Humoral Immune Response with Focus on IgG Glycosylation

In Murine Models

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UNIVERSITY OF GOTHENBURG

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To Mom and Dad

Research is what I'm doing when I don't know what I'm doing.

Wernher von Braun

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ABSTRACT

The humoral immune system orchestrates a vital defense mechanism through the secretion of antibodies, especially Immunoglobulin G (IgG), which actively targets and neutralizes foreign particles and pathogens. Glycosylation is a post-translational modification of proteins that affects their size, shape, and folding. IgG glycosylation, plays a pivotal role in mediating both pro- and anti-inflammatory effects in diseases, thereby regulating pathogenicity through alterations in interaction with fragment crystallizable gamma receptors (FcγRs). Despite the recognized importance of IgG glycosylation, the influence of various factors such as estrogen, inflammation, and aging on the humoral immune response remains unexplored in functional models. Therefore, the primary objective of this Ph.D. thesis is to unravel the impact of these factors, with a particular focus on IgG glycosylation, in murine models. First, we investigated whether Bazedoxifene, a 3rd generation selective estrogen receptor modulator (SERM) exhibits estrogenic characteristics in IgG glycosylation under immune-induced postmenopausal conditions. Results demonstrated that Bazedoxifene did not mimic estrogenic effects on IgG glycosylation during pathogenic immune responses. Second, we investigated estrogen's effects on IgG glycosylation in healthy postmenopausal mice. The findings revealed that estrogen treatment in healthy postmenopausal states increased IgG glycosylation, thereby mitigating IgG pathogenicity. Finally, we investigated the impact of aging and toll like receptor 2 (TLR2) on the humoral immune response to bacteremia. Utilizing young and old wild-type (WT) and $TLR2^{-/-}$ mice under both healthy and bacteremia conditions, the study showed that TLR2 and aging significantly altered immunoglobulin levels. Additionally, bacteremia induced a limited response in aged mice, with increased IgG glycosylation observed in healthy and infected conditions in wild type old mice. In summary, this thesis demonstrated the regulation of humoral immune response and the factors including age, sex hormones, and the presence of TLR2, can markedly influence the humoral immune response and IgG glycosylation, leading to a shift from pro- to anti-inflammatory states or vice versa beyond diseased environments.

Keywords: Humoral immunity, Antibodies, IgG-glycosylation, Estrogen, Inflammaging.
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SAMMANFATTNING PÅ SVENSKA

Antikroppar, även kallade immunoglobuliner (Ig), produceras av B-celler och plasmaceller och är den viktigaste komponenten i kroppens humorala immunsvar. I de här studierna fokuserar vi främst på IgG antikroppar. IgG består av två olika funktionella delar där den ena delen kallade Fab-delen, binder och neutraliserar specifika antigen och den andra delen, den så kallade Fc-delen, binds till Fc-gamma receptorer presentera på ytan av olika immunceller. IgG kan antigen dra i gång eller dämpa immunsystemet beroende på dess bindningsförmåga till olika Fc-gamma receptorer. Glykosylering är en post-translationell modifiering av bland annat IgG, där sockerstrukturer påverkar storlek och form vilket direkt påverkar bindningen mellan IgG och Fc-gamma receptorer och därigenom hur sjukdomsframkallande IgG är. Här studerar vi om östrogen, inflammation och åldrande påverkar det humorala immunsvaret och glykosyleringen av IgG i olika djurmodeller. Den första artikeln visar att Bazedoxifen, ett läkemedel som binder östrogenreceptorer på ett selektivt sätt, inte påverkar nivåer eller grad av glykosylering av IgG på samma sätt som östrogen i en modell av immunstimulerade postmenopausala möss. I den andra artikeln studerar vi hur östrogen påverkar IgG i friska, ej immunstimulerade, möss. I denna modell så påverkar östrogen inte nivåerna av IgG, men däremot graden av glykosylering. Detta innebär att östrogen reglerar hur glykosylerade och därmed sjukdomsframkallande IgG är på samma sätt i både immunstimulerade och friska postmenopausala möss. Slutligen, visar vi att både åldrande samt om man har en specifik receptor som känner igen bakterier, TLR2, påverkar det humorala immunsvaret vid en bakterieinfektion i mus. Åldrande friska möss har högre nivå av IgG och samtidigt högre glykosyleringsgrad av IgG. Möss som saknar TLR2 har högre nivå av IgG och samtidigt högre glykosyleringsgrad av IgG jämför med vildtypsmöss, men i dessa möss ses begränsad effekt av ålder. Äldre möss har även vid en bakterieinfektion, högre glykosyleringsgrad av IgG, men nivåerna av IgG vid en bakterieinfektion påverkas varken av TLR2 eller ålder. Sammanfattningsvis visar denna avhandling att humoral immunitet och glykosylering av IgG inte bara regleras av infektioner eller sjukdomar utan även av andra faktorer såsom ålder, könshormoner och brist på TLR2. Detta kan ha betydelse för att balansera immunsvaret och påverka inflammatoriska sjukdomar.

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Priti Gupta**, Karin Horkeby, Hans Carlsten, Petra Henning, Cecilia Engdahl. **Bazedoxifene does not share estrogen effects on IgG sialylation.** *PLoS One. 2023 May 18;18(5):e0285755*

II. **Priti Gupta**, Tibor Sághy, Jauquline Nordqvist, Jonas Nilsson, Hans Carlsten Karin Horkeby, Petra Henning, Cecilia Engdahl. **Impact of Estrogen on IgG Glycosylation and Serum Protein Glycosylation in a Murine Model of Healthy Postmenopause.** *Front Endocrinol. 2023 Sep 11:14:1243942*

III. **Priti Gupta**, Zhicheng Hu, Pradeep Kumar Kopparapu, Meghshree Deshmukh, Tibor Sághy, Majd Mohammad, Tao Jin*, Cecilia Engdahl*. **The impact of TLR2 and aging on the humoral immune response to *Staphylococcus. aureus* bacteremia in mice.** *Sci Rep. 2023 May 31;13(1):8850*

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ABBREVIATIONS

ADCC	Antibody dependent cellular ctotoxicity
APC	Antigen presenting cell
B4Galt1	Galactosyltransfarase 1
BM	Bone marrow
BMD	Bone mineral density
BZA	Bazedoxifene
CDC	Complement-dependent cytotoxicity
CIA	Collagen induced arthritis
DC	Dendritic cell
E2	Estradiol
ER	Estrogen receptor
ERE	Estrogen response element
Fut8	Fucosyltransferase 8
FcγR	Fragment crystallizable gamma receptor
Gal	Galactose
GalNac	N-acetyl galactosamine
Glu	Glucose
HRT	Hormone replacement therapy
HSC	Hematopoietic stem cell
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	Interleukin-6
LC-MS	Liquid chromatography-mass spectrometry
Man	Mannose
Neu5Ac	N-Acetylneuraminic acid

Neu5Gc	N-Glycolylneuraminic acid
OVX	Ovariectomy
pQCT	Peripheral quantitative computer tomography
PAMP	Pathogen associated molecular pattern
RA	Rheumatoid arthritis
S.Aureus	Staphyloccus Aureus
SERM	Selective estrogen receptor modulator
SNA	Sambacus Nigra Lectin
ST6gal1	Sialyltransferase 1
TACI	Transmembrane Activator and Calcium-modulator and CAML Interactor
TLR	Toll like receptor
TNF	Tumor necrosis factor

1. INTRODUCTION

The Immune system

The immune system is a well-organized mechanism for defending individuals against pathogens—bacteria, viruses, and other microorganisms—that invade the body's microenvironment. In addition, the body's damaged cell products and non-infectious foreign particles can also elicit an immune response. The immune system is divided into two parts: innate and adaptive immune systems.

