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Sex differences in SARS-CoV-2 infection

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Abstract

Coronavirus Disease 2019 (COVID-19) is an infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Females generally mount a more robust immune response to infections and vaccination. The COVID-19 pandemic highlighted this sexual bias, with men being at higher risk of death and severe manifestations of disease. However, the underlying mechanisms remain understudied. We evaluated how B an T cells respond to SARS-CoV-2 infection and to COVID-19 vaccination. Here we show that upon SARS-CoV-2 infection, there is a spike-specific B and T cell response against the virus. Our data demonstrate that spike-specific T cells have a Tfh-like phenotype, characterized by high expression of CXCR5 and ICOS. Our findings indicate that spike-specific T cells produce IL-10 at high concentrations, which is a feature of hyperinflammation during severe SARS-CoV-2 infection. Moreover, our data reveal the presence of anti-spike IgG, IgA and IgM antibodies in circulation. IgG levels correlated with the days of symptoms. Further studies are needed to understand better the sex bias and the mechanisms underlying SARS-CoV-2 infection. The type and quantity of sex hormones vary throughout a woman's life, especially during pregnancy and breastfeeding. Initial clinical trials of mRNA COVID-19 vaccines excluded lactating women, causing a scarcity of data to guide decision-making. We evaluated how BNT162b2 and mRNA-1273 vaccines impact the immune response of lactating women and the protective profile of breastmilk. We show that, upon vaccination, immune transfer to breastmilk occurs through a combination of anti-spike secretory IgA (SIgA) antibodies and spike-reactive T cells. Our data suggest that cumulative transfer of IgA might provide the infant with effective neutralization capacity. These findings put forward that breastmilk might convey both immediate, through anti-spike SIgA, as well as long-lived, via spike-reactive T cells, immune protection to the infant. Further studies are needed to determine spike-T cells functional profile.

Keywords: COVID-19 infection and vaccination; sex bias; sex hormones, milk transferred cellular and humoral protection

Resumo

O sistema imunitário é responsável por proteger o nosso organismo contra invasões externas, tais como vírus, bactérias, fungos, parasitas e células cancerígenas. Para proteger o organismo contra estes invasores, o sistema imunitário tem a capacidade de reconhecer o patogénio e ativar mecanismos de defesa com o objetivo de o eliminar. O sistema imunitário é composto por 2 tipos de respostas principais: resposta imunitária inata e resposta imunitária adaptativa. A resposta imunitária inata é a primeira linha de defesa do organismo, atuando nos locais de infeção e inflamação de forma rápida (minutos ou horas) e pouco específica, através da produção de citoquinas e quimiocinas. Este tipo de resposta não possui memória imunitária, ou seja, não tem a capacidade de reconhecer o mesmo patogénio caso o organismo seja exposto a este no futuro. As principais células da resposta imune inata são: os fagócitos (macrófagos e neutrófilos), células dendríticas, mastócitos, basófilos, eosinófilos, células *natural killer* (NK) e células linfoides inatas. Pelo contrário, a resposta imunitária adaptativa é antigénio-específica e antigénio-dependente. Esta especificidade faz com que a resposta seja mais demorada, levando dias ou até semanas a ser atingida. Este tipo de resposta possui memória, o que permite uma resposta mais rápida e robusta, caso o organismo volte a ser exposto ao antigénio. As principais células da resposta imunitária adaptativa são: as células T antigénio-específicas (imunidade celular) e as células B que produzem anticorpos (imunidade humoral). A vacinação é uma forma de desencadear uma resposta imunitária. Através da administração de pequenas doses de um antigénio, a resposta imunitária é induzida, assim como a memória imunitária.

A Doença por Coronavírus-19 (COVID-19) é uma doença infeciosa causada pelo Coronavírus da Síndrome Respiratória Aguda Grave-2 (SARS-CoV-2). Desde o aparecimento do primeiro caso em Wuhan, na China, em dezembro de 2019, tem vindo a disseminar-se mundialmente e a afetar um grande número de pessoas. Os coronavírus pertencem a uma vasta família de vírus que podem causar infeção no Homem, mas também noutros mamíferos e aves. Estas infeções afetam o sistema respiratório, nomeadamente o trato superior, podendo ser semelhantes às constipações comuns ou evoluir para uma forma de doença mais grave, como a pneumonia, podendo levar à morte. Os principais sintomas incluem febre, tosse, dor de garganta, desconforto torácico e dores musculares e, em casos graves, dispneia, infiltração pulmonar bilateral e eventual morte.

Uma das primeiras constatações estatísticas desta pandemia foi o facto de os homens apresentarem com maior frequência manifestações graves da COVID-19 e, por consequência, um maior risco de morte do que as mulheres. O estudo de outras doenças infeciosas já tinha demonstrado que os homens estão frequentemente associados a respostas imunitárias mais fracas, assim como a uma maior suscetibilidade no que diz respeito a infeções virais. Para além disso, as mulheres geralmente apresentam uma resposta imunitária mais forte e robusta aquando da vacinação, como está demostrado no caso da vacina contra a gripe. Todas estas evidências demonstram o papel preponderante do sexo de um indivíduo na resposta imunitária. No entanto, é importante distinguir os conceitos de "Sexo" e "Género", pois são muitas vezes confundidos. Enquanto o sexo de um indivíduo é uma variável biológica definida pela diferente organização dos cromossomas, órgãos reprodutivos e hormonas sexuais, o género inclui comportamentos e atividades que são definidos pela sociedade ou cultura, ou seja, são os fatores sociais que determinam o "masculino" e "feminino".

A pandemia da COVID-19 veio evidenciar este viés entre sexos no que toca às defesas contra doenças infeciosas, ou seja, as diferenças na resposta imunitária entre homens e mulheres infetados com SARS-CoV-2. No entanto, os mecanismos que estão subjacentes a estas diferenças ainda se encontram pouco estudados. Com este trabalho nós avaliámos a resposta das células B e T à infeção por SARS-CoV-2, bem como da vacinação contra a COVID-19. Após a infeção por SARS-CoV-2, verificámos que existe

uma resposta das células B e T específicas para a proteína espícula do vírus. Para além disso, os nossos resultados demonstraram também que as células T específicas para a espícula apresentam um fenótipo semelhante às células T foliculares (Tfh, do inglês *T follicular helper*), que são caraterizadas pela elevada expressão de CXCR5 e ICOS. Verificámos que o ICOS, de entre as moléculas estudadas, é a única molécula que consegue ser modulada pela progesterona, hormona sexual feminina. Os nossos resultados demonstraram que as células T específicas para a espícula produzem concentrações elevadas de IL-10, o que já se sabe ser uma caraterística particular de hiperinflamação em casos graves de infeção por SARS-CoV-2. Para além disso, os nossos dados revelam a presença de anticorpos IgG, IgAe IgM contra a espícula em circulação. Os níveis de anticorpos IgG estão correlacionados com os dias de sintomas da doença. No entanto, continuam a ser necessários mais estudos para melhor compreender este viés sexual e os mecanismos subjacentes a este após infeção por SARS-CoV-2.

É sabido que o tipo e a quantidade de hormonas sexuais variam ao longo da vida da mulher, nomeadamente em períodos como a gravidez e a amamentação. Na verdade, a tolerância imunitária é obrigatória entre a mãe e o feto para que ocorra uma gravidez completa. Uma vez que o feto possui genes de origem paterna, isto poderia provocar uma resposta de rejeição por parte da mãe. A tolerância imunitária é o que permite ao feto sobreviver durante os noves meses de gestação. Os bebés têm um sistema imunitário imaturo, dependendo da transferência de células imunes maternas e anticorpos através da amamentação para adquirirem imunidade. O leite materno contém células B e T, mas também uma grande variedade de imunoglobulinas (Ig, do inglês *Immunoglobulins*), incluindo IgG, IgA e IgM. Enquanto a IgG do leite materno é principalmente proveniente do sangue, a IgA e IgM do leite são provenientes do tecido linfoide associado às mucosas (MALT, do inglês *mucosa-associated lymphatic tissue*). Nas mucosas, tanto a IgA como a IgM são produzidas localmente sobre a forma de anticorpos poliméricos, ligadas a proteínas da cadeia j e componentes secretores.

Inicialmente, os ensaios clínicos de vacinas de mRNA contra a COVID-19 excluíram mulheres lactantes, o que causou uma grande escassez de informação para orientar as tomadas de decisão por parte das autoridades de saúde. Isto é preocupante, uma vez que, dentro da população pediátrica, os bebés são o grupo mais afetado pela COVID-19. Este trabalho permitiu-nos avaliar o impacto das vacinas BNT162b2 e mRNA-1273 na resposta imune de mulheres lactantes e o perfil protetor do leite materno. Conseguimos mostrar que após a vacinação, a transferência imunológica para o leite materno ocorre através de uma combinação de anticorpos IgA secretores contra a espícula (SIgA, do inglês *secretory IgA*) e células T específicas contra a espícula. Embora tenhamos descoberto que a concentração de IgA contra a espícula no leite materno possa não ser suficiente para neutralizar diretamente o vírus SARS-CoV-2, os nossos dados sugerem que a transferência cumulativa de IgA pode fornecer ao bebé uma capacidade efetiva de neutralização. Os nossos dados sugerem ainda que o leite materno pode transmitir proteção imunitária imediata, através de anticorpos IgA secretores contra a espícula, e também de longa duração, através de células T contra a espícula, para o bebé. O facto de mostrarmos que o IgA é produzido na glândula mamária na forma secretora é de extrema importância, uma vez que esta forma consegue resistir ao conteúdo ácido do estômago do bebé e ser assim distribuído pelo organismo. No entanto, mais estudos são necessários para avaliar esta possibilidade e determinar o perfil funcional das células T específicas contra a espícula.

Palavras-chave: infeção e vacinação em COVID-19; viés entre sexos; hormonas sexuais, leite transfere proteção celular e humoral

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ACE2	Angiotensin-Converting Enzyme 2
AIM	Activation Induced Marker
APC	Antigen-Presenting Cell
Bcl6	B Cell Lymphoma 6
CCR6	C-C Chemokine Receptor 6
CD40L	CD40 Ligand
COVID-19	Coronavirus Disease 19
CXCR5	C-X-C Chemokine Receptor Type 5
DC	Dendritic cell
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
E_2	Estradiol
FBS	Fetal Bovine Serum
FCS	Flow Cytometry Standard
FoxP3	Forkhead Box P3
FPLC	Fast Protein Liquid Chromatography
HIV	Human Immunodeficiency Virus
ICOS	Inducible T-cell COStimulator
ICU	Intensive Care Unit
IFN	Interferon
IFNα	Interferon-α
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile Range
IRF5	IFN Regulatory Factor 5
GC	Germinal Center
MALT	Mucosa-Associated Lymphatic Tissue
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MFI	Median Fluorescent Intensities
MOI	Multiplicity of Infection
mRNA	Messenger RNA
NT50	Half-Maximal Neutralization Titre
NK	Natural Killer
PBMC	Peripheral Mononuclear Cell
PBS	Phosphate-Buffered Saline
Pdc	Plasmacytoid Dendritic Cell
PD1	Programmed Cell Death Protein 1
P_F	Progesterone in Follicular Phase
pIgR	Polymeric Immunoglobulin Receptor
P_L	Progesterone in Luteal Phase
PROM	Premature Rupture of Membranes
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction

S	Trimeric Spike Protein
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SEC	Size Exclusion Chromatography
SIgA	Secretory Immunoglobulin A
SLE	Systemic Lupus Erythematosus
Tfh	T Follicular Helper
Th1	T Helper Type 1
TLR	Tool Like Receptor
TLR7	Tool Like Receptor 7
TLR8	Tool Like Receptor 8
TMB	3,3′,5,5′-Tetramethylbenzidine
TMPRSS2	Transmembrane Serine Protease 2
Treg	Regulatory T
XCI	X-Chromosome Inactivation

1. Introduction

1.1 Immune responses

The immune system protects us from environmental threads, including infections, and detects and removes abnormal cells that can potentially lead to malignancies. Optimal immunological homeostasis is achieved when the threat is removed with high efficiency and at low cost (collateral tissue damage) for the host[1].

The immune system can be divided in two "lines of defense": innate immunity and adaptive immunity[2]. Innate immunity is the host's first line of defense to an invading pathogen. The innate immune response is initiated within minutes or hours after aggression and is an antigen-independent defense mechanism used by the host. The innate immune response has no immunologic memory and, consequently, it is unable to recognize the same pathogen if the body is exposed to it in the future. Innate immunity is characterized by the rapid recruitment of immune cells to sites of infection and inflammation through the production of cytokines and chemokines. The cells of the innate immune system include: phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid cells[3]. By contrast, adaptive immunity is antigen-dependent and antigen-specific. This specificity involves a time lag between exposure to the antigen and maximal response, with the immune response taking days or weeks to become established. The hallmarks of adaptive immunity are: specificity, memory, and self–non-self-recognition[4]. The capacity of memory enables the host to mount a faster response of greater magnitude on subsequent exposure to the antigen. The cells involved in the adaptive immune response include: antigen-specific T cells, which are activated to proliferate through the action of antigen presenting cells (APCs) (cell-mediated immune response), and B cells, which differentiate into plasma cells to produce antibodies (humoral immune response)[5]. Effective immunization against infectious diseases is achieved through adaptative immune responses. Innate and adaptive immunity are not mutually exclusive mechanisms of host defense, but rather are complementary, with defects in either system resulting in occasional failure, host vulnerability or inappropriate responses[3,5].

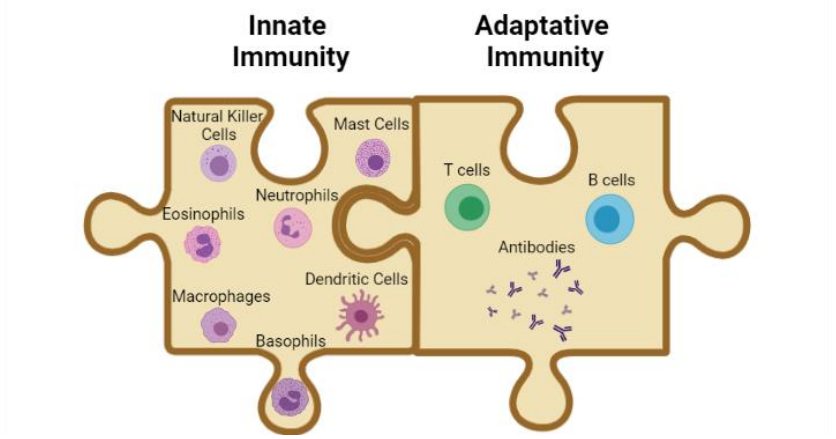

Figure 1.1 Human immune system. The immune system can be divided into two "lines of defense": innate immunity and adaptive immunity.

T cells are further grouped into lineages based on their function. Helper $CD4^+$ T cells that help coordinate the activity of other immune cells by secreting specific cytokines[6]. Cytotoxic $CD8^+$ T cells, on the other hand, directly kill infected or malignant cells[7]. Regulatory T (Treg) cells keep immune responses in check by preventing autoimmunity, suppressing the immune response and maintaining peripheral tolerance[8]. Memory T cells that derive from previous antigen activation and maintain long term immunity are a differentiation stage of T cells. T follicular helper (Tfh) cells are a specialized subset of $CD4^+$ T cells and play a key role in helping B cells in antibody production. Tfh are essential for germinal center (GC) formation, affinity maturation, the development of memory B cells[9,10]. Tfh are defined by expression of the transcription factor Bcl6 and cell surface markers including CXCR5, PD1 and ICOS[10].

