple comparisons test. Results were significant when $p \leq 0.05$. (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$)

The greatest differences between treatments were seen within the first two centimeters (Fig 2.4). Agglutinated sperm were observed at the entrance of the cervical mucus column (<1 cm depth) with both HCA and HCA-LALAPG treatments, but since the antibody concentration was low, individual sperm were also able to penetrate into the cervical mucus column. In HCA-treated samples, very few progressively motile sperm were observed; the majority of individual sperm appeared to be trapped in the cervical mucus as visualized by rapid vibration of the sperm without any forward motility. HCA-LALAPG also agglutinated sperm at the interface with cervical mucus, but there were far more individual sperm with forward progression. As shown in Table 2.1A, the number of progressively motile sperm that penetrated the cervical mucus column was comparable between the medium-only control and HCA-LALAPG-treatment groups at 90 minutes; there were significantly fewer progressive sperm in the HCA treatment group compared to both no antibody and HCA-LALAPG (Table 2.1B). Sperm progression through cervical mucus varied between the four cervical mucus donors (Fig 2.S2); one subject had inhospitable mucus that did not permit sperm penetration regardless of treatment group, whereas three subjects had hospitable mucus that showed similar patterns of sperm penetration in medium-only control and HCA-LALAPG-treated samples, and significant trapping in HCA-treated samples.

HCA can induce ADCP whereas HCA-LALAPG cannot

Fc effector functions of HCA have not previously been fully characterized. We are particularly interested in the capacity of HCA to mediate antibody-dependent cellular phagocytosis (ADCP) since antigen presentation of sperm antigens could lead to antisperm immunity and infertility. Differentiated U937 macrophage-like cells were incubated for 30 minutes with antibody-treated sperm, after which they were washed for 30 minutes in PBS and treated with trypsin for 5 minutes to remove non-specifically bound spermatozoa. Fixed slides were stained with Diff Quik stain kit, a morphology dye stain, and observed under a light microscope to quantify the number of associated spermatozoa per macrophage-like cell. Associated spermatozoa were characterized as those that were either attached to the surface of a cell (i.e., in the beginning of the phagocytosis process), or partially engulfed. The positive control antibody, Campath, elicited the greatest level of sperm association with more than one spermatozoon per effector cell (Fig 2.5A). The number of sperm associated with U937 macrophages after HCA treatment was ~ 30% of the mean in the Campath condition and was significantly higher than both medium-only control and HCA-LALAPG treatment ($p = 0.013$ for both comparisons). HCA-LALAPG treatment was not significantly different from the medium-only control ($p > 0.99$). Attached sperm were clearly visible by microscopy in the HCA- and Campath-treated conditions, while no sperm were visible in the HCA-LALAPG- or medium-only-treated conditions (Fig 2.5B).

A phagocytosis time-course experiment was conducted to determine whether there was an effect of incubation time on the number of HCA-coated sperm that were phagocytosed by U937 macrophages. Internalized sperm were scored as either partially (head or tail) or wholly phagocytosed. The number of internalized spermatozoa per macrophage increased over time (Fig 2.S3) and reached a maximum after 2.5-hours ($p = 0.018$). The number of phagocytosed sperm was significantly higher in HCA treated cultures ($p=0.037$) than in medium-only-, HCA-LALAPG-, or VRC01-N- (negative control antibody) treated cultures (Fig 2.5C). U937 macrophages were occasionally observed with numerous internalized HCA-treated sperm (Fig 2.5D), whereas this was not observed with HCA-LALAPG, medium or VRC01-N treatment.

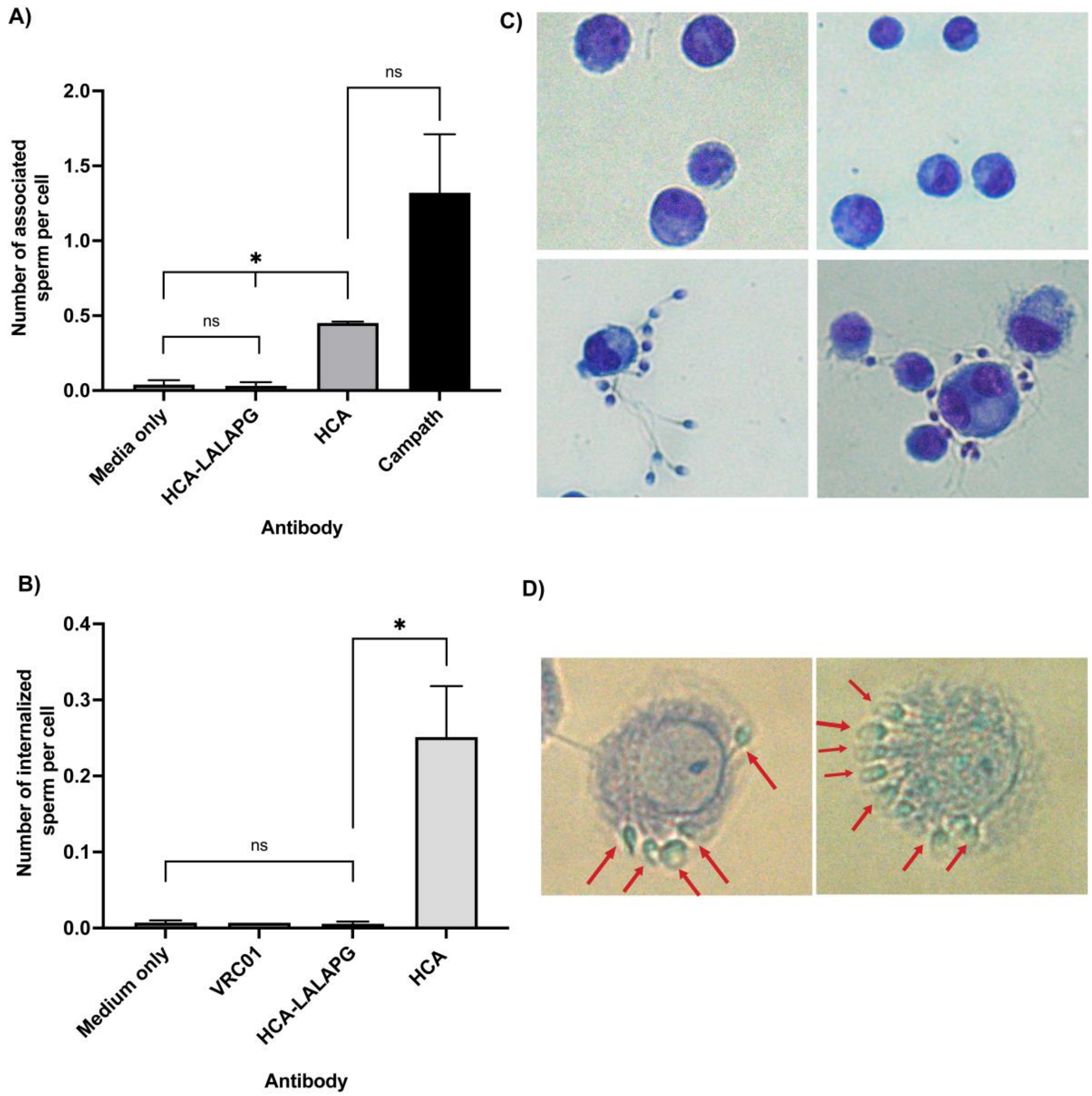

Fig 2.5- Antibody-dependent sperm phagocytosis. A) Significantly more sperm were associated with U937 macrophages in HCA-treated cultures than in cultures treated with HCA-LALAPG or medium-only control (p=0.0128, p=0.0131, respectively). "Associated sperm" were defined as those either attached to the surface of the macrophage (i.e., at the beginning of the

phagocytosis process), or inside the cell. Campath, an anti-CD52 monoclonal antibody known to mediate ADCP effector function, was used as a positive control. Final antibody concentrations were 50 µg/mL. B) The number of internalized sperm per macrophage was quantified following a 2.5-hour incubation with antibody-treated sperm. Final antibody concentrations were 50 µg/mL. The number of internalized sperm was significantly higher following treatment with HCA than with HCA-LALAPG, medium-only control, or VRC01 isotype control ($p = 0.037$; negative control run twice, isotype control run once). (C) Images of macrophages incubated with sperm exposed to either medium-only (top left), HCA-LALAPG (top right), HCA (bottom left), or Campath (bottom right). Images were taken at 200x magnification. (D) Sperm internalization post-HCA treatment (red arrows) by U937 macrophages. Images were taken at 400X magnification. Data are expressed as mean ± SEM of three independently performed experiments. Statistical analyses were conducted using either repeated measures one-way ANOVA or mixed-effects analysis of log-transformed data followed by Tukey multiple comparisons tests.

Discussion

HCA is an antisperm IgG monoclonal antibody currently being studied in preclinical and clinical studies for its contraceptive activity. Here, we characterized an Fc-engineered variant (HCA-LALAPG) to delineate Fab and Fc-mediated functions and to evaluate an alternate vaginal film candidate that may reduce undesirable inflammatory and immune side effects. No differences in sperm agglutination potency were detected. Agglutination, mediated by the Fab region, is thought to be the main contraceptive mechanism of HCA. Both HCA and HCA-LALAPG rapidly agglutinated sperm within 60 seconds[96] even at low antibody concentrations. Thus, engineering and customization of the Fc region of HCA does not affect Fab-mediated sperm agglutination.

Differences in Fc-mediated functions between HCA and HCA-LALAPG were observed. Some antisperm antibodies can immobilize sperm in the presence of a complement source[92,93] . We have previously shown the ability of HCA to immobilize sperm in a concentration dependent manner in the presence of human serum (complement source)[29]. HCA did not immobilize sperm in the presence of heat-inactivated complement (HiC), demonstrating that sperm immobilization requires enzymatically active complement. With its mutations in the Fc region, HCA-LALAPG has significantly reduced binding to C1q[84] so it was not surprising that it did not immobilize sperm in the presence of complement. While immobilizing sperm may increase HCA's potential contraceptive ability, complement activation and its cytotoxic effect can also lead to cytokine production and immune cell recruitment[97]. The female reproductive tract is reported to have low levels of complement, so it is unclear how much CDC contributes to the HCA contraceptive effect, but use of HCA-LALAPG could prevent unwanted inflammation in the genital tract, a condition which can increase barrier permeability and pathogen translocation[98]. On the other hand, complement-mediated sperm immobilization could contribute to the contraceptive mechanism of HCA, and the use of HCA-LALAPG could diminish this beneficial effect.

The ability of HCA and the HCA-LALAPG variant to trap sperm in cervical mucus was also compared as this mechanism is thought to contribute to the HCA contraceptive effect. While the exact mechanism of mucus trapping is not fully understood, antibodies may bind to cervical mucus through sugar-sugar hydrogen bonds or Fc receptor-like molecules[99,100]; other studies indicate that antibodies interact with mucus via weak electrostatic interactions between mucins and the Fc region[53,76]. As antibody-mucin interactions are weak, transient, and low affinity, it has been difficult to determine the exact mechanism behind mucus trapping. A previous study showed that removing the Fc region on antisperm antibodies decreased cervical mucus trapping[76]. Based on these reports we hypothesized that HCA-LALAPG, with its altered Fc region, would show decreased sperm-cervical mucus interactions. As conception risk is highest around the time of ovulation, midcycle cervical mucus was used for the mucus trapping studies, and a low antibody concentration (12.5 µg/mL) was used to increase the likelihood of finding individual motile sperm in the cervical mucus. Nearly all individual (non-agglutinated) HCA-treated sperm were trapped in cervical mucus whereas few HCA-LALAPG-treated sperm were trapped, suggesting that the mutations in the Fc region negatively affect the ability of the HCA-LALPG variant to interact with cervical mucus. Sperm trapping in cervical mucus could represent an important contraceptive function of HCA, and use of HCA-LALAPG could diminish this function.

Lastly, we demonstrated that HCA, but not HCA-LALAPG, mediates sperm phagocytosis by macrophage-like cells. The ability of HCA to mediate ADCP was previously unknown. The inability of HCA-LALAPG to elicit ADCP was unsurprising since this effector function is dependent upon FcγR binding. HCA-mediated sperm phagocytosis may have serious implications in the context of the female reproductive tract (FRT). Macrophages are widely distributed in the FRT and fluctuate with changes in hormone levels throughout the menstrual cycle[48]. These phagocytic cells make up about 10% of the total number of leukocytes in the FRT[101] and have been shown to be the major mucosal tissue Fc-receptor bearing effector cell and potentially mediate clearance of HIV via interaction with antibodies[102]. As phagocytes are abundant and have potent activity in the FRT, there is the potential for host production of antisperm antibodies following HCA-mediated sperm phagocytosis and antigen presentation. In men with obstructive azoospermia, increased numbers of spermiophages (macrophages that have ingested sperm) have been associated with the formation of autoantibodies against sperm[103]. Host formation of antisperm antibodies would be a highly undesirable immune response in women seeking a reversible method of contraception. However, it is unclear how agglutination contributes to phagocytosis; it is plausible that large agglutinates may be difficult for macrophages to envelop and digest. HCA-LALAPG did not trigger ADCP and is thus less likely to induce immunity to sperm, providing an advantage over HCA in this regard.

Antibody engineering technology continues to improve, offering greater possibilities for antibody design and customization. The challenge is determining the optimal antibody profile in the desired context, especially one as specialized as the vaginal mucosa. Understanding the potential Fc effector functions of an antibody-based drug is critical to the development of a safe and effective product. We have established that the HCA-LALAPG variant had decreased Fc-mediated functions compared to HCA. Some Fc functions such as mucus trapping and sperm immobilization may contribute to the contraceptive efficacy of HCA and may be desirable. Other Fc functions such as ADCP may promote detrimental immune responses through antigen presentation and host production of antisperm antibodies. Fc-engineered variants of HCA may provide safer, more conservative alternatives to HCA, but results from this study as well as ongoing clinical trials will inform if agglutination is an effective contraceptive mechanism alone or if HCA is more effective with a functional Fc domain and that HCA-LALAPG may not be optimal for this role. Use of high resolution maps of FcR binding sites on human IgG1[55] could provide further insight for designing more specific HCA variants with desired functions. We speculate that the ideal contraceptive antibody candidate would retain an ability to trap sperm in cervical mucus while demonstrating reduced ADCP activity. Functional studies using other Fc variants may be performed in the future as we seek to improve HCA for clinical use.

Acknowledgements

Our sincerest gratitude to our study participants and the staff at the General Clinical Research Unit (GCRU) at Boston University for use of their facilities and assistance.

Supporting Information

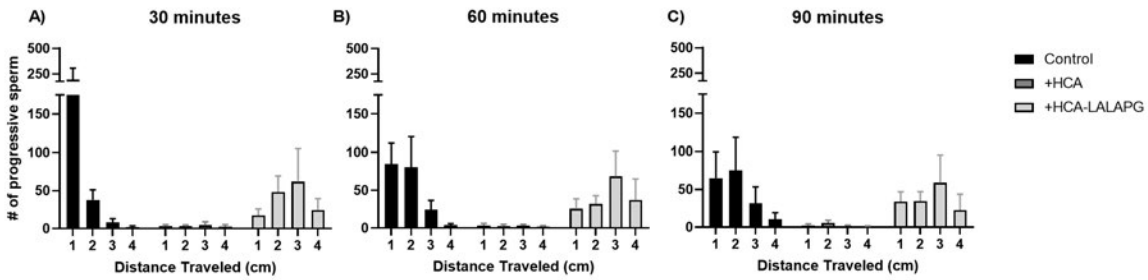

Figure 2.S1- Cervical mucus penetration test timecourse. The number of progressively motile sperm at 30 minutes (A), 60 minutes (B), and 90 minutes (C) in ovulatory cervical mucus, at capillary tube depths of 1, 2, 3 and 4 cm. The number of progressive sperm was significantly lower in the HCA treatment group than with HCA-LALAPG at all timepoints. Statistics are given in S2 Table. Sperm agglutination was observed for HCA and HCA-LALAPG-treated sperm at the initial centimeter, but non-agglutinated, progressive sperm were commonly observed at 2, 3 and 4 cm with HCA-LALAPG. Data are shown as mean ± SEM and is representative of four independent experiments, each with a different cervical mucus donor. Statistical analyses were conducted using repeated measures ANOVA of log transformed data followed by a Tukey multiple comparisons tests. Results were significant when $p \leq 0.05$. (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$)

A)

Condition	30 minutes				60 minutes				90 minutes			
	1cm	2cm	3cm	4cm	1cm	2cm	3cm	4cm	1cm	2cm	3cm	4cm
Control (-HCA)	194.00 ±112.70	37.75 ±13.40	8.00 ±5.05	2.25 ±1.31	84.50 ±27.11	80.25 ±39.93	24.3 ±12.21	3.75 ±2.17	65.00 ±34.59	75.00 ±43.16	31.5 ±21.74	10.75 ±8.43
+HCA	3.25 ±1.97	3.00 ±1.73	4.50 ±4.50	2.75 ±2.43	3.25 ±2.93	2.75 ±2.10	3.25 ±1.70	1.75 ±1.44	2.5 ±1.89	5.75 ±3.33	1.75 ±0.85	1.00 ±0.71
+HCA-LALAPG	17.25 ±8.66	48.25 ±21.09	61.75 ±43.76	24.5 ±14.93	25.5 ±13.32	32.00 ±10.87	68.25 ±33.50	37.00 ±27.88	33.75 ±12.99	34.5 ±12.48	58.75 ±36.54	22.5 ±21.17

B)

Comparison	30 minutes				60 minutes				90 minutes			
	1cm	2cm	3cm	4cm	1cm	2cm	3cm	4cm	1cm	2cm	3cm	4cm
Control vs. HCA	p=0.003	p=0.02	p=0.34	p=0.97	p<0.0001	p<0.0001	p=0.01	p=0.50	p<0.0001	p=0.0001	p=0.002	p=0.02
Control vs. LALAPG	p=0.007	p=0.92	p=0.12	p=0.14	p=0.04	p=0.32	p=0.66	p=0.06	p=0.38	p=0.75	p=0.35	p=0.10
HCA vs. LALAPG	p=0.33	p=0.01	p=0.007	p=0.09	p=0.001	p=0.0004	p=0.002	p=0.006	p<0.0001	p=0.0007	p<0.0001	p=0.03

Table 2.S1- Penetration of ovulatory cervical mucus at 30, 60, and 90 minutes. A) Number of progressively motile sperm in the cervical mucus penetration test (shown as mean ± SEM). B) Statistical differences between treatment groups at each distance. Significant comparisons indicated by shaded cells.