The innate immune system

Innate immunity is the body's first line of defense against foreign pathogens. Every time they encounter microbes and foreign substances, they respond the same way. The primary components of innate immunity are: 1) epithelial barriers, both physical (like skin and mucus) and chemical (gastric juice, saliva enzymes, and others), 2) cells including phagocytes such as macrophages, dendritic cells, neutrophils, and directly cytotoxic cells like mast cells, and natural killer cells, and 3) soluble complement protein components. Pathogen recognition receptors, such as Toll-like receptors (TLRs), recognize pathogen-associated molecular patterns (PAMPs) and activate the immune response by promoting inflammation and destroying the microbes. TLRs are widely expressed in all hematopoietic cells and recognize gram-positive (Staphylococcus family) and gram-negative bacteria (e g, Vibrio cholera and E. coli). Mice have 13 distinct types of TLRs compared to humans just 10 TLRs [1]. TLR-2 is an essential receptor for gram-positive bacteria, such as Staphylococcus aureus (*S. aureus*). Activation of TLR-2 occurs during the interaction with lipoproteins synthesized by S. aureus and substances produced or released by stressed or dying cells [2, 3]. Moreover, TLR2 forms a heterodimer with either TLR1 or TLR6 and initiates the signaling [4].

Innate immunity initiates with the activation of macrophages that first release alarm signals such as cytokines, and chemokines, to alert the immune system, and facilitate the recruitment of immune cells to the infection site. Neutrophils, the first responders, destroy pathogens by releasing cytotoxic granules and phagocytosis. Monocytes, recruited from circulation, differentiate into macrophages and engage in pathogen engulfment. At the same time, Dendritic cells (DCs) start transporting antigens they have engulfed and present them to specialized cells of adaptive immunity that provide more specific immune responses.

The link between innate and adaptive immune response

Professional antigen-presenting cells (APC) including macrophages, DCs, and B-cells, initiate the specific immune response, providing a crucial link between the innate and adaptive arms of the immune system. These versatile cells actively participate in the continuous intake of extracellular antigens, which allows them to carefully absorb various chemical signals from the environment. They then effectively process these peptide antigens within and, in an organized manner, transfer the peptide through the major histocompatibility complex (MHC) molecules. Additionally, when APCs encounter bacterial antigens, and these antigens bind to the TLRs on the surface of immune cells for eg: DCs, it triggers a response that makes the DCs highly potent APCs. This activation enables them to efficiently present accumulated antigens to T-cells and coordinate immunological responses to initiate an effective targeted adaptive immune response.

The adaptive immune system

Adaptive immunity, also known as acquired immunity, is made up of specialized cells called B- and T-lymphocytes. These B- and T-lymphocytes possess the capability to recognize a unique antigen specificity, which is determined by the structure of its antigen-binding sites. Adaptive immunity is further classified into humoral immunity (mediated by B-cells-produced antibodies in the circulation and mucosal secretion) and cell-mediated immunity (mediated by T-cells) (Figure 1).

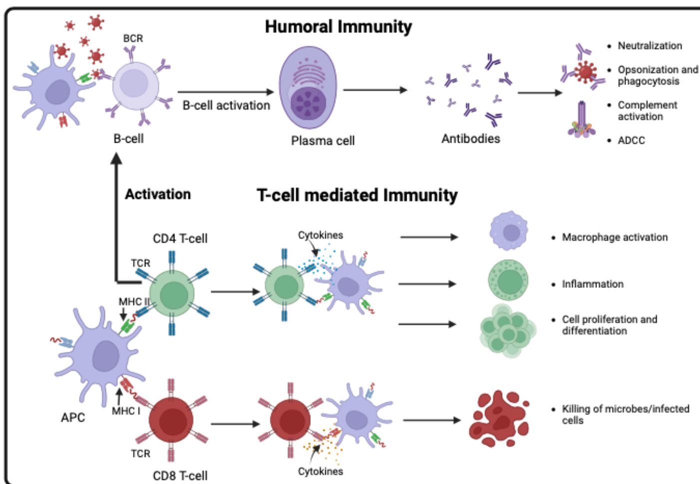

Figure 1: Overview of adaptive immune response. APC: antigen-presenting cells, BCR: B-cell receptor, TCR: T-cell receptor, MHC: major histocompatibility class, ADCC: antibody dependent cellular cytotoxicity.

T-cells

T-cells, arising from the bone marrow and maturing in the thymus, are characterized by their diverse T-cell receptor, enabling recognition of specific antigens. There are two main types of T-cells: cytotoxic T-cells (CD8/T_C), which directly attack infected or abnormal cells, and helper T-cells (CD4/T_H), which assist in coordinating immune responses by releasing signaling molecules called cytokines. CD8/ T_C can recognize antigens presented by MHC I while CD4/ T_H only interacts with MHC II presented peptides on APCs. CD4/ T_H cells are central for regulating other immune cells such as B-cells. T-helper cells can further divide into different subtypes: T_H1, T_H2, and T_H17 depending on the interaction with specific antigens and their functions. T-cells contribute to the body's defense against pathogens, regulate immune balance, and participate in the memory response for long-lasting protection.

B-cells

B-cells are important in the humoral immune response by producing antibodies. B-cells develop from the bone marrow compartment in different stages: pro-B cells, pre-B cells, and later immature B-cells. To complete further development, immature B-cells migrate to secondary lymphoid tissue, such as the spleen or lymph node, and then mature into transitional, follicular, and marginal B-cells (Figure 2). Follicular B-cells, upon antigen activation, become plasma cells and memory cells. Plasma cells release antibodies into the bloodstream [5]. During B-cell development, the B-cell receptor (BCR) is assembled on the surface of B-cells through highly ordered gene recombination events and can simply explained as a membrane-bound antibody.

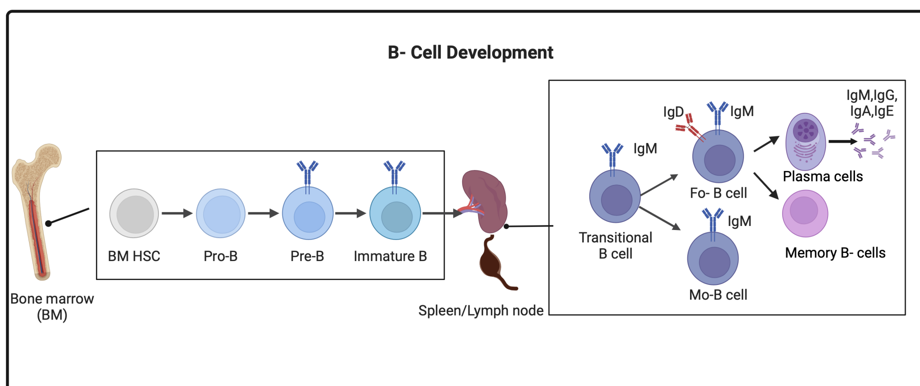

Figure 2: Overview of B-cell development. BM: bone marrow, HSC: hematopoietic stem cells. Pro B-progenitor B-cells, Pre-B: precursor B- cells, Fo: Follicular, MoZ: Marginal zone.