Vaccination is a way to trigger the immune response, representing the greatest contribution of immunology to human health[11]. Vaccines work by giving small doses of an antigen, such as dead or weakened live viruses, to elicit an immune response and consequently immunological memory (activated B cells and sensitized T cells)[11]. Memory mediates protection from infection or disease and allows our body to react quickly and efficiently to future exposures.

1.2 Sex bias in immune responses

Sex is considered a major determinant of an immune response. The concept of "Sex" and "Gender" is commonly confounded, even though each term has a distinct connotation[12]. Sex of an individual is a biological variable defined by the differential organization of chromosomes, reproductive organs, and sex steroid levels[13]. Gender includes behaviors and activities that are determined by society or culture in humans, that is, social factors that determine "masculine" and "feminine"[14]. Immunological responses differ between males and females, which can be influenced by both sex and gender. Sex contributes to physiological and anatomical differences that influence exposure, recognition and transmission of microorganisms[13]. Conversely, gender may reflect behaviors that influence exposure to microorganisms, access to healthcare or health-seeking behaviors that affect the course of the disease[13,14].

Accumulating evidence supports a role of sex-based differences in susceptibility to vaccination and medications, and in both the incidence and pathogenesis of autoimmune diseases[15,16]. Women in general generate a stronger immune response to infections and vaccinations; on the other hand, they suffer more from inflammatory and autoimmune diseases[1].

It is known that typically males are more susceptible to infectious diseases than females. Several studies have documented this sex bias in susceptibility to certain bacterial, parasitic, and viral infections (e. g women with acute human immunodeficiency virus (HIV) infection have 40% less viral ribonucleic acid (RNA) in their blood than men)[13,17]. Women have historically been under-represented in clinical trials, including vaccine clinical trials[18]. The lack of women in vaccine clinical trials may have led to unnecessarily high dosing of vaccines for women[19]. Vaccines for a large variety of pathogens are commonly administrated identically to males and females. However, studies analyzing variation in vaccine-induced immunity have pointed to different responsiveness depending on the sex of the recipients. Healthy women (aged 18–64 years) typically generate a higher and more robust antibody response to an influenza vaccine and, notably, their antibody response to a half dose of the vaccine was equivalent to the antibody response to a full dose in men[20]. The most striking sex differences in autoimmune diseases are observed in Sjogren's syndrome, systemic lupus erythematosus (SLE), autoimmune thyroid disease (Hashimoto's thyroiditis and well as Graves' disease) and scleroderma, which represent a spectrum of diseases in which the patient population is >80% women[21].

Sex bias in immune response could result from direct effects of an individual's sex chromosome and from sex hormones[22]. Several genes that play an important role in regulating immune responses in humans are located on the X chromosome (e. g. genes encoding FoxP3, CD40L, TLR7, TLR8 and IL-2 receptor subunit gamma)[1]. Females carry two copies of the X chromosome, resulting in a potentially double dose of X-linked genes, which can lead to an imbalance in the amount of gene products[23]. To correct this imbalance, mammalian females developed a unique mechanism of dosage compensation, called X-chromosome inactivation (XCI)[24]. Female mammals transcriptionally silence one of their two X chromosomes[24]. Recent data showed that portions of the X chromosome manage to escape this inactivation, which results in higher expression of TLR7 levels, and possibly other immune genes, in women than in men[25]. This could explain the predisposition of women to TLR7-driven autoimmune diseases, such as systemic lupus erythematosus[1]. Exposure of peripheral blood mononuclear cells (PBMCs) to TLR7 ligands *in vitro* causes higher production of interferon-α (IFNα) in cells from women than from men[26]. In addition, plasmacytoid dendritic cells (pDCs) from female humans and mice have higher basal levels of IFN regulatory factor 5 (IRF5) and IFNα production following TLR7 ligand stimulation[27].

It is known that sex hormones have potent effects on immune cells. The major sex hormones that have been studied in immunity are estrogens, testosterone, and progesterone[12]. All these hormones and their associated receptors are present in both males and females on many immune cells. Sex hormones bind to specific nuclear and membrane-associated protein receptors resulting in diverse and often contradictory effects in innate and adaptive immunity[12,28]. For example, estrogen can both promote immunosuppressive Treg activation and proinflammatory $CD4^+$ Th1 cell responses[12]. The hormone concentration, the types, and concentrations of hormone receptors in immune cells and the activation environment during the immune can have a major impact on the ultimate hormone immunomodulatory effect[12]. The global gene expression profile of PBMCs from young and elderly men and women has revealed age- and sex-dependent alterations in immune cell transcriptomics[29]. With ageing, a general decline in immune function is observed due to loss of sex hormones. Several of these changes are gender specific and affect postmenopausal women[12]. Testosterone increases susceptibility to infections, while estrogen decreases that susceptibility[22]. Another important hormone that has immune reactivity and plays an important role in immune activation is prolactin. Prolactin, the hormone that promotes lactation, also functions as a cytokine stimulating lymphocyte proliferation and cytokine production that plays an important role in immune activation[30].

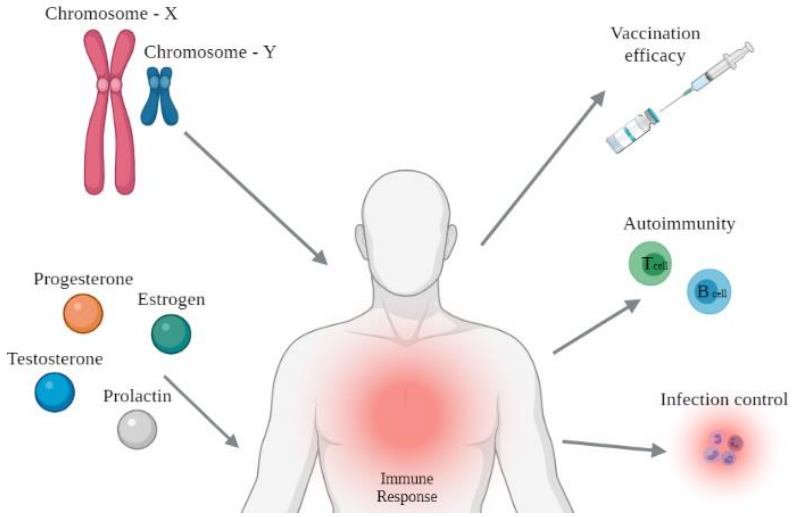

Figure 1.2 Human immune response. Sex bias in immune response results from direct effects of sex chromosomes and sex hormones (progesterone, estrogen, testosterone, and prolactin). These influences alter the immune response toward vaccination, affect autoimmunity and susceptibility to infections in a sex-specific manner.

1.3 COVID-19

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late December 2019 in Wuhan, Hubei province, China[31]. The rapid spread of this highly transmissible and pathogenic coronavirus has caused a pandemic disease, named "coronavirus disease 2019" (COVID-19)[31]. The COVID-19 pandemic threatens human health and public safety and has had a catastrophic effect on the world's demographic, which has resulted in millions of infections and hundreds of thousands of deaths worldwide[32].

Coronaviruses are a large family of viruses that usually cause mild to severe upper-respiratory tract infections in humans[33]. Over the past 20 years, three new coronaviruses have emerged from animal reservoirs, causing severe disease and global concerns[34]. Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), two highly transmissible and pathogenic coronaviruses with zoonotic origin[31], emerged in humans and caused epidemic and local outbreaks in 2002 and 2012, respectively, making coronaviruses a new public health concern of the twenty-first century[35]. The third novel coronavirus, SARS-CoV-2, was declared a global pandemic by the World Health Organization on March 11, 2020.

SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as an entry receptor, with virus entry enhanced by cellular transmembrane serine protease 2 (TMPRSS2), which primes the spike protein of the virus[36]. The spike protein mediates viral entry into host cells by first binding to a host receptor through the receptor-binding domain (RBD) in the S1 subunit and then fusing the viral and host membranes through the S2 subunit[37]. ACE2 is expressed in numerous tissues, including nasal, respiratory, and intestinal epithelial cells, kidney, and blood vessels[36]. The main way by which people are infected with SARS-CoV-2 appears to be the respiratory route with initial infection of the upper respiratory tract[31]. Infected individuals suffer from symptoms similar to those of other upper respiratory tract infections like fever, dry cough and chest discomfort, and in severe cases, dyspnea and bilateral lung infiltration[38–40].

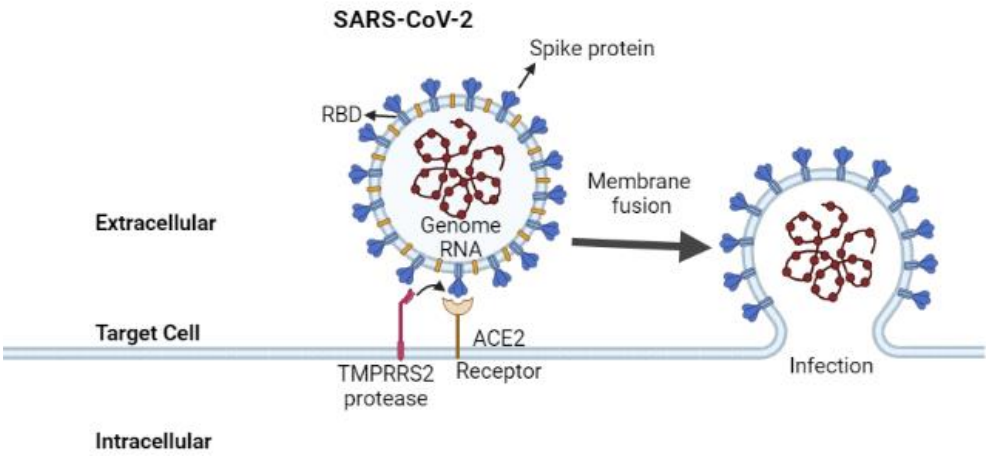

Figure 1.3 Mechanism of SARS-CoV-2 cellular entry.

According to the severity of symptoms, COVID-19 patients can be classified as: asymptomatic (committed to the hospital for other complaints, subjected to routine PCR-testing for SARS- CoV-2 but upon medical examination do not exhibit any signs or symptoms), mild (display fever, cough, myalgias, or loss of taste and smell but does not require oxygen supplementation), moderate (require non-invasive oxygen supplementation and hospitalization) and severe (require invasive oxygen supplementation and patients are committed to intensive care)[41].

1.4 Sex bias in COVID-19

Human biological sex plays a fundamental role in COVID-19 outcomes[36]. SARS-CoV-2 causes significantly more hospitalizations, higher rates of intensive care unit (ICU) admission, and more deaths in males than in females, across diverse countries and age groups[42]. Globally, for every 10 hospitalizations of adult females there are 13 in males[42,43]. Male bias in COVID-19 mortality is observed in 37 of 38 countries that have provided sex-disaggregated data and the risk of death in males is ~1.7 times higher than in females[36]. Aging is strongly associated with higher risk of death in both sexes, but at all ages above 30 years, males have a significantly higher mortality risk than females[36]. On the other hand, the differences between males and females in the rate of confirmed SARS-CoV-2 infections are age dependent, being greater among females compared to males between the age of 10 and 50 years and greater among males before the age of 10 years and after the age of 50 years[44]. This suggest that biological sex differences contribute to a male-biased death, but gender-associated risk of exposure may affect rates of infection differently for males and females[36].

COVID-19 is characterized by strong innate immune cytokine and chemokine responses despite the disproportionately low antiviral defense signature mediated by IFNs[45]. Patients with severe COVID-19 exhibit high serum concentrations of proinflammatory cytokines and chemokines, with particularly high concentrations of IL-6 and the inflammasome-associated cytokines IL-1β and IL-18[46]. Male patients have higher plasma concentrations of innate immune cytokines and chemokines, such as IL-8 and IL-18, compared with female patients[43]. By contrast, female patients have higher plasma levels of IFNα over the disease course[43]. Autoantibodies that neutralize type I IFN signaling have been reported in severe cases of SARS-CoV-2 infection, the majority of whom in older males patients[47]. Conversely, even older female patients have more robust T cell activation than males, whereas male patients have a significant decline with age[43]. Male patients with poor T cell activation at the early phase of disease onset have worse COVID-19 outcomes, whereas no such difference is observed in female patients[43].

Focusing on genetic differences, one small case series identified loss-of-function variants of the pattern-recognition receptor TLR7 in male patients requiring mechanical ventilation[48]. Upon stimulation in vitro with imiquimod, a TLR7 agonist, peripheral blood mononuclear cells isolated from the patients showed no increase in TLR7 messenger RNA (mRNA) expression, decreased expression of transcripts in the type I interferon (IFN) pathway, and decreased production of IFN-γ, as compared to healthy controls[42]. TLR7 is X-linked and is known to escape X-inactivation[49], suggesting a mechanism whereby men expressing a single copy of TLR7 are at increased risk of severe disease compared to women expressing two copies[42]. ACE2 is an X chromosome-encoded gene that is downregulated by estrogens[50]. TMPRSS2 is regulated by androgen receptor signaling in prostate cells[51]. Knowing that, further research is needed to understand if the sex- biased expression of ACE2 together with the regulation of TMPRSS2 by androgens, increases SARS- CoV-2 susceptibility of males compared to females[36].

Sex differences in the immune system also have implications in SARS-CoV-2 response to vaccination[52]. Analysis of convalescent plasma showed that male sex, advancing age, and hospitalization for severe COVID-19 were associated with greater SARS-CoV-2 antibody titers[53]. This could be related to the increased risk of more severe COVID-19 outcomes in male patients, which could drive to higher B cell activation and, consequently, more antibody production[52,53].

1.5 COVID-19 during pregnancy

Given the important modulatory effects of sex hormones on immune cell functions, it is not surprising that hormonal fluctuations accompanying the menstrual cycle, pregnancy, and menopause have an impact on immune responses and susceptibility to infectious diseases[22]. In fact, induction of a tolerogenic immune response is an obligate step in a normal pregnancy, allowing the mother to tolerate an allograft expressing paternal antigens, the fetus[54]. Immunological changes favoring tolerance can also be detected in the blood of pregnant women[55].

The disease course of COVID-19 in pregnant women seems to be at increased risk for severe illness compared to non-pregnant women with COVID-19[56]. In pregnant women presenting with severe symptoms (e.g. hypoxia, pneumonia, etc.), enhanced ACE2 expression is thought to be linked to severity of symptoms[57]. A recent meta-analysis reported that less than 20% of pregnant women need admission to the ICU[58]. Pneumonia is one of the most common outcomes in pregnant women with COVID-19[59–61].

Recent data showed that while the expression pattern of ACE2 decreases gradually over gestational age in placentas derived from healthy pregnancies, ACE2 protein is present at significantly higher levels in term placenta collected from COVID-19 cases[62]. Importantly, ACE2-mediated risk for placental susceptibility to SARS-CoV-2 may vary throughout gestation, with detection of higher ACE2 levels in the first and second trimesters suggesting the most vulnerability may exist prior to term[62].

The risk of vertical transmission can theoretically exist in COVID-19 since ACE2 receptors are significantly expressed in the placenta with which SARS-CoV-2 may bind and enter. Intrauterine vertical transmission may typically occur through trans-placenta, or ingestion or aspiration of cervicovaginal secretions[63,64]. Obstetric management of COVID-19 positive pregnant women is essential to procure a good prognosis for the mother and prevent infection in the newborn[65].

1.6 Lactating woman

Infants have an immature immune system and depend on the transfer of maternal immune cells and antibodies via the breastmilk to provide them with immunity[66–71]. During lactation, breastmilk humoral and cellular content changes, reflecting the development of the infant's own immune system and digestive tract, although it is present in the milk during at least the first year of the infant life [67,72,73]

Human breastmilk contains a wide variety of immunoglobulins (Ig), including IgA (~90%), IgM (~8%) and IgG (~2%)[74]. While human milk IgG mostly originates from the blood, milk IgA and IgM originate from mucosa-associated lymphatic tissue (MALT)[75,76]. At the mucosa sites, IgA and IgM are secreted in the form of polymeric antibodies complexed to j-chain and secretory component proteins[76]. The secretory component plays a critical role in protecting secretory IgA (SIgA) and IgM from proteolytic cleavage in the gut, facilitating their digestive traffic, systemic uptake and tissue distribution in the infant[75].