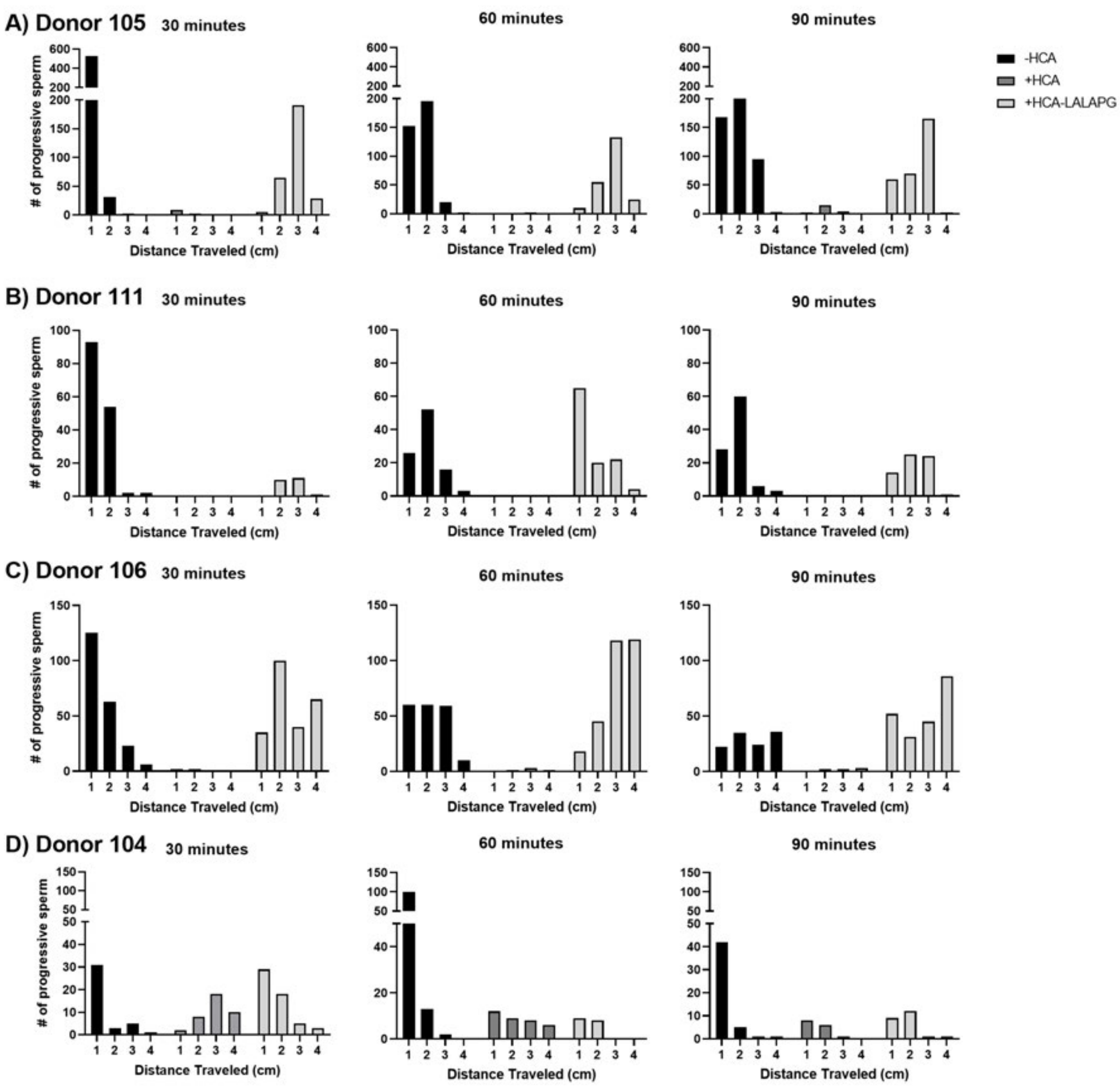

Figure 2.S2- Cervical mucus penetration test by donor. Number of progressively motile sperm at 30 minutes, 60 minutes, and 90 minutes in ovulatory cervical mucus. Three out of four donors showed far few progressive sperm in the HCA treatment group compared with HCA-LALAPG and medium-only treatment (Figure 2.S2 A-C) while Donor 104 had fewer progressive sperm in any of the treatment groups (Figure 2.S2 D).

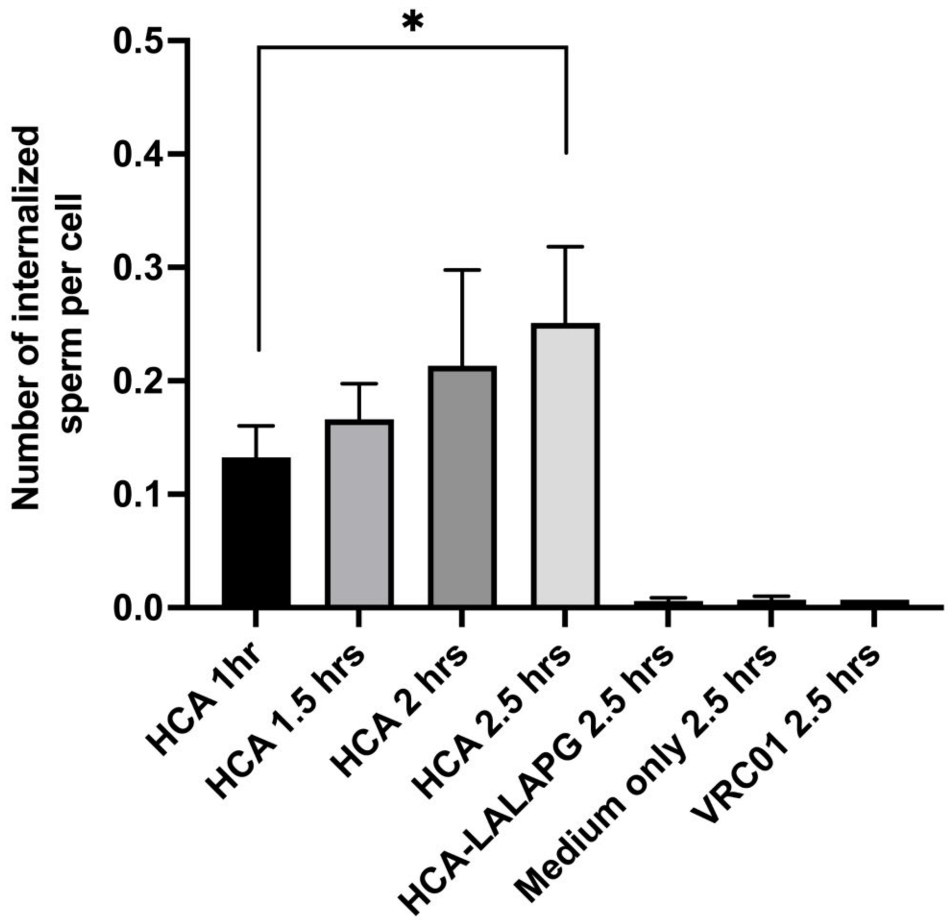

Figure 2.S3- Antibody-dependent sperm phagocytosis timecourse. The number of internalized sperm per macrophage increased in HCA-treated cultures overtime; a significant increase was observed between 1 hour and 2.5-hour timepoints ($p = 0.018$). HCA-LALAPG, medium-only control, and isotype control (VRC01) did not mediate sperm phagocytosis even at the 2.5-hour time point. Final antibody concentrations were 50 µg/mL. Data are expressed as mean ± SEM of three independently performed experiments. Statistical analyses were conducted using repeated measures one-way ANOVA of log-transformed data followed by Tukey multiple comparisons tests.

CHAPTER THREE: Establishing synthetic mRNA-mediated antibody expression as a novel topical delivery system of HCA

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Author Contributions:

EN performed the experiments and wrote the manuscript. JYJ contributed to the manuscript and provided resources. DV provided resources and experimental support. DJA and PS provided advice, oversight, and resources.

Data in this chapter will be included in a manuscript co-written with collaborators at Emory University, currently in preparation for publication.

Abstract

Human Contraception Antibody (HCA), an IgG1 antisperm monoclonal antibody, is currently in development as a nonhormonal female contraceptive. Though previous studies demonstrated potent contraceptive properties, the efficacy of HCA as a product is partly dependent on a suitable topical delivery system. Here, we explored an atomized, mRNA-based delivery method to induce expression of HCA in the female reproductive tract (FRT). The use of synthetic mRNA for contraception is novel and could provide several advantages including reversibility, safety, durability, and cost-effectiveness. Transfection experiments were conducted in a human vaginal epithelial cell line and a 3D vaginal tissue model using mRNA encoding GPI-anchored and secreted HCA. Vaginal cells were conducive to mRNA uptake and expressed functional and sperm-specific HCA. Expression was reversible and did not affect cell viability or tissue permeability. The GPI-anchored HCA demonstrated enhanced retention compared to secreted HCA and may be especially suitable for longer-lasting contraception. Furthermore, we showed that mRNA-expressed multivalent variants of HCA were more potent than IgG1 HCA. These data suggest that aerosolized mRNA may be a feasible topical delivery platform of HCA though further in vivo characterization is needed.

Introduction

High rates of unintended pregnancy continue to be a major public health issue worldwide[1]. Unplanned pregnancy may lead to health, social, and economic risks for both mother and child, especially in low- and middle-income countries[15]. Many sexually active women who wish to delay or limit childbearing stop using or do not use contraception due to undesirable side effects, high cost, and inconvenience of use[3]. Rates of discontinuation and nonuse of contraception suggest that there is an urgent need to develop novel contraceptive methods that align with user preferences. Recent studies show that factors contributing to the continuation of vaginally inserted contraceptive methods include an acceptable side effect profile, user-control, and reversibility[15]. The development of new, nonhormonal contraceptive products that fit this description could help to significantly reduce the number of unintended pregnancies.

Antisperm antibodies have been of considerable interest since the late 20th century due to their ability to affect fertility through a variety of mechanisms[27,25]. Human Contraception Antibody (HCA) is an antisperm monoclonal IgG1 antibody derived from a naturally-occurring IgM in the serum of an infertile woman[104]. The main contraceptive function of HCA is the agglutination of sperm cells via binding of glycoprotein CD52g, a male reproductive tract-specific epitope found on the surface of mature sperm cells[26,105]. Agglutination prevents sperm cells from progressively swimming through the female reproductive tract (FRT), ultimately blocking fertilization. HCA also facilitates Fc effector functions like the trapping of sperm in cervical mucus, complement-dependent sperm immobilization, and antibody-dependent sperm phagocytosis[29,106].

Localized vaginal delivery of HCA provides the most efficient route of administration and allows for lower drug doses and reduced side effects[30]. Our group recently completed a phase 1 clinical trial assessing the safety and efficacy of a topical vaginal film containing HCA[35]. HCA was expressed in a transgenic *Nicotiana* platform (HCA-N) and formulated into polymer films. Postcoital tests demonstrated that a single dose of the film applied prior to intercourse was safe and significantly reduced the number of progressively motile sperm in cervical mucus. This provides evidence not only for the efficacy of HCA as a contraceptive but also for the suitability of vaginal administration.

Expanding to other modes of vaginal HCA delivery systems would provide women with more options and account for differences in preference and behavior among contraceptive users. Exploring alternative modes of delivery by harnessing new and innovative tools in biotechnology could also help to improve the product's efficiency and retention. Over the past few years, synthetic mRNA has been at the forefront of therapeutics due to transience, non-integration, acceptable safety profile, rapid expression in target cells, cost-effectiveness, and accelerated developability[32]. Synthetic mRNA can be utilized to express monoclonal antibodies for a number of applications[62]. Here, we propose to express HCA using mRNA for contraception.

The efficacy of synthetic mRNA-mediated antibody expression has previously been described[68–70]. Transfections were performed with two mRNA strands: one for the heavy chain (HC) and one for the light chain (LC) (Fig 3.1A). The 3' end of the LC can be fused with a luminescent reporter, nanoluciferase (Nluc), for expression detection and visualization. In addition, a GPI-linker can be included on the HC to anchor the assembled antibody to the cell membrane for longer retention. These mRNAs can be topically delivered through atomization, i.e. “spraying” the mRNAs directly onto the tissue surface (Fig 3.1B). The principle behind atomization is that hypotonic solutions such as water, when delivered via aerosol, alter the pressure at the host membrane, facilitating pore formation and direct access of the mRNA to the cytosol[69,70]. Once in the cytosol, the heavy and light chain mRNAs have access to the cell’s ribosomal machinery and are translated, correctly assembled, and either secreted or, with the inclusion of a GPI-linker, anchored to the plasma membrane.

Tiwari et al. and Lindsay et al. demonstrated the use of mRNA atomization to successfully transfect mucosal surfaces in lung and vaginal epithelia, respectively[69,70]. GPI-anchored mRNA-expressed anti-RSV antibodies were retained on the plasma membrane of transfected murine lung epithelial cells and protected against infection in vitro and in vivo[69]. In an HIV-1 model, the entire lower female reproductive tract of sheep and macaques was shown to be conducive to transfection by atomized mRNA[70]. GPI-anchored PGT121 resulted in neutralizing genital secretions and tissue explants as long as 28 days post-transfection. Here, we plan to apply this same concept to provide prolonged contraceptive protection by expressing mRNA-encoded HCA in several models of the female reproductive tract.

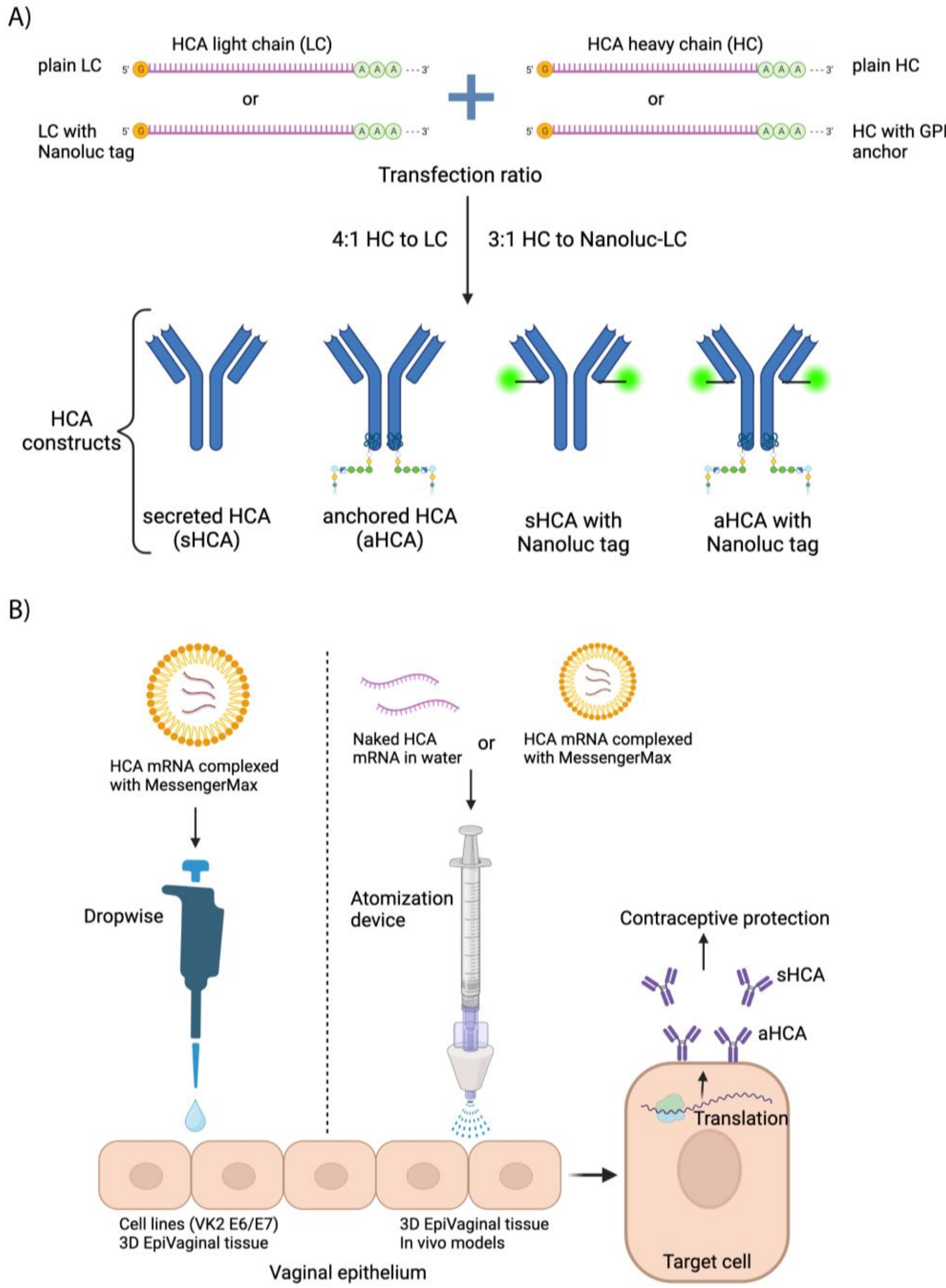

Figure 3.1- HCA mRNA constructs and transfection methodology. A) Potential mRNA constructs following transfections with optimized ratios. B) mRNA transfections in models of the female reproductive tract using either dropwise or aerosol transfection methods. Figure generated in Biorender.com.

Methods

Cell lines

Human alveolar epithelial (A549) and vaginal epithelial (VK2 E6/E7) cell lines were obtained from ATCC. A549 were cultured in DMEM media supplemented with L glutamine, 10% fetal bovine serum (FBS), and antibiotics. VK2 E6/E7 were cultured in Keratinocyte-Serum Free medium (GIBCO-BRL 17005-042) with 0.1 ng/ml human rEGF, 0.05 mg/ml BPE, and additional calcium chloride 44.1 mg/L (final concentration 0.4 mM). Purified mRNA HCA expressed in A549s were used for functional testing. Expi293F cells (ThermoFisher Scientific) were used to produce multimeric mRNA-expressed HCA variants and were cultured in Expi293 expression medium (ThermoFisher Scientific).

MatTek EpiVaginal™ tissue model

The partial-thickness EpiVaginal tissue model (MatTek Cat# VEC-100, MatTek Corporation, Ashland, MA, USA) was used as a physiologically and functionally relevant transfection model of the female reproductive tract[107]. Three-dimensional EpiVaginal tissues were cultured in 24-well inserts from human primary vaginal-ectocervical cells and are highly-differentiated to contain stratified basal and apical epithelial cell layers.

Antibodies

Human Contraception Antibody (HCA):

HCA is an IgG monoclonal antibody that binds to sperm-specific epitope CD52g. Parent HCA, also known as HCA-N, was produced using DNA templates in transgenic *Nicotiana benthamiana* (fucosyl- and xylosyl-transferase knockouts) by Kentucky

Bioprocessing Inc (KBio; Owensboro, KY) as previously described[33]. HC4-N was a commercial-grade positive control in functional testing.

Campath-1:

Campath-1, also known as alemtuzumab, is a humanized IgG monoclonal antibody against CD52 (ThermoFisher Scientific Cat# MA5-16999, Waltham, MA, USA, RRID: AB_2538471). Campath-1 was used as a capture antibody in the HCA ELISA and a positive control in sperm specificity staining.

Synthetic mRNA in vitro transcription

Plasmids were prepared and used as templates for in vitro transcription (IVT). Briefly, the plasmids included the sequences for the T7 promoter, 5’ and 3’ untranslated regions (UTRs), open reading frame (ORF) and two stop codons. Heavy or light chain sequences of HCA were inserted into each ORF. The plasmids were then linearized by NotI-HF restriction enzymes (New England Biolabs), followed by purification with sodium acetate (ThermoFisher Scientific) and rehydration with nuclease-free water. A HiScribe T7 kit (New England Biolabs) with N1-methyl-pseudouridine modification was utilized for IVT from the purified, linearized plasmids. Resultant RNA strands were isolated from the plasmids by adding DNase I (Aldevron) for 30 min and were purified using lithium chloride (ThermoFisher Scientific) precipitation. The RNA was denatured, capped on the 5’ end with a Cap-1 structure using guanylyltransferase and 2’-O-methyltransferase (Aldevron), and given poly-A tails on the 3’ end. The RNA was purified again, treated with alkaline phosphatase (New England Biolabs) and resuspended in nuclease-free water. These mRNAs were quantified with a nanodrop and analyzed by gel electrophoresis to ensure purity. The mRNAs were aliquoted and stored at -80 °C until use.

mRNA transfections

Cell line transfections:

Lipofectamine MessengerMAX reagent (ThermoFisher Scientific) was used to transfect cells according to the manufacturer's instructions. The mRNA solutions were prepared by mixing heavy and light chain-encoding mRNAs with a 4:1 ratio in Opti-MEM (Gibco) or 3:1 when using light chain with Nanoluc inclusion. Cells in 24-well plates at 80% confluency were treated with 1 µg total mRNA and incubated at 37 °C. VK2-transfected supernatants were collected and run on an HCA ELISA to quantify expression. For production of HCA variants, 75 µg of mRNAs were added to 75 x 10^6 of Expi293F cells in 125 mL flasks under a shaker with 125 rpm and incubated for 2 days at 37 °C after which antibodies were purified either by resin-based spin columns, molecular weight cutoff, or tangential flow filtration.

EpiVaginal tissue aerosol delivery:

Tissues were equilibrated in MatTek media upon arrival and incubated overnight at 37 °C, 5% CO_2. After 24 hours, MessengerMAX-mRNA solutions in Opti-MEM were prepared as described above and loaded into a Teleflex MAD Nasal™ atomization device. The atomization device was then fixed onto a clamp stand and used to either aerosol-transfect tissues with 20 µg mRNA or spray tissues with Opti-MEM as a negative control. The tissues were then incubated at 37 °C, 5% CO_2 and supernatants were collected periodically for functional readouts.

Immunofluorescence

Specificity staining:

mRNA-expressed antibodies were tested for specificity by staining human sperm cells and PBMCs as a control. Washed cells were air-dried on glass slides and fixed in acetone. After blocking for 45 min (DAKO Cat# X0909), primary antibodies (i.e. mRNA HCA, commercial-grade HCA, or Campath) were applied to slides and incubated at RT for 1 hr. Antibodies were detected with a 1:1000 dilution of Cy3-conjugated anti-human IgG. Slides were mounted with Vectashield AntiFade Mounting Medium containing DAPI (Vector Laboratories Cat# H120010) and imaged under an Olympus microscope fitted with epifluorescence imaging.