In addition to antibody synthesis, B-cells also serve as potent antigen-presenting cells and can produce cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-10 (IL-10). B-cells can generate immunomodulatory effects with the help of CD4/T_H and DC, as well as regulate lymphoid tissue organization and influence tumor development [6-8]. B-cells in the form of B regulatory cells can act as a regulatory element in autoimmunity, possibly helping to modulate the immune response to prevent the excessive inflammation of the body's own tissue and maintain self-tolerance. These regulatory properties are generally linked to the induction of IL-10 via B regulatory cells as described [9, 10] and promote the formation of autoantibody-producing plasma cells that trigger T-cell responses through antigen presentation [11]. Additionally, B-cells also exhibit the expression of TLRs, as discussed in a review [12]. However, compared to other TLRs, the level of TLR2 expression is relatively low in B-cells. Studies involving mice have explored TLR2 and TLR adapters (eg; MyD88), revealing their capacity to orchestrate B-cell responses to diverse antigens and infections [13, 14].

Plasma cells

Plasma cells are terminally differentiated B-cells that provide antigen defense by continuously producing antibodies. Plasma cells are generated due to antigen activation in secondary lymphoid organs [15] and at the site of infection. Plasma cells are large lymphocytes that contain a lot of cytoplasm and are responsible for synthesizing and secretion of antibodies. Plasma cells are subclassified as either short-lived (unable to migrate from tissue to tissue) or long-lived. The half-life of short-lived plasma cells is three to four weeks. IgM and IgG are produced by short-lived plasma cells and peak up 8-10 days following immunization. Short-lived plasma cells secrete low-affinity antibodies, whereas long-lived plasma cells are further developed and secrete high-affinity antibodies. Notably, plasma cells exhibit a diverse array of TLRs, including TLR2. Previous studies in humans have demonstrated that the expression of TLR2 is elevated in plasma cells derived from peripheral blood as opposed to those derived from tonsillitis [16].

Antibodies

Antibodies are very diverse in structure and can interact with a wide range of foreign molecular structures. Their basic structure is Y-shaped and comprises four polypeptide chains: two identical heavy chains (50–77 kDa) and two identical light chains (25 kDa) connected by disulphide bonds. The light and heavy chains consist of variable domain (V) and constant

domain (C). The antibody is divided into two parts known as Fab (F stands for fragment and ab stands for antigen binding) and Fc (crystallizable fragment) (Figure 3). The Fab part is mainly engaged in antigen recognition and binding domain, while Fc comprises the heavy chain region and mediates the effector function by interacting with Fc gamma receptors (FcγRs). Antibodies are subdivided into five different types: IgG, IgM, IgA, IgE, and IgD. The function and different features of these classes are determined by the part of the heavy chain within the hinge and Fc region. IgM and IgD are predominantly found on the surface of B cells as BCR. IgA mediates mucosal immunity and IgE is involved in allergic response. They possess a distinctive ability to initiate signaling responses when exposed to various types of antigens [17]. Antibodies mediate their effector function in line with the defense mechanism by neutralizing the effect of antigen, by opsonization and phagocytosis, by activating the complement cascades, and by antibody-dependent cellular cytotoxicity (ADCC).

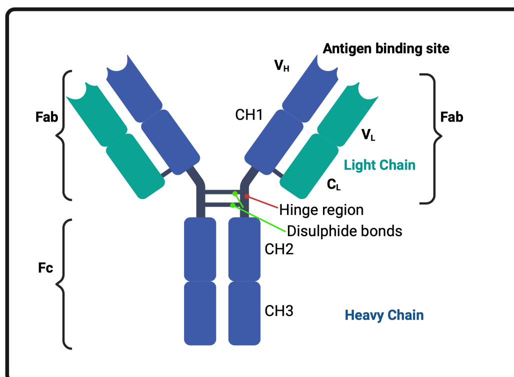

Figure 3: General Structure of antibodies.CH: constant heavy, V: variable, C: constant, L: light chain.

IgM is the first antibody to manifest in the body's defense reaction to an antigen exposure. Plasma cells release IgM, which is pentameric in structure. Antigens can be targeted for opsonization through the activation of the complement system by IgM via a classical pathway. It can be used to diagnose infectious disorders and determine recent infections by looking for IgM antibodies in a patient's serum. Natural antibodies in mice are mainly IgM but in humans, they could be IgM, IgG, and IgA. They are generally low affinity polyreactive antibodies, capable of binding multiple structurally unrelated antigens. They are present in the body without any overt immunization, although there is a possibility for some antigen sources from the gut microbial biome [3, 18].

IgG is the most abundant antibody comprising about 10-20% of all plasma proteins [19]. IgG antibodies are known for their extended presence in the bloodstream, with a half-life of approximately 21-28 days. IgG can be further subclassified into IgG1, IgG2, IgG3, and IgG4 in humans. In mice, IgG isotype is divided into IgG1, IgG2a, IgG2b/2c, and IgG3 subclasses. IgG

contains the ability to penetrate a wide range of tissues and can activate both pro- and anti-inflammatory responses by engaging FcγRs in both cellular and humoral pathways of the immune system [20].

Fragment crystallizable gamma receptors (FcγRs)

FcγRs are a class of hematopoietic cell surface glycoproteins found on almost all immune cells. There are four classes of FcγRs in mice: I, III, and IV known as activating FcγRs, and one inhibitory receptor, FcγRIIb (Figure 4). In humans, there are five activating, FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcγRIIIb, and one inhibitory, FcγRIIb, receptor. FcγRIIb and FcγRIII mostly interact with mouse IgG1, IgG2a, and IgG2b. FcγRIV has a higher binding affinity for IgG2a and IgG2b. Human IgG2, IgG4, and IgG1 subtypes, on the other hand, demonstrate more restricted binding to FcγRs [20, 21]. In addition, inhibitory FcγRIIb, on B-cells, is important for the maintenance of humoral tolerance as well as has an important role in the regulation of plasma cell homeostasis and survival [22]. Additionally, all antibodies mediate the effector function via the engagement of Fc part to the FcγRs. Regulation of humoral and cellular responses including phagocytosis and ADCC is mediated by FcγRs [22, 23].

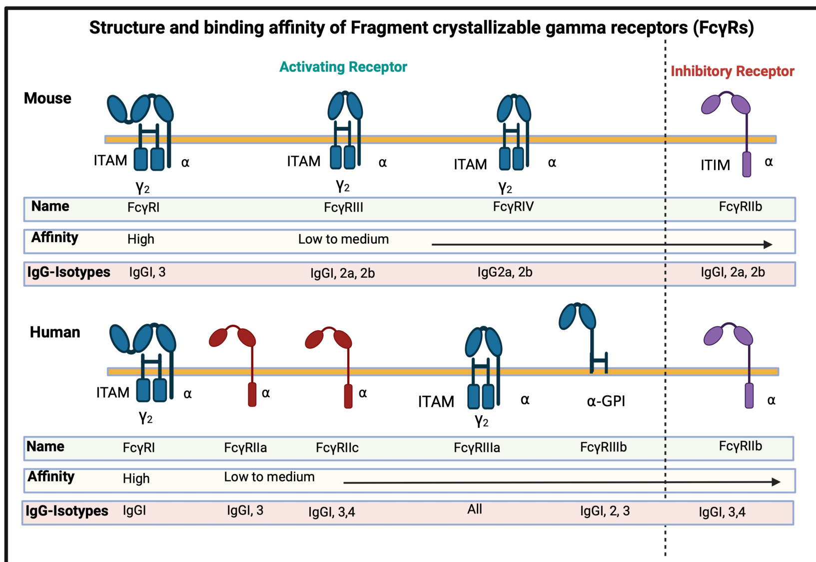

Figure 4: Family and structure of FcγRs and binding affinity with IgG-Isotypes. ITAM: Immunoreceptor tyrosine-based activation motif, ITIM: Immunoreceptor tyrosine-based inhibitory motif.