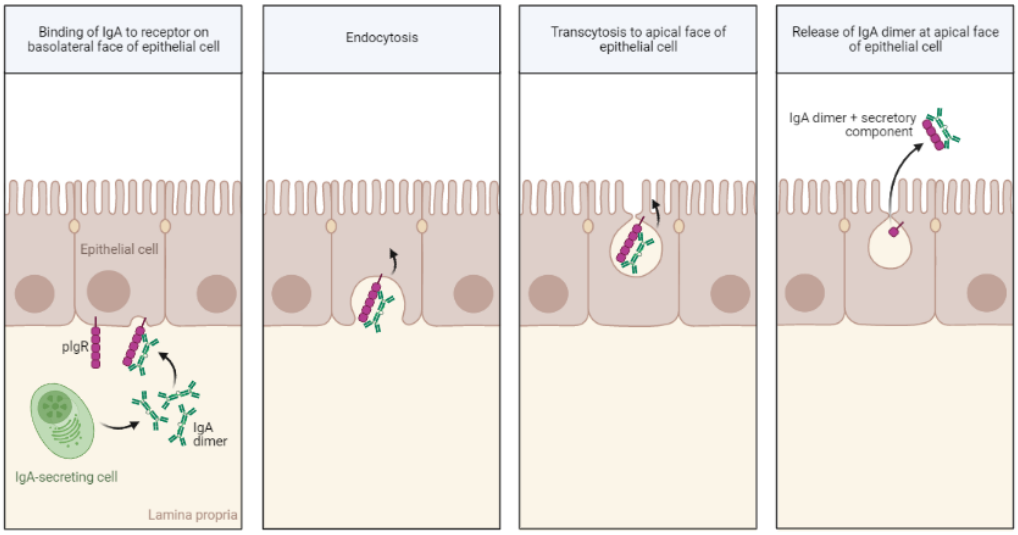

Figure 1.4 Transfer of IgA to breast milk. Plasma cells produce IgA (SIgA), which is passed into breast milk by the polymeric Ig receptor (pIgR) on the mammary epithelium.

Breastmilk also contains several types of maternal immune cells, including B and T cells, beyond antibodies[72,77]. Milk lymphocytes are more activated and/or differentiated than their blood counterparts, with milk T cells including almost exclusively of effector memory cells and with B cells containing mainly class-switched IgD^- memory B cells and plasma cells[78,79]. Accumulating evidence supports that milk B and T cells are capable to resist the gastric environment, enter blood circulation and be distributed into infant tissues[80–82]. Recent studies, including those based on humans, have indicated that transfer of maternal lymphocytes via breastmilk significantly assists the newborn's immune system[75].

Prolactin, the hormone that promotes lactation, also functions as a cytokine stimulating lymphocyte proliferation and cytokine production[30]. Consequently, prolactin drives a unique immune profile, composed by increased phagocytic and cytolytic activities and B and T cell activation.

1.7 Vaccination of lactating woman

Initial COVID-19 clinical trials of mRNA vaccines excluded lactating women, causing an absence of data to guide vaccine decision-making by health authorities[83]. Even after vaccine authorization, breastfeeding health care workers (HCW) were advised to discontinue breastfeeding upon receiving COVID-19 mRNA vaccine[84]. This is especially worrisome since infants are the children's age group most affected by COVID-19[85,86]. Given the physiological changes observed in lactating women and the crucial role of breastmilk in providing infant immunity, there is an urgent need to predict how mRNA vaccines impact immune responses in lactating mothers and to discover the effector profile of breastmilk transferred immunological protection.

It has been previously shown that mRNA vaccines induce spike reactive B and T cells in the blood, but it remains to be addressed whether those vaccines can elicit local mucosal T and B cell responses which could be transferred to the suckling infant via breastmilk[87,88]. Few recent reports have documented IgA presence in the breastmilk in response to COVID-19 mRNA vaccines and have shown that antibody production between lactating and non-lactating women receiving COVID-19 mRNA vaccines is similar[89,90].

2. Objectives

The nature and strength of immune responses differ between women and men, resulting in sex-specific differences in the prevalence, manifestations, and outcomes of vaccination and infectious and autoimmune diseases. It is known that females exhibit a stronger immune response against infections and the role of sex hormones in the immune system, but the mechanisms underlying that remain understudied. The COVID-19 pandemic highlighted this sexual bias. Men present more frequently severe manifestations of disease and are at higher risk for death, emphasizing the need to develop treatment strategies for COVID-19 that take these differences between the sexes into account. Sex hormones have potent effects on immune cells and vary throughout a woman's life, especially during pregnancy and breastfeeding. Women have historically been under-represented in clinical trials, including vaccine clinical trials. The clinical trials of mRNA COVID-19 vaccines were not an exception, excluding lactating women. For that reason, how the mRNA vaccines impact on the cellular immune response of lactating women has so far remained unaddressed. Moreover, it is currently unknown whether vaccine elicited milk IgA is produced in the mammary mucosa in its secretory SIgA form or if it is provided as monomeric IgA form by the blood. Thus, the main goals of this project were as follows:

- Identify the role of female sex hormones in protecting from severe disease through modulation of spike-specific B (humoral) and T (cellular) cell response;
- Dissect the crosstalk between female sex hormones in the modulation of X-chromosome immune genes and how it impinges T cell function;
- Evaluate the effects of mRNA COVID-19 vaccine on the humoral and cellular immune responses of breastfeeding women;
- Uncover breastmilk effector immune composition upon COVID-19 mRNA vaccination.

3. Methodology

3.1 Subject recruitment and sample collection

For the COVID-19 patients, blood was collected with sodium heparin anticoagulant from 31 individuals that were hospitalized in the Hospital Fernando Fonseca and were confirmed positive for SARS-CoV-2 by RT-PCR from nasopharyngeal and/or oropharyngeal swabs in a laboratory certified by the Portuguese National Health Authorities (Table 7.1).

For the vaccination lactating cohort study[71], blood and breastmilk from 23 nursing mothers were collected on two separate occasions. Coinciding with the priority vaccination of health care workers, 14 paired samples of breastmilk and blood were collected between December 2020 and February 2021, a median of 10 days after first and second mRNA vaccine administration, as previously described[91] (Table 7.2). The second collection interval occurred upon opening of the vaccination to the general population from June to September 2021 and participants were recruited through social media platforms, pre-natal support groups and/or word of mouth. In this second period, 9 paired samples of breastmilk and blood were collected, ~11 days after the first and ~15 days after the second mRNA vaccine administration (Table 7.3). Of the 22 control participants, 20 received with Pfizer BTN162b2 and 2 the Moderna mRNA-1273 vaccine. Blood was collected by venipuncture in EDTA tubes and breastmilk was collected with breast pump into sterile containers. Both biospecimens were immediately processed. All participants provided informed consent and all procedures were approved by NOVA Medical School ethics committee (11/2021/CEFCM and 112/2021/CEFCM), in accordance with the provisions of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization.

PBMCs were isolated via Ficoll density gradient. Blood was 1:2 diluted with PBS 1x, layered over Ficoll and centrifuged at 1200g for 30min without brake. PBMCs were washed twice with PBS 1x and cryopreserved (10% DMSO in FBS (Fetal Bovine Serum) and stored at -80°C) or cultured as described below. Breastmilk was centrifuged at 3000g for 30 min and cells were collected and cryopreserved. Plasma from both studies was stored at -80°C and skim milk at -20°C for subsequent analysis.

3.2 Cell stimulation

PBMCs from COVID-19 patients were resuspended in RPMI1640 media supplemented with 10% of FBS + 1% of antibiotic + 1% glutamine and cultured in 96-well plates at 2 x 10^6 cells /ml in the presence of IL-2 (20 U/ml) (NIH). Cells were cultured either unstimulated or stimulated for 5 days with trimeric spike protein (1 μg/ml) and hormones to mimic the different phases of the menstrual cycle: luteal phase with estradiol (4 ng/ml) + progesterone (15 ng/ml) (Sigma) and follicular phase with estradiol (4 ng/ml) + progesterone (1.5 ng/ml). In the stimulated conditions we added α-CD28 (5 μg/ml) (BioLegend) cross-linked with anti-mouse IgG1 (2.5 μg/ml) (RMG1-1) (BioLegend). On day 3 we added hormones and IL-2 again and on day 4 we added spike again. On day 1 and day 5 the cells were analyzed by flow cytometry.

3.3 Flow cytometry staining and acquisition

For both COVID-19 patients and the vaccination lactating cohort study, an Activation Induced Marker (AIM) assay was performed using OX40 and CD25 dual expression to detect spike reactivity[92]. In this assay we stimulated PBMCs overnight with 1 μg/ml of spike protein plus 5 μg/ml of α-CD28 (CD28.2)

(BioLegend) cross-linked with 2.5 µg/ml of anti-mouse IgG1 (RMG1-1) (BioLegend). On the following day we used OX40 and CD25 dual expression to detect spike reactivity through flow cytometry.

For detection of SARS-CoV-2 reactive T cells from COVID-19 patients on day 1 cells were stained for viability with AmCyan Fixable Viability Dye 506 for 20 min at 4°C in the dark for both stainings. In the first staining cells were washed twice with FACS buffer (2% FBS in PBS) and primary antibodies were incubated for 20 min at 4°C in the dark. Antibodies for surface staining included α-CXCR5 (J252D4), α-CD4 (RPA-T4), α-CD3 (SK7), α-CCR6 (G034E3), α-CD40L (24-31), α-CD25 (BC96) and α-OX40 (ACT35). Cells were washed twice with FACS buffer and fixed with 1% of PFA. In the second staining monoclonal antibodies for surface staining included α-CD3 (SK7), α-CD4 (RPA-T4), α-OX40 (ACT35), α-CD25 (M-A251), α-CD38 (HIT2) and α-ICOS (C398.4A) and were incubated for 20 min at room temperature in the dark. Cells were fixed with 1% PFA for 20 min and permeabilized with Saponin 0,1% for 20 min at room temperature in the dark. Antibody for intracellular staining was α-TLR7 (4G6) and was incubated for 30 min at room temperature in the dark. Cells were washed once with Saponin (0,1%) and once with FACS buffer and acquired on a BD FACSCanto II cytometer (BD Biosciences) and analyzed with FlowJo v10.7.3 software (Tree Star). Day 5 was performed as day 1.

For detection of SARS-CoV-2 reactive B and T cells in the vaccination lactating cohort study[71] RBD was labelled with an available commercial kit according to manufactor's instructions (life technologies, A20181). PBMCs were stained with a fixable viability dye eFluor™ 506 (invitrogen) and surface labelled with the following antibodies all from BioLegend: α-CD3 (UCHT1), α-CD4 (SK3), α-OX40 (Ber-ACT35), α-CD25 (M-A251), α-CD69 (FN50), α-CXCR-5 (J252D4), α-CCR6 (G034E3), α-CD19 (SJ25C1), α-IgD (IA6-2), α-CD27 (O323) and α-CD20 (2H7) and also with the RBD labelling as described above. Cells were washed, fixed with 1% PFA and acquired in BD FACS Aria III equipment (BD Biosciences) and analyzed with FlowJo v10.7.3 software (Tree Star).

3.4 ELISA Assay

The hosting lab customized an in-house ELISA[41] developed by Krammer and collaborators[93]. All plasma samples were heat-inactivated at 56°C for 15 min before use in the in-house ELISA. High-binding 96-well ELISA plates (Nunc) were coated with spike protein at 0.5µg/ml or RBD at 0.5µg/ml overnight at 4°C. After washing three times with 0.1% PBS/Tween20 (PBST) using an automatic plate washer (ThermoScientific), plates were blocked with 3% milk in 0.05% PBS-T or with 3% BSA in 0.05% PBST for 1 h at room temperature. In a 96-well plate samples diluted 1:50 in 1% milk powder PBST or in 1% BSA PBST were added, as well as calibrators. Seriated dilutions 1:3 starting at 1:50 and ending at 1:109350 were performed in all samples. Calibrators included sera from PCR-tested SARS-CoV-2 infected individuals classified in three groups according to their antibody titers: high-, moderate and low-antibody producers. Two individual samples from each group were used. Negative controls included one pre-pandemic sample and one blank well. Finished the blocking hour the samples were transfered from the dilution plates to the ELISA plates and incubated for 1h at room temperature. Plates were washed and incubated for 30 min at room temperature with 1:25000 dilution of HRP-conjugated anti-human IgG, IgA and IgM antibodies (Abcam, ab97225/ab97215/ab97205) or with 1:10000 dilution of anti-human SIgA (Abcam ab3924) followed by a 30 min incubation with HRP-labelled secondary antibody (Biorad, 706516) at 1:5000 dilution in 1% BSA 0.05% PBS-T. Plates were washed with PBST and 50 µL of TMB substrate (BioLegend) was added to the wells for 7 min. The reaction was stopped by adding 25µL of 1 M phosphoric acid (Sigma) and read at 450nm on a plate reader (BioTek). Endpoint titters were defined as the last dilution before the absorbance dropped below OD_{450} of 0.15. This value was established using plasma from pre-pandemic samples collected from subjects not exposed to SARS-

CoV-2[41]. For samples that exceeded an OD_{450} of 0.15 at last dilution (1:109350), end-point titter was determined by interpolation[94]. For IL-10 detection a commercial kit was used and the protocol was performed as manufactor's instructions (Invitrogen, 88-7106-22).

3.5 Purification of milk IgA and IgG

IgA and IgG from breast milk samples were purified through Peptide M/Agarose (Invivogen) or Protein G (ThermoScientific), respectively, according to the manufacturer's instructions. Briefly 1 ml of skim milk was incubated with 2 ml of Peptide M/Agarose or Protein G and incubated for 20 min. Peptide M/Agarose and Protein G beads were washed 3 times with wash buffer (10mM sodium phosphate 150mM sodium chloride; pH 7.2) and eluted in fractions of 500 µl with 0.1M glycine pH 2.76. The pH of the collected fractions was adjusted to 7 with 1M TRIS; pH 8.83, pooled IgA and IgG fractions were washed with PBS and concentrated using Amicon Ultra with a 100 kDa membrane (Millipore). All steps were carried out at 4ºC. Skim milk and IgA and IgG milk fractions were analysed on non-reducing polyacrylamide gel electrophoresis (BN-PAGE) on NativePAGE 4–16% Bis-Tris gels (ThermoFisher Scientific) with NativeMark (ThermoFisher Scientific) as molecular weight marker and stained with ProBlue Safe Stain (Giotto Biotech).

3.6 Production of 293T cells stably expressing human ACE2 receptor

Production of 293T cells stably expressing human ACE2 receptor was done as previously described[95]. Briefly, VSV-G pseudotyped lentiviruses encoding human ACE2, 293ET cells were transfected with pVSV-G, psPAX2 and pLEX-ACE2 using jetPRIME (Polyplus), according to manufacturer's instructions. Lentiviral particles in the supernatant were collected after 3 days and were used to transduce 293T cells. Three days after transduction, puromycin (Merck, 540411) was added to the medium, to a final concentration of 2.5 µg/ml, to select for infected cells. Puromycin selection was maintained until all cells in the control plate died and then reduced to half. The 293T-Ace2 cell line was passaged six times before use and kept in culture medium supplemented with 1.25 µg/ml puromycin.