Vaginal tissue staining:

Epivaginal tissues were paraffin-embedded, sectioned at 5 µm, de-waxed, rehydrated, and subjected to antigen retrieval prior to immunofluorescent staining to visualize and detect mRNA-expressed antibodies. Sections were blocked with 10% donkey serum in TBS. A mouse anti-Nanoluc (Promega) primary antibody diluted 1:50 in diluent (DAKO Cat# S3022) was applied to tissues followed by a 1:200 donkey anti-mouse Cy3-conjugated secondary antibody. Slides were mounted with Vectashield AntiFade Mounting

Medium containing DAPI and imaged under an Olympus microscope fitted with epifluorescence imaging.

Semen collection and processing

Human semen samples were acquired from healthy males aged 18-45. All donors met the WHO criteria for fertility and provided written informed consent before participation as set out by the Institutional Review Board at Boston University (Human Subjects Protocols H36843 and H41454). Prior to sample collection, males were instructed to remain sexually abstinent for at least 48 hours. After collection, samples were incubated at 32 °C for 30-60 min to allow for liquefaction. Motile sperm cells were separated from seminal plasma by overlaying whole semen on an equal volume of ISolate density gradient (FUJIFILM Irvine Scientific; Santa Ana, CA, USA) and centrifuging at 300 x g for 20 min. The sperm pellet was retrieved, resuspended, and washed in sperm multipurpose handling medium (MHM; FUJIFILM Irvine Scientific; Santa Ana, CA, USA). Sperm quality parameters including percent motile sperm, percent progressive sperm, and concentration were assessed using the Computer-Assisted Sperm Analysis system (CASA; Human Motility II software, CEROS II, Hamilton Thorne, Beverly, MA, USA). Sperm cells were diluted in MHM to 20-40 x 10^6/ml for functional assays.

Functional assays

Agglutination kinetics assay:

The sperm agglutination kinetics assay was used to measure time elapsed for anti-sperm antibodies to completely agglutinate sperm as previously described[29]. Briefly, two microliters of sperm cells in MHM were added evenly onto a multi-spot slide 9 mm well (ThermoScientific, Waltham, MA, USA) and observed under an Olympus inverted microscope at 10X. An equal volume of antibody in MHM was added onto the well and pipetted up and down three times after which a timer was started. Seconds to complete sperm cell agglutination within the field of view was recorded. The contraceptive threshold was set at two minutes and thirty seconds, which is slightly under the 3-5 minute time interval for sperm to reach the endocervix[90,108]. Each antibody condition was performed in triplicate. Experiments were performed three times with different semen donors to account for donor variability.

Sperm escape assay:

The sperm escape assay was used to measure percent of progressive sperm cells that escape agglutination relative to media control as previously described[29]. Briefly, 30 µl of sperm cells in MHM were added to an equal volume of antibody in MHM and were mixed by pipetting up and down three times. Sperm-antibody suspensions were incubated at a 45-degree angle for 5 minutes at RT. Sperm agglutinates fall to the bottom of the tube while escaped sperm swim freely to the top. After incubating, 2.5 µl of the suspension was sampled from the top millimeter of the tube and added to a 4-chamber slide (Microcell 15424, Vitrolife, San Diego, CA, USA). Percent progressively motile sperm relative to the media-only control was determined using the CASA. Each antibody condition was performed in triplicate. Experiments were performed three times with different semen donors to account for donor variability.

HCA enzyme-linked immunosorbent assay (ELISA)

The HCA ELISA is an in-house indirect sandwich ELISA to detect and quantify HCA in supernatant and vaginal secretions. Campath-1, the capture antibody, was diluted in PBS to 10 µg/ml to coat the plate overnight. Nonspecific binding to the plate was blocked using 5% FBS in PBS. Plates were washed three times in between steps with 1X PBS containing 0.05% Tween-20 (PBST). Aliquots of seminal plasma from previous semen donors that had been stored at –80 °C were used as antigen source. Seminal plasma diluted 1:1 in sample diluent (1X PBS, 10% FBS, 0.5% Triton X-100) was added to plates and incubated for 2 hours at 37 °C. Primary antibody, either HCA-N standard dilutions in sample diluent or supernatant/secretions containing mRNA-expressed HCA, was applied to plates and allowed to incubate for 1 hour at 37 °C. A 1:5000 dilution of HRP-conjugated anti-human IgG was used to detect HCA by incubation for 1 hour at 37 °C. TMB substrate was then added for a maximum of 30 min at room temperature. Colorimetric reactions were stopped using sulfuric acid. Plates were read immediately at 450 nm on a BioTek Synergy HTX multi-mode plate reader (Agilent, Santa Clara, CA, USA). Unknown concentrations of HCA were quantified by interpolating the HCA-N standard curve using sigmoidal 4-parameter logistics (4PL) regression analysis.

Safety profile analyses

Transepithelial electrical resistance (TEER):

Vaginal tissue barrier integrity/permeability was measured at various timepoints using an electrode volt-ohm meter (EVOM3, World Precision Instruments, Sarasota, FL, USA).

Vaginal tissue proinflammatory response:

The MatTek EpiVaginal™ tissue model was also used to study proinflammatory effects of acute mRNA exposure and mRNA HCA-sperm immunocomplexes. For acute mRNA proinflammatory effects, apical and basal tissue supernatants were collected 6 hrs post-mRNA transfection. For mRNA HCA-sperm immunocomplex effects, 50 µl of whole semen were applied to the apical side of tissues 24 hrs post-mRNA transfection. Tissues were incubated for 6 hrs at 37 °C after which mRNA HCA-sperm suspensions were washed off and replaced with fresh MatTek media. Twenty-four hrs post-semen exposure, apical and basal supernatants were collected. Cytokines in all supernatants were measured using a custom 6-plex Procartaplex™ immunoassay kit (Invitrogen, ThermoFisher Cat# PPX-06, ThermoFisher Scientific., Waltham, MA, USA). Analyte levels were compared to media controls and included: IL-1α, IL-1β, IL-6, IL-8, IL-1RA, MCP-1.

mRNA vaginal interaction dynamics

pH exposure:

VK2s were seeded at 1 x 10^5 cells/well in a 24-well plate. Prior to transfection, sHCA-Nluc mRNA in MessengerMAX vehicle was exposed to 0.5% lactic acid, around the physiologically relevant value in women with Lactobacillus-dominated microbiota[109], with an adjusted pH of 3 or 3.8 for 50 min at 37 °C after which they were used to transfect cells. Following a 24-hr transfection, luminescent reporter signal in supernatant was compared among experimental conditions.

Female sex hormone exposure:

VK2 cells were seeded at 5 x 10^4 cells/well in a 24-well plate and grown for 7 days in either 17ß-estradiol (E2, 10^{-9}M), progesterone (P4, 10^{-7} M), medroxyprogesterone acetate (MPA, 10^{-9} M), E2 + P4, or media only. Hormone concentrations correspond to the highest serum levels during the menstrual cycle[110]. Hormones were replaced every 48 hours. Following a 24 hr-transfection, luminescent reporter signal in supernatant was compared among experimental conditions. Conservation of cell viability and hormone receptor expression was validated (data not shown).

mRNA efficiency:

VK2s were seeded at 1 x 10^5 cells/well in a 24-well plate and treated with either media or 1 µg aHCA in triplicate. After 24 hours, cells were harvested, fixed in acetone, and stained with 1:1000 Cy3-conjugated anti-human IgG. Ten random fields per replicate were imaged and counted (at least 200 cells per replicate) and percent of cells transfected was calculated based on the number of Cy3-positive cells over total.

Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism (Version 10.0.0; GraphPad Software Inc.; San Diego, CA, USA). Data were analyzed by either repeated measures analysis of variance (ANOVA), mixed-effects analysis, or Kruskal-Wallis analysis. A significant analysis was followed by a multiple comparison tests as indicated by the type of analysis. If data used to perform parametric tests were not normally distributed, they were log (natural) transformed prior to analysis. Differences were considered to be statistically significant when $p<0.05$.

Results

Vaginal cells are conducive to mRNA transfections in vitro and express functional and sperm-specific HCA

Secreted HCA was detected in VK2 supernatant as early as 2 hours following a 1 µg mRNA transfection. Peak expression occurred at 24 hours with a mean HCA concentration of 18.4 µg/ml, and concentrations declined thereafter with almost no new production by 72 hours (Fig 3.2A). mRNA-expressed HCA was purified following in vitro mRNA cell transfections and used to assess sperm specificity and function. Positive staining was observed on sperm using mRNA-expressed HCA, commercial-grade HCA, and Campath (anti-CD52). No non-specific binding was observed on PBMCs for mRNA HCA and the commercial-grade control, though staining was seen in the Campath condition as PBMCs heterogeneously express CD52[111] (Fig 3.2B).

mRNA HCA demonstrated sperm agglutination comparable to commercial-grade HCA (HCA-N) at 25-100 µg/ml (Fig 3.2C). HCA-N is GMP-produced in *Nicotiana* and is the current platform for the HCA vaginal film. Although agglutination time was longer than commercial-grade control at 6.25 µg/ml, the mRNA Abs agglutinated 100% of sperm within the time limit. The contraceptive threshold of the mRNA Abs was 1.56 µg/ml, as was seen with the control. There was no difference in agglutination between sHCA and sHCA-Nluc. The mRNA Abs also performed well in the sperm escape assay, another measure of agglutination activity, where number of progressive sperm that "escape" agglutination is quantified. In fact, there were no differences between mRNA HCA and commercial-grade HCA (Fig 3.2D). Overall, vaginal epithelial cells were conducive to mRNA transfection with peak expression at 24 hours, and purified mRNA HCA was functional and sperm-specific.

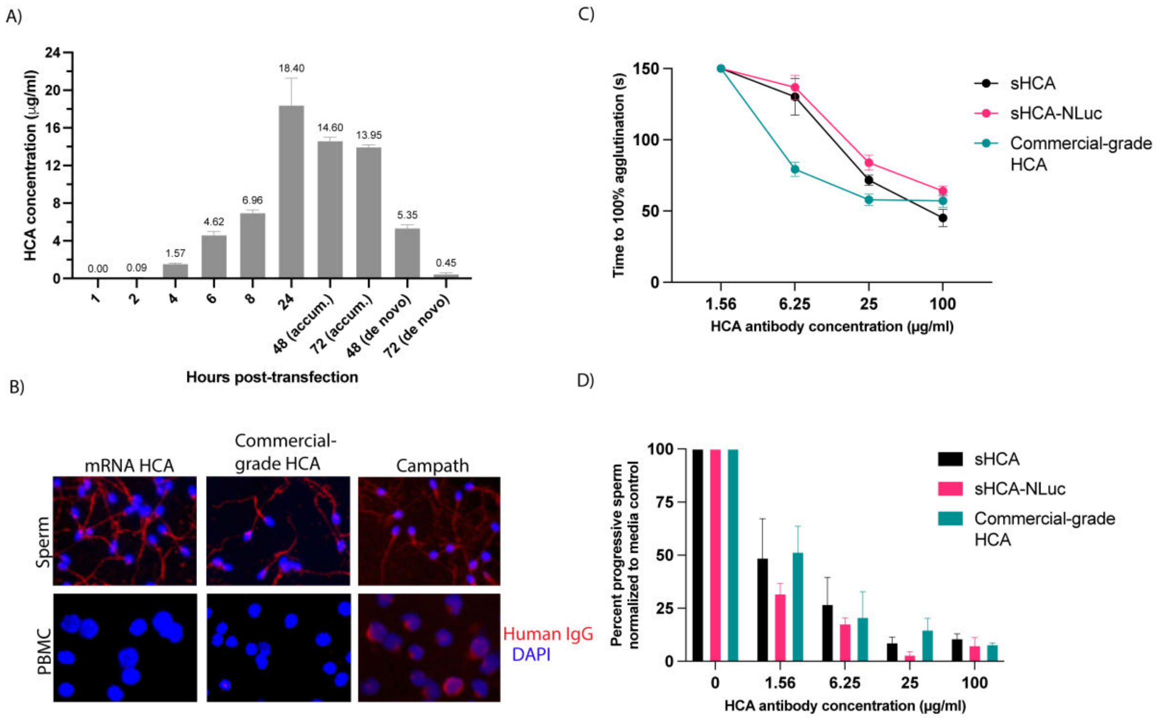

Figure 3.2- Cell line mRNA transfections: mRNA HCA expression, function, and specificity. A) VK2 transfection time course. HCA concentration was determined by ELISA. "Accum" denotes accumulated antibody over specified time (i.e., no supernatant collection until that timepoint) and "de novo" denotes new antibody production following supernatant collection and media replacement. B) Immunofluorescent staining of human sperm and human peripheral blood mononuclear cells (PBMCs). All antibodies were applied at a concentration of 50 µg/ml (mRNA purified from Expi293F transfection). C) Kinetic agglutination assay. "Nluc" denotes HCA with a Nanoluciferase tag (mRNA Abs purified from A549 transfection). D) Sperm escape assay.

mRNA transfections are not affected by vaginal-specific environmental conditions and demonstrate efficient vaginal cell uptake

Next, we determined whether conditions within the female reproductive tract affect mRNA integrity and subsequent mRNA transfection. We recapitulated the acidic vaginal environment that occurs via lactic acid production by *Lactobacilli* which dominate a healthy vaginal microbiome[109]. Though the average vaginal pH in humans is 4.5, normal pH ranges as low as 3.8[112]. sHCA-Nluc mRNA complexed with MessengerMAX was incubated with 0.5% lactic acid titrated to two different pH levels, 3 and 3.8, at 37 ºC for 50 minutes and then used to transfect VK2 cells. There was no significant difference in Nanoluc detection between the neutral and pH 3.8 conditions (Fig 3.3A). However, a pH of 3.0 resulted in a significantly lower Nanoluc signal compared to the neutral transfection, though this pH is not physiologically relevant.

The primary female sex hormones are estrogen and progesterone, which fluctuate throughout the menstrual cycle. We assessed the effect of female sex hormones on mRNA expression. VK2 cells were treated with estradiol (E2), progesterone (P4), or medroxyprogesterone acetate (MPA, hormonal birth control) for 7 days at physiologically relevant concentrations[110], after which cells were transfected. MPA was tested to ensure there were no confounding effects with hormonal contraceptives, since these may be used in tandem with the mRNA platform. No reduction of Nanoluc detection was seen in any hormone treatment (Fig 3.3B).

Transfection efficiency was examined by calculating percent cell expression (i.e. cell uptake) following mRNA transfection. VK2 cells were transfected with aHCA mRNA and fluorescently stained to visualize and quantify aHCA on cell surfaces (Fig 3.3C, D). At 24 hours post-infection, cells were harvested, imaged, and scored. Transfection efficiency averaged to 74.3%. Fluorescent aHCA "halos" were clearly visible via microscopy in which a majority of VK2s were positive. Overall, vaginal pH and hormone conditions did not reduce mRNA expression and the vast majority of cells transfected with mRNA expressed HCA.

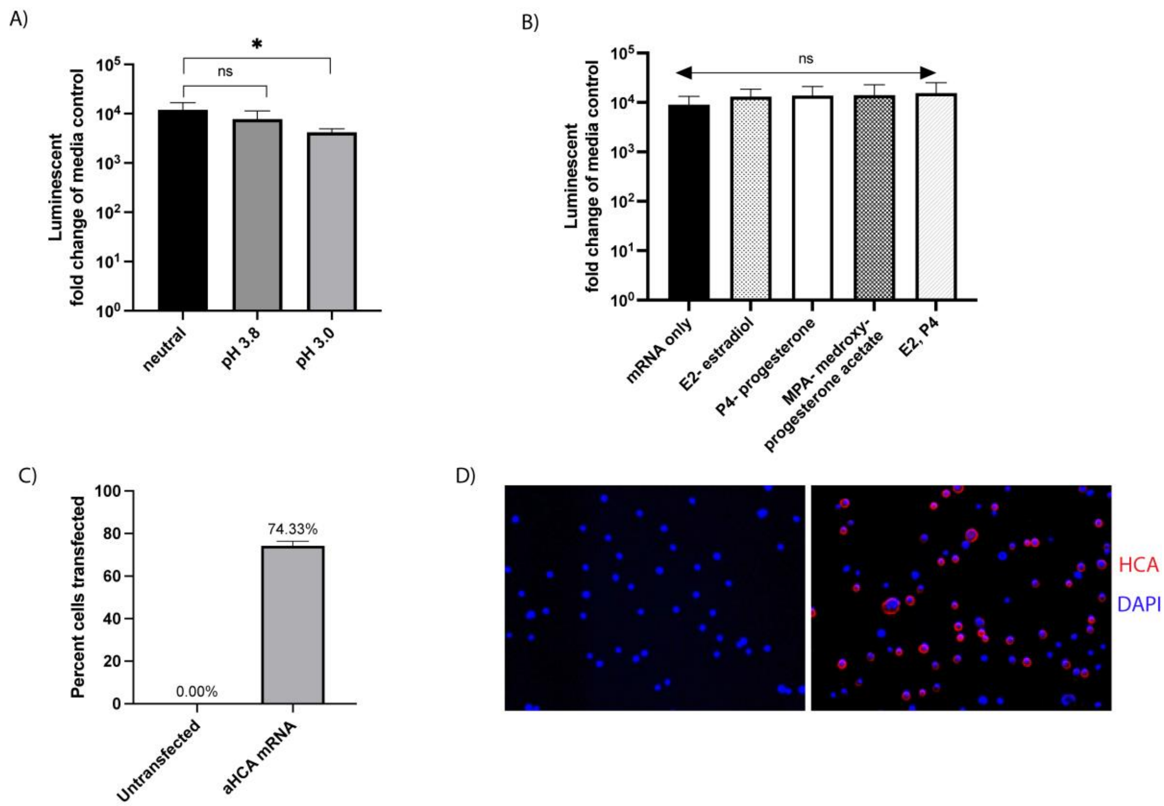

Figure 3.3- mRNA interaction dynamics within the vaginal microenvironment. A) Detection of sHCA-Nluc in VK2 supernatant following transfections with mRNA pretreated with 0.5% lactic acid-supplemented media titrated to low pH values (*p<0.05). B) Detection of sHCA-Nluc from VK2 cells pretreated with female sex hormones for 7 days prior to mRNA transfection. C) mRNA transfection efficiency. Quantification of percent cell uptake of mRNA following immunofluorescence experiments (mean of 3 replicates per condition). D) Representative images (100X total magnification) of fluorescent anti-human IgG antibody tagging anchored HCA on cell membranes of VK2 cells following 24-hour mRNA transfections. NS, nonsignificant.

Atomization is an efficient and safe delivery method of mRNA in a 3D vaginal tissue model

The MatTek 3D EpiVaginal™ tissue model histologically and functionally resembles native vaginal tissue and is an apt model for atomized mRNA delivery. Twenty µg of naked mRNA in water or LNP-complexed mRNA were loaded into a Teleflex atomization device and were sprayed directly onto tissue inserts. Tissues were incubated at 37 ºC for up to 4 days (tissue viability limit) after which supernatants were collected and tissues were processed to determine HCA expression. Both sHCA-Nluc- and aHCA-Nluc-treated tissue supernatants produced a luminescent signal that averaged around 10^3-fold higher than the media control for three independent experiments (Fig 3.4A). Anchored HCA expression was detected even at 4 days post-transfection (18 µg/ml), as shown by HCA ELISA quantification and immunohistochemistry, suggesting the aHCA construct may contribute to a longer antibody retention time (Figs 3.4B, C).

Next, assessed the safety profile of our atomized mRNA delivery system. Vaginal irritation was evaluated within two contexts: acute mRNA exposure (i.e. analysis 6 hours post-transfection) and mRNA/semen co-exposure (6-hour semen exposure 24 hrs post-transfection, analysis 24 hours post-semen application) to recapitulate a physiologically relevant scenario since HCA/semen immunocomplexes can be proinflammatory. Transepithelial electrical resistance (TEER) was used to measure tissue integrity and permeability. There were no significant differences in TEER among tissues treated with culture media only, transfection media only, sHCA mRNA, or aHCA mRNA at any of the timepoints (Fig 3.4D). Proinflammatory markers traditionally used to define female genital inflammation[113] were measured in both apical and basal supernatants using a Luminex multiplex kit. There were no significant increases in analyte levels compared to media control in either apical or basal supernatants following acute mRNA exposure (Fig 3.4D).