Modulation of humoral immune responses by infection

The humoral immune responses are important for protection against invading pathogens. B-cell activation through binding of the BCR to a cognate antigen together with additional signals regulates both proliferative and differentiation processes. This results in the expansion of effector cells that can secrete abundant amounts of antibodies as well as long-lived populations of memory B cells that can activate during secondary infections as extensively reviewed in [24]. Successful vaccination/immunization strategies against a large number of pathogens, including pathogenic bacteria and virus infections, show the dependency on the humoral immune response [24-26]. The initiation of humoral immunity through bacterial infection predominantly relies on the innate immune response by recognizing, engulfing, and presenting bacterial antigens to activate the adaptive immune system. This sets in motion the production of antibodies that contribute to the elimination and defense against bacterial invaders. The importance of antibodies is further strengthened by mice studies, where immunoglobulin levels were substantially increased following bacterial infection [27, 28]. Additionally, some viruses may be able to lower humoral immunity by the induction of a strong inflammatory response, which suppresses B-cell differentiation and antibody production [29].

Modulation of humoral immune responses by TLR2

The identification of TLRs and their role in detecting infections has emerged as a significant development in immunology. TLRs are pivotal in recognizing microbes within the innate immune system and also influencing and improving the quality of the adaptive immune response [30]. A longitudinal study involving malaria-naive human individuals suggests that immunization against malaria with a protein antigen and TLR ligands leads to a higher induction of antigen-specific antibodies. Additionally, a recent study in mice demonstrates that, apart from TLR2 signaling in DCs, TLR-mediated stimulation directly on B cells is also necessary for inducing strong antibody responses [31]. A previous study on $TLR2^{-/-}$ mice reveals an insufficient antibody repertoire to induce intestinal ischemia/reperfusion (IR) tissue damage. This suggests that, in addition to the inflammatory response, TLR2 is necessary for the natural production of antibodies in response to IR-induced injury [32]. Subsequent investigations in mice indicate that TLR2 signaling shapes the specific antibody response to the Salmonella typhi antigen. These studies reveal that $TLR2^{-/-}$ mice exhibit reduced IgG titers, with a more

pronounced impairment in producing IgG3 anti-porins antibodies compared to healthy control [33]. Collectively, these studies provide evidence of the modulation of the humoral immune response by TLR2. Beyond its impact on humoral immunity, TLR2 can influence IgG glycosylation by activating adaptive immunity through PAMP. Nonetheless, insufficient evidence remains to establish a clear relationship between IgG glycosylation and TLR2.

Glycosylation

Glycosylation is a post-translational modification wherein newly synthesized proteins undergo additional modification through the attachment of oligosaccharides (glycans). In eukaryotes, a majority of extracellular proteins, including cell-surface membrane protein receptors and adhesion molecules, undergo glycosylation. During this process, monosaccharides are sequentially attached through glycosidic linkages, forming glycoconjugates. These glycoconjugates are covalently linked to target macromolecules, often proteins or lipids. Many proteins in blood plasma are glycosylated, providing solubility, hydrophilicity, and a negative charge, and reducing the nonspecific intermolecular interactions. Additionally, glycosylation protects against proteolysis and enhances the proper function of the protein by promoting correct folding, protein stability, and biological processes [34].

The glycosylation process takes place in the secretory pathway of the endoplasmic reticulum (ER) and Golgi apparatus catalyzed by the glycosyltransferase reactions. The glycosyltransferases transfer sugar directly to the polypeptide and the growing glycan chains of the glycoproteins and are secreted within the secretory pathway [35]. Thus, glycosylation is a complex cellular process further divided into N-linked and O-linked. O-linked glycosylation links glycan N- acetylgalactosamine and the amino acid serine or threonine, where O-glycans are covalently attached to proteins.

N-linked Glycosylation

N-glycans are covalently linked with polypeptides on the asparagine residue by an *N*-glycosidic bond between an *N*-acetylgalactosamine and an asparagine amino acid residue (GlcNAcβ1-Asn). The peptide sequence to become N-glycosylated contains asparagine followed by another amino acid, except for proline, and then with a serine or threonine residue (Asn-X-Ser/Thr). *N*- glycans have been categorized into three types: 1) oligomannose or high mannose, in which only mannose (Man) residues are added to the core, 2) hybrid, in which only Man residues are attached to one arm of the core and one GlcNAc are added to the other arm, and 3) complex structures in which

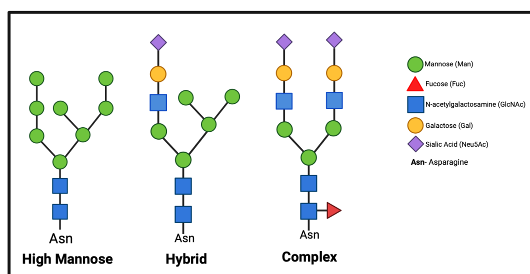

Figure 5: N-Glycan structure.

two arms (antennas) initiated by *N*-acetylglucosamine (GlcNAc) are attached to the core with sialic acid commonly found as the terminal residue (Figure 5) [36].

Biosynthesis of N-glycan in the ER-Golgi pathway

Following protein synthesis, naïve protein carrying dolichol-phosphate (Dol-P) sugar, translocate into the surface of ER via the ER-Golgi pathway (Figure 6) [35]. Once the naive protein enters the ER, it flips around on the ER lumen, where Man and glucose units are added to form a 14-sugar structure $Glc_3Man_9GlcNAc_2$ in the presence of the enzyme called oligosyltransferases (OST), which catalyzes the transfer of the oligosaccharides from Dol-P to Asn-X-Ser/Thr through the linkage of GlcNAc to Asn [37]. This nascent carbohydrate–protein conjugate undergoes further processing to remove the glucose residues as part of a quality-control check in the ER. Following that, further modification occurs by the hydrolytic removal of glycan residues in the various compartments. At the Golgi apparatus, including *cis-Golgi* Man is added, followed by *medial-Golgi* for the addition of N-acetylgalactosamine (GalNAc), galactose (Gal), fucose (Fuc) (Figure 6). The proteins are finally transported to the *trans-Golgi*, where sialic acid (Neu5Ac) could be attached as the terminal motive. The addition of all these sugar motives requires the presence of mannosyltransferase, N-acetyl galactosamine transferases, galactosyltransferase, fucosyltransferases, and sialyltransferase respectively [36]. Moreover, highlighting the significance of aberrant glycosylation in serum or plasma proteins, and IgG, reveals its correlation with a range of diseases, including bacterial infections, cancer, and autoimmune conditions [38].

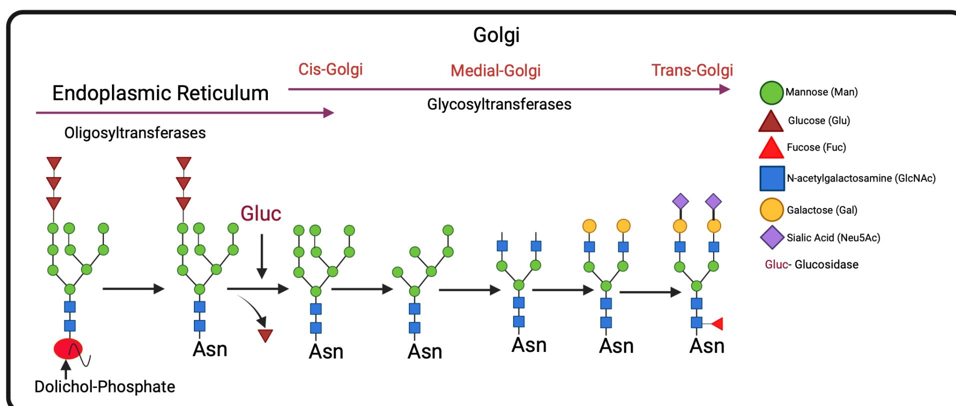

Figure 6: Biosynthesis of N-glycosylation in ER-Golgi pathway. Asn: asparagine.