3.7 Production and titration of spike pseudotyped lentiviral particles

To generate spike pseudotyped lentiviral particles, 6×10^6 293ET cells were co-transfected with 8.89ug pLex-GFP reporter, 6.67µg psPAX2, and 4.44µg pCAGGS-SARS-CoV-2-Strunc D614G, using jetPRIME according to manufacturer's instructions. The virus-containing supernatant was collected after 3 days, concentrated 10 to 20-fold using Lenti-XTM Concentrator (Takara, 631231), aliquoted and stored at -80°C. Pseudovirus stocks were titrated by serial dilution and transduction of 293T-Ace2 cells. At 24h post transduction, the percentage of GFP positive cells was determined by flow cytometry, and the number of transduction units per ml was calculated.

3.8 Neutralization assay

Heat-inactivated skim breast milk and plasma samples were four-fold serially diluted and then incubated with spike pseudotyped lentiviral particles for 1h at 37°C. The mix was added to a pre-seeded plate of 293T-Ace2 cells, with a final MOI (multiplicity of infection) of 0.2. At 48h post-transduction, the fluorescent signal was measured using the GloMax Explorer System (Promega). The relative fluorescence units were normalized to those derived from the virus control wells (cells infected in the

absence of plasma or skim breast milk), after subtraction of the background in the control groups with cells only.

3.9 Quantification and statistical analysis

Statistical analysis was performed by using GraphPad Prism v9.00. First, we tested the normality of the data by using D'Agostingo & Pearson normality test, by checking skewness and kurtosis values and visual inspection of data. Then, if the samples followed a normal distribution, we chose the appropriate parametric test; otherwise, the non-parametric counterpart was chosen. In two groups comparison: for paired data the Wilcoxon matched-pairs signed-rank test and paired t test were used; for unpaired data, Man-Whitney test and the unpaired t test were used. For multiple groups comparison, repeated measures one-way analysis of variance (ANOVA) with posttest Turkey's and Holm-Sidák's multiple comparisons or Friedman or Kruskal-Wallis tests with posttest Dunn's multiple comparisons were used as indicated. For correlations, Pearson or Spearman tests were used as described. The half-maximal neutralization titre (NT50), defined as the reciprocal of the dilution at which infection was decreased by 50%, was determined using four-parameter nonlinear regression (least squares regression without weighting; constraints: bottom=0). Spearman and Pearson correlation test were used in correlation analysis. The choice of each test was dependent on the underlying distribution and is indicated in the legend of the figures.

4. Results

4.1 COVID-19 patients

The nature and strength of immune responses differ between women and men, resulting in sex-specific differences in the prevalence, manifestations, and outcomes of vaccination and infectious and autoimmune diseases. The current SARS-CoV-2 pandemic highlights the clinical consequences of these sex differences.

4.1.1 Spike-specific T cells have a Tfh-like cell phenotype

We received blood samples from 31 hospitalized patients in Hospital Fernando Fonseca with positive RT-PCR test for COVID-19. Demographic and clinical data are contained in Table 7.1.

We used an AIM assay to detect spike specific $CD4^+$ T cells, through the upregulation of OX40 and CD25[96,97]. All donors had spike-specific T cells ($OX40^+CD25^+$) in circulation and there were no significant differences in their frequency between day 1 and day 5 (Fig. 4.1 A, B).

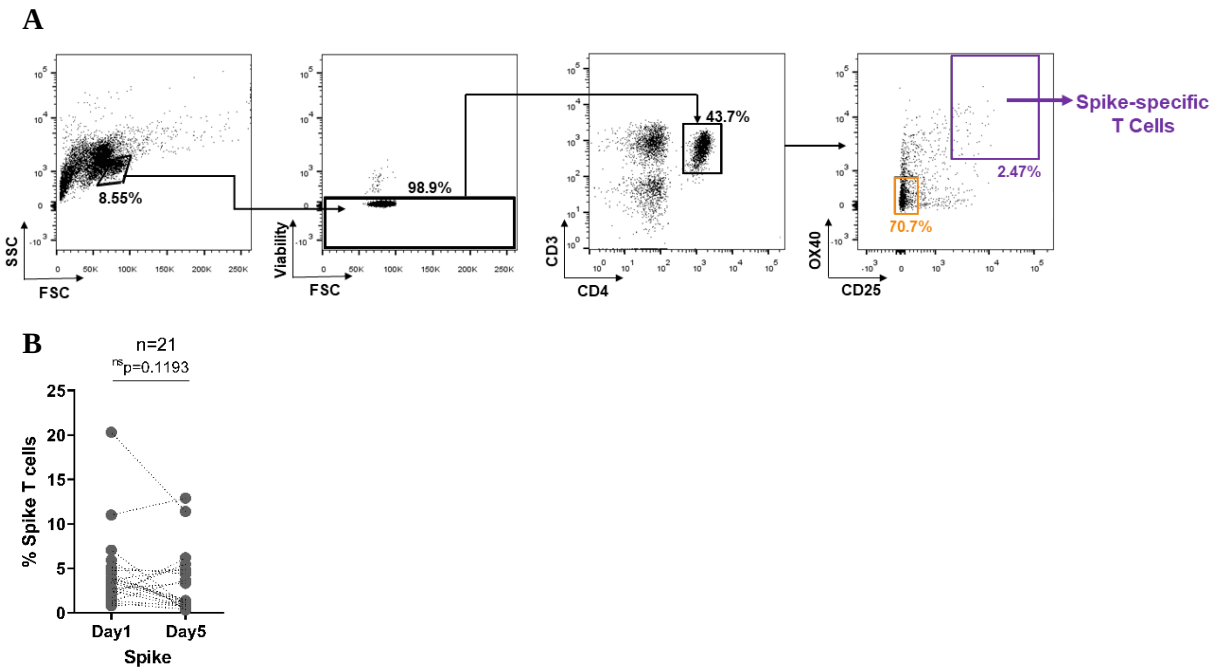

Figure 4.1 Spike-specific T cells are present in blood samples. (A) Gating strategy for $CD3^+CD4^+OX40^+CD25^+$ spike-specific T cells in blood samples. We started by looking for lymphocytes, inside them we looked for viability, inside viability we identify the $CD3^+CD4^+$ T cells and inside them we were able to identify the population of $CD3^+CD4^+OX40^+CD25^+$ spike-specific T cells (purple) and we can also detect a non-specific population (orange). (B) Donor matched analyses of spike-specific T cells between day 1 and day 5. p value determined by non-parametric paired Wilcoxon test. ns: not significant.

In order to phenotype the spike-specific T cell population we evaluate the expression of CXCR5, ICOS, CD40L, CD38 and TLR7. CXCR5 and ICOS identify the presence of Tfh cells in circulation, while CD40L guides antibody production. CCR6 identifies T cells that are migrating to infection sites, CD38 measures the activation state of the cells and TLR7 is involved in viral recognition. Both CD40L and TLR7 are located in X chromosome and it is known that TLR7 escapes X-chromosome inactivation[49], so we also wanted to see if this had any influence on our results.

Through AIM assay we evaluated these molecules inside of the specific population on day 1 (Fig. 4.2A). We observed a higher expression of CXCR5 and ICOS inside spike-specific T cells, which indicates a

Tfh phenotype for this population. Spike T cells seems to have a higher propensity to migrate to sites of inflammation, since there was a significant increase in CCR6 expression. Spike-specific T cells displayed more CD40L than the negative population, which guides to antibody production by B cells. There were no differences between spike-specific T cells and non-specific regarding activation state, as there were no significant differences on CD38 expression. There was a significant increase in TLR7 expression in spike-specific population, which is line with the fact that TLR7 is the virus ligand. For these molecules the results were the same at day 5 (Fig. 4.2B).

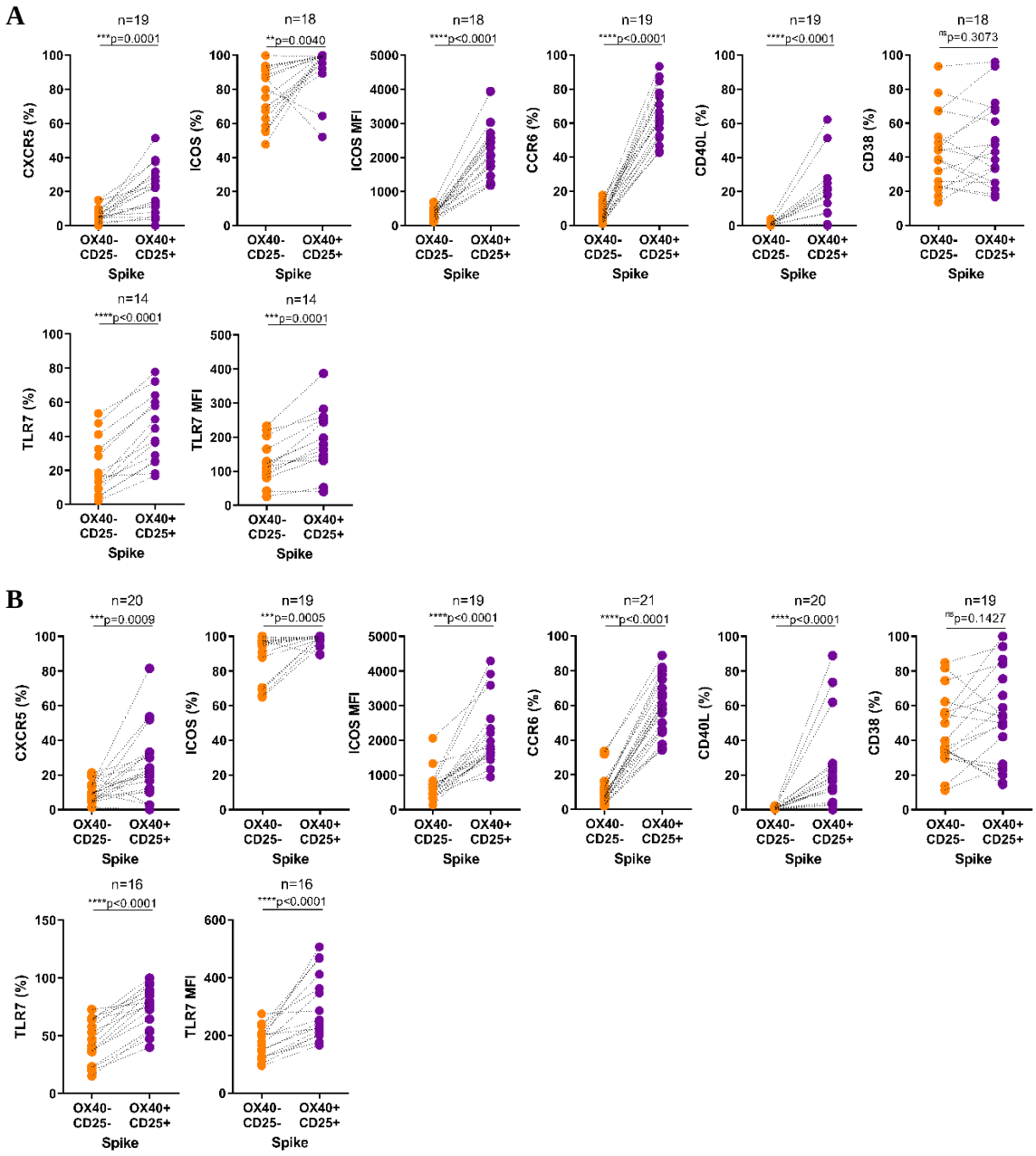

Figure 4.2 Spike-specific T cells have a Tfh-like cell phenotype. (A) Donor matched analysis of molecules inside spike-specific T cells ($OX40^+CD25^+$) and outside this population ($OX40^-CD25^-$), on day 1. (B) Donor matched analysis of molecules inside spike-specific T cells and outside this population, on day 5. Non-specific T cells (orange), spike-specific T cells (purple). Median fluorescent intensity (MFI). p values determined by parametric paired t test and by non-parametric paired Wilcoxon test when appropriate. ****p<0.0001, ***p<0.001, **p<0.01, ns: not significant.

We next sought to evaluate if hormones were modulating the spike-specific population. In addition to spike, the cells were also stimulated with estradiol and progesterone for 5 days. We observed no significant differences between spike-specific T cells frequency with or without hormones (Fig. 4.3A), meaning that hormones are not modulating the spike-specific population. The frequency of spike T cells did not correlate with donor's age neither with the days of symptoms (Fig. 4.3 B, C). We observed a higher frequency of spike-specific T cells on male patients (Fig. 4.3D). Then we evaluated the molecules inside of the specific population to see if hormones made a difference. The results were the same, hormones were not modulating the molecules inside the spike-specific population (Fig. 4.3E). ICOS was the one molecule that can be modulate by progesterone in follicular and luteal phases (Fig. 4.3E).

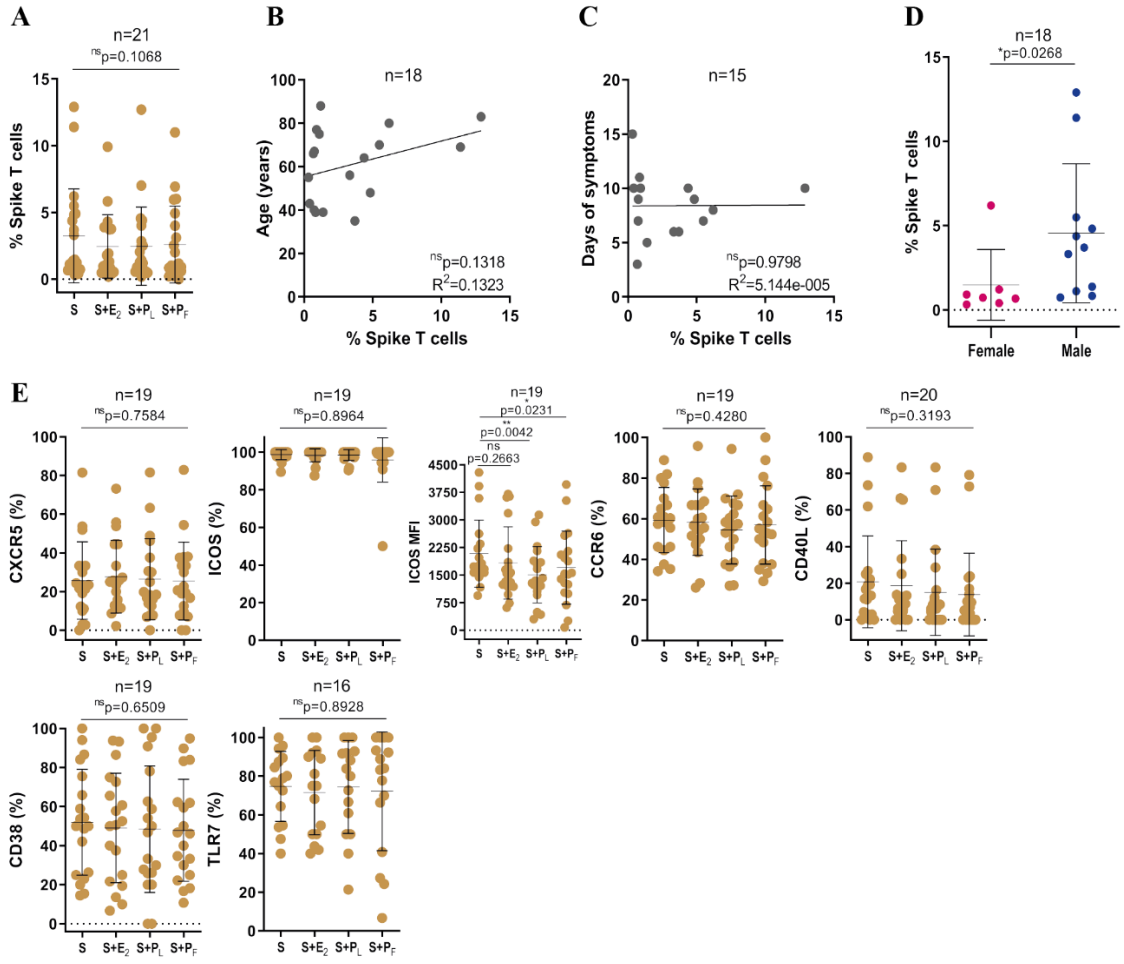

Figure 4.3 Progesterone modulates ICOS expression. (A) Cumulative frequency of spike-specific T cells stimulated with spike (S) or with spike plus estradiol (E_2), or progesterone in luteal phase (P_L), or progesterone in follicular phase (P_F), on day 5. (B) Correlation between the frequency of spike-specific T cells and donor's age, on day 5. (C) Correlation between the frequency of spike-specific T cells and the days of symptoms, on day 5. (D) Comparation between the frequency of spike-specific T cells and donor's sex. (E) Cumulative frequency of the molecules inside the spike-specific T cell population when cells were stimulated with spike or with spike and hormones, on day 5. Median fluorescent intensity (MFI). p values determined by parametric paired and unpaired t test, by non-parametric paired Wilcoxon test and Man-Whitney test when appropriate, by ANOVA, post-hoc Holm-Sidák's and by Friedman, post-hoc Dunn's when comparing 4 groups. Pearson correlation. **p<0.01, *p<0.05, ns: not significant.