Similarly, mRNA/semen co-exposure did not reduce tissue permeability at either 6 or 24 hours post-semen application (Fig 3.4E). Tissue integrity in Nonoxynol 9-treated tissues, a spermicide and positive control for irritation, was significantly reduced. Analytes in mRNA/semen co-exposed tissue supernatants were compared to a semen-only control group, since semen is naturally inflammatory and can elevate proinflammatory markers[114]. No significant differences were seen compared to the semen-only control. Overall, atomized mRNA delivery resulted in positive HCA expression that was retained longer with the anchored construct, and the delivery system had an acceptable safety profile.

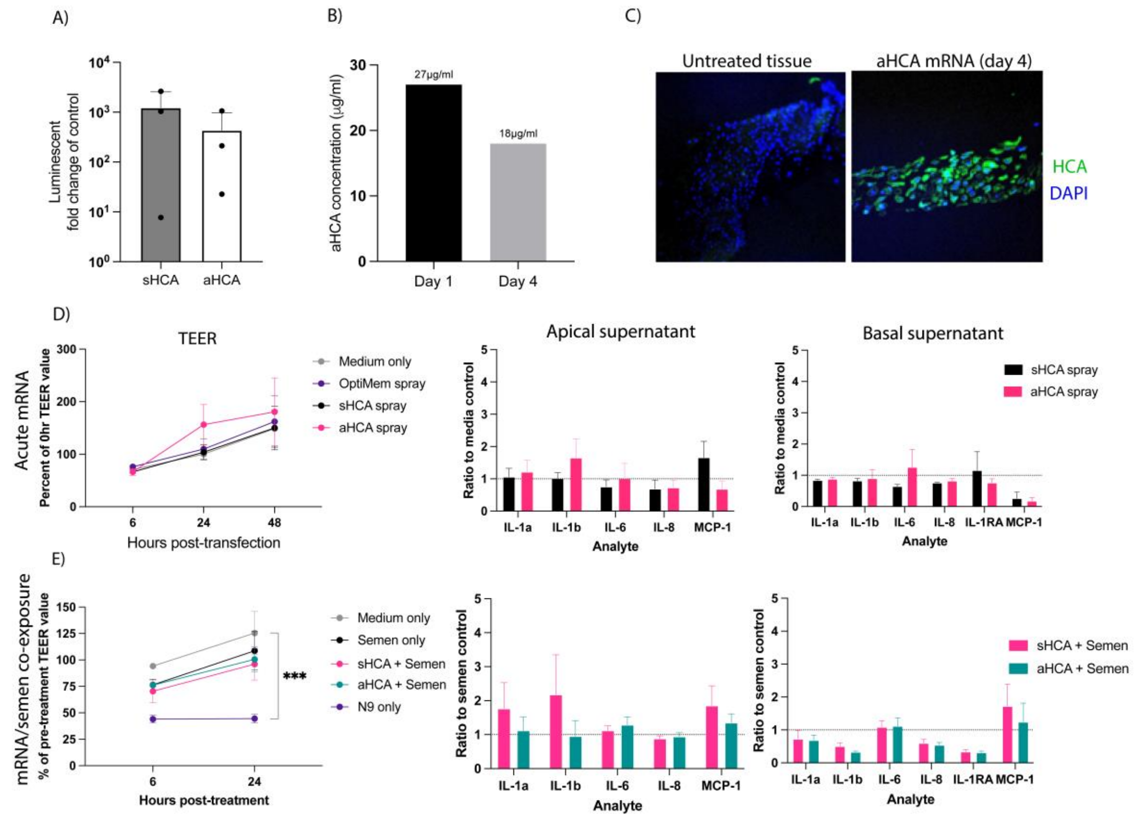

Figure 3.4- 3D EpiVaginal tissue transfections: mRNA HCA retention and safety profile. A) Luminescent signal in tissue supernatants 24 hours post-aerosol transfection. B) HCA quantification via ELISA following an aHCA mRNA tissue transfection. C) Immunohistochemistry of aHCA mRNA-treated tissue. HCA expression was screened using an anti-Nanoluc antibody. D) Safety profile of acute (6-hour) mRNA exposure. TEER, transepithelial electrical resistance. Proinflammatory markers were quantified in both apical and basal tissue supernatants. E) Safety profile of mRNA/semen co-exposure. Tissues were transfected with mRNA for 24 hours after which they were exposed to semen for 6 hours. Supernatants were collected 24 hours after semen application. Proinflammatory markers were quantified in both apical and basal tissue supernatants. N9, Nonoxynol-9 (***p<0.001).

Multimeric engineered constructs of mRNA-expressed HCA are more potent than the parent IgG

mRNA design is highly customizable and thus conducive to antibody engineering. Therefore, we wanted to explore multimeric constructs of HCA for increased potency. We developed several mRNA-expressed HCA variants including dimeric IgA HCA and IgG HCA engineered with the tailpiece of IgM called “IgG/t” (Fig 3.5A). IgG/t is pentameric with the inclusion of the J chain and hexameric without the J chain. Multimeric constructs of HCA were mRNA-expressed in Expi293F cells and purified. We compared the agglutinating function among the mRNA HCA variants using HCA-N as a positive control. At 12.5 µg/ml, all antibodies agglutinated 100% of sperm cells in less than 50 seconds, well below our contraceptive threshold cutoff of 150 seconds (Fig 3.5B). Multimeric IgA and IgG/t variants were significantly more potent than IgG HCA at lower concentrations. Specifically, dimeric IgA and IgG/t HCA completely agglutinated sperm at a concentration as low as 0.78 µg/ml, whereas the mRNA-expressed IgG HCA control had a functional limit of around 3.16 µg/ml. This demonstrates increased agglutinating potency of HCA multimers compared to parent IgG.

Similarly, we observed fewer escaped progressive sperm in the sperm escape assay with IgA and IgG/t HCA compared to IgG HCA (Fig 3.5C). In fact, no progressive sperm were observed in the IgG/t condition. Progressive sperm pose the highest risk of fertilization as they sperm are able to move in a forward progression and thus transverse the cervix. Overall, multimeric variants are of great interest because increased potency could reduce the mRNA dose needed to achieve contraceptive protection[115].

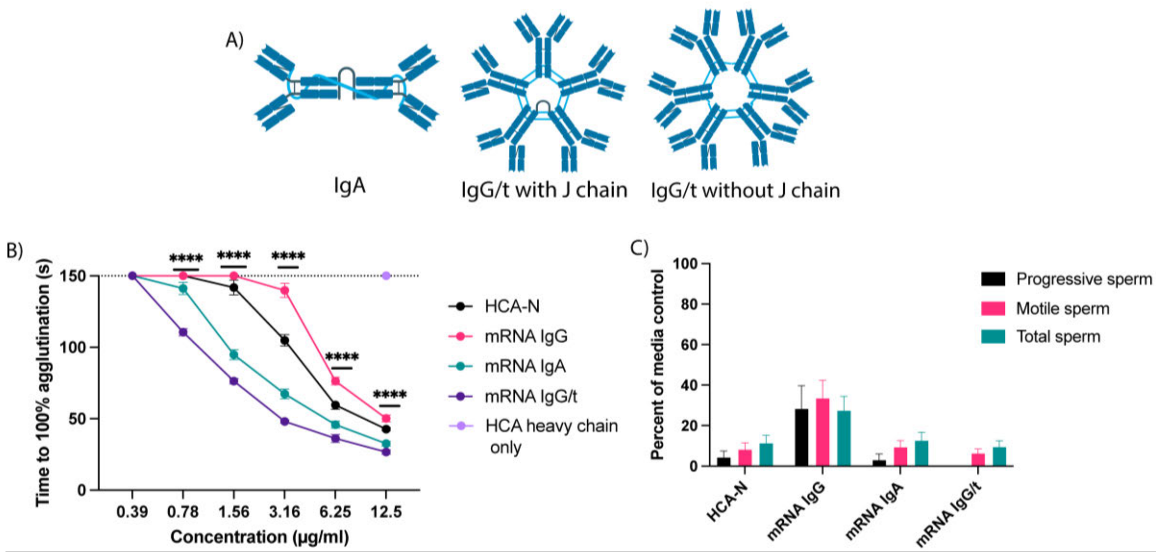

Figure 3.5- Engineered mRNA-expressed multimeric variants of HCA. A) Schematic of multimeric constructs. From left to right: dimeric IgA, pentameric HCA engineered using the tailpiece of IgM and assembled with the J chain, hexameric HCA engineered using the tailpiece of IgM and assembled without the J chain. Generated in biorender.com. B) Kinetic agglutination assay. Statistics denote multiple comparisons test between mRNA IgG and mRNA IgG/t following a two-way ANOVA, though there are other statistical significances between variants not shown (****$p<0.0001$). C) Sperm escape assay. All antibodies were diluted to 25 μg/ml prior to assay.

Discussion

mRNA is a versatile biological tool that allows for efficient protein expression in vivo. While it has most recently been utilized to develop vaccines against cancer and infectious diseases[67,32], mRNA can be harnessed for a diverse range of therapeutic applications. Here, we proposed to establish an mRNA-based topical delivery system of HCA, an antisperm antibody, for nonhormonal contraception. The vaginal mucosa is an especially receptive candidate for localized mRNA delivery due to easy compartment access and permeability[30]. Using mRNA as an alternative delivery system of HCA would allow for certain advantages over protein delivery including cost-effectiveness, favorable safety profile, preservation of host glycosylation, scalability, and pharmacokinetic control.

In this study, in vitro models of the female reproductive tract were conducive to mRNA transfection and subsequent HCA expression, with an average concentration of 18.4 µg/ml 24 hours after a 1 µg mRNA transfection in VK2 cells. Previous studies indicate that HCA-N is capable of 100% sperm agglutination at concentrations as low as 6.25 µg/ml[29]. mRNA-expressed HCA was sperm-specific and demonstrated potent agglutination similar to HCA-N. Certain physiological conditions within the FRT such as low pH and hormone exposure did not affect the ability of vaginal cells to express mRNA-mediated HCA. mRNA transfection efficiency in the presence of abnormal vaginal conditions such as bacterial vaginosis is currently unknown and may need to be explored. Moreover, a 3D vaginal tissue model was conducive to delivery of mRNA using an atomization device. This aerosol delivery system and subsequent expression of HCA appeared to be both noninflammatory and nondetrimental to vaginal tissue integrity, suggesting an acceptable safety profile.

Because mRNA design can be easily customized, we explored multimeric variants of HCA to increase potency and reduce agglutination time. Dimeric and hexameric variants of HCA were successfully designed and expressed in vitro. Of interest, these multimers completely agglutinated sperm at as low as 0.78 µg/ml and were clearly superior in function to the parent IgG. Further studies and optimization of these designs may provide an alternative HCA variant to be used in vivo to achieve greater function, e.g. lower IC_{50} values, using lower doses of mRNA.

By tethering HCA to the vaginal cell surface with a GPI anchor, we were able to prolong retention of antibody titers for up to 4 days. Because of the limited duration of viability of EpiVaginal tissues, future studies are needed to more completely characterize aHCA pharmacokinetics, though previous in vivo studies demonstrated retention of mRNA-expressed PGT121 in the FRT out to 28 days[70]. GPI anchors can be naturally cleaved in vivo by endogenous membrane-associated enzymes such as phospholipases[116]. It may be possible to co-transfect vaginal cells with mRNA encoding these types of enzymes for tighter control over release kinetics.

In vivo studies using animal models with FRTs similar in anatomy and size to humans are ongoing and necessary to determine the mRNA dose needed to achieve sufficient HCA expression for contraception. It is unknown how much HCA is required in the vaginal epithelium to provide complete contraceptive protection, however our recent phase 1 clinical trial using a 20 mg HCA-N film was sufficient to block virtually all progressive sperm in the cervix[35]. Fortunately, mRNA production and purification is extremely scalable[117] and higher doses should be tolerable due to the transient and nonimmunogenic nature of modified mRNA[64]. Additionally, cost of manufacture of mRNA is inexpensive compared to protein production[118].

HCA expression was achieved as quickly as 2 hours in vitro. Thus mRNA-mediated contraception may serve as an on-demand method to be self-administered by the user and has the potential to remain active in the FRT for multiple days, especially with the incorporation of a membrane anchor. The mRNA in vivo applicator is currently undergoing design optimization. Long-term storage and shelf-life are possible via lyophilization which increases mRNA stability[63].

Furthermore, this paradigm may be applicable to the development of multipurpose prevention technologies (MPTs), all-in-one products for the prevention of both sexually transmitted infections (STIs) and pregnancy[31]. For example, co-transfecting with mRNA strands that express both HCA as well as neutralizing antibodies like PGT121 could provide both contraception and HIV protection in one product (see Appendix 2 for preliminary data). Overall, this topical mRNA mechanism is a cost-effective, durable, safe, and accessible alternative to protein delivery of HCA and may provide a novel nonhormonal and reversible method of contraception, though further characterization is needed.

Acknowledgements

Many thanks to Jess Barmine for assisting with the hormone experiments and to ZabBio, Inc. for kindly providing HCA-N.

CHAPTER FOUR: Platform-specific Fc *N*-glycan profiles of two current HCA production methods and their implications

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Author Contributions: EN led conceptualization of the study, performed the phagocytosis assays, and wrote the manuscript. CX performed the mass spectrometry analyses and contributed to the manuscript. PS and KW provided resources. DJA and CEC provided advice, oversight, and resources and contributed to the manuscript.

This manuscript is being prepared for publication.

Abstract

Fc *N*-glycosylation of IgGs is necessary for effector functions and is a crucial component of quality control. The choice of manufacturing platform has the potential to significantly influence the Fc glycans of an antibody and consequently alter its clinical profile. Human Contraception Antibody (HCA) is an IgG1 monoclonal antisperm antibody currently in clinical development as a novel, nonhormonal contraceptive. Part of its development is selecting a suitable expression platform specific to the female reproductive tract. Here, we compared two HCA platforms of interest, namely *Nicotiana*-based manufacturing (HCA-N) and mRNA-mediated expression (HCA_{mrna}) in vaginal cells. Fc *N*-glycan profiles of HCA produced by these two platforms were determined using mass spectrometry which revealed major differences in site occupancy, glycan types, and glycoform distribution. Analysis of antibody-dependent cellular phagocytosis (ADCP), an Fc-mediated function, to address how these discrepancies may affect biological properties revealed significant differences in sperm phagocytosis between the platform-specific HCAs. Overall, we provide evidence that these two platforms produce functionally distinct HCAs which may inform platform decisions not only for HCA clinical development but for monoclonal antibodies in general.

Introduction

Co- and post-translational modifications (PTMs) are compositional changes to proteins that occur within or outside the cell following translation. These changes are most commonly additions of modifying groups such as methyl, glycosyl, and phosphoryl groups. PTMs can modify the structure and behavior of a protein[119]. Like most proteins, monoclonal antibodies (mAbs) are subject to multiple PTMs as they transit the cell prior to secretion. Glycosylation is the most common mAb PTM and has a significant role in biological activity, stability, and antigenicity[120,60]. Glycosylation can alter pharmacokinetics and pharmacodynamics of an antibody through interactions with the neonatal Fc receptor and the endocytic mannose receptor[57]. Glycans on mAbs expressed by platforms such as non-human mammalian cell lines have proven to cause unwanted immunogenic responses[121]. Thus glycan analysis is crucial to the quality control process of therapeutic antibodies, though there is little clarity over the extent to which glycosylation in this context should be regulated[59].

The majority of monoclonal antibodies available on the market are of the isotype subclass IgG1[122]. Human IgGs contain a conserved *N*-linked glycosylation on asparagine residue 297 (Asn^{297}) in the heavy chain constant domain 2 (CH2) of the Fc region, a crucial element in IgG structure and function[123] (Fig 4.1A). *N*-linked glycosylation features an oligosaccharide consisting of several sugar moieties attached to the amide nitrogen of an asparagine residue via a trimannosyl chitobiose core. In humans, these sugar moieties are limited to fucose, mannose, *N*-acetylglucosamine (GlcNAc), galactose, and sialic acid residues. The *N*-glycan lipid precursor, dolichol phosphate, is synthesized in the

Endoplasmic Reticulum (ER)[124]. Subsequent transferase reactions complete the assembly of *N*-glycan precursor $Glc_3Man_9GlcAc_2$ on the dolichol phosphate (Fig 4.1B). The precursor glycan is transferred to nascent proteins at an asparagine reside within the sequence Asn-X-Ser/Thr, where X is any amino acid except proline. The proteins undergo proper folding and trimming of glucose and mannose residues and are eventually transported to the Golgi for *N*-glycan maturation. Here, stepwise actions of highly selective glycosidases and glycotransferases can replace mannose branches with GlcNAc and additional glycan residues including fucose, galactose, and sialic acid can be added to the glycan structure (Fig 4.1C).

There are three main classes of *N*-glycan: oligomannose, complex, and hybrid. Each contains the $Man_3GlcNAc_2$ core; they differ in their terminating branch patterns[125]. Beyond the chitobiose ($GlcNAc_2$) core, oligomannose (high mannose) types contain only mannose residues, up to nine, and occur when the mannose moieties in the *N*-glycan precursor are preserved. Complex types are generated by substituting the $\alpha3$- and $\alpha6$-linked mannose residues with GlcNAc. Other moieties can be added to these branched GlcNAc residues. Hybrid glycans retain one unsubstituted mannose branch and have one or more GlcNAc linkages.

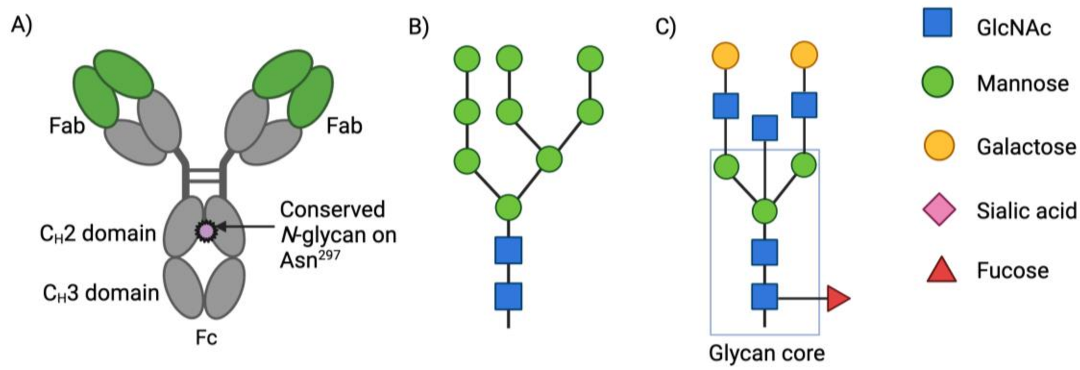

Figure 4.1- The conserved IgG Fc *N*-glycan. A) Structure of an IgG antibody. B) *N*-glycan precursor. C) Example of a mature *N*-glycan with each of the most likely moiety additions. The rectangle indicates the trimannosyl chitobiose glycan core. Purple diamond indicates *N*-Acetyl sialic acid. Created in Biorender.com.

Antibodies can function through several mechanisms facilitated by the Fc region including opsonization, promotion of phagocytosis via macrophage activation, and complement-mediated cytotoxicity. Depending on the therapeutic goal of an antibody, triggering effector function may be desirable or undesirable. While effector functions are beneficial for efficient clearing of antigens, an exceedingly strong and persistent immune response, especially in sensitive mucosal areas like the vagina, can be damaging. *N*-glycosylation is a key factor that modulates IgG Fc-mediated functions. The IgG-Fc glycan at residue 297 is necessary for effector function through Fcγ receptor (FcγR) binding[123]. Since the Fc region is the site of contact for Fc receptors, changes in the structure of the Fc domain conferred by the conserved Fc *N*-glycan at Asn^{297} can have a major impact on receptor binding and thus effector function[126]. Efforts in glycoengineering are of increasing interest as a means to customize therapeutic antibody effector functions[127].

Certain glycan moieties can affect binding to Fc receptors differently and thus influence an antibody's potential for Fc-mediated functions. For example, oligomannose glycoforms demonstrate rapid clearance and enhanced Fc binding[128,129]. Hypergalactosylated glycans can enhance receptor binding and antibody-dependent cellular cytotoxicity (ADCC) activity[74]. A bisecting GlcNAc has also been shown to induce strong cytotoxicity. A terminal sialic acid can contribute to longer half-life and anti-inflammatory effects. The addition of a core fucose moiety can inhibit interactions between the Fc and Fc receptors, thus reducing the ability of IgG to trigger effector functions. Overall, Fc *N*-glycans can play a significant role in fine-tuning the pharmaceutical properties of an antibody.