IgG-Glycosylation

All IgG molecules are glycosylated with both N-linked and O-linked glycans. 15-20% of IgG-Fab carry one or more N-glycosylation sites consensus on the variable region [39-41]. IgG-Fab does not include a conserved site for glycosylation but is generated during the somatic hypermutation. This Fab glycan is known to modulate the antigen binding affinity and antibody stability, affecting its serum half-life, and even leading to antibody aggregation [42, 43]. IgG-Fc N-glycosylated at the conserved Asn-297, located on the CH2 domain, contributes to the stability, and maintains the IgG's quaternary structure. IgG-Fc glycosylation regulates the ability of IgG to bind with FcγRs, displaying noncovalent interactions that affect the conformation of the stability of the IgG antibody [44-46]. The attached conserved glycans on IgG-Fc contain a pentasaccharide core containing two -GlcNAc and three- Man residues (Figure 7). This structure can be further modified by adding GlcNAc, core fucose, bisecting GlcNAc, Gal, and sialic acid at the terminal part.

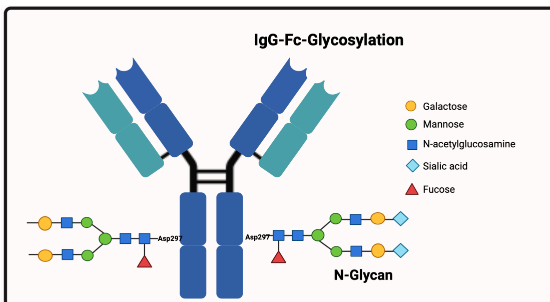

Figure 7: Representative image of IgG-Fc Glycosylation.

The regulation of IgG-Fc glycosylation is mostly focused on the four key glycosyltransferases that are important for glycan addition on the glycan chain. The glycosyltransferase responsible for core fucosylation on IgG is Fucosyltransferase 8 (*FUT8*), a modification that strongly affects the inflammatory potential of IgG [47]. The addition of galactose and sialic acid on the glycan chain is mediated by Galactosyltransferase 1 (*B4GALT1*) and Sialyltransferase 1 (*ST6GAL1*), respectively. Additionally, many Genome-wide association studies of IgG-N glycosylation have shown that glycosyltransferase expression is regulated by other genomic regions [48, 49].

Modulation of immune responses by IgG-glycosylation

IgG modification by the glycosylation mechanism impacts biological function in various ways. Fucosylation and sialylation of IgG-Fc play an important role in the effector function of IgG in the inhibition of ADCC [50-52] and limit the interaction capability with FcγRs [53]. While increasing pathogen engulfment, destruction, and complement activation, providing a balance

between pro- and anti-inflammatory immune responses. A bacterial infection could significantly differ in the IgG glycosylation pattern, including increased A-galactosylated IgG (G0) [54]. Patients with Meningococcal sepsis show lower IgG1 fucosylation and higher bisection than healthy control [55]. It has been reported that changes in glycosylation patterns during systemic inflammation are associated with an increased risk of mortality [56]. Significant differences in antibody glycosylation have also been observed in the experimental setting of tuberculosis [57] and human studies of tuberculosis [54]. Further, a terminal Gal residue on the glycan chain has been demonstrated to decrease and activate a proinflammatory response by increasing the production of proinflammatory cytokines in aging and autoimmune disorders such as RA [51, 58, 59]. Decreased IgG glycosylation has been observed as a predictive biomarker during early autoimmune inflammation [60, 61]. An experimental model of collagen-induced arthritis (CIA) revealed a decrease in IgG galactosylation and sialylation. Nonetheless, treatment with phytoestrogen increases both IgG galactosylation and sialylation, leading to CIA protection and reduced disease severity [62]. In healthy individuals, approximately 10-15% of serum IgG is sialylated, with the majority being mono-sialylated [63], but this decreases during inflammation and autoimmune disease flare-ups. Additionally, sialylation with only Neu5Ac α -2,6 sialylated IgG has an important role as a biological component of immunomodulatory intravenous immunoglobulin (IVIG), which has been shown to develop an anti-inflammatory, immune response in different autoimmune diseases including systemic lupus erythematosus (SLE) [64-66]. Furthermore, sialylated IgG has been demonstrated in mouse models to reduce IgG-mediated anaphylaxis allergic reactions and contribute to remission in autoimmune diseases [67, 68]. Recognizing the important role of changes in IgG glycosylation and IgG in immune induction, whether triggered by autoantibodies, inflammation, or environmental factors, has opened new therapeutic possibilities. Techniques such as glycoengineering or precise cleavage of N-linked IgG Fc-glycans using bacterial enzymes, along with the modulation of downstream or upstream effector pathways, offer the development of therapeutic approaches [69].

Hormones

Hormones are chemical messengers coordinating different functions, controlling and maintaining the body's homeostasis [70]. They are released directly into the bloodstream from ductless endocrine glands and mediate their effects by binding to specific receptor proteins, often linked to various intracellular effector processes. Lipophilic (lipid-attractive) compounds, like steroids (eg; estrogen) can enter the cells directly by permeabilization through the membrane lipid bilayer, binding with intracellular receptors that modulate the gene expression by regulating transcriptional mechanisms. On the other hand, hydrophilic (hydrogen-loving) substances, like peptide hormones, bind to cell surface receptors initiating intracellular signal transmission through interaction with membrane proteins [70].

Estrogen

Estrogen refers to a class of female sex hormones that includes estrone (E1), estradiol (E2), estriol (E3), and estretrol (E4) [71, 72]. Estrogen is also known as C18 steroids because it contains 18 carbons ($C_{18}H_{24}O_2$) [72]. All estrogen levels fluctuate throughout the life stage in females [73]. Furthermore, all four estrogen groups can bind to nuclear and membrane estrogen receptors [72, 73]. E2 is the most potent estrogen secreted by the granulosa cells of ovarian follicles and corpora lutea [70, 72] in non-pregnant females between menarche and menopause. The primary source of E2 biosynthesis is cholesterol. Cholesterol is converted into progesterone and then converted by several steps, resulting in androgens and testosterone, which are then aromatized into estradiol. Not only ovaries but adipose tissue as well as the liver, adrenal glands, and breast produced E2 to a lesser degree [74].

Estrogen signaling pathways

The estrogen mainly exerts its effect through the estrogen receptors (ERs). There are two main types of ERs: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estrogen can enter the plasma membrane and interact with intracellular ERα and ERβ to exert direct binding to DNA. Conformational changes occur upon estrogen binding to ERs, including receptor dimerization, and then the ER-estrogen complex is translocated to the nucleus. There it initiates binding to the estrogen response element (ERE) sequence and then promotes the transcription of the target genes, called the estrogen classical genomic pathway [72, 75, 76]. In the non-classical signaling pathway, the ER-estrogen complex acts through protein-protein interaction, such as stimulating protein-1 or activator protein-1 with transcription factors and

response elements rather than binding directly to the ERE sequence [77, 78]. Different coregulators can enhance or decrease the transcriptional activity of estrogen [72]. The activation of ERs in the cytoplasm or plasma membrane could initiate signaling cascades in non-genomic estrogen signaling, which can directly impact a cell's ability to survive and function. Non-genomic ERα signaling frequently involves protein-kinase cascades, such as the phosphatidylinositol 3-kinase and mitogen-activated protein kinase (MAPK) signaling pathway. It may indirectly affect gene transcription through the phosphorylation of transcription factors. The release of intracellular calcium and an increase in the synthesis of cyclic adenosine monophosphate (cAMP) can also result from the activation of ERs at the cell membrane. Furthermore, another type of ER has been found: a membrane-bound G-protein coupled estrogen receptor-1 (GPER-1). It mediates signaling through rapid non-genomic intracellular signaling mechanisms.