4.1.2 Estradiol seems to increase IL-10 concentration

After phenotype spike-specific T cells we performed a functional assay to evaluate cytokine production. On day 5 we collected the supernatants from all conditions, and we performed an ELISA assay with them to evaluate IL-10 concentration. We observed no significant differences, although there appeared to be a trend to estradiol increase IL-10 concentration (Fig. 4.4A). We also observed that spike-specific T cells produced IL-10 in higher concentrations and progesterone had no effect on IL-10 concentration (Fig. 4.4A). To evaluate the differences between sexes we looked for IL-10 production in females and males individually. Despite no significant differences, in female patients we observed the same trend, estradiol increased IL-10 concentration (Fig. 4.4 B, C).

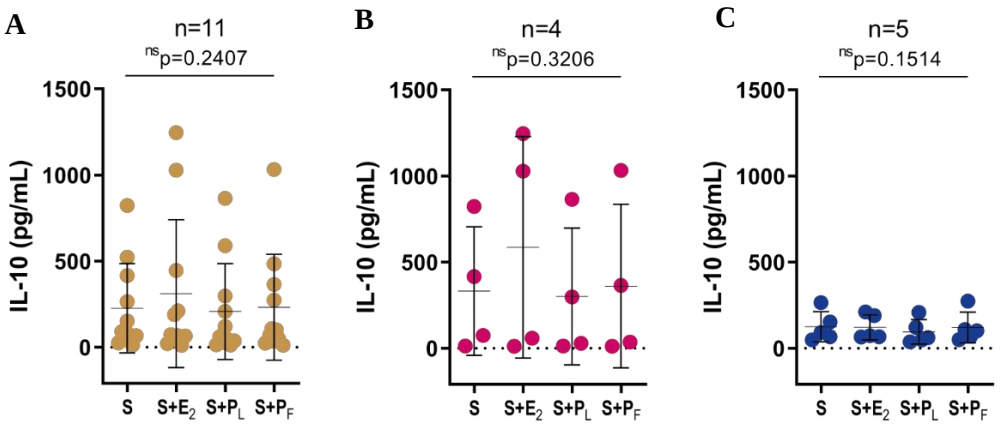

Figure 4.4. Estradiol seems to increase IL-10 concentration. (A) Cumulative frequency of IL-10 concentration when cells were stimulated with spike or with spike and hormones. (B) Cumulative frequency of IL-10 concentration when cells were stimulated with spike or with spike and hormones, in females (pink). (C) Cumulative frequency of IL-10 concentration when cells were stimulated with spike or with spike and hormones in males (blue). Spike (S), estradiol (E_2), progesterone in luteal phase (P_L), progesterone in follicular phase (P_F). p value determined by by ANOVA, post-hoc Holm-Sidák's and by Friedman, post-hoc Dunn's when comparing 4 groups. ns: not significant.

4.1.3 Anti-spike IgG and IgM levels corelate with donor's days of symptoms

In order to evaluate B cell response through antibody (IgG, IgA and IgM) production, we performed an ELISA assay. All donors had anti-spike antibodies in circulation (Fig. 4.5A). We observed higher IgG titers compared to IgA and IgM (Fig. 4.5B). Anti-spike IgG, IgA and IgM antibody levels did not correlate with the frequency of spike-specific T cells neither with donor's age (Fig. 4.5 C, D). We detected a correlation between the days of symptoms and both anti-spike IgG and IgM levels, but not with IgA levels (Fig. 4.5E). We next wanted to assess if sex was determinant for antibody production, but we observed no significant differences between anti-spike IgG, IgA and IgM antibody levels and donor's sex (Fig.4.5F).

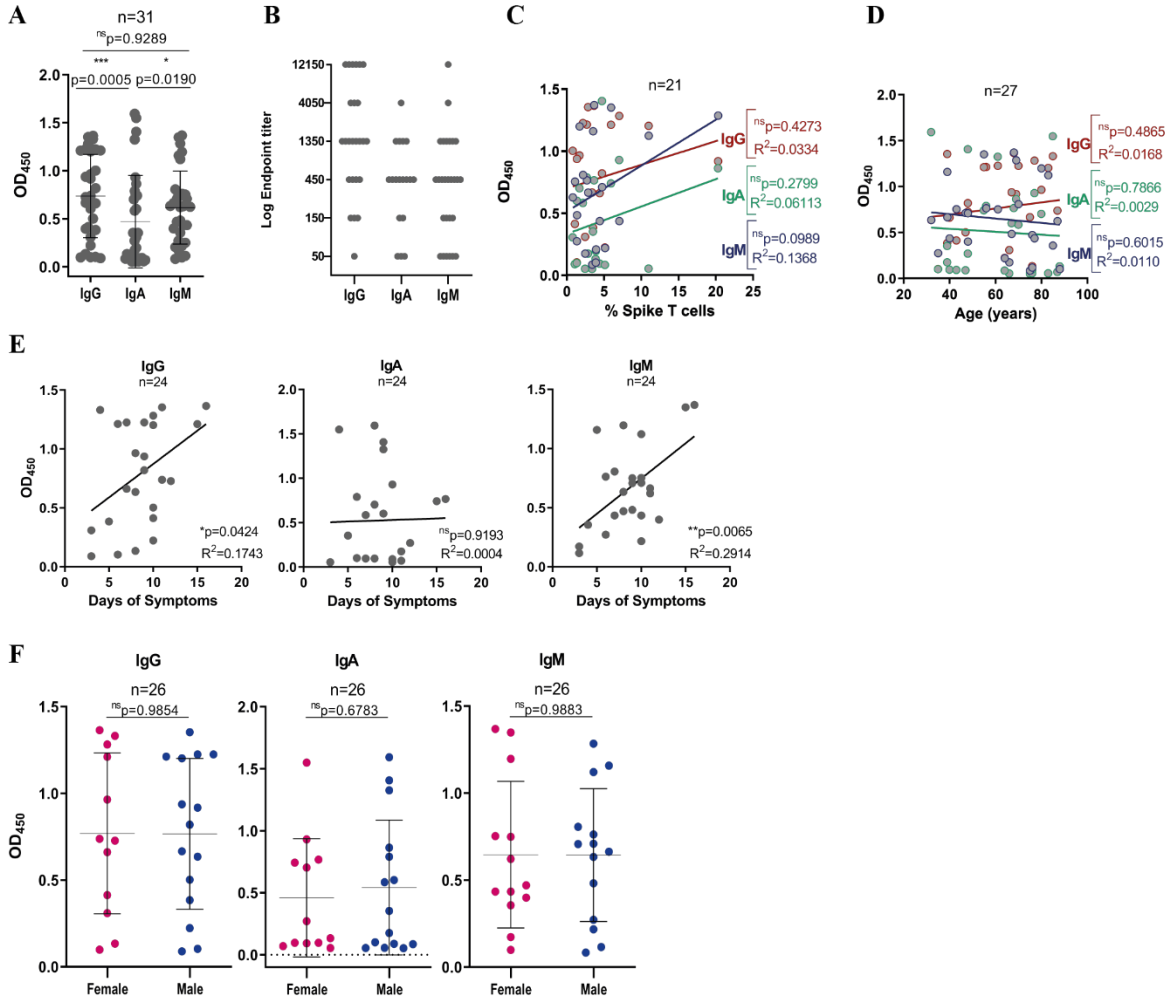

Figure 4.5 Anti-spike IgG and IgM levels corelate with donor's days of symptoms. (A) Anti-spike IgG, IgA and IgM in blood samples, measured by absorbance at 450 nm (OD_{450}). (B) Endpoint titers for anti-spike antibodies in blood samples (IgG, IgA and IgM). (C) Correlation between anti-spike IgG, IgA and IgM with spike-specific T cells frequency. (D) Correlation between anti-spike IgG, IgA and IgM with donor's age in years. (E) Correlation between anti-spike IgG, IgA and IgM with the days of symptoms. Correlation between anti-spike IgG, IgA and IgM with donor's age in years. (F) Comparation of OD_{450} between females and males for anti-spike IgG, IgA and IgM in blood samples. p values determined by Friedman, post-hoc Dunn's when comparing 3 groups, by parametric paired and unpaired t test, when appropriate. Pearson and Spearman correlations. ***p<0.001, *p<0.05, ns: not significant.

4.2 COVID-19 vaccination in lactating woman

Sex hormones have potent effects on immune cells and vary throughout a woman's life, especially during pregnancy and breastfeeding. Initial COVID-19 clinical trials of mRNA vaccines excluded lactating women. Even after vaccine authorization, breastfeeding HCW were advised to discontinue breastfeeding upon receiving COVID-19 mRNA vaccine.

Experiments and data generated for figure 4.6. A-G, figure 4.7 A-F and figure 4.8D were performed by Juliana Gonçalves and A. Margarida Juliano. Experiments and data generated for figure 7.1 were performed by Diogo Athayde. Experiments and data generated for figure 4.6H and figure 4.7 G-K were performed by Marta Alenquer and Filipe Ferreira. All the remaining experiment were performed by Juliana Gonçalves. This section is part of a paper published in Cell Reports Medicine: Gonçalves J, *et al*. doi: doi.org/10.1016/j.xcrm.2021.100468

4.2.1 COVID-19 mRNA vaccines induce production of SIgA by mammary mucosa early upon 1st dose administration

Altogether were collected 23 paired samples of breastmilk, pre-vaccination and after first and second mRNA vaccine administration. Demographic data are contained in Tables 7.2 and 7.3.

We looked at humoral response in breastmilk and blood ~10 days post first vaccine dose, when protection conferred by mRNA vaccines is starting[98,99]. All lactating women had anti-spike antibodies in circulation with 17/23 $IgA^+IgG^+IgM^+$, 2/23 IgG^+IgM^+, 1/23 IgA^+IgG^+ (Fig. 4.6.A). Similarly, 22/23 lactating women presented anti-spike antibodies in breastmilk, with 2/23 $IgA^+IgG^+IgM^+$, 3/23 IgA^+, and 17/23 IgA^+IgG^+ (Fig. 4.6A). Anti-spike IgG, IgA and IgM antibody levels did not correlate with donors' age (Fig. 4.6B). We detected a trend between milk and blood anti-spike IgA levels and within milk samples a correlation between anti-spike IgA and IgG (Fig. 4.6 C, D). As mRNA vaccines are better suited at inducing systemic monomeric IgA rather than polymeric mucosal SIgA, we sought to identify the source of IgA in the breastmilk through detection of SIgA reactive against spike and its RBD domain. Anti-spike SIgA was present in 70% of milk samples and correlated with anti-spike IgA in milk (Fig. 4.6 E, F).

To gain insight into the possible neutralizing properties of milk and blood antibodies, we first assessed RBD endpoint titers[41]. Milk anti-RBD IgA endpoint titer was lower when compared to the corresponding anti-RBD endpoint titers for IgA, IgG and IgM in blood (Fig. 4.6G). None of milk samples were neutralizing and even though several blood samples possessed moderate (1:450) and high (>1:1350) antibody endpoint titers only three blood samples were neutralizing (Fig. 4.6H).

Altogether our data shows that SIgA is produced early (d10) by the mammary mucosa in response to the first dose of mRNA vaccine and indicate that mRNA vaccines are capable of inducing a local immune response by the mammary MALT.

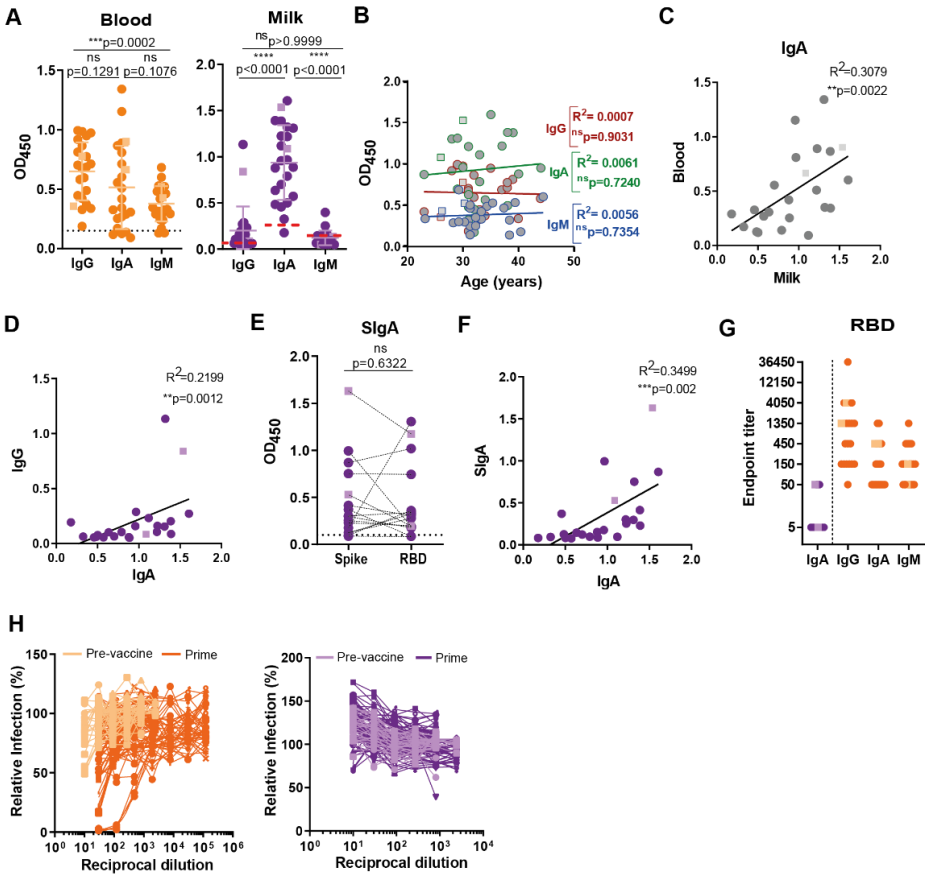

Figure 4.6 (In previous page) mRNA vaccines induce production of SIgA into the breastmilk early upon 1st dose administration. (A) Anti-spike IgG, IgA and IgM in plasma and skim milk of breastfeeding women (n=23), measured by absorbance at 450 nm (OD_{450}). (B) Correlation between anti-spike IgG, IgA and IgM with donor's age in years. (C) Correlation between anti-spike IgA in plasma and skim milk. (D) Correlation between anti-spike IgA and IgG in skim milk. (E) Donor matched analysis between anti-spike and anti-RBD SIgA in skim milk. (F) Correlation between anti-spike IgA and SIgA in skim milk. (G) Endpoint titers for anti-RBD antibodies in skim milk (IgA), and plasma (IgG, IgA and IgM). (H) Neutralization curves for plasma and skim milk. Plasma (orange), skim milk (purple). Circles: Pfizer; squares: Moderna. Dashed line: assay cut off. n=23 nursing women and n=22 controls. p values determined by ANOVA, post-hoc Turkey's and Friedman, post-hoc Dunn's when comparing 3 groups; by parametric paired t test and by non-parametric paired Wilcoxon test when appropriate. Pearson and Spearman correlations. ****p<0.0001, ***p<0.001, **p<0.01, ns: not significant.