The main contraceptive mechanism of Human Contraception Antibody (HCA), an IgG1 antisperm mAb in development as a novel nonhormonal contraceptive, is sperm agglutination, however one Fc-mediated function of HCA that is of considerable interest is antibody-dependent cellular phagocytosis (ADCP). We previously demonstrated that HCA is capable of mediating certain effector functions such as complement-mediated sperm immobilization and sperm phagocytosis via Fc-Fc receptor interactions[106]. Though ADCP could potentially serve as a secondary contraceptive mechanism to clear sperm, subsequent antigen presentation which may be possible due to the presence of antigen presenting cells within the vaginal epithelium[130,50], would be highly undesirable. Multiple Fc gamma receptors are responsible for eliciting ADCP including FcγRI, FcγRlla and FcγRIII[131]. Interactions between platform-specific HCA Fc *N*-glycans and these Fc receptors may modulate HCA-mediated sperm phagocytosis and thus clinical profile.

Differences in glycan composition among expression platforms and glycan heterogeneity within a single platform can significantly impact the biology of an antibody. Protein glycosylation is cell type-dependent, inherently heterogeneous, and relies on a number of factors that contribute to final structure[132]. These factors include enzyme levels within a cell, availability of monosaccharide nucleotides, and Golgi architecture such as organization and localization of enzymes within the Golgi apparatus. Thus, selection of an appropriate expression system is an important quality control component in antibody production.

Advances in monoclonal antibody engineering have led to the exploration of novel production methods to increase yield and lower costs. Our group has been exploring several expression systems for HCA. One such platform is *Nicotiana benthamiana*, a close relative of the tobacco plant, for production of "plantibodies" [27]. This platform allows for rapid, low-cost, large-scale production of monoclonal antibodies. It is a viral-based, transient expression system that leads to accumulation of antibodies within days (Fig 4.2A). Whole mature plants (genetically modified to knock out xylosyl- and fucosyl-transferase) are infiltrated with a highly dilute *Agrobacterium* suspension carrying t-DNAs encoding viral replicons. The result is a high copy number of RNA molecules encoding the antibody. The *Nicotiana* plants used in this system are transgenic strains with altered glycosylation pathways so that produced antibodies contain mammalian glycoforms. This production method is currently being used to manufacture HCA-formulated topical vaginal films for clinical trials[133,134].

Another platform for HCA is topical delivery of synthetic mRNA encoding HCA in the female reproductive tract (Fig 4.2B). mRNA platforms provide several advantages including: cost-effectiveness, high efficiency, reversibility, safety, and durability[62]. Synthetic mRNAs encoding HCA are generated via in vitro transcription and modified with a 5' cap and N1-methylpseudouridine substitution to increase stability and evade innate immune sensors[64]. The HCA mRNA strands are then used to transfect cells in vitro using lipid nanoparticles or in vivo by aerosol delivery directly to vaginal epithelium. Once mRNA is taken up by cells, host translational machinery translates and secretes HCA. This method has previously been established in an animal model for expression of anti-HIV antibodies in vaginal mucosa[70]. mRNA HCA expression bypasses the need to eliminate non-human glycans, as is required in *Nicotiana* expression. mRNA uptake in human vaginal cells leads to antibody production with glycans native to the host.

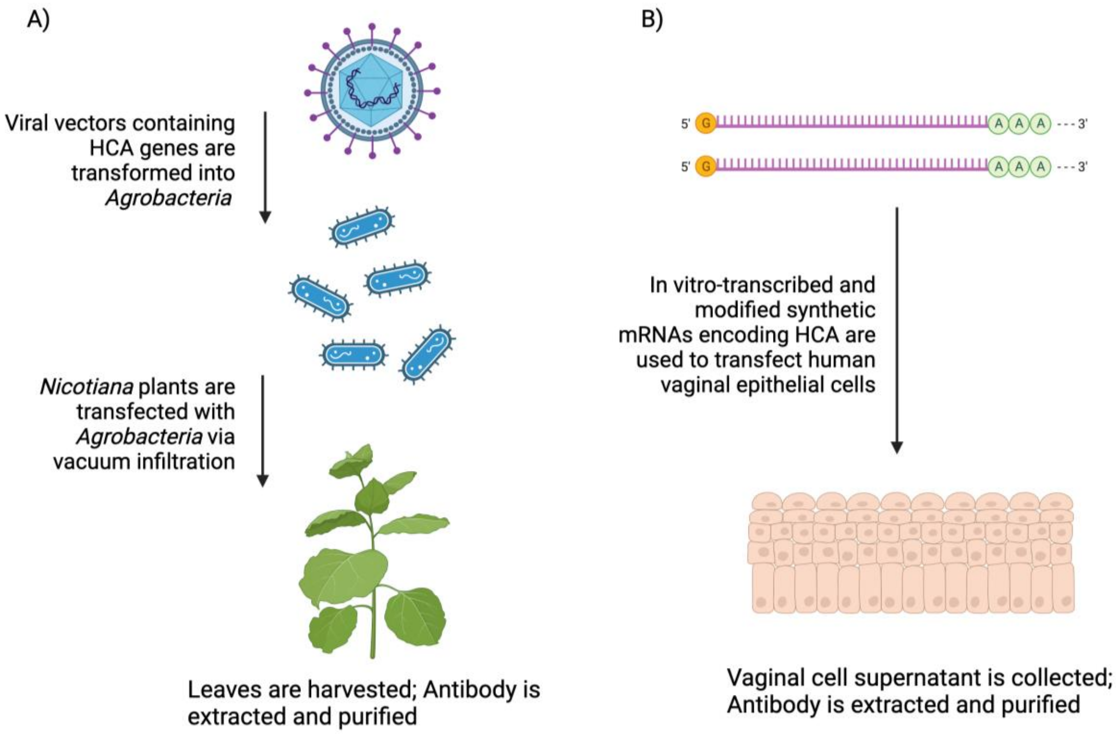

Figure 4.2- Novel HCA expression platforms. A) HCA produced in *Nicotiana benthamiana* (HCA-N). B) HCA produced in mRNA-transfected vaginal epithelial cells (HCA_{mRNA_VK2}). Created in Biorender.com

Glycosylation is one of the most critical quality attributes that impacts efficacy, safety, and stability of monoclonal antibody therapeutics. Choice of production platform and consequently types of posttranslational modifications can dramatically impact the safety and biophysical properties of antibodies in solution. Antibody production in different systems can result in a variety of heterogeneous glycoforms at site Asn^{297}. The most widely used host of biopharmaceutical production, the Chinese hamster ovary cell (CHO), yields heterogeneous glycans which can lead to inconsistency of the functional profile of an antibody. With novel expression systems emerging, the extent to which their respective glycosylation patterns affect mAb function is important to understand.

We applied mass spectrometry-based glycoproteomic techniques to characterize and compare the HCA Fc *N*-glycan compositions produced by two current expression systems, namely *Nicotiana benthamiana* and topical synthetic mRNA. We also explored whether differences in Fc *N*-glycans influence the biological properties of HCA. Potential for Fc-mediated function, specifically antibody-dependent cellular phagocytosis, was compared between mRNA- and *Nicotiana*-produced HCA. The overall goal of this work is to elucidate how the HCA expression platforms influence glycan composition and thus quality control of biological activity.

Methods

mRNA HCA production (HCA$_{mRNA_VK2}$)

VK2 E6/E7 (human vaginal epithelial) cells were obtained from ATCC and cultured in complete Keratinocyte-Serum Free medium supplemented with human recombinant epidermal growth factor (rEGF), bovine pituitary extract (BPE), and Pen-Strep (GIBCO-BRL 17005-042). mRNA strands encoding heavy and light chains of HCA were generated in the Santangelo lab at Emory University. Sequences were ordered as a DNA gBlock with 5’ and 3’ UTRs and cloned into a vector. Vectors were purified and in vitro transcribed (IVT) with an N1-methyl-pseudouridine modification. Resultant mRNAs were purified and capped prior to use. Lipofectamine MessengerMAX reagent (ThermoFisher Scientific) was used to transfect VK2 cells with mRNAs in Opti-MEM

(Gibco). Following a two-day incubation at 37 ºC, IgG molecules were purified from cell supernatant either by 100K molecular weight cut-off spin filtration, tangential flow filtration, or with Melon™ Gel IgG Purification Kit (ThermoFisher Scientific).

Nicotiana HCA production (HCA-N)

HCA was produced in *Nicotiana benthamiana* (HCA-N) by KBio, Inc. (Owensboro, KY) as previously described[135,136]. Transgenic strains of *Nicotiana* plants subjected to fucosyl- and xylosyl-transferase knockout (**Δ**XF) were used. Xylosyltransferase (XylT) knockout prevents addition of xylose, a non-mammalian glycan residue. Fucosyltransferase (FucT) knockout prevents core α1,3-fucose, a non-mammalian linkage of fucose[137]. Briefly, whole mature plants were vacuum-infiltrated with an *Agrobacteria* suspension carrying t-DNAs encoding viral replicons resulting in a high copy number of RNA molecules encoding HCA. Plants were than harvested to extract and purify HCA-N.

Mass spectrometry analysis

HCA IgG protein was characterized by liquid chromatography tandem mass spectrometry (LC-MS). Prior to analysis, 5 µg of each IgG sample were reduced with 10 mM of dithiolthreitol, alkylated with 50 mM iodoacetamide, then digested with Glu-C (C-terminal cleavage at of glutamic acid, 1:50, enzyme:protein) and trypsin (C-terminal cleavage at arginine and lysine, 1:50, enzyme:protein) at 37 °C for 16 hours and the digest mixture was cleaned up with a C18 SPE cartridge. Next, samples were analyzed by nanoUPLC-MS/MS for determination of peptide molecular masses followed by fragmentation in order to locate the glycosylation site(s) and assign glycoform compositions. For determination of the *N*-glycosylation site occupancy, an aliquot of the above digest mixture was dissolved in water (^{18}O, 97%, Cambridge Isotope Laboratories, Inc., Tewksbury, MA) and deglycosylated with PNGase F (New England Biolabs Inc., Ipswich, MA) at 37 °C for 16 hours prior to MS analysis. NanoUPLC-MS/MS analyses were performed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher Scientific, Waltham, MA) coupled with an ACQUITY UPLC M-Class system (Waters Corp., Milford, MA) via a TriVersa NanoMate (Advion, Ithaca, NY). A nanoEase Symmetry C18 UPLC Trap Column (100 Å, 5 µm, 180 µm × 20 mm, Waters) was used as the trapping column and a nanoEase MZ HSS C18 T3 UPLC Column (100 Å, 1.8 µm, 75 µm × 100 mm, Waters) was used as the analytical column for LC separation. Peptides were trapped at 4 µL/min for 4 min with 1% acetonitrile and 0.1% formic acid (Solvent A). Then, the peptide/glycopeptide mixtures were separated on the analytical column according to the following conditions: 0–1 min: 2% B, 1–3 min: 2–5% B, 3–43 min: 5–40% B (Solvent B: 99% acetonitrile and 0.1% formic acid).

MS analyses were performed in the positive mode with the RF lens set to 30% and scans were acquired with the following settings: 120,000 resolution @ *m/z* 200, scan range *m/z* 370–2000, 1 µscan/MS, normalized AGC target 250%, and a maximum injection time of 50 ms. For high energy collisional dissociation (HCD) analyses, initial MS^2 scans (normalized collision energy (NCE) 30%) were acquired with the following settings: 15,000 resolution @*m/z* 200, scan range *m/z* 100–2000, 1 µscan/MS, AGC target $1x10^6$, and a maximum injection time of 100 ms. For analysis of samples that had not been treated with PNGase F, oxonium ions were used to sense the presence of a glycopeptide and then trigger the generation of an HCD MS/MS spectrum. If two of six common oxonium ions (*m/z* 204.0867 (HexNAc ion), *m/z* 138.0545 (HexNAc-CH_6O_3 ion), *m/z* 366.1396 (HexNAcHex ion), *m/z* 168.0653 (HexNAc – 2 H_2O fragment ion), *m/z* 186.0760 (HexNAc - H_2O fragment ion), *m/z* 292.1031 (NeuAc ion), *m/z* 274.0927 (NeuAc – H_2O fragment ion) were detected in the HCD spectrum within 15 ppm mass tolerance, MS^2 spectra were acquired. For HCD-triggered-HCD, the triggered-HCD scan was set to 30,000 resolution @*m/z* 200, scan range *m/z* 100-2000, 1 µscan/MS, AGC target $1x10^6$, and a maximum injection time of 150 ms. MS/MS data were searched against 20352 entries in a UniProtKB database restricted to *Homo sapiens* (downloaded in May, 2021) by PMI-Byonic (version v3.8-11, Protein Metrics Inc., CA, USA). Carbamidomethylation (C) was set as fixed modification, whereas Met oxidation and protein N-terminal acetylation were defined as variable modifications. Mass tolerance was set to 10 and 20 ppm at the MS and MS/MS levels, respectively. Enzyme specificity was set to C-terminal of glutamic acid and C-terminal of arginine and lysine with a maximum of two missed cleavages. The Protein Metrics 172 human *N*-glycan library was used for assignment of *N*-linked glycosylation. For samples that had been treated with PNGase F, HCD MS/MS data were obtained for the 20 most abundant peaks in the MS1 spectrum. Ratios of unlabeled and ^{18}O-labeled peptides were based on peak heights in the MS1 spectra.

Antibody-dependent sperm phagocytosis

U937 pro-monocytes were obtained from ATCC and cultured in RPMI 1640 complete medium supplemented with L-Glutamine, Fetal Bovine Serum (FBS), and Pen-Strep (GIBCO-11875093). Cells were seeded at a density of 0.5 x 10^6/ml in 6-well plates containing sterile glass coverslips. Cells were treated with 100 ng/ml of phorbol-12-myristate-13-acetate (PMA) to stimulate macrophage differentiation for 48-72 hours at 37 ºC, after which activated U937 cells adhered to coverslips at around 1 x 10^6 cells per coverslip. Sperm cells were obtained and isolated from human semen samples from healthy men aged 18-45 years. All semen donors provided informed consent prior to collection (Human Subjects Protocols H36843 and H41454).

The ADCP assay was performed as described by Oren-Benatoya, et al. with modifications[95]. Briefly, 1 x 10^6 sperm cells were suspended in sperm multipurpose handling medium (MHM; FUJIFILM Irvine Scientific, Santa Ana, CA) supplemented with either HCA-N, HCA_{mRNA_VK2}, Campath (anti-CD52, positive control), VRC01 (anti-HIV, isotype control), or medium only (negative control). Sperm-antibody suspensions were added to wells and were incubated at 37 ºC for 30 min. Following a wash step, cells were incubated in PBS at 37 ºC for 30 min and treated with trypsin for 5 min to remove non-specifically bound sperm. The coverslips were retrieved and treated with Differential Quik III dye kit (Polysciences, Warrington, PA, USA) to visualize phagocytosis under a light microscope. The number of associated antibody-opsonized sperm per macrophage was counted for each condition. Associated sperm were considered those either opsonized/engaged with the Fc receptor (attached) or partially engulfed.

Statistical analysis

Data analysis and graph creations were performed using GraphPad Prism (Version 9.4.1; GraphPad Software Inc.; San Diego, CA, USA). Statistical significance between HCA-N and HCA_{mRNA_VK2} ADCP assay results was determined by two-tailed paired t-test. Data were natural log-transformed prior to analysis. The definition of statistical significance was $p<0.05$.

Results

Sequence coverage was sufficient for both HCA-N and HCA_{mRNA_VK2}

To address our questions, we applied RP UPLC-MS/MS to investigate the glycosylation of both HCA-N and HCA_{mRNA_VK2}. Data obtained for HCA-N provided exceptional sequence coverage of HCA heavy and light chains and showed little to no contamination by other proteins (Table 4.1), owing to the fact that *Nicotiana* HCA production was performed in a commercial, GMP-grade facility (KBio, Inc., Owensboro, KY). HCA_{mRNA_VK2}, produced from cell culture in an academic lab setting, contained a high percentage of additional proteins. The high abundance of serotransferrin, which made up 66% of the sample, can be attributed to its endogenous expression by VK2 cells and subsequent transfer into the culture supernatant (Supplemental Table 4.1) (Serotransferrin is also among the 153 proteins produced in the female reproductive tract (FRT) that are included in the "Normal Pap Test Core Proteome"[138]). Even so, the sequence coverage of HCA heavy and light chains in HCA_{mRNA_VK2} was sufficient to confirm their production and the abundances of peaks corresponding to glycopeptides containing Asn^{297} were sufficient to generate the glycan profile.

Table 4.1- Sequence coverage and protein composition of HCA-N and HCA_{mRNA_VK2}

	Protein	Sequence coverage	Percentage of total sample (%)
HCA-N	IgG Lambda Heavy Chain	99.6	-
	IgG Lambda Light Chain	80.8	-
HCA_{mRNA_VK2}	IgG Lambda Heavy Chain	93.84	10
	IgG Lambda Light Chain	65.54	5
	Serotransferrin	80.69	66
	Bovine Serum Albumin	84.02	4
	Glutathione S Transferase	84.69	2
	Keratin, Type II Cytoskeletal	45.10	2
	Clostridial Collagenase	N/A	< 1
	Bovine Glutamate Dehydrogenase 1, Mitochondrial	N/A	< 1

Compositional differences in N-glycan profiles between HCA-N and HCA_{mRNA_VK2}

Human IgGs usually contain *N*-glycans only at the highly conserved *N*-glycosylation site on the Fc region of the heavy chain. There are two *N*-glycosylation sites (NXS/T, X ≠ P) on the heavy chain of HCA (N^{71} and N^{297}) but there are no *N*-glycosylation sites on the light chain. The first heavy chain site is largely unoccupied (Fig 4.S1). The second, highly conserved glycan site is Asn^{297} (Fig 4.3). The presence of b- and y-type fragment ions provided the full peptide sequence information; existence of the b- and y-type product ions in this MS/MS spectrum allowed assignment of the peptide sequence as ^{293}EEQYNSTYR^{301}. Moreover, the b_5/Y_1, b_5/Y_1 and y_5/Y_2 fragment ions (e.g.,

b_5+HexNAc, y_5+HexNAc, y_5+HexAc$_2$ ions) precisely defined the glycosylation site at N[297].

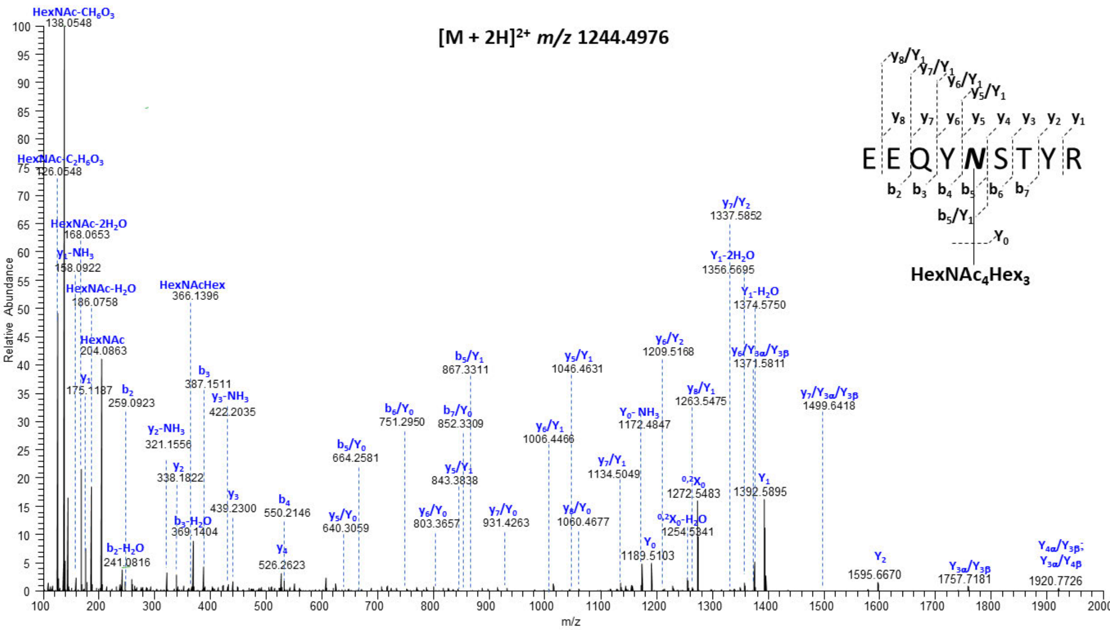

Figure 4.3- Representative HCD tandem mass spectrum of the $[M + 2H]^{2+}$ precursor ion observed at m/z 1244.4976, corresponding to the *N*-glycopeptiform obtained from IgG HCA, consisting of the peptide [293]EEQYN297STYR[301], with its backbone modified at N[297] by HexNAc4Hex3.