Hormone Replacement Therapy (HRT)

As women transition to menopause between 45 to 50 years of age, the production of estrogen, mainly estradiol (E2) and progesterone from ovaries, starts to cease. In clinical practice, HRT is often used to control the symptomatic challenges of menopause, simply giving back the hormones estrogen and progesterone. HRT offers the long-term benefit of reducing the progression of postmenopausal osteoporosis but is also associated with cardiovascular disease [79].

Selective estrogen receptor modulators (SERMs)

Apart from the estrogens that are naturally produced by gonadal, there are several synthetic organic and inorganic molecules, including selective estrogen receptor modulators (SERMs), and phytoestrogen, which can recognize the ER's ligand-binding domain as estrogen [80]. This active complex could mediate both non-classical and classic estrogen signaling. In comparing the molecular structure of SERM with E2, they contain long bulky side chains that impact ER conformational change upon interaction. The complex with ERs and SERMs differs from the estrogen-ER complex, altering the coregulator’s interaction capability to either push or inhibit the action of the complex. The coregulators differ depending on the status of the cells as well as between different tissues, which leads to the activation or repression of estrogen-inducible genes and mediates the cellular response [81]. E2 and SERMs appear to have tissue-specific effects by regulating distinct target genes through ERα and ERβ. Most genes ERα regulated

differ from those ERβ regulated in response to E2 and SERMs [82-84]. SERMs are classified based on their development; 1st generation, Tamoxifen is used as a treatment option for ER-positive breast cancer, 2nd generation, Raloxifene is approved for the prevention and treatment of postmenopausal osteoporosis, and also for the reduction of invasive breast cancer risk in women, and 3rd generation, Bazedoxifene (BZA) [85] used as a treatment for postmenopausal osteoporosis and postmenopausal symptoms.

BZA is structurally different from other SERMs and exhibits a unique tissue specificity with ER activity [86]. BZA has less specificity with ERα and has been shown to down-regulate ERs in vitro by increasing receptor degradation in breast cancer cells [87, 88]. BZA is currently used in the EU, Japan, and USA (conjugated estrogen/BZA as DUAVEE), to prevent vertebral fractures and non-vertebral fractures in women with higher fracture risk after menopause [89]. Pre-clinical studies have shown that BZA acts as an ER antagonist in the uterus [90, 91] and breast [89] while agonistic effect on bone [92]. BZA treatment is also associated with an increased risk of venous thromboembolism [93].

Modulation of humoral immune responses and IgG glycosylation by Estrogen

In recent years, numerous studies have gathered evidence highlighting estrogen's role in regulating many aspects of the inflammatory immune response [94-97]. Estrogen plays an important role in inhibiting T-cell and B-cell lymphopoiesis [91, 98-102] while increasing the production of plasma cells and circulating IgG [101, 103-105]. Additionally, for most respiratory diseases including influenza as well as evidence during the SARS-CoV-2 pandemic, women appeared to be better responders in mounting the invasion of respiratory viral pathogens than males, suggesting that this protective action might be influenced by estrogen and other sex hormones on cellular and humoral immunity [106].

Many studies indicate that factors such as gender, sex hormones (estrogen), and menopause contribute to the change in IgG-Fc glycosylation. This alteration leads to a shift in a pro-inflammatory response and plays a significant role in modulating disease outcomes [53, 62, 101, 107-113]. The change in IgG glycan, mostly the A-galactosylated IgG, is generally lower in females as they age compared to males in healthy serum, indicating a role of estrogen [113]. As females reach menopause, there is a rapid increase in the A-galactosylated and desialylated forms of IgG [53, 111, 114]. A study on premenopausal women explored the effects of E2 by suppressing estrogen production using leuprolide, an analog of gonadotropin-releasing

hormone, demonstrating a shift in IgG glycan profile similar to postmenopausal women. After restoring estradiol levels, they observed the IgG glycosylation was fully recovered to its original state [53]. Pre-clinical studies further support that estrogen treatment increases IgG glycosylation in healthy animals and those experiencing inflammation [62, 101]. Moreover, alterations in IgG glycosylation, mainly the shifting to a pro-inflammatory stage, are observed during pre-, peri-, and post-menopausal women [115].

The peak incidence of RA in females consists of menopause, characterized by increased A-galactosylated IgG glycoform and decreased galactosylated, contributing to disease severity, as extensively reviewed in [116]. HRT-containing estrogen increases IgG galactosylation and sialylation in RA patients [101]. This shift towards an anti-inflammatory response is also observed in pregnant women with RA, due to an elevation in estrogen levels [107, 117-119]. Maternal IgG becomes more glycosylated during pregnancy [120, 121]. These studies have collectively established a connection between estrogen and IgG glycosylation in females, suggesting that sex hormones modulate immunity and generate an anti-inflammatory response in postmenopausal conditions.

The impact of SERMs on humoral immunity has not yet been explored in detail. However, few experimental studies have been performed that suggest an important role of SERM in B and T-cell lymphopoiesis [91, 100, 102, 122]. How SERMs modulate IgG-glycosylation is still an open question. However, a few studies have demonstrated the role of SERMs in glycosylation, including Raloxifene [53], and phytoestrogen [62] in both humans and mice respectively. Raloxifene treatment in healthy premenopausal women, whose gonadal hormone production was deprived by leuprolide, reduced A-galactosylated IgG [53]. Additionally, phytoestrogen treatment increased the IgG galactosylation and sialylation in an experimental setup of CIA mice [62].

Aging

The concept of aging refers to the gradual decline in the physiological functions essential for survival and fertility. As people age, their immunological responses decrease, leading to physical, psychological, and changes in the social life balance. Approximately two-thirds of the roughly 150,000 daily global deaths result from age-related factors [123]. Aging impacts both innate and adaptive immunity. The decrease in immunity during aging is known as immunosenescence. It is a complex phenomenon linked to an increased frequency and severity of infections, decreased immune responses, and decreased efficacy of immunization in the elderly [124]. The decline in immune function can result in a chronic, low-grade inflammatory state, known as inflammaging [125]. As individuals age, various components accumulate in the body, such as nucleic acids, mitochondrial DNA, mitochondria, and heat shock proteins, originating from cell death or damage. Several conditions are linked to aging such as susceptibility to infection, diabetes, osteoporosis, vascular disease, and neurodegenerative diseases (Figure 8), limiting the survival of elderly individuals. Additionally, aging and age-related diseases are associated with disruptions in protein homeostasis, also known as proteostasis. Studies suggest that in aged individuals, unfolded or aggregated proteins cannot be further degraded, accumulating in tissues, contributing to the development of age-related pathologies, including septicemia, tuberculosis, Alzheimer's disease, and Parkinson's disease [126-128]. These components can be detected by immune receptors such as TLRs, triggering the production of pro-inflammatory cytokines. Hallmarks of inflammaging are identified by the elevated levels of proinflammatory markers in the bloodstream, such as IL-10, IL-6, TNF-α, and

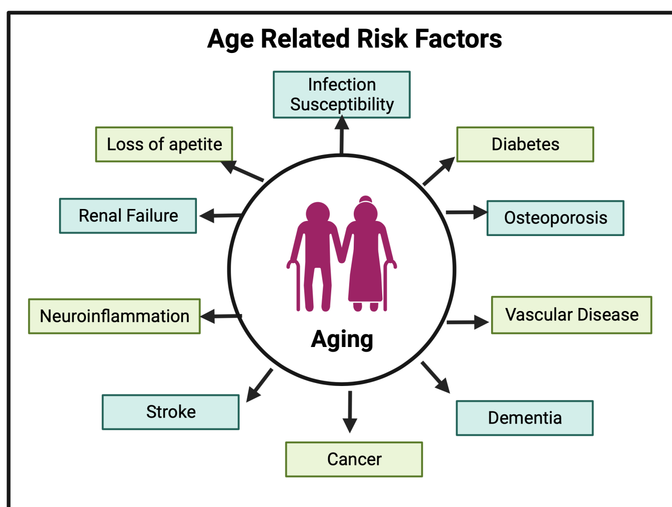

Figure 8: Age-related risk factors.

immune cell chemokines. This poses a heightened vulnerability to persistent morbidity and disability, associated with conditions such as dementia, depression, cancers, diabetes, and premature mortality [129, 130]. Additionally, as people age, the expression of TLRs decreases [131, 132].