4.2.2 Unconcentrated neutralizing IgA can be found in milk after vaccine second dose

While circulating neutralizing IgG to mRNA vaccine are optimally detected after boost[100], it is currently unclear whether vaccine induced milk antibodies are neutralizing[89,90]. Contemporaneously with IgG surge in the blood at ~10 days post vaccine boost, we detected anti-spike and anti-RBD IgG in milk (Fig. 4.7 A, B). In contrast, the frequency and levels of spike-reactive IgA remained constant in blood and milk, even though we observed a slight increase in milk anti-RBD IgA (Fig. 4.7 A-E). Similarly, milk SIgA levels recognizing spike and its RBD domain remained constant upon vaccine boost (Fig. 4.7D). Interestingly, anti-spike IgA in breastmilk inversely correlated with circulating anti-spike IgG (Fig. 4.7C). This might be due to the fact that higher induction of circulating IgG might prevent spike protein to reach the mammary MALT and effectively induce local antibody production. Donor paired analysis shows that while milk anti-spike IgG was boosted, milk anti-spike IgA and SIgA remained constant following vaccine second dose (Fig. 4.7E). Nonetheless the frequency of milk samples with spike-SIgA increased from 70% to 87%. Vaccination induced a similar antibody profile in blood, with vaccine boost increasing anti-spike IgG but not affecting IgA (Fig. 4.7F)[101]. IgM was poorly induced upon vaccination (Fig. 4.7F).

As expected, all the plasmas from fully vaccinated lactating women were neutralizing (NT50: 238.69; IQR, 148.47–383.36) and comparable to an aged-matched female cohort (NT50: 215.39; IQR, 135.34–303.51) (Fig. 4.7 G, H) and to previous reports for mRNA vaccines[88,100,102]. Importantly, only 1 milk sample displayed weak neutralizing activity (Fig. 4.7I). To identify which milk Ig could potentially neutralize SARS-CoV-2, we purified IgA and IgG by affinity chromatography and concentrated 5-fold prior to running neutralization assays. Weak neutralization could only be detected in three IgA fractions, which clustered around highest SIgA levels (Fig. 4.7 J, K, Table 7.4). Next, we purified milk IgA through size exclusion chromatography (SEC) (Fig. 7.1 A, B). Purified IgA from pre- and post-vaccination samples eluted in a single peak corresponding to polymeric SIgA, suggesting that vaccination did not result in an influx of monomeric IgA from the blood (Fig. 7.1 C, D).

Our data indicate that anti-spike-SIgA titers were unaffected by vaccine boosting. While the concentration of spike-reactive IgA in breastmilk might not be sufficient to directly neutralize viral infection, our data suggest that cumulative transfer of IgA through feeding might provide the infant with effective SARS-CoV-2 neutralization.

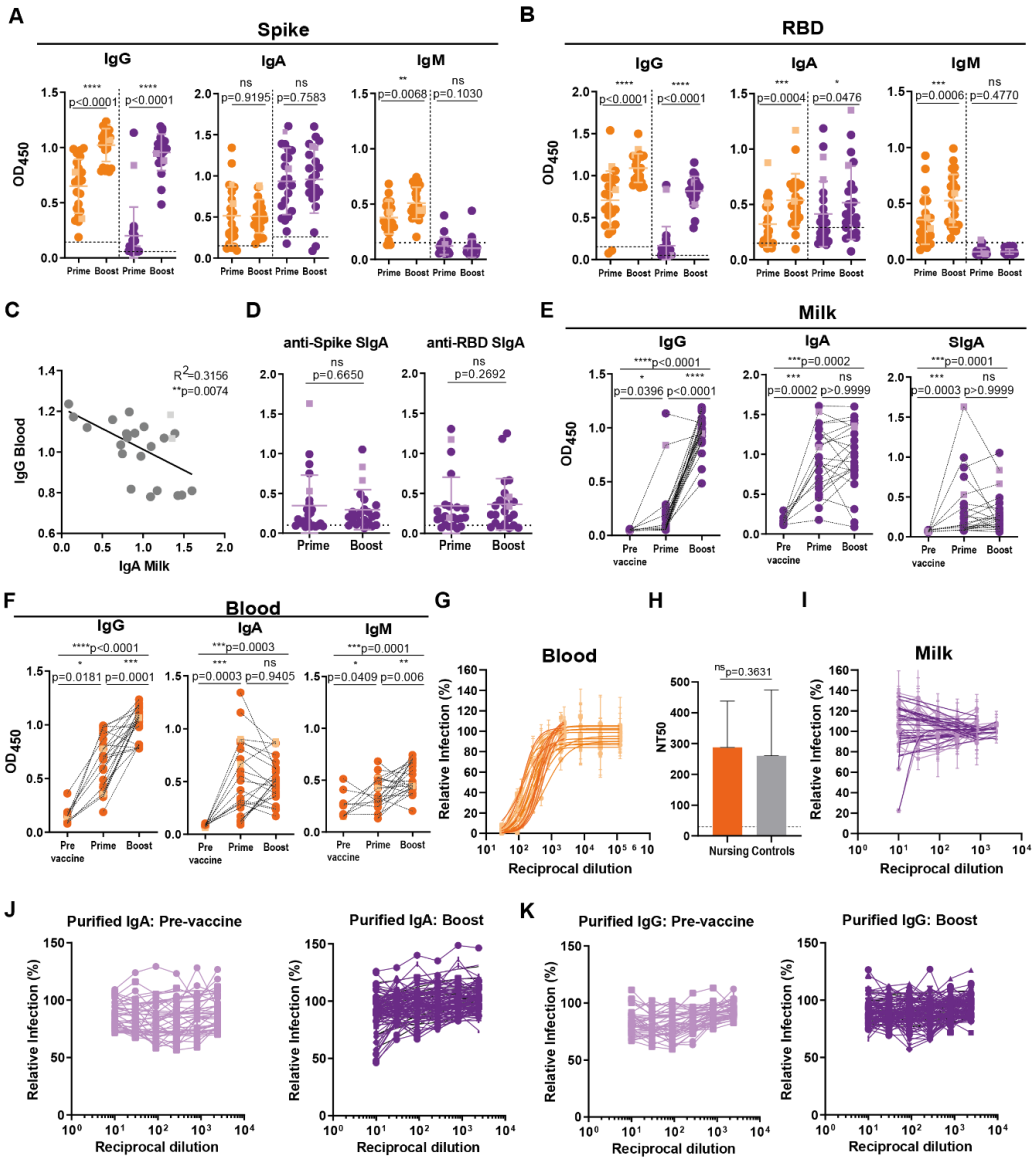

Figure 4.7 Neutralizing antibodies are found in blood and less frequently in milk after vaccine second dose. (A) Anti-spike IgG, IgA and IgM, at ~10 days after first (prime) and second (boost) vaccine doses in plasma and in skim milk, measured by absorbance at 450 nm (OD_{450}). (B) Anti-RBD IgG, IgA and IgM performed as in (A). (C) Correlation between anti-spike IgA in skim milk versus anti-spike IgG in the blood. (D) Comparison between anti-spike- and anti-RBD SIgA, at ~10 days after first (prime) and second (boost) vaccine dose, measured as in (A). (E) Donor matched analysis of anti-spike IgG, IgA and SIgA, pre-vaccination, after vaccine first (prime) and second (boost) doses, in skim milk. (F) Donor matched analysis of anti-spike IgG, IgA and IgM, pre-vaccination, after vaccine first (prime) and second (boost) doses, in blood. (G) Plasma neutralization curves. (H) Plasma neutralization titers (NT50) in nursing and control women. (I) Skim milk neutralization curves. (J) Neutralization curves for skim milk purified IgA concentrated 5-fold, pre-vaccination and ~10 days after vaccine boost. (K) Neutralization curves for skim milk purified IgG concentrated 5-fold, pre-vaccination and ~10 days after vaccine boost. Plasma (orange), skim milk (purple). Circles: Pfizer; squares: Moderna. Dashed line: assay cut off. n=23 nursing women and n=22 controls. p values determined by parametric paired t test and by non-parametric paired Wilcoxon test when appropriate, by ANOVA, post-hoc Holm-Sidák's and Kruskal-Wallis, post-hoc Dunn's when comparing 3 groups. Spearman correlation. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns: not significant.

4.2.3 Lactating women have higher frequency of circulating RBD-reactive memory B cells and anti-RBD antibodies

While human IgA secreting B cells are preferentially retained in the mammary gland, functional IgG secreting B cells can be found in higher frequencies in breastmilk[75,78]. We sought to evaluate the presence of RBD-reactive B cells in the milk following vaccination. We could only detect B cells in 5/23 milk samples, which were overwhelming IgD^{-}[78] (Fig. 4.8A). Due the limited number of B cells detected we were not able to assess the presence of RBD-reactive milk B cells.

Mainly due to hormonal changes, lactating women display distinct immune responses[30]. In the blood, a clear population of RBD-binding IgD^{-} B cell population could be detected post vaccine first dose, which remained unaltered upon boost (Fig. 4.8 B, C). Both RBD-reactive plasmablasts and memory B cells were detectable after vaccine prime, with only plasmablasts increasing in frequency upon boost (Fig. 4.8 B, C). Compared to controls, breastfeeding women have higher frequency of memory B cells and higher titers of circulating anti-RBD IgA and IgG (Fig. 4.8 C, D). Moreover, RBD-reactive memory B cells, and overall RBD-reactive B cells, correlated with anti-spike IgG levels (Fig. 4.8 E, F), but not with neutralization titers (Fig. 4.8 E, F).

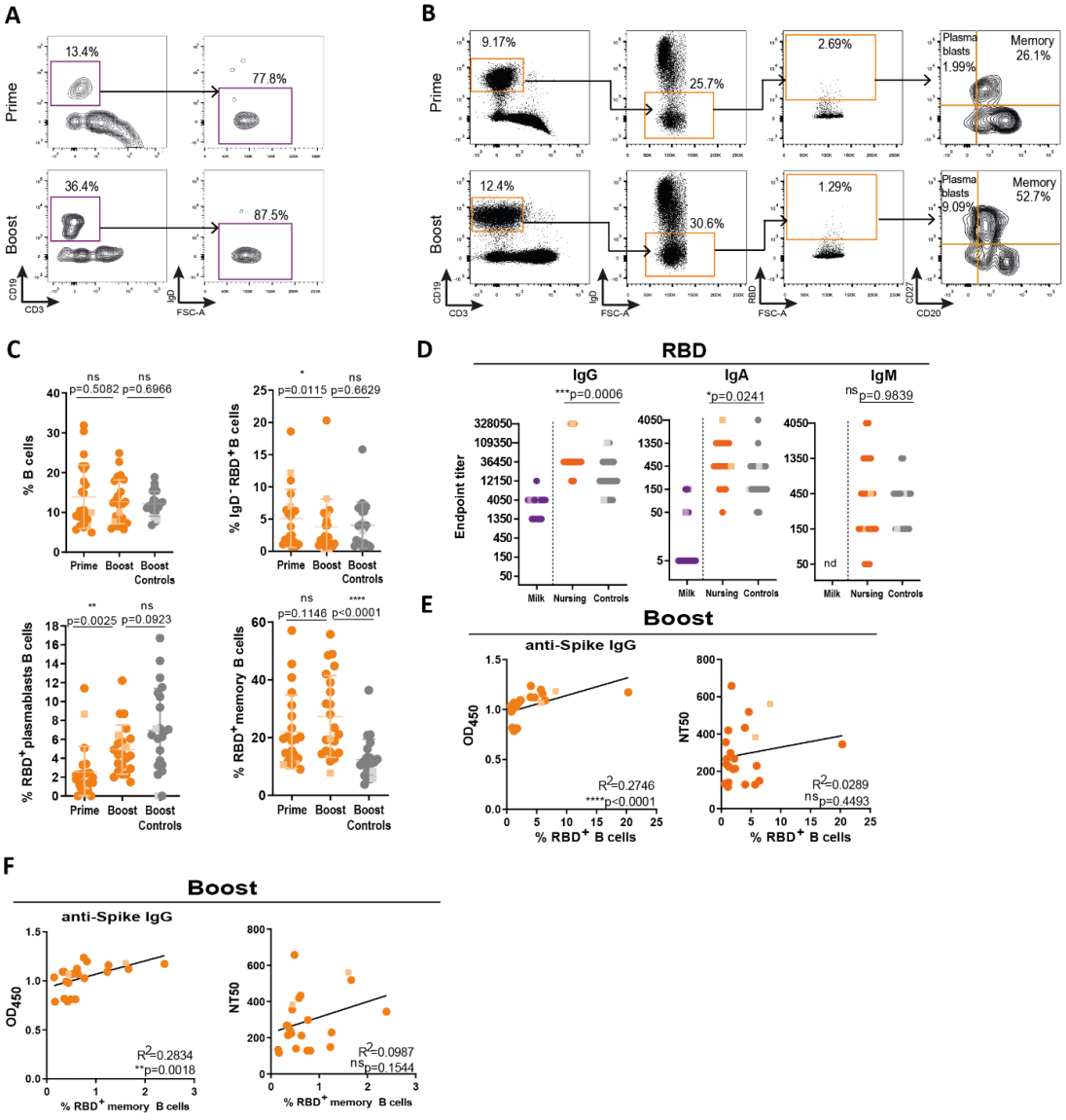

Figure 4.8 (In previous page) Lactating women have higher frequency of RBD-reactive memory B cells and anti-RBD antibodies in circulation. (A) Gating strategy for $CD3^{-}CD19^{+}IgD^{-}$ B cells in skim milk, after first (prime) and second (boost) vaccine doses. (B) Gating strategy for circulating RBD-reactive $CD3^{-}CD19^{+}IgD^{-}CD20^{-}CD27^{+}$ plasmablasts and $CD3^{-}CD19^{+}IgD^{-}CD20^{+}CD27^{+}$ memory B cells. (C) Cumulative frequency of circulating total B cells (top left), RBD-reactive B cells (top right), plasmablasts (bottom left) and memory B cells (bottom right) after first (prime) and second (boost) vaccine doses for nursing and after the second (boost) vaccine dose for controls. (D) Endpoint titers for anti-RBD IgG, IgA and IgM in skim milk and plasma of nursing women and in control's plasma. nd: non-detectable. (E) Correlation between anti-spike IgG and neutralization titers with the frequency of RBD-reactive B cells, upon vaccine boosting. (F) Correlation between anti-spike IgG and neutralization titers (NT50) with the frequency of RBD-reactive memory B cells, upon vaccine boosting. Circles: Pfizer vaccine; squares: Moderna vaccine. n=23 nursing women and n=22 controls. p values determined by non-parametric paired Wilcoxon test, t test and Man-Whitney test when appropriate. Pearson and Spearman correlations. ****p<0.0001, ***p<0.001, **p<0.01, * p<0.05, ns: not significant.