Mass spectrometry-based glycoproteomic analysis revealed 13 unique glycoforms of all three glycan types (i.e. oligomannose, hybrid, complex) at the Asn[297] site on HCA-N (Fig 4.4). The quantitative analysis of *N*-glycosylation site occupancy in the *Nicotiana*-generated antibody revealed that only 49.1% of the glycosylation site was occupied (Table 4.2). Among samples with an occupied site, the three glycan classes were generally well distributed. Interestingly, despite knockout of α1,3 FucT and the absence of other fucosyltransferases within the transgenic *Nicotiana* platform, around 1% of the sample was fucosylated, suggesting incomplete knockout. The most abundant glycan within this sample was G0, consistent with previous literature on *Nicotiana* manufacturing[139,140].

The Fc *N*-glycan profile of HCA-N was compared to HCA_{mRNA_VK2} which contained 11 unique glycoforms at the Asn^{297} site (Fig 4.4). Interestingly, the *N*-glycosylation site occupancy on HCA_{mRNA_VK2} was 96.0% (Table 4.2). All three glycan types were represented, however, there was a bias toward complex types containing a fucose moiety. Specifically, the glycans of highest abundance were G0F, G1F, and G2F, which are common among human IgGs. As mentioned earlier, core fucose can potentially reduce binding affinity to FcγRs[141]. Of note, HCA_{mRNA_VK2} contained a sialylated glycan (G2S1F), also known to reduce Fc receptor binding but promote a longer half-life. Thus, there were considerable differences in site occupancy, glycan types, and glycoform distributions between these platform-specific HCAs.

Additionally, we observed that a monoHexNAc modified the Asn^{297} site of the Asn-Ser-Thr sequon of both HCA-N and HCA_{mRNA_VK2}. This modification is denoted as "other" in Figure 4.4. Unlike a typical *N*-glycosylation of the Asn on NXS/T with a $HexNAc_2Hex_3$ core, the monoHexNAc as an *N*-glycan modifying the Asn^{297} site of this sequon is uncommon and not well understood.

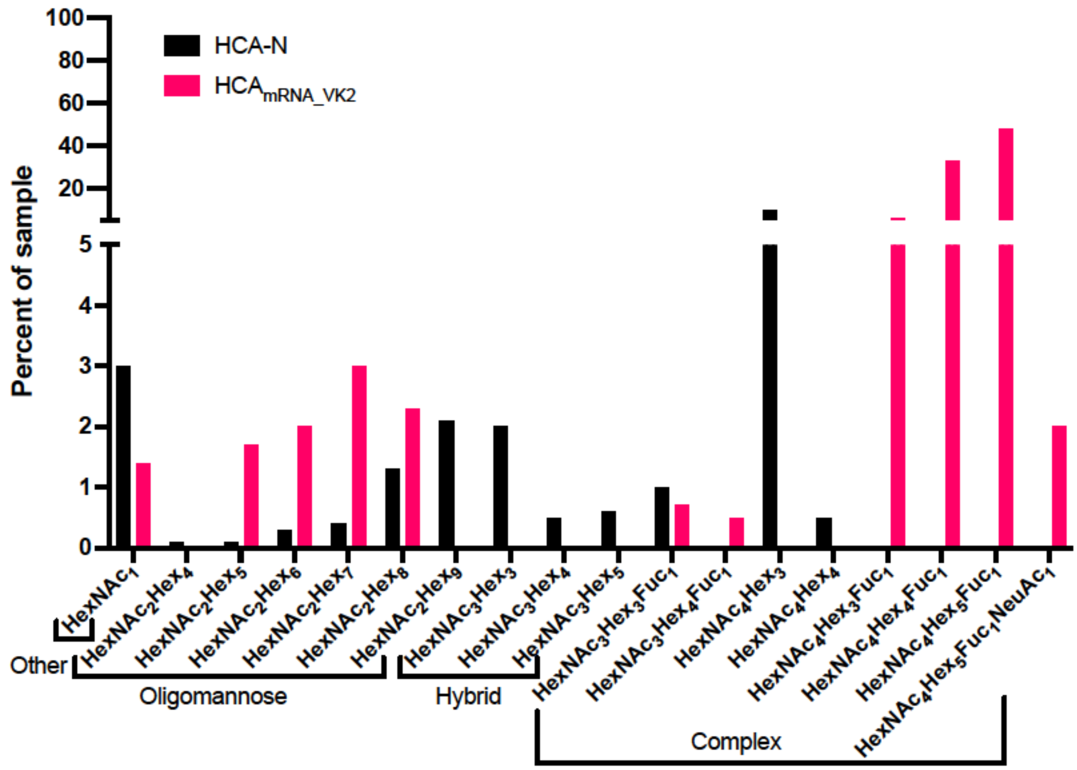

Figure 4.4- Composition of Fc *N*-glycans on platform-specific HCAs. A) Fc *N*-glycan profiles of HCA-N (black) and HCA_{mRNA_VK2} (pink). Glycans are located on the second potential *N*-glycosylation site on the HCA heavy chain (N^{297}) at amino acid sequence R.EEQYNSTR.V.

Table 4.2- Quantification of the *N*-glycosylation site occupancy.

	HCA-N	**HCA_{mRNA_VK2}**
Occupied	49.1%	96.0%
Not occupied	50.9%	4.0%

Prior to quantifications, digested samples were dissolved in water and deglycosylated with PNGase F.

HCA-N and HCA_{mRNA_VK2} exhibit different levels of sperm phagocytosis

Both HCA-N and HCA_{mRNA_VK2} were able to mediate sperm opsonization and phagocytosis at a low concentration of 3.33 µg/ml (Fig 4.5A, B). However, HCA_{mRNA_VK2} was able to induce significantly more sperm phagocytosis than HCA-N (0.42 and 0.09 sperm per macrophage, respectively, $p = 0.0108$). We have previously shown that at higher concentrations (e.g., 25-50 µg/ml), HCA-N can induce a greater degree of ADCP[106], but given that a large percentage of the Fc *N*-glycan site on HCA-N is unoccupied, as seen in Table 2, it is possible that at a lower concentration there are not enough *N*-glycans present to interact with FcγRs and induce a stronger response, whereas HCA_{mRNA_VK2} is highly glycosylated. Overall, there are biological differences, specifically differences in Fc function, between platform-specific HCAs.

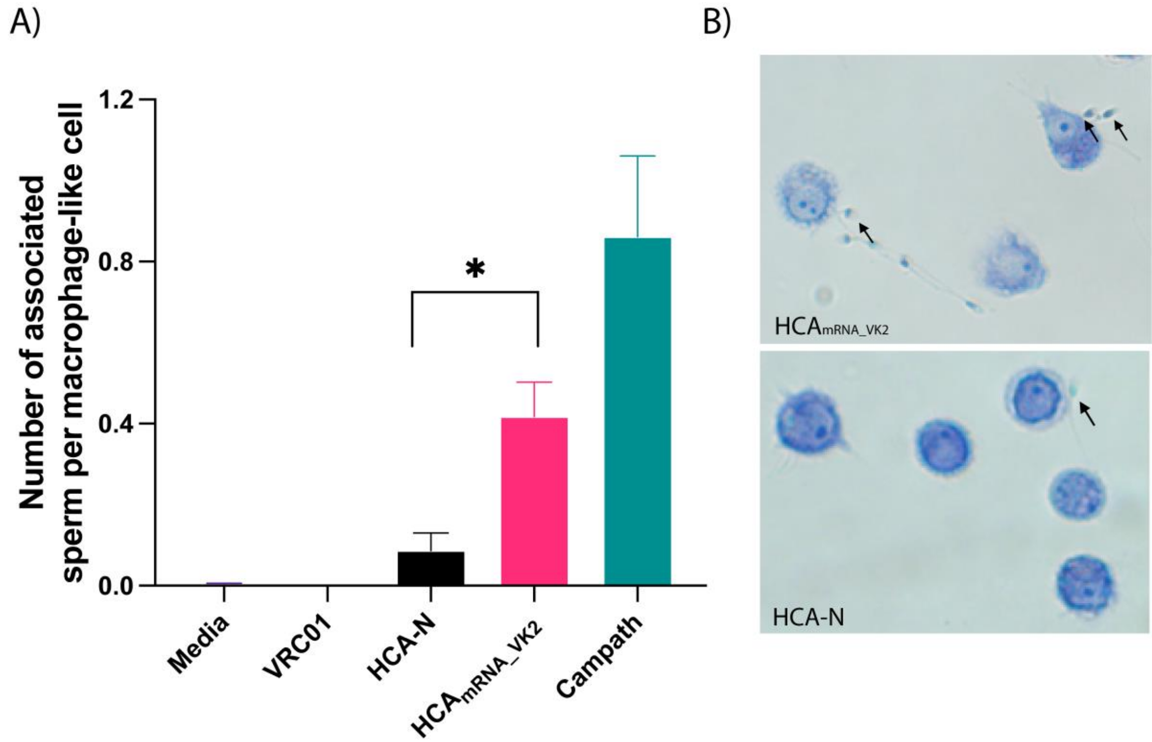

Figure 4.5- Antibody-dependent sperm phagocytosis of platform-specific HCAs. A) Number of associated sperm per macrophage-like cell (i.e. engaged with Fc receptor/opsonized or partially internalized) for each antibody treatment (3.33 µg/ml). Assay controls are as follows: Media-only negative control (n=2), Campath positive control (n=2), and VRC01 isotype control (n=1). HCA-N and HCA_{mRNA_VK2} data are expressed as mean ± SEM of three independently performed experiments. HCA-N and HCA_{mRNA_VK2} log-transformed data were analyzed by two-tailed paired t-test (p = 0.0108). B) Images of sperm cells associated with macrophage-like cells during the phagocytosis process. Images were taken at 200X magnification.

Discussion

As the IgG-Fc glycan at residue Asn^{297} is needed for effector function through FcγR binding, it is important to characterize occupancy and glycoform distribution and how they may differ among expression platforms. Here, we have shown that two current production methods of HCA, namely transgenic *Nicotiana benthamiana* and mRNA-mediated expression in vaginal cells, have distinct Fc *N*-glycan profiles. Though mRNA-mediated expression of HCA in vaginal cells eliminates the need for the knockdown of certain transferases for unwanted non-human glycans and confers native glycans to the antibody, HCA-N had a simpler glycan profile than that of HCA_{mRNA_VK2}, which is a preferable characteristic for quality control in antibodies. Mass spectrometry analysis confirmed that the Asn^{71} site on the heavy chain of HCA IgG is rarely occupied by a glycan, while the Asn^{297} site is glycosylated with a variety of glycoforms. Moreover, most of these glycoforms are classified to complex type in mRNA-expressed HCA. It is worth noting that HCA_{mRNA_VK2} has a higher degree of fucosylation than HCA-N. Additionally, we observed that an uncommon monoHexNAc modified the Asn^{297} site of the Asn-Ser-Thr sequon of both HCA-N and HCA_{mRNA_VK2}. It is unclear how the monoHexNAc modification is produced and whether it has a biological function.

We also found that HCA-N and HCA_{mRNA_VK2} produced varying levels of antibody-dependent sperm phagocytosis, an important Fc-mediated function of interest within the FRT. This was not surprising since the *N*-glycan composition and biodistribution were distinct for each platform. ADCP may be an undesired Fc mechanism of HCA because antigen presentation of sperm proteins may lead to host production of antisperm antibodies and contraceptive irreversibility. However, the potential for this scenario in vivo is still unclear and will need to be further evaluated in future HCA clinical trials. We provide evidence that different expression platforms, even for the same biologic, can impact bioactivity and safety profile. Therefore, when selecting an expression system for antibodies, platform-specific glycans may be an important consideration among quality control parameters.

Glycan profiles of the Fc region are critical antibody quality attributes. For this reason, the FDA and other regulatory agencies require pharmaceutical companies to characterize and maintain drug glycosylation within defined acceptance criteria that limit deviation from the pattern present in the drug candidate used in clinical trials[142]. Current efforts in glycoengineering and tailoring glycosylation patterns may facilitate the development of mAbs with more homogeneous IgG glycans that elicit the desired set of effector functions for their respective therapeutic uses[143]. Several methods are in development to tailor glycosylation. One method is enzymatic remodeling of IgG glycosylation, either by residue alteration via knockout or recombinant expression of glycosyltransferases, to achieve addition or removal of the core fucose and/or sialic acids, or through use of transglycosylation via endoglycosidases and glycosynthases[58]. Another method is treatment with endoglycosidases EndoS or EndoS2 for the hydrolysis and removal of the Fc glycan to block certain effector functions[144]. Though certain challenges and limitations persist in this growing field, glycoengineering may be an important tool and future direction that would allow for optimization and homogenization of the Fc *N*-glycans on HCA within a given expression platform.

Supporting Information

Table 4.S1- Protein composition of untransfected VK2 culture supernatant.

Protein	Percentage of total sample	Sequence coverage (%)	Molecular weight of unmodified protein (kDa)
Serotransferrin	97	66	77
HCA IgG Lambda Heavy Chain	<1	11.2	48
HCA IgG Lambda Light Chain	<1	9.9	26
Bovine Serum Albumin	1	78.9	66
Trypsin	1	20.8	24

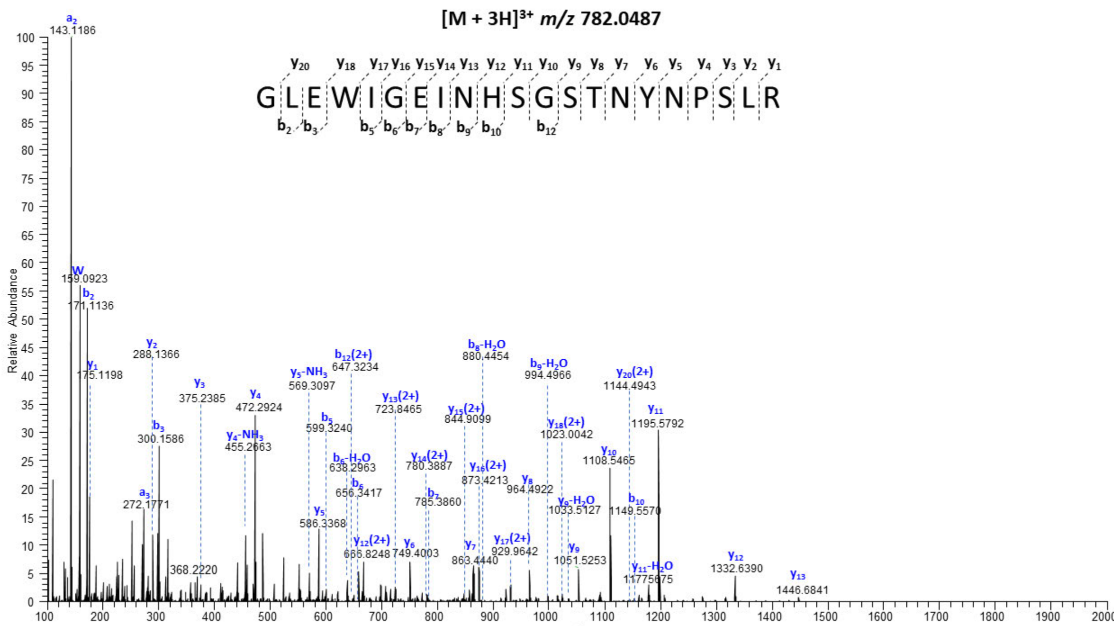

Figure 4.S1- HCD tandem mass spectrum of the $[M + 3H]^{3+}$ precursor ion at *m/z* 782.0487, ^{63}GLEWIGEINHSGSTNYN-PSLR^{83}, obtained from IgG HCA, corresponding to the non-glycosylated tryptic peptide containing the unoccupied potential *N*-linked site.

CHAPTER FIVE: Discussion

Summary

While several contraceptive methods are available on the market today, obstacles contributing to the high rate of global unintended pregnancies still exist. Hormonal options are the most effective but they do not address the needs of all women; many groups cannot use hormonal methods due to intolerable side effects or contraindications. Developing new modes of nonhormonal contraception would help to close this gap. Our group has seen success over the past few years in both preclinical and clinical studies of Human Contraception Antibody (HCA), a male reproductive tract-specific monoclonal antibody and potent agglutinator of sperm. This body of work aims to further optimize HCA as a nonhormonal option of contraception for women by harnessing innovative methods in antibody design, delivery, and production.

The female reproductive tract, the site of mucosal HCA delivery, is residence to several types of widely distributed immune cells including macrophages, NK cells, neutrophils, and dendritic cells[48]. It is imperative to determine immune cell interactions between HCA and the vaginal environment to fully characterize its profile for clinical use. Though sperm agglutination mediated by the Fab region has previously been described[29], Fc effector functions of HCA were not as clearly defined. Here, we demonstrated that HCA is capable of mediating such effector functions including complement-dependent cytotoxicity/sperm immobilization, mucus trapping, and antibody-dependent cellular phagocytosis[106].

HCA-LALAPG, an Fc-silenced variant[54], was used both as a control for HCA effector function studies and as a potential candidate for a more functionally conservative version of HCA. Though parent HCA and HCA-LALAPG exhibited no difference in sperm agglutination, HCA-LALAPG was unable to immobilize sperm due to reduced C1q binding and showed reduced sperm trapping in cervical mucus. Impairment of sperm trapping was expected since this mechanism is speculated to be dependent on electrostatic interactions between the Fc region and mucins in cervical mucus[53]. HCA-LALAPG was also incapable of eliciting phagocytosis of sperm by macrophages. Thus, the Fc-engineered HCA-LALAPG variant, with conserved agglutination potency, could be a more conservative alternative to HCA to reduce undesirable antibody-mediated inflammation by CDC and antigen presentation by ADCP within the female reproductive tract. However, postcoital test results in the phase 1 HCA film clinical trial suggest that certain Fc functions of HCA, especially CDC, are important for contraception[35] and completely ablating these secondary functions could be detrimental. This point will be discussed further in the study limitations section.

Local delivery of antibodies to the female reproductive tract is favorable over systemic delivery for a host of reasons including fewer side effects and better efficiency. One way to achieve topical delivery of concentrated HCA protein is through formulation in a topically insertable product such as dissolvable film or intravaginal ring[27]. For our phase 1 clinical trial, HCA produced in *Nicotiana* (HCA-N) was formulated into a highly concentrated vaginal film. Though these methods are feasible and efficacious, antibody production is costly and retention of contraceptive titers following administration via these methods is short-lived. Therefore, we are interested in establishing an mRNA-based HCA delivery platform to achieve cost-effective, host-native, and prolonged expression. Preclinical studies conducted by collaborators at Emory University demonstrated successful aerosolized delivery of mRNA to mucosal sites in vivo for protection against RSV and HIV-1[69,70]. Here, we applied this concept to the expression of HCA in the vaginal mucosa for contraception. We were able to express sperm-agglutinating titers of HCA following mRNA transfection in both a vaginal cell line and 3D vaginal tissue model using an atomization device. We demonstrated longer retention time with the inclusion of a GPI anchor on the heavy chain of the antibody as well as a favorable safety profile. Further optimization of this method and ongoing in vivo studies could lead to the inception of a new delivery paradigm for HCA.

Advancements in antibody engineering approaches have contributed to the growing field of monoclonal antibodies and have helped to put more candidates into the late-stage clinical pipeline. In fact, over two-thirds of antibodies in late-stage clinical trials in 2023 have been engineered to improve their therapeutic effect in some way[73]. In this study, we employed approaches in antibody engineering to multimerize IgG HCA and to establish a dimeric IgA HCA isotype. A hexameric IgG1 HCA multimer containing the tailpiece of IgM was designed to increase the number of epitope binding sites on sperm and thus increase agglutination potency. Both hexameric IgG1 and dimeric IgA HCAs were expressed via mRNA and were significantly faster at agglutinating sperm compared to parent HCA. Moreover, mRNA-expressed hexameric HCA had a low IC_{50} of 1.969

compared to the IgG1 HCA-N IC_{50} of 5.62. Variants with superior potency could be used in a product to increase efficacy and lower required doses.