Modulation of humoral immune responses and IgG glycosylation by Inflammaging.

Age-related changes in humoral immunity have shown a discrepancy in the regulation of strong immune responses. Although the number of B-cells diminishes with age, serum levels of antibodies, particularly IgG and IgA, increase [133]. Both in aged humans and aged mice, B-cell production begins to decline in the bone marrow [134, 135], which could be linked to age-related changes in the bone marrow microenvironment [136]. A reduction in organ-specific autoantibodies, followed by an increase in non-organ-specific autoantibodies and a decrease in high-affinity defensive response, is a fairly common shift in the humoral immune response during aging [127]. In old individuals, the cell division capability is reduced compared to young individuals, which could also lead to reduced clonal expansion after exposure to antigen stimuli [133]. Elderly individuals often respond poorly to vaccines compared to young individuals [137-139]. The levels of IgG subclasses, specifically IgG1, IgG2, and IgG3, show a significant increase in elderly humans [140]. Meanwhile, the levels of IgM remain unchanged. These alterations in the IgG subclasses may elevate the susceptibility of the elderly to bacterial and viral infections, contributing to increased hospitalization rates. Biological age has been shown to alter the composition and function of IgG-glycosylation [63]. IgG glycosylation predominantly galactosylation in humans and sialylation in mice, gradually varies with age [21, 46, 111, 141, 142]. The first study of IgG glycosylation, conducted by Parekh et al. in 1988, demonstrated that the abundance of A-galactosylated IgG-N glycans increased after the age of 25 [143]. Notably, di-galactosylated IgG was decreased, while there was no change in mono-galactosylated IgG [141]. Aging is highly associated with changes in IgG galactosylation and sialylation, especially reduced amounts, which can be seen in old age. Furthermore, bisecting GlcNac on IgG has been shown to change with age although the change is inconsistent. Some studies have reported an increase [113], some no change [144, 145] but others demonstrate a decline in bisecting GlcNac [146].

2. AIMS

The overall aim of this thesis is to elucidate how estrogen, aging, and TLR2 affect the humoral immune response and IgG glycosylation in experimental murine models.

Paper I

To study whether Bazedoxifene, a selective estrogen receptor modulator, shares the estrogenic characteristics of IgG glycosylation and total serum protein sialylation in an immune-induced post-menopausal mouse model.

Paper II

To study whether estrogen treatment has an impact on the humoral immune response and IgG glycosylation in a healthy postmenopausal mouse model.

Paper III

To study how TLR2 and aging affect the humoral immune response in an *S. aureus*-induced bacteremia in mouse model.

3. METHODS

Mouse models

Mice are excellent study subjects to understand diseases, physiological processes, treatment, development, and vaccine response. Although mice and humans are not exactly alike, the genetical, anatomical, and morphological similarities between them and humans allow researchers to learn important things about human biology. Our knowledge of the pathophysiology of numerous systemic and infectious disorders has been improved by the use of animal models. There are many benefits of using an animal model to be able to study the *in vivo* effect in animals of different ages, sexes, and species. However, there are significant disadvantages when employing a genetically modified mouse model, including diseases (neurological conditions), cost, limitations on resources, strain variability, ethical issues, and translational challenges.

Postmenopausal or Ovariectomy mouse model

The ovariectomy (OVX) model is a model to induce post-menopause in rodents and is often utilized for describing the roles of sex hormones. Rodents, including mice, do not naturally experience menopause and maintain their ability to reproduce throughout their lives. To replicate the hormonal changes seen in postmenopausal women, female mice have undergone a situation resembling post-menopause in women. In this OVX model, mice's ovaries are surgically removed (Figure 9). Since ovaries are the main source of estrogen production, removing them can reduce the amount of endogenous estrogen production. This mouse model has been utilized in papers I and II.

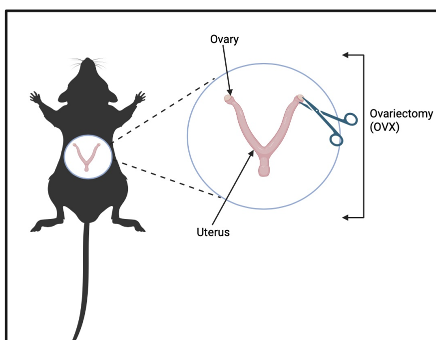

Figure 9- Postmenopausal mouse model. OVX: ovariectomy.

Sex hormone treatment

Bazedoxifene (24 µg/mouse), 17-β estradiol-3 benzoate (1 µg/mouse), and vehicle (Migloyl oil) were given 5 days per week, starting 10 days after the first OVA immunization, and treatment lasted until the termination. In paper II, three separate sets of experiments were performed, and various treatment methods were used. In experiment -I, a subcutaneous slow-release pellet with 17β-estradiol (167 ng/mouse/day) was inserted in OVX mice, or corresponding

placebo pellets were inserted in OVX and Sham-operated mice. In experiment -II, a subcutaneous injection of 17-β estradiol-3 benzoate (1 µg/mouse) or vehicle, was subcutaneously injected 5 days per week. Both experiments -I and -II were carried out until day 36. In experiment -III, mice were either sham or OVX operated on and then monitored for a longer period until day 105 after OVX.

Immunization mouse model

In paper I, mice's immune system was first activated by injecting 100 µg ovalbumin (OVA) emulsified in complete Freund's adjuvant subcutaneously on day 0 (10 days after OVX) at the tail base, followed by a subcutaneous booster injection of 100 µg OVA, emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) on day 28, to induce IgG production.

Mouse model for S.aureus bacteremia

Bacteremia is a systemic infection in the bloodstream caused by the invasion of gram-positive bacteria *Staphylococcus aureus* (S. aureus). Bacteremia is a very common occurrence in around 10-15% of patients aged between 60-65 [147], increasing the risk of disease severity and often leading to an increased risk of mortality [25, 148, 149]. It is an increasing issue among the elderly with many comorbidities that require rapid medical attention [150]. TLR2 is a critical receptor for lipoprotein (Lpp) synthesized from *S. aureus*. Bacteremia also affects the humoral immune responses by impacting the IgG levels. Paper III utilized a bacteremia mouse model. In brief, 200 µl of *S. aureus* suspension (1.5 x 10^6 CFU/mouse) was injected intravenously into the tail vein of WT and TLR2$^{-/-}$ mice of C57BL6 background. The animals were monitored daily for up to 10 days post-infection then blood serum and organs were collected.

Aged mouse model

An aged mouse as a model can be used to mimic processes of aging and age-related disease mechanisms in humans. Mice usually live for about 18 to 24 months. The life phase equivalencies between mice and humans are presented in **Table 1**. The aged mouse model is widely used in research applications such as neurodegenerative diseases, postmenopausal models, immune system alterations, and many more. However, these aged mouse models can have many limitations such as higher variability than in young mice, health issues, limited life span, and translational challenges. In papers III, young/aged mice were utilized. In paper III, the mouse age range of both WT and TLR2$^{-/-}$ old mice were 73-89 weeks and young mice were 13-28 weeks old.