4.2.4 Spike-specific T cells are transferred through breastmilk

Emerging evidence suggests the requirement of both antibody-mediated and T cell-mediated immunity for effective protection against SARS-CoV-2[103]. To detect spike specific $CD4^{+}$ T cells, we used an AIM assay using OX40 and CD25 dual expression to detect spike reactivity[96,97]. We could only robustly detect $CD4^{+}$ T cells in the milk of 12 (52%) donors (Fig. 4.9 A, B). The absence of T cell detection in the other 11 samples was likely due to insufficient milk volume available. After vaccine second dose, spike-reactive T cells could be identified in all milk samples with detectable T cells, with frequencies ranging from 0.7% to 9.1% (Fig. 4.9 A, C). Indicating that spike-T cells are transferred to breastmilk, upon mRNA vaccination.

All lactating women possessed spike-T cells (median, 0.76%; IQR, 0.5–1.19) in circulation after vaccine prime, and their frequency was not altered by subsequent boost (Fig. 4.9 D, E), even though their activation state, measured by CD69 expression, was decreased (Fig. 4.9 D, E). Curiously, it appears that after vaccine boost, breastfeeding women have less spike-reactive $CD4^{+}$ T cells when compared to vaccinated controls (Fig. 4.9 D, E). In view of the role of $CD4^{+}$ T cells in B cell effector differentiation, we looked if there was an association between circulating spike-reactive T cells and RBD-reactive B cells. There was no correlation between spike-T cells and RBD-plasmablasts or memory B cells (Fig. 4.9F).

Altogether, our data show that, in addition to antibodies, milk also contains spike-reactive T cells. These spike-reactive T cells might transfer long-lived immunity to the suckling infant.

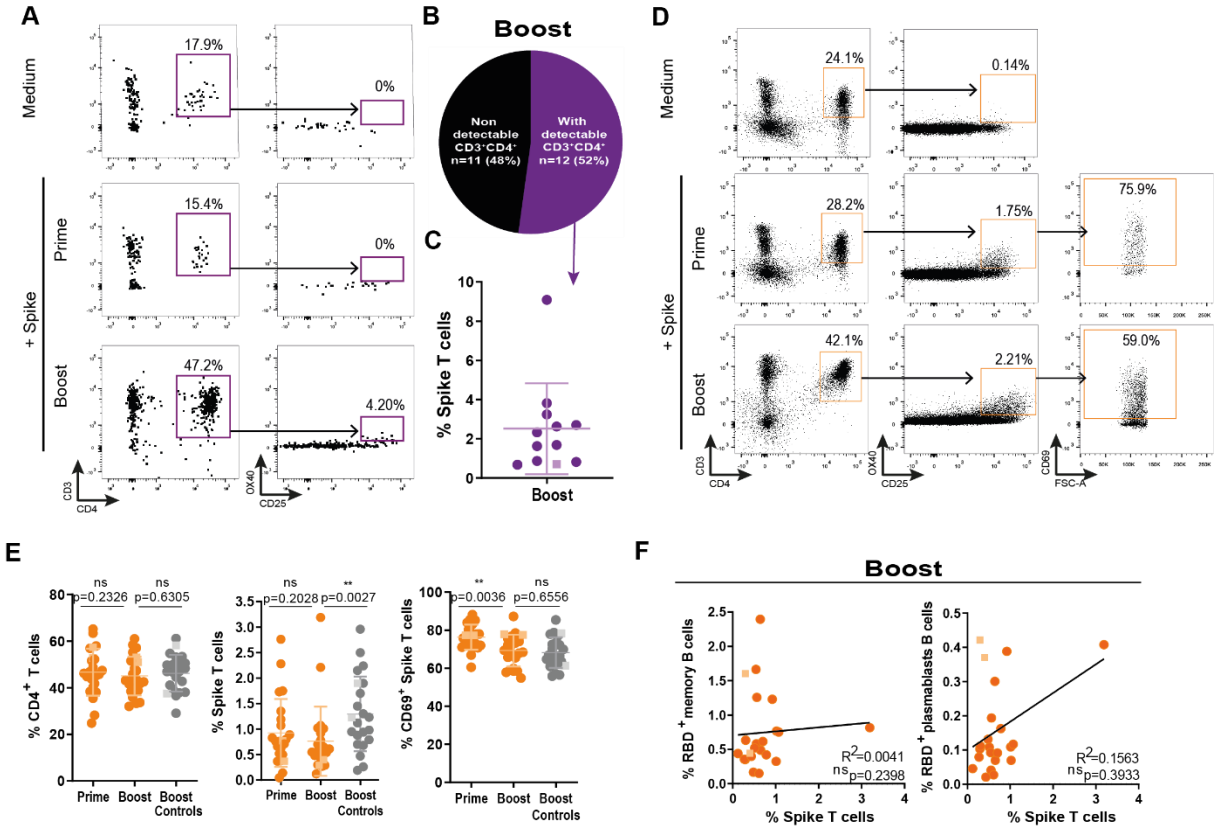

Figure 4.9 Spike-specific T cells are transferred to breastmilk after vaccine boost. (A) Gating strategy for $CD3^+CD4^+OX40^+CD25^+$ spike-specific T cells in skim milk, after first (prime) and second (boost) vaccine doses. (B) Frequency of milk samples with detectable versus non detectable $CD3^+CD4^+$ T cells. (C) Cumulative frequency of spike-specific T cells in skim milk after vaccine boost. (D) Gating strategy of circulating for spike-specific T cells, after first (prime) and second (boost) vaccine doses. (E) Cumulative frequency of T cells (left), spike-specific T cells (middle) and of $CD69^+$ spike-specific T cells (right) after first (prime) and second (boost) vaccine doses in nursing women and post boost for controls. (F) Correlation between the frequency of RBD- reactive memory B cells (right) and plasmablasts (left) with the frequency of spike-specific T cells after boost. Circles: Pfizer vaccine; squares: Moderna vaccine. n=23 nursing women and n=22 controls. p values determined by parametric paired and unpaired t test, by non-parametric paired Wilcoxon test and Man-Whitney test when appropriate. Spearman correlation. **p<0.01, ns: not significant.

5. Discussion

The immunological balance is different between women and men. Women, in general, generate a stronger immune response to infections and vaccinations, although they suffer more from inflammatory and autoimmune diseases[1]. The current SARS-CoV-2 pandemic highlights the clinical consequences of these sex differences. Sex differences in COVID-19 severity and morbidity exist, with the male sex being a risk factor[42,43]. Compared to females, male COVID-19 patients have an increased risk of ICU admission and in hospital mortality. The risk of death in males is ~1.7 times higher than in females[36]. Hospitalized patients with severe disease have increased production of inflammatory cytokines and chemokines. Female patients have more robust T cell activation than males when first admitted into hospital. This poorer T cell response is correlated with worse disease outcomes in males only[104]. However, the precise causes of death by COVID-19, regardless of sex, remain unknown. Through a combination of flow cytometry and serology we show that upon SARS-CoV-2 infection there is a spike-specific B (humoral) and T (cellular) cell response against the virus.

Hereupon, to better understand the SARS-CoV-2 infection, the first aim of our project was to phenotype the spike-specific T cell population. For that, we performed an AIM assay, which allowed us to detect spike-specific T cells in circulation. We observed no significant differences in spike T cells frequency between day 1 and day 5. We next phenotype the spike-specific T cell population through the expression of CXCR5, ICOS, CD40L, CD38 and TLR7 using an AIM assay. We observed a higher expression of CXCR5 and ICOS inside spike-specific T cells, suggesting a Tfh-like phenotype for these cells. Tfh cells play a vital role in providing help to antigen-specific B cells to initiate and maintain humoral immune responses against SARS-CoV-2[105]. For the antibody production is essential the secretion of cytokines IL-10 and IL-4 and the expression of co-receptors ICOS and CD40L. Spike T cells also appeared to guide antibody production by B cells and to have a higher propensity to migrate to sites of inflammation, since there was a significant increase in CD40L and CCR6 expression, respectively. CD38 measures the activation state of the cells and we observed no significant differences between spike-specific T cells and non-specific, regarding activation state. Finally, there was a significant increase in TLR7 expression in spike-specific population, which is in line with the fact that TLR7 is a virus receptor. Some sex differences may be caused by the inherent imbalance in the expression of genes encoded on the X and Y chromosomes of a host[106]. Many genes on the X chromosome regulate immune function and play an important role in modulating sex differences in the development of immune-related diseases. Both CD40L and TLR7 are located on the X-chromosome. TLR7 recognizes viruses with RNA genomes, which include SARS-CoV-2, and has higher expression levels in cells from female than males[107]. Due to the limited number of T cells detected and the imbalance of gender in the samples we were not able to evaluate these differences in our results.

Sex hormones, including testosterone, estrogens, progesterone, and prolactin, occur in different concentrations between the sexes, with males typically having greater levels of testosterone and females often having greater levels of estrogen and progesterone at reproductive ages. Sex hormones have potent effects on immune cells[22]. Estrogen and progesterone and their associated receptors are present in many immune cells. And is already known that estrogen and progesterone are capable to modulate T cells through these receptors[12]. Estrogen increases T cell number and decrease the susceptibility to infections, while progesterone increases this susceptibility since has an immunosuppressive effect on the immune system[22]. We next evaluated if female sex hormones, namely estradiol and progesterone, were modulating the T cell response. We observed no significant differences between spike-specific T cells frequency with or without hormones, meaning that hormones are not modulating the spike-specific population. Then we evaluated the expression of the same markers inside the specific population to see

if hormones made a difference. ICOS was the one molecule that can be modulated by progesterone in follicular and luteal phases. ICOS is an inducible costimulatory molecule expressed on activated $CD4^+$ and $CD8^+$ T cells and plays an important role in the formation and function of Tfh cells, being known as a surface marker for them[108,109]. Despite progesterone concentration being different between the two phases of the menstrual cycle (higher in luteal phase) they both decrease ICOS expression. It is known that females have higher frequencies of $CD3^+$ and $CD4^+$ T cells as well a higher $CD4^+/CD8^+$ ratio compared to males. In contrast, $CD8^+$ T cells and NK cells frequencies are greater in males[110–114]. T cell frequencies and activation remains greater in females than males during SARS-CoV-2 infection[43,115]. However, we observed a higher frequency of spike-specific T cells on male patients, which is not consistent with reported studies. This result can be explained by the small number of samples that we have from females (n=7) and males (n=11) and possibly from distinct disease presentation in this small number of samples. We need to increase our cohort before any definite conclusion can be drawn. The frequency of spike T cells did not correlate with donor's age neither with the days of symptoms.

In order to evaluate T cell response through cytokine production we performed a functional assay, in which we evaluated IL-10 concentration by ELISA. IL-10 is a cytokine known for its potent anti-inflammatory and immunosuppressive effects. A primary function of IL-10 during infection is to inhibit the host immune response to pathogens and microbiota, thereby mitigating tissue damage and immunopathology[116]. IL-10 also promotes B cell differentiation, proliferation, survival, and antibody production[117,118]. We observed that spike-specific T cells produced IL-10 at high concentrations. The dramatic elevation of IL-10 concentration appears to be a peculiar feature of hyperinflammation during severe SARS-CoV-2 infection and several studies indicate that IL-10 levels predict poor outcomes in patients with COVID-19[119–121]. Despite there were no significant differences, it seems to be a trend to estradiol increase IL-10 concentration. Next to evaluate the differences between sexes we looked for IL-10 production in females and males individually. Despite no significant differences, in female patients we observed the same trend, estradiol increased IL-10 concentration. As what happened in T cells, also here we have a small cohort (females n=4 and males n=5). For that reason, we are not able to evaluate the differences in IL-10 production between women and men.

In order to evaluate B cell response through antibody (IgG, IgA and IgM) production, we performed an ELISA assay. All donors had anti-spike antibodies in circulation, which is in line with other studies that already demonstrated the presence of these antibodies in COVID-19 patients[122,123]. We observed that IgG had higher antibody titers compared to IgA and IgM, which is correlated with the days of symptoms for IgG. We also detected a correlation between the days of symptoms and IgM levels, but not with IgA levels. We next wanted to assess if sex was determinant for antibody production, since there were other studies that described higher antibody levels in females compared to males[124]. However, we observed no significant differences between anti-spike IgG, IgA and IgM antibody levels and donor's sex. Due to the imbalance of gender in our samples we were not able to evaluate these differences in our results. In the future, it will be important to evaluate the production of cytokines with antiviral activity and immunoregulatory functions. Moreover, it will be also important to conduct functional assays to assess B cell response at cellular level.

Within pediatric population, infants are among the most susceptible to COVID-19 and also present the highest COVID-19 fatality rate[69,85,125]. Lactating women were excluded in initial clinical trials of mRNA COVID-19 vaccines and lactating HCW were advised to discontinue breastfeeding upon receiving COVID-19 mRNA vaccine by health authorities[84]. It is known that lactating women display a unique immune activation state, in order to provide immunity to the suckling infant. However, how mRNA vaccines impact lactating women and which is the breadth and effector profile of milk transferred immune response remains poorly understood. Through a combination of serology, virus neutralization,

flow cytometry and SEC we identified the secretory and neutralizing properties of breastmilk transferred antibodies and cellular immunity induced by mRNA vaccination.

Infants' immunity is achieved through the secretion of polymeric antibodies complexed to j-chain and secretory component proteins by the mammary MALT, which includes polymeric mucosal SIgA[76]. The secretory component is essential to ensure that milk antibodies survive the gastric environment and are effectively transferred to the infant[76]. Spike-reactive SIgA was detected in breastmilk of COVID-19 patients[126]. However, it remained unknown whether SIgA was similarly present upon mRNA vaccination. In order to evaluate the source of IgA in the breastmilk upon mRNA vaccination we performed an ELISA assay, in which we evaluated spike-reactive SIgA secretion. We detected spike-reactive SIgA in 87% of milk samples, indicating that mRNA vaccines can elicit local immune responses by mammary and oral mucosa[127]. Next, to explore the possibility that milk antibodies neutralize SARS-CoV-2, we performed an ELISA assay. Although it was weak, 1/23 milk samples exhibited neutralizing activity. Nevertheless, the purification and concentration of IgA increased its neutralization capacity. Our data suggest that the transfer of IgA through breastmilk might provide the infant with effective SARS-CoV-2 neutralization. Compared to COVID-19 infection[128,129], milk neutralizing capabilities post-vaccination appear to be weaker. This is in line with the fact that spike-reactive SIgA levels remained constant following vaccine second dose, indicating a T cell-independent production. It has been described that T cell-independent IgA responses resulted in the production of SIgA with low affinity and polyreactive[76,130]. As SARS-CoV-2 infection effectively primes airway and gut mucosa immunity, the two sources of mammary T and B cells[75], it is likely that milk SIgA production is primed in a T cell dependent manner, and thus presents higher neutralization capacity[131,132].

T cells are an important and so far unexplored route for milk transferred COVID-19 immunity. Previous studies have shown that milk transferred lymphocytes can survive the adverse environment of the digestive tract and seed in infant's tissues[81,82,87,88,133–135]. This is due to a combination of a biochemical reaction between infant's saliva and breastmilk that protects lymphocytes from acid injury[66–68], a decrease in enzyme and acid content in the infant's digestive tract[136,137], and an increase in gut permeability[134]. $CD4^+$ T cells are crucial in mediating mRNA vaccine protection[102,138,139], especially in suboptimal neutralizing antibodies settings[140]. It is possible that milk transferred spike-reactive T cells might mediate protection from infection by seeding in the infant's upper respiratory tract and gut.