When exploring different topical delivery methods for HCA, it is important not only to compare cost of production, ease of manufacture, and antigen binding potency, but also to investigate differences in antibody posttranslational modifications like glycosylation. The conserved IgG Fc *N*-glycosylation is necessary for binding to Fc receptors to elicit effector functions and clearance and can also contribute to antigenicity. We compared Fc *N*-glycan profiles on HCA from our two platforms of HCA currently under evaluation, i.e. protein production in *Nicotiana* (HCA-N) and mRNA transfection of vaginal cells (HCA_{mRNA_VK2}). Using mass spectrometry, we were able to show that there are, in fact, differences in Fc glycan occupancy, type, and distribution between these two platforms. Glycans from all three classes (i.e. hybrid, complex, oligomannose) were represented. However, half of Fc *N*-glycan sites on HCA-N were unoccupied (50.9%) and HCA_{mRNA_VK2} was highly glycosylated (96.0%) with a high abundance of the glycans G0F, G1F, and G2F, which are fucosylated complex type glycans.

Because variations of Fc glycans can influence Fc-mediated function, we then investigated whether the glycan differences seen between HCA-N and HCA_{mRNA_VK2} modulate ADCP activity, an Fc function. Both HCA-N and HCA_{mRNA_VK2} were able to mediate sperm opsonization and phagocytosis by macrophages at a low concentration of 3.33 µg/ml. HCA_{mRNA_VK2} was able to induce significantly more sperm phagocytosis than HCA-N. We have previously shown that at higher concentrations (i.e. 25-50 µg/ml), HCA-N can induce a greater degree of ADCP[106], but given that a large percentage of the Fc *N*-

glycan site on HCA-N is unoccupied, it is possible that at lower concentrations testes here there are not enough *N*-glycans present to interact with FcγRs and mount a strong ADCP response. Confirming that different HCA platforms of interest can influence the functional profile of the antibody further solidifies glycosylation as a critical quality attribute to be considered in further production efforts and may help to better select a suitable platform of HCA.

In conclusion, HCA is capable of both potent sperm agglutination and Fc-mediated effector functions including ADCP, CDC, mucus trapping, and sperm immobilization. Antibody engineering makes it possible to customize both the Fab and Fc regions through multimerization, isotype, and mutations to design an optimal variant with desired functions to be implemented within the female reproductive tract. Different delivery approaches and platforms including mRNA expression are possible and can offer improvements but can also introduce variations in glycosylation. These are all factors to be strongly considered and evaluated in further development and optimization of HCA as a nonhormonal contraceptive.

Study Limitations

An optimal HCA variant has still not been identified. Ideally, this variant would potently agglutinate sperm with a low IC_{50} and would be unable to elicit phagocytosis while still maintaining the ability to immobilize and trap sperm. Sperm phagocytosis poses the risk of antigen presentation and consequently host production of anti-sperm antibodies which would be highly undesirable in the effort to develop reversible contraception. We have characterized several engineered variants of HCA such as the superior agglutinator IgG/t and Fc-silenced HCA-LALAPG. However, these each fall short is some aspect; we have shown in preliminary studies that IgG/t exhibits higher levels of ADCP and that HCA-LALAPG, though incapable of ADCP, is also incapable of mucus trapping and sperm immobilization.

Our HCA film phase 1 clinical trial suggests that mucus trapping and sperm immobilization are especially important for the efficacy of the product. In the postcoital test following film use, there were virtually no progressive sperm in cervical mucus; these sperm were presumably agglutinated prior to reaching the cervix. Substantial counts of sperm in almost all participants were seen that fell into two other sperm populations: nonprogressively motile and immotile sperm. It is hypothesized that the nonprogressively motile sperm, i.e. moving in place, are sperm trapped in cervical mucus and that immotile sperm are either dead or immobilized as a result of complement-dependent cytotoxicity. In most participants, the immotile sperm category was the most represented in cervical mucus. The presence of complement proteins in the FRT suggests that the classical complement cascade is functional in cervical mucus and can interact with antibodies for Fc functions. Overall, these data suggest that CDC and mucus trapping play crucial contraceptive roles and should not be excluded by use of Fc mutants like LALAPG.

In developing and producing HCA products for in vivo use, the minimum dose required to achieve contraception has yet to be determined. Future studies are needed since users can vary in vaginal fluid volume that may dilute antibody concentration. In our phase 1 study, the film contained 20 mg of HCA which was sufficient to achieve our primary endpoint of <5 progressively motile sperm per high power field (PMS/HPF), though it is possible that this benchmark could have been achieved with a lower dose. Additionally, there have been some inconsistencies surrounding the primary endpoint in PCTs for vaginal contraceptive testing. Studies have defined cut-offs as <1, <5, and <10 PMS/HPF[145]. PCTs are crucial for contraceptive efficacy testing, however these types of studies are difficult to conduct because of logistical reasons. Participants must have tubal ligations to eliminate risk of contraception and must come into the clinic on multiple occasions for evaluation. Furthermore, it is not possible to determine the actual change in the rate of pregnancy from PCT trials.

The mRNA experiments in this body of work were performed in vitro. Though the 3D EpiVaginal tissue model is superior to immortalized cell lines because it is differentiated and more physiologically relevant, it only survives in optimal conditions for about one week and thus it is difficult to completely characterize mRNA-expressed HCA pharmacokinetics. Additionally, the lack of PBMCs in this 3D tissue model may have excluded the possibility of PBMC-derived inflammation in the safety profile experiments.

Our collaborators are currently conducting several in vivo HCA mRNA delivery experiments. However, mRNA-mediated expression in the non-human primate rhesus macaque model has posed several issues pertaining to HCA detection and functional analysis. Because macaques and humans have a high degree of genome similarity[146], we have had considerable trouble with background signal in untransfected animals. We plan to go forward in two ways. Firstly, we plan to add a 6xHis tag to the heavy chain of the mRNA HCA as a better way of purifying mRNA-expressed HCA from macaque secretions for quantification and agglutination studies. Secondly, we plan to conduct in vivo experiments in a sheep model to reduce species-based cross-reactivity. The Santangelo and Villinger groups have had previous success using the sheep model for mRNA delivery to the FRT to express PGT121 for HIV protection[70]. While sheep and rhesus macaques are considered acceptable FRT models due to anatomical similarities in size and architecture to humans[147,148], they pose several limitations including near-neutral vaginal pH[149,150], differences in vaginal microbiomes[151], and discrepancies in reproductive cycles[152].

Implications

The most apparent implication for this body of work is the introduction of a new, nonhormonal method of female contraception onto the market. Following years of preclinical studies and characterization, HCA has successfully completed a phase 1 clinical trial demonstrating safety and efficacy. We have also shown that the parent HCA used in the clinical trial has an extremely favorable stability profile (see Appendix 1). Our hope is for the continual drug development and progression of HCA through the clinical stage pipeline and the eventual availability of HCA as a cost-effective, over-the-counter, accessible product.

The efficacy of topical administration of a monoclonal antibody to the FRT for contraception also implies the potential use of other antibody-based drugs at the vaginal mucosa. Because fully humanized antibodies pose low risk of immunogenicity, they may be used in combination with others to promote protection against multiple indications as an MPT. Our group has previously completed a phase 1 clinical trial using MB66, a combination product containing anti-HIV-1 and anti-HSV-2 monoclonal antibodies[133], though future products could contain other anti-STI mAbs. Similar to the HCA phase 1 clinical trial, MB66 was formulated into a dissolvable vaginal film. However, these mAbs can be formulated into several types of alternative platforms like vaginal rings for extended release and even lubricant to be applied to the penis as a male product, currently in development.

This work also illuminates a non-obvious application for mRNA therapeutics. Often-cited potential applications of mRNA-based therapeutics include infectious diseases, metabolic genetic diseases, cancer, and cardiovascular diseases[67]. We demonstrate that mRNA-mediated applications at the vaginal mucosa, like contraception and STI protection, are feasible and should not be overlooked. We also validate the use of mRNA delivery via aerosol for topical delivery at mucosal sites. Moderna is similarly exploring an intranasally administered mRNA-LNP vaccine against SARS-CoV-2 infection to target mucosa locally[153]. In theory, our end-product device to administer atomized mRNA to the FRT could contain lyophilized mRNA for maximal stability and shelf-life, would be easy to use, self-applied, and cost-effective.

Lastly, we provide new evidence that alternative platforms of antibody production can impact the quality and predictability of a biologic's clinical profile. Production of HCA using two platforms of interest, namely *Nicotiana* and mRNA, resulted in distinct Fc *N*-glycans and consequently inconsistent levels of ADCP, though HCA-N had a simpler glycan profile likely attributed to prior XylT and FucT knockouts. Thus, when selecting a suitable mAb platform, though cost and ease of manufacture are important factors, glycosylation and other post-translational modifications should also be key considerations for quality control. Overall, our mass spectrometry data and comparison of glycan profiles may be of interest to the manufacturing sector for production considerations.

Future Directions

The HCA vaginal film is slated for further testing in phase 1 and phase 2a clinical trials to assess safety of repeated use and to determine optimal dose, respectively. An alternative HCA variant may be selected to replace the parent IgG1 HCA to improve antigen binding, promote thermostability, and/or customize the Fc region to exclude ADCP. We are interested in further engineering the HCA Fc region for tighter control over effector functions. New constructs are in development to enhance CDC but prevent ADCP. Though there is some overlap in residues comprising the binding sites for Fc receptors responsible for these effector functions, high resolution maps can help expose residues that improve binding to one type of receptor while reducing binding to another[55]. We also plan to determine if HCA can mediate ADCC, another important effector function of interest, in which antibodies bound to target cells elicit NK cell-induced cell death.

Glycoengineering is a potentially powerful method of further controlling Fc-mediated functions and overall production quality. Glycosylation profiles are commonly heterogeneous, and batch-to-batch, even within the same production method, exhibit profile changes and are thus susceptible to variations in function. Chemo-enzymatic glycoengineering is among recent innovative developments in the field. This allows for the generation of monoclonal antibodies with well-defined, uniform Fc *N*-glycans, a characteristic which results in consistency and allows customization of biological properties for the specific application[154]. It is possible to employ these approaches to improve upon the glycan profiles of HCA.

In addition to the currently ongoing in vivo mRNA studies encoding secreted and anchored IgG HCA with collaborators at Emory University and the University of Louisiana at Lafayette, we also plan to conduct in vivo studies with mRNAs encoding HCA multimers described in Chapter 3 for increased potency. Furthermore, we are designing transfection experiments that include co-delivery with mRNAs encoding phospholipase C (PLC) for tighter control over release kinetics of the GPI-anchored HCA. We hope to further optimize the mRNA constructs for better efficiency and pharmacokinetics with these future studies.

MPTs may also be achievable through our described mRNA-mediated platform. Preliminary data using a single-chain mRNA containing a linker[155] to join the heavy and light chain strands demonstrate that simplified transfections containing one strand of mRNA instead of two can lead to more efficient translation and assembly of the protein. Not only do single-chain transfections improve expression, but they also enable transfections using two or more mRNAs encoding different antibodies. For example, transfecting cells with single chain HCA and PGT121 mRNAs resulted in the presence of both HCA and PGT121 mAbs in cell supernatant that provided contraceptive and HIV-neutralizing activity (see Appendix 2). We plan to conduct experiments with three mRNAs: HSV-8 mRNA, PGT121 mRNA, HCA mRNA for triple protection against HSV-2, HIV-

1, and pregnancy. In theory, mRNAs can be used in this way in any combination for protection against a variety of STIs.

Conclusion

HCA has previously been described as having strong contraceptive activity through potent agglutination of sperm. The studies within this work further identify, characterize, and engineer certain aspects of the antibody's clinical profile including Fc function and glycosylation to improve upon the parent HCA (Fig 5.1). By harnessing innovative methods of mRNA therapeutics, we also established an alternative mRNA-mediated delivery system that could potentially represent a new paradigm for contraception and MPTs. Overall, this work helps to inform further drug development of HCA for a more efficacious and safer product, i.e. a novel nonhormonal and cost-effective contraceptive to aid in lowering rates of unintended pregnancy.

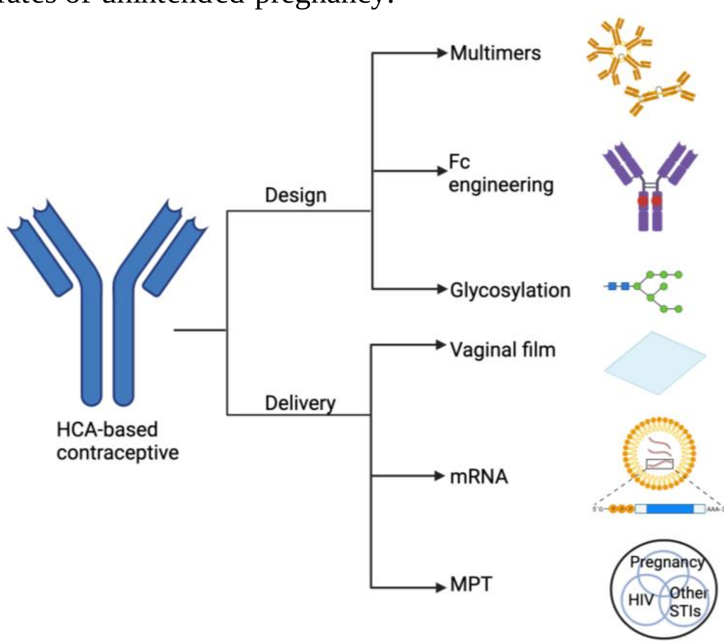

Figure 5.1- Development strategies for an HCA-based contraceptive. Generated in Biorender.com

APPENDIX ONE: HCA-N vaginal film drug product development: stability testing and select phase 1 clinical trial data

Thurman A, Moench T, Hoke M, Politch J, Cabral H, Mausser E, Nador E, et al. (2023). ZB-06, a vaginal film containing an engineered human contraceptive antibody (HC4-N), demonstrates safety and efficacy in a phase 1 postcoital test and safety study. Am J Obstet Gynecol 228(6): P716.E1-716.E12. https://doi.org/10.1016/j.ajog.2023.02.024

Data in Figure A1.3 were collected by EN and data in Table A1.1 were collected by EN and EM.

Drug product stability testing

As mentioned previously, HCA is manufactured in *Nicotiana benthamiana* (HCA-N), a close relative of the tobacco plant, to address certain challenges of antibody manufacturing including scalability, cost, and speed of production[33]. The drug product (DP) form of HCA, the HCA-N vaginal film used in clinical trials, is manufactured in a GMP facility at KBio, Inc. (Owensboro, KY). To assess drug product stability and shelf-life, the phase 1 formulation was tested for agglutination function against a reference standard at several timepoints. The kinetic agglutination test as described previously was used to determine function and potency and was qualified as an analytical procedure beforehand in conjunction with ZabBio, Inc. Aliquots were stored at both 2-8 ℃ (refrigerated temperature) and 22-28 ℃ (room temperature). Timepoints included 1, 3, and 6 months for room temperature storage and 1, 3, 6, 12, 18, 24, and 36 months for refrigerated storage. Test samples were deemed acceptable if the IC_{50} value was within 50-150% of the reference standard. R^2 values ≥ 0.8 using a 4PL fit were deemed valid. The assay was performed side-by-side by two analysts to achieve blindedness.

Due to the COVID-19 pandemic, the room temperature DP sample was only run at 1- and 3-month timepoints. At both timepoints, agglutinating function was maintained and the acceptance criterion was met since percent of reference standard was 133.2% and 84.1%, respectively (Fig A1.1). Differences in IC_{50} values seen between timepoints are likely due to semen donor variability. These data suggest that the phase 1 DP is stable at room temperature for at least 3 months and may be stable even longer.

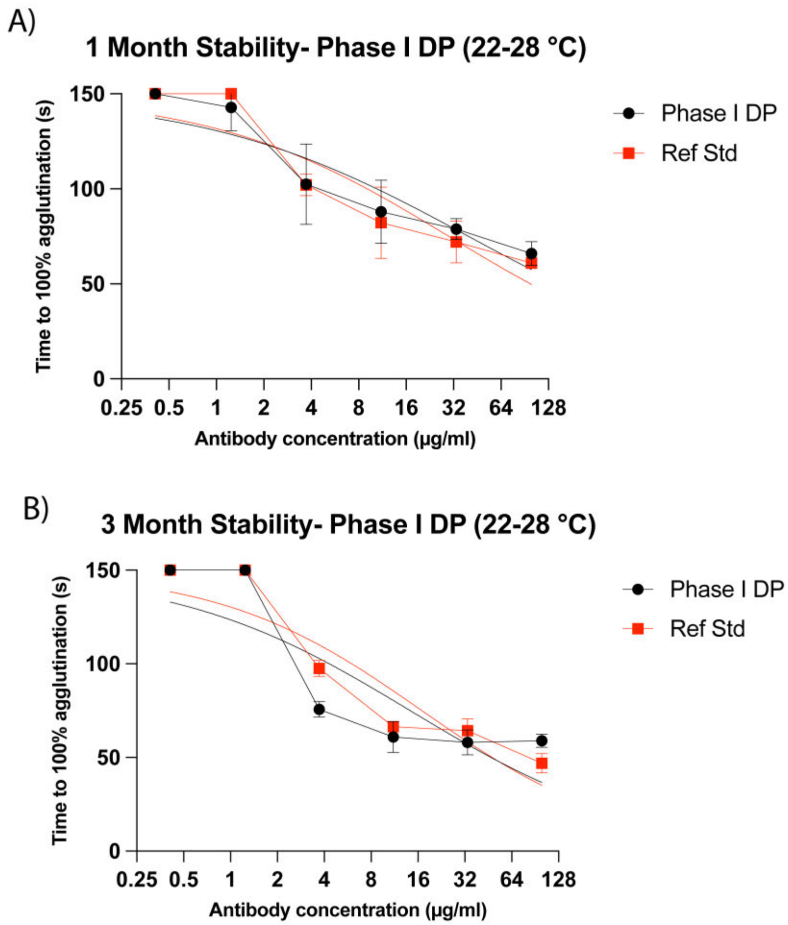

Figure A1.1- Phase 1 DP stability at 22-28 ℃. A) 1 month timepoint. B) 3 month timepoint. Smooth curves represent nonlinear fit models using sigmoidal 4PL used to calculate IC_{50} values. Error bars represent standard deviation of three trials.

Table A1.1- IC_{50} Values of phase 1 DP stability at 22-28 ℃.

	Sample IC_{50}	**Ref Std IC_{50}**	**% of Ref Std**
1 Month	39.80	29.88	133.2
3 Months	14.28	16.98	84.1

The refrigerated phase 1 DP sample was run out to 36 months. Even after storage for 3 years, the sample showed no evidence of degradation according to the potency assay. The 1-month timepoint sample was 91.3% of the reference standard (Fig A1.2A) and the 36-month timepoint was 113.09% of the reference standard (Fig A1.2B). Intermediate timepoints also achieved the acceptance criterion (data not shown). Again, differences in IC_{50} values seen between timepoints are likely due to semen donor variability. According to these data, the phase 1 DP is very stable when refrigerated and could contribute to a favorable shelf-life in real-world use. A 48-month timepoint is planned for December 2023.

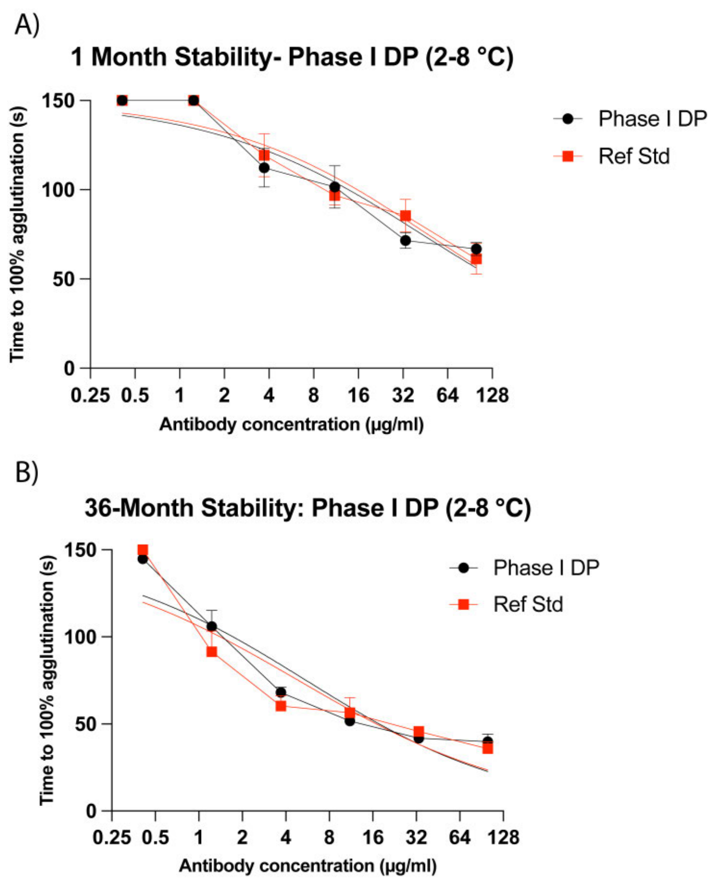

Figure A1.2- Phase 1 DP stability at 2-8 ℃. A) 1 month timepoint. B) 36 month timepoint. Smooth curves represent nonlinear fit models using sigmoidal 4PL. Error bars represent standard deviation of three trials.

Table A1.2- IC_{50} Values of phase 1 DP stability at 2-8 ℃.

	Sample IC_{50}	Ref Std IC_{50}	% of Ref Std
1 Month	42.97	47.06	91.3
36 Months	5.53	4.89	113.09

HCA phase 1 clinical trial pharmacokinetics

A phase 1, first-in-woman, proof-of-concept clinical trial was performed to assess safety and efficacy of the HCA-N vaginal film[35]. The film contained 20 mg of HCA (i.e. HC4-N). Enrolled participants had 10 in-person clinic visits in which postcoital tests (PCTs) were performed to collect vaginal fluid (VF) and cervical mucus (CM) across three menstrual cycles. Visit 4 consisted of a baseline PCT measurement (no film), visit 7 was a film-use PCT 2 hours after intercourse, visit 8 was a product safety check PCT 24 hours after intercourse, and visit 10 was a post-product recovery PCT (no film). Pharmacokinetics or presence of HCA across visits was detected in VF and CM using the HCA sandwich ELISA as previously described.

In VF, peak HCA detection was seen at visit 7 as expected (Fig A1.3A) and was significantly higher than the no-product cycle (visit 4) and the recovery cycle (visit 10). Residual HCA was detected 24 hours post film use (visit 8) though much lower than visit 7. In CM, peak HCA detection was seen at visit 8, though levels were similar to visit 7, and was significantly higher than the recovery cycle, suggesting CM is capable of retaining HCA longer perhaps due to higher viscosity and slower rate of discharge. Overall, these data demonstrate that the film locally delivers high concentrations of HCA and is retained for as long as 24 hours post-use.

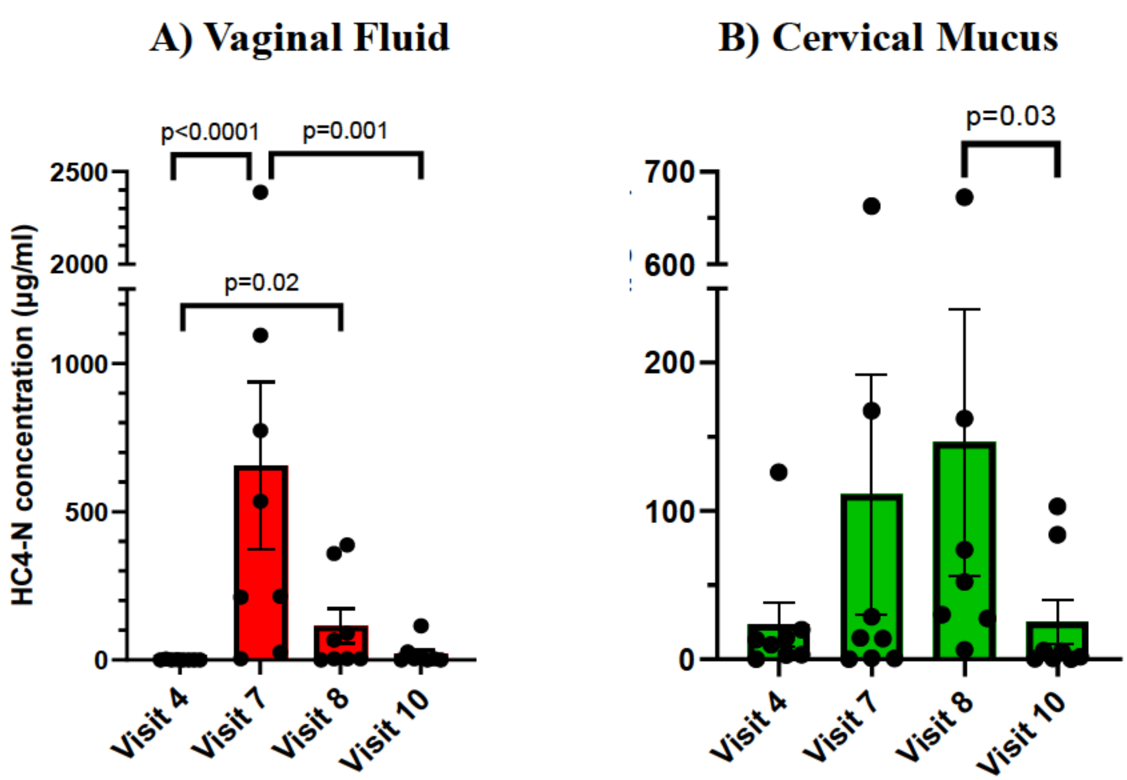

Figure A1.3- HC4-N concentrations (Mean ± SEM µg/ml) in vaginal fluid (A) and cervical mucus (B). Visit 4=Baseline PCT (no product), Visit 7=2-3 hrs post ZB-06 film PCT, Visit 8=24 hrs post ZB-06 film PCT, Visit 10=Follow-up PCT (no product). In vaginal fluid, concentrations were significantly elevated 2-3 hours (Visit 7; $p<0.0001$) and 24 hours (Visit 8; $p=0.02$) following intercourse with ZB-06 film compared to intercourse with no product baseline Visit 4. HC4-N concentrations were also significantly elevated at Visit 7 compared to follow-up Visit 10 (no product PCT; $p=0.001$). HC4-N concentrations in No-Product Visit 10 did not differ significantly from baseline Visit 4 ($p=0.48$) (Repeated Measures One-Way ANOVA with Tukey's multiple comparison tests). In cervical mucus, HC4-N concentrations were significantly elevated 24 hours (Visit 8; $p=0.03$) following intercourse with ZB-06 film compared to follow-up Visit 10 (no product PCT; $p=0.03$). No other comparisons were statistically significant (Mixed-effects analysis with Tukey's multiple comparison tests). *Thurman. Human contraceptive antibody film. Am J Obstet Gynecol 2023.*

HCA phase 1 clinical trial pharmacodynamics

Vaginal fluid, cervical mucus, and serum were also assessed in vitro for sperm agglutinating function as a surrogate pharmacodynamics marker using the kinetic agglutination assay as previously described. All eight participant vaginal swabs from visit 7 contained sperm-agglutinating samples (Table A1.1). Six of eight participants had sperm-agglutinating cervical mucus at visit 7. No other samples from any other visits were able to agglutinate sperm. Sperm agglutination was not observed in serum samples from any participant at any visit.

Table A1.3- Assessment of sperm agglutination in participant serum, vaginal fluid, and cervical mucus. *Thurman. Human contraceptive antibody film. Am J Obstet Gynecol 2023.*

Agglutination kinetics Assay

	Serum					Vaginal swab								Cervical mucus							
Patient	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10
101	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
102	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
104	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
105	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
106	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
112	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
113	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–
114	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–

APPENDIX TWO: Improving transfection efficiency and developing mRNA-based MPTs using single-chain mRNAs

Single-chain mRNA HCA variants

Our collaborators in the Santangelo lab at Emory University have moderated the HCA mRNA to include a G4S linker to link the heavy and light chain mRNA strands together[155]. Reducing the number of necessary mRNA strands from two to one not only eases manufacturing efforts but also enhances expression efficiency. Single-chain mRNAs allow for simultaneous translation of HC and LC; a single mRNA strand diminishes assembly error post-translation. All HCA variants were expressed using a single mRNA, including secreted HCA (sHCA), anchored HCA (aHCA), IgA HCA, and hexameric IgG with IgM tail (IgG/t). Function of these variants was tested in the kinetic agglutination assay following antibody purification using HCA-N as a control.

The single-strand mRNA Abs exhibited exceptionally fast sperm agglutination times (Fig A2.1). IgG sHCA and HCA-N had superimposed dose-dependent curves whereas IgA HCA agglutinated sperm as low as 0.39 µg/ml and hexameric HCA was fully agglutinating as low as 0.1 µg/ml. The single-chain multimeric variants demonstrated faster agglutination times than that of their multi-chain counterparts (see Chapter 3). In fact, the multi-chain IgG/t had a functional limit of 0.78 µg/ml whereas the single-chain IgG/t had a functional limit of 0.1 µg/ml. This suggests that including an mRNA linker so that transfection only consists of one mRNA strand improves efficiency and results in more potent antibody products.

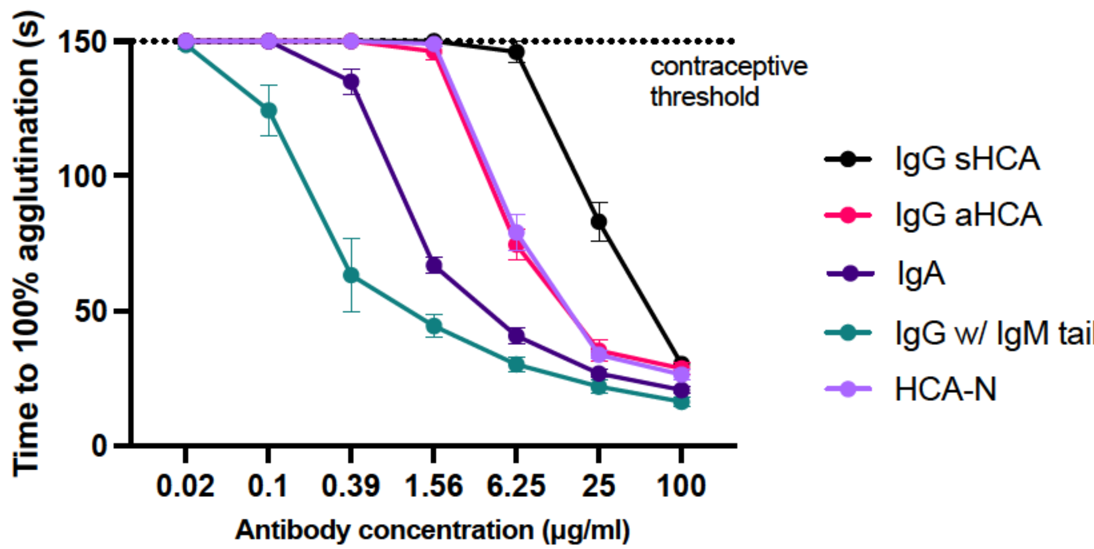

Figure A2.1- Kinetic agglutination assay using single-chain mRNA HCA and variants. Bars represent standard deviation of three independent experiments using different semen donors.

Single-chain mRNA co-transfections as MPTs

Reducing assembly issues via single-chain mRNAs also makes it easier to combine HCA mRNA with other therapeutic or prophylactic antibody mRNAs. One important application is the production of multipurpose prevention technologies (MPTs). MPTs address multiple sexual reproductive health issues with one product, e.g. contraception and STI protection[27]. We combined HCA with PGT121, a broadly neutralizing anti-HIV-1 monoclonal antibody. Cells were transfected with both an HCA single-chain mRNA and a PGT121 single-chain mRNA of various isoforms (i.e. IgG, IgA, hexameric IgG/t). In theory, after the target cell is transfected with both mRNAs, they will be simultaneously translated and cell supernatant should contain both HCA and PGT121 Abs.

After the cell supernatants were purified, agglutinating and neutralizing activities were assessed using kinetic agglutination and TZM-bl assays, respectively. All samples agglutinated sperm (Fig A2.2A) and neutralized 100% of Q23-17 virus at around 100 ng/ml (Fig A2.2B). As expected, the IgG/t variant had both the lowest agglutination and neutralization IC_{50} values. It is unclear what ratios of antibodies are present in the co-transfection supernatant (e.g. 1:1 HCA and PGT121) and if such ratios are consistent. The presence of both HCA and PGT121 titers in purified supernatant may explain slower agglutination times compared to HCA-only single-chain agglutination data. Overall, single-chain mRNA co-transfections exhibit great potential and may represent a new paradigm of MPTs, though future studies are needed for optimization.

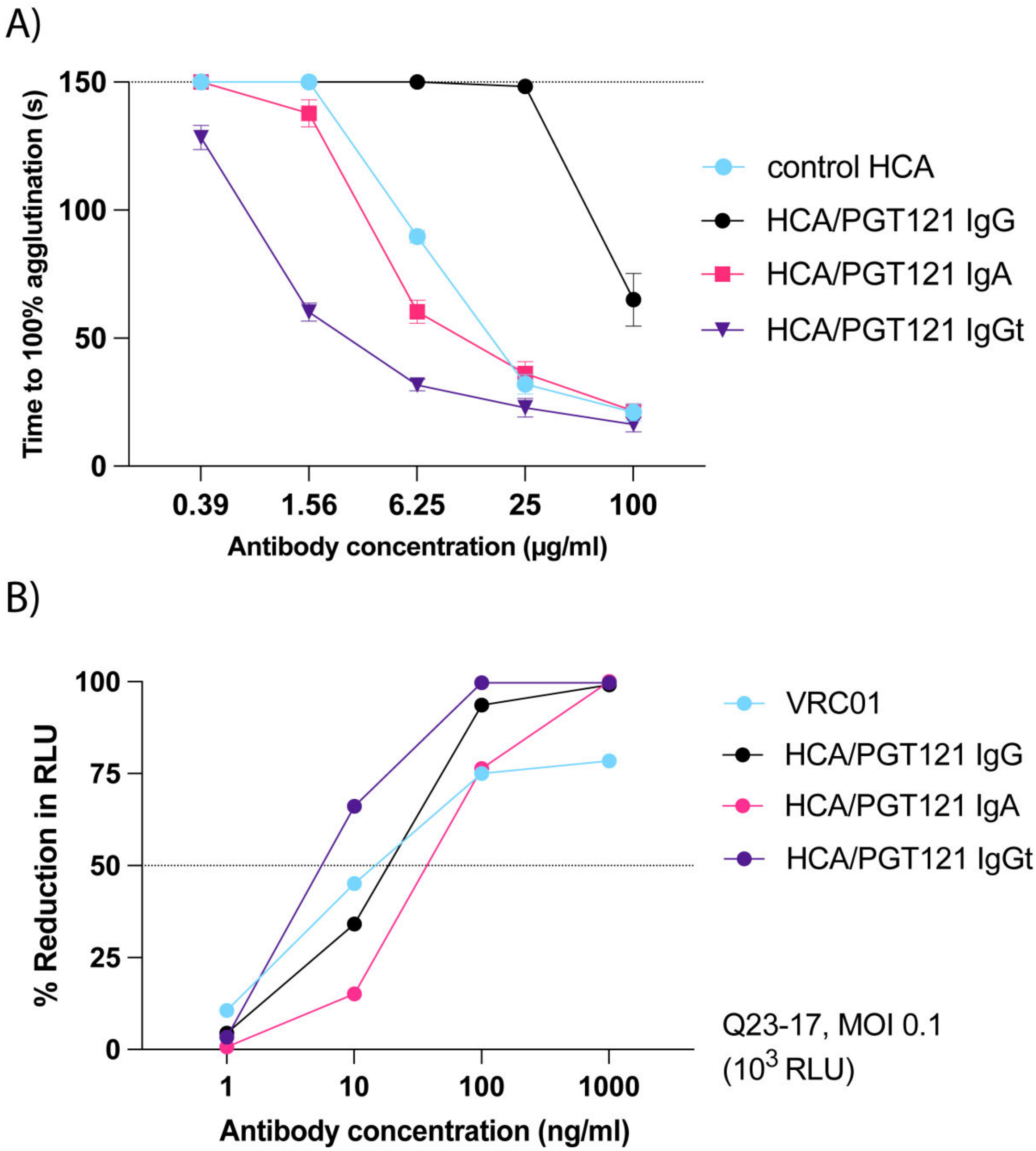

Figure A2.2- Functional studies of mRNA HCA-PGT121 co-transfections. A) Kinetic agglutination assay. Control HCA is HCA-N. Bars represent standard deviation of three independent experiments using different semen donors. B) TZM-bl neutralization assay. Q23-17 at a MOI of 0.1 was used to infect TZM-bl cells for 48 hours. VRC01, a broadly neutralizing anti-HIV-1 IgG1 antibody, was used as a positive control.

JOURNAL ABBREVIATIONS

Acta Biomater .... Acta Biomaterialia

Adv Biosci Biotechnol .... Advances in Bioscience and Biotechnology

Am J Obstet Gynecol ....American Journal of Obstetrics and Gynecology

Am J Reprod Immunol .... American Journal of Reproductive Immunology

Anim Reprod Sci .... Animal Reproduction Science

Annu Rev Biochem ....Annual Review of Biochemistry

Biochim Biophys Acta .... Biochimica et Biophysica Acta

Biol Reprod ....Biology of Reproduction

Biotechnol ....Biotechnology

Biotechnol Prog .... Biotechnology Progress

BMJ Glob Heal ....British Medical Journal Global Health

Clin Chem Lab Med .... Clinical Chemistry and Laboratory Medicine

Clin Exp Immunol ....Clinical and Experimental Immunology

Clin J Am Soc Nephrol .... Clinical Journal of the American Society of Nephrology

Clin Proteomics ....Clinical Proteomics

Commun Biol .... Communications Biology

Curr Opin Pharmacol ....Current Opinion in Pharmacology

Curr Opin Virol ....Current Opinion in Virology

Curr Pharm Biotechnol ....Current Pharmaceutical Biotechnology

Exp Hematol .... Experimental Hematology

Fertil Steril ....Fertility and Sterility

Front Cell Dev Biol....................................... Frontiers in Cell and Developmental Biology

Front Immunol .... Frontiers in Immunology

Front Vet Sci.. Frontiers in Veterinary Science

Gynecol Obstet Invest... Gynecologic and Obstetric Investigation

Hum Reprod...Human Reproduction

Hum Vaccin .... Human Vaccines

Hum Vaccin Immunother..................................... Human Vaccines & Immunotherapeutics

Immune Netw...Immune Network

Immunol Rev... Immunological Reviews

Int J Androl.. International Journal of Andrology

Int J Gynecol Obstet..............................International Journal of Gynecology & Obstetrics

Int J Mol Sci...International Journal of Molecular Sciences

Int Urogynecol J...International Urogynecology Journal

J Biol Chem... Journal of Biological Chemistry

J Clin Endocrinol Metab .... Journal of Clinical Endocrinology and Metabolism

J Control Release ....Journal of Controlled Release

J Exp Med ....Journal of Experimental Medicine

J Gen Intern Med... Journal of General Internal Medicine

J Immunol ....Journal of Immunology

J Med Primatol...Journal of Medical Primatology

J Mol Biol.. Journal of Molecular Biology

J Pharm Sci..Journal of Pharmaceutical Sciences

J Reprod Immunol..Journal of Reproductive Immunology

J Sex Res ....Journal of Sex Research

J Transl Med ....Journal of Translational Medicine

JAMA...Journal of the American Medical Association

Lancet Glob Heal ....The Lancet Global Health

Methods Enzymol ....Methods in Enzymology

Mol Hum Reprod ....Molecular Human Reproduction

Mol Ther ....Molecular Therapy

Mucosal Immunol ....Mucosal Immunology

N Engl J Med ....New England Journal of Medicine

Nat Biotechnol ....Nature Biotechnology

Nat Commun .... Nature Communications

Nat Rev Drug Discov..Nature Reviews Drug Discovery

Nat Rev Immunol...Nature Reviews Immunology

Nucleic Acids Res..Nucleic Acids Research

Obstet Gynecol..Obstetrics and Gynecology

Obstet Gynecol Clin North Am ....Obstetrics and Gynecology Clinics of North America

Plant Biotechnol J ....Plant Biotechnology Journal

PLOS Med.. Public Library of Science Medicine

PLOS One..Public Library of Science One

Proc Natl Acad Sci................................Proceedings of the National Academy of Sciences

Protein Cell ....Protein & Cell

Protein Eng Des Sel .... Protein Engineering, Design & Selection

Sci Rep .... Scientific Reports

Sci Trans Med .... Science Translational Medicine

Semin Immunol .... Seminars in Immunology

Signal Transduct Target Ther .... Signal Transduction and Targeted Therapy

Stud Fam Plann .... Studies in Family Planning

Toxicol Vitr .... Toxicology In Vitro

Transl Res .... Translational Research

Trends Biotechnol .... Trends in Biotechnology

Trends Immunol .... Trends in Immunology

Trends Microbiol .... Trends in Microbiology

Wiley Interdiscip Rev RNA .... Wiley Interdisciplinary Reviews RNA

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