We sought to evaluate the presence of RBD-reactive B cells in both milk and blood samples following vaccination by flow cytometry. In addition, we performed an ELISA assay to evaluate anti-RBD antibodies production. We could only detect B cells in 5/23 milk samples, which were overwhelming IgD^-. Due to the limited number of B cells detected, we were not able to assess the presence of RBD-reactive milk B cells. In the blood, a clear population of RBD-binding IgD^- B cell population was detected post vaccine first dose and remained unaltered upon boost. We detected higher RBD-reactive IgG and IgA titers in lactating women. However, consistent with previous studies, their neutralization titers were undistinguishable from controls[89]. In addition, we found that lactating women had higher frequencies of RBD-reactive memory B cells in circulation, which correlated with their anti-spike IgG levels, but not with neutralization titers. Recent studies suggest the requirement of both antibody-mediated and T cell-mediated immunity for effective protection against SARS-CoV-2[103]. We next performed an AIM assay, which allow us to detect spike-specific $CD4^+$ T cells. We could only detect $CD4^+$ T cells in the milk of 12 (52%) donors and this could be due to insufficient milk volume available in the other 11 samples. After vaccine boost spike-reactive T cells could be identified in all milk samples, indicating that spike-T cells are transferred to breastmilk, upon mRNA vaccination. All lactating women possessed spike-T cells in circulation after vaccine prime, and their frequency was not altered by subsequent boost. We looked if there was an association between circulating spike-reactive T cells and

RBD-reactive B cells. There was no correlation between spike-T cells and RBD-plasmablasts or memory B cells. Even though the reasons for increased frequencies of RBD-reactive memory B cells are likely multifactorial, increased levels of milk inducing prolactin has been associated with increased antibody titers and activated "memory"-like B and T cells[141–146]. As memory B cells have been proposed to play a key role in mounting recall responses to COVID-19 mRNA vaccines[100], further studies will be needed to determine if these cellular differences are maintained in medium term and whether they will impact long term protection.

Sex is considered a major determinant of an immune response. The nature and strength of immune responses differ between women and men, resulting in sex-specific differences. Women in general generate a stronger immune response to infections and vaccinations; on the other hand they suffer more from inflammatory and autoimmune diseases[1]. As is well known, women have historically been under-represented in clinical trials, including vaccine clinical trials[18]. Initial clinical trials of mRNA COVID-19 vaccines were not an exception, excluding lactating women[83]. Altogether, both results from COVID-19 vaccination in lactating women and COVID-19 patients reinforce the urgent need to bridge the gap between men and women regarding the immune response to vaccination or infectious diseases and how these can be affected by the different stages of a woman's life. Collecting sex-disaggregated data is essential to better understand the features of the disease and to better guide treatment strategies. Moreover, taking a sex-informed approach in research and medicine should be mandatory, which will result in more equitable health outcomes. Further studies are needed to assess the impact of hormonal variation during a woman's lifetime, namely during two very important stages: pregnancy and lactation.

6. References

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7. Supplementary Data

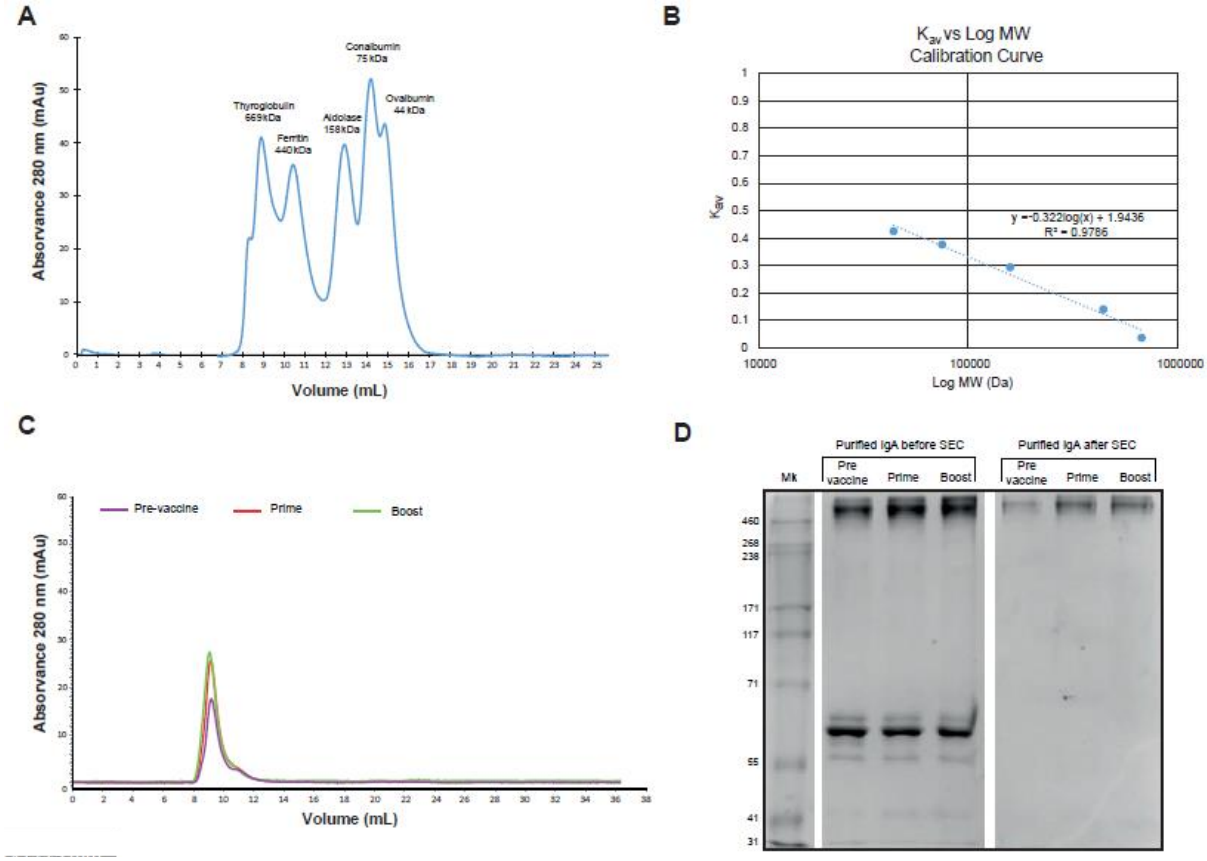

Figure 7.1 Size exclusion chromatography (SEC) of IgA purified milk fractions. Related to Figure 4.7. (A) Chromatogram of all standard proteins run in a Superdex 200 increase 10/300 GL. HWM Filtration Calibration Kit (Cytiva) were used with the following proteins: thyroglobulin (669 kDa); ferritin (450 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (44 kDa). These standard proteins were dissolved in bi-distilled water and their chromatographic profiles were obtained using an UV detector. (B) Graphical representation of calibration curve of partition coefficient (Kav) of each protein versus their respective molecular weight in Daltons. The Kav was calculated through the following formula: Kav=(Ve–V0)/(Vc-V0) where Ve is the elution volume of the protein, V0 is the void volume and Vc is the column bed volume. A dispersion graph Kav vs logMW was constructed. The equation obtained for the calibration curve is: Kav = -0.322log(MW) + 1.9436, where Kav is the partition coefficient and MW is the protein molecular weight (Da). (C) Size exclusion chromatogram of skim milk purified IgA fraction collected pre-vaccine (purple), or after first (red) and second (green) vaccine doses. (D) Non-reducing polyacrylamide gel electrophoresis of purified IgA fractions before SEC and after SEC of milk samples collected pre-vaccine, or after first (prime) and second (boost) vaccine doses. For the sake of clarity we removed gel lines 2 and 5 which pertained to the paired blood sample.

Table 7.1 Demographic and clinical data of COVID-19 patients. Related to Figure 4.1.

Parameters	COVID-19 patients n=31	
	Females	**Males**
Sex*	12 (44.5%)	15 (55.5%)
Age categories-n (%)*		
30-44	2 (16.7%)	4 (26.7%)
45-64	2 (16.7%)	5 (33.3%)
65+	8 (66.6%)	6 (40%)
SARS-CoV-2 PCR+	100%	100%
Days of symptoms**	10 (3-16)	8.5 (3-11)

*Demographic data missing for four donors.

**Clinical data missing for six donors. Median calculated out of total available data.

Table 7.2 Demographic data of nursing women. Related to Figure 4.6.

Nursing	Age (years)	Feeding duration (months)	Days post 1st dose	Days post 2nd dose	Vaccine	COVID-19 diagnostic
1	31	16	10	10	BNT162b2	No
2	44	12	10	11	BNT162b2	No
3	34	7	8	7	BNT162b2	No
4	37	13	13	13	BNT162b2	No
5	39	21	13	10	BNT162b2	No
6	31	23	8	10	BNT162b2	No
7	33	13	8	8	BNT162b2	No
8	35	11	16	9	BNT162b2	No
9	38	4	10	10	BNT162b2	No
10	28	15	12	12	BNT162b2	No
11	33	9	9	7	BNT162b2	No
12	38	13	8	9	BNT162b2	No
13	31	3	8	9	BNT162b2	No
14	29	13	7	10	BNT162b2	No
15	30	6	12	12	BNT162b2	No
16	32	5	15	26	BNT162b2	No
17	32	4	11	21	BNT162b2	No
18	40	4	10	9	BNT162b2	No
19	30	4	15	15	mRNA-1273	No
20	31	4	11	25	BNT162b2	No
21	26	6	10	10	mRNA-1273	No
22	29	14	11	11	BNT162b2	No
23	23	9	10	10	BNT162b2	No
24*	30					
25*	28					
26*	27					

* Only pre-vaccination samples were provided and used to calculate milk Ig cut-offs.

Table 7.3 Demographic data of controls. Related to Figure 4.6.

Controls	Age (years)	Days post 2nd dose	Vaccine	COVID-19 diagnostic
1	26	10	BNT162b2	No
2	34	16	BNT162b2	No
3	26	10	BNT162b2	No
4	28	10	BNT162b2	No
5	40	10	BNT162b2	No
6	31	10	BNT162b2	No
7	30	10	BNT162b2	No
8	34	11	BNT162b2	No
9	36	11	BNT162b2	No
10*	62	11	BNT162b2	No
11	31	16	BNT162b2	No
12	31	18	BNT162b2	No
13	32	18	BNT162b2	No
14	31	21	BNT162b2	No
15	37	20	BNT162b2	No
16	39	11	BNT162b2	No
17	36	13	BNT162b2	No
18	43	9	BNT162b2	No
19	40	12	BNT162b2	No
20	23	21	mRNA-1273	No
21	25	12	mRNA-1273	No
22	43	22	BNT162b2	No
23	41	12	BNT162b2	No

* Excluded from the study due to post-menopausal status.

Table 7.4 OD_{450} for spike-reactive SIgA, IgA, IgG in breastmilk, and NT50 for purified milk IgA, after vaccine second dose. Related to Figure 4.7.

Nursing	OD450 anti- Spike SIgA	OD450 anti- Spike IgA	OD450 anti- Spike IgG	NT50 purified IgA
1	0.491	1.39	1.156	n.d.
2	0.249	0.899	0.988	n.d.
3	0.097	0.806	0.923	n.d.
4	0.216	1.092	0.992	n.d.
5	0.353	1.427	0.961	n.d.
6	0.159	0.853	1.088	n.d.
7	0.226	1.174	1.042	n.d.
8*	1.052	1.6	1.032	-
9	0.424	1.476	0.957	7.45 (4.29-9.57)
10	0.085	0.726	0.973	n.d.
11	0.269	1.258	1.174	n.d.
12	0.204	1.099	1.057	n.d.
13	0.137	0.746	0.963	n.d.
14	0.266	0.975	1.043	n.d.
15	0.102	0.145	0.86	n.d.
16	0.062	0.087	0.763	n.d.
17	0.205	0.313	1.037	n.d.
18	0.167	0.796	0.869	n.d.
19	0.834	1.339	0.945	2.15 (0.14-6.47)
20	0.301	0.831	0.484	n.d.
21	0.665	1.359	0.881	9.15 (4.22-39.97)
22	0.109	1.012	0.615	n.d.
23	0.133	0.627	1.191	n.d.

* This sample could not be used to purify milk IgA due to insufficient volume.

Table 7.5 Key resource table.

REAGENT OU RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD28 (Clone CD28.2)	BioLegend	302914/302933
Anti-mouse IgG1 (Clone RMG1-1)	BioLegend	406602
Anti-human CD134 (Clone Ber-ACT35 (ACT35))	BioLegend	350012
Anti-human CD4 (Clone RPA-T4)	BioLegend	300506
Anti-human CD4 (Clone SK3)	BioLegend	344665
Anti-human CD3 (Clone UCHT1)	BioLegend	300424
Anti-human CD3 (Clone SK7)	BioLegend	344824
Anti-human CD25 (Clone BC96)	BioLegend	302610
Anti-human CD25 (M-A251)	BioLegend	356112/356108
Anti-human TLR7 (Clone 4G6)	Novus Biologicals	NBP2-27251
Anti-human CD154 (Clone 24-31)	BioLegend	310834
Anti-human CD185 (Clone J252D4)	BioLegend	356904
Anti-human CD196 (Clone G034E3)	BioLegend	353432
Anti-human CD38 (Clone HIT2)	BioLegend	303534
Anti-human CD278 (Clone C398.4A)	BioLegend	313510
Anti-human CD19 (Clone SJ25C1)	BioLegend	363026
Anti-human CD20 (Clone 2H7)	BioLegend	302342
Anti-human CD27 (Clone O323)	BioLegend	302810
Anti-human CD69 (Clone FN50)	BioLegend	310930
Anti-human CD134 (Clone Ber-ACT35)	BioLegend	350028
Anti-human IgD (Clone IA6-2)	BioLegend	348249
Goat anti-Human IgG	Abcam	ab97225
Goat anti-Human IgA	Abcam	ab97215
Goat anti-Human IgM	Abcam	ab97205
Anti-IgA Secretory Component	Abcam	ab3924
Anti-mouse IgG1 (Clone RMG1-1)	BioLegend	406601
Anti-Mouse IgG HRP	Bio Rad	1706516
Anti-Goat IgG	Invitrogen	A-11057
Fixable Viability Dye eFluor™ 506	Invitrogen	65-0866-18
Chemicals, peptides, and recombinant proteins		
Peptide M / Agarose	InvivoGen	gel-pdm-2
Protein G Agarose	Thermo Scientific	20398
SARS-CoV-2 Spike CS + PP	iBET Bioproduction Unit	N/A
SARS-CoV-2 RBD	iBET Bioproduction Unit	N/A
SEB	Sigma-Aldrich	S4881
rh-IL-2	NIH	136
Progesterone	Sigma-Aldrich	P8783
PBS 10x	Alfa Aesar	J62036/K183
Ficoll	Biowest	L0560
FBS	Biowest	S181B
DMSO	Corning	25-950-CQC
RPMI1640 media	Gibco	21975-034

Antibiotic-Antimycotic	Gibco	15240-062
L-Glutamine	Gibco	2916801
PFA	Sigma-Aldrich	P6148
Saponin	Roth	4185.1
Tween 20	Sigma-Aldrich	P1379
TMB	BioLegend	421101
Phosphoric acid	Sigma-Aldrich	P5811
Non-fat dry milk	Santa Cruz	sc-2325
BSA	HyClone	SH30574.02
Critical commercial assays		
Alexa Fluor™ 488 Antibody Labeling Kit	Invitrogen	A20181
Human IL-10 Uncoated ELISA	Invitrogen	88-7106-22
Software and algorithms		
FlowJo V10.7.3	BD Biosciences	https://www.flowjo.com/solutions/flowjo; RRID: SCR_008520
Prism V9.00	GraphPad	http://www.graphpad.com/; RRID: SCR_002798