Table 1: Life history stages in C57BL/6J mice in comparison to humans.
Life phase equivalencies between mice and human

	Mouse (age)	Human (age)
Mature adults	3-6 months	20-30 years
Middle age	10-14 months	38-47 years
Old	18-24 months	56-69 years

Serum analyses

Serum analysis is a method to analyze the blood serum obtained from the liquid portion of blood after coagulation and centrifugation. Serum analysis can provide valuable health information such as levels of antibodies, cytokines, hormones, or metabolites. Enzyme-linked immunosorbent assay (ELISA) is a commonly and widely used method to quantify specific proteins or antibodies in the serum. There are several types of ELISA tests, including direct, sandwich, competitive, and reverse ELISA. In direct ELISA, an antigen is coated directly onto the well plate, followed by blocking and then the addition of serum samples and a detection antibody coupled with a Horseradish peroxidase (HRP) antibody, followed by tetramethylbenzidine (TMB) substrate addition and absorbance measurement.

In sandwich ELISA a capture antibody is coated onto 96 well plates. The nonspecific binding sites are then blocked with a blocking buffer, after which samples are placed on the plate, the plate is washed, and a secondary antibody that binds to the protein of interest is added. The protein of interest or antibody may be present in the serum sample. As detecting antibodies, secondary antibodies linked to enzymes such as HRP are used. The unattached antibody-enzyme conjugates are then washed away, and the substrate is added to be transformed into a color signal by the enzyme. A microplate absorbance reader is used to determine the presence and quantity of the specified protein by measuring the absorbance of the plate's wells. In all papers, I, II, and III, the ELISA method was widely utilized to quantify the levels of IgG, IgG isotypes, IgM, and content of sialic acid on IgG as well as to measure the total protein sialylation (papers I and II).

Mass spectrophotometry

Mass spectrometry is an analytical method that is used to measure mass to charge ratio of ions. The results are presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio (m/z). The most used method for the analysis and purification of any glycoprotein in general is liquid chromatography (LC). The principle of LC is the distribution or separation of individual molecules in two different phases, mobile and stationary phase according to their relative affinities. Glycans have a high level of structural heterogeneity, with different linking configurations and monosaccharide branching within the molecule. Such complexity increases isomer separation and makes structural analysis difficult. There are two techniques to study glycans; 1) Glycomics, which is the definition of the entire repertory of glycans produced by a cell, tissue, or organism under particular circumstances, at a given time, and 2) Glycoproteomics identifies the glycosylated sites on each glycoprotein in a cell and, ideally, quantifies the diverse glycan structures present at each site [151].

In paper II, a glycoproteomic approach and liquid chromatography-masss pectrophotometry/mass spectrum (LC-MS/MS) were utilized to profile the IgG glycosylation in the serum samples. We chose the glycoproteomic approach to analyze the IgG-Fc glycosylation because this allowed us to characterize the site-specific glycan heterogeneity and the correlation of glycan compositions with their protein attachment sites.

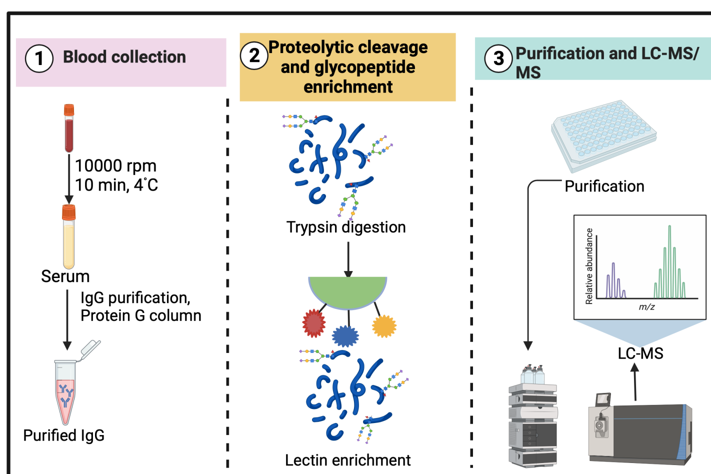

Figure 10- The flowchart of Glycoproteomic and Liquid chromatography-mass spectrometry/MS method. rpm: revolution per minute.

In paper II, we detected the highest level of IgG2b-Fc glycosylated subtype (Uniprot entry P01867, peptide sequence EDYNSTIR). LC-MS/MS method is an efficient way to distinguish isomeric structures. First, protein G is used to purify the IgG from the serum sample since

protein G has a higher binding affinity with all IgG isotypes. The flowchart of LC-based approaches for N-glycosylation analysis of IgG is summarized here in Figure 10. Although glycoproteomic is a rapidly developing and specific tool to identify site-specific glycosylation but there are some limitations as well. One limitation is that the current glycoproteomic method cannot identify the full complements of glycans on every site of a heterogeneous glycoprotein [151].

Flow cytometry

Flow cytometry is a technique that allows a rapid and multiparametric analysis of single cells in a liquid suspension. It uses lasers as light sources to generate scattered and fluorescent light signals that photomultiplier tubes can read. These signals are turned into electrical signals that are analyzed by a computer, and data is created into a file in a standardized format (fcs). Cell populations can be analyzed or purified based on their fluorescent or light-scattering characteristics. A variety of fluorescent reagents are utilized in flow cytometry. These include fluorescently conjugated antibodies, DNA binding dyes, viability dyes, and fluorescent expression proteins [152]. The flow cytometry analysis in this thesis was mainly utilized to detect the frequency and mean fluorescence intensity of immune cells such as B-cell, and plasma cells, and all the quantitative analyses were performed by using FlowJo (version 10.8.2).

Single-cell suspensions were made from BM and spleen from the mice. Cells were stained with fluorescent conjugated specific antibodies required to detect cells of interest. In paper I, the frequency of B-cells, plasma cells, and mean fluorescent intensity (MFI) of Sambuca nigra lectin (SNA) in total live cells, B-cells, and plasma cells from BM cells were determined. In paper II, the MFI of SNA in total live cells, B-cells, and plasma cells from BM and spleen cells were determined. SNA is a lectin, isolated from elderberry bark, which preferentially binds to sialic acid attached to terminal galactose in α-2,6 but also to a lesser degree, with α-2,3 linkage. In paper III, the frequency of T-cells, B-cells, and plasma cells of naïve healthy mice from both old and young WT and TLR2$^{-/-}$ mice from spleen cells were calculated.

Bone analysis

A quantitative computed tomography (QCT) scan is a medical technique used to measure bone mineral density (BMD) using an X-ray computed tomography (CT) scanner to generate the

image of bone mineral density values. Peripheral quantitative computed tomography (pQCT) analysis is a type of QCT used to measure a volumetric BMD and provide a three-dimensional structure. The pQCT can separate between cortical and trabecular bone, by scanning the bone dissected in the mid-diaphyseal for cortical bone and metaphyseal region for trabecular bone parameters. In papers I, and II, the XCT Research M (v4.5B; Norland Stratec) was used for pQCT scans, at a voxel size of 70 µm.

Real-Time PCR (RT-PCR)

RT-PCR is a sensitive method for the quantification of specific mRNAs in a biological sample. Isolated RNA is reversibly transcribed into complementary-DNA (cDNA), then amplified by PCR using specific probes and primers. Specific probes are conjugated with a fluorescent reporter. This fluorescent reporter emits a fluorescent signal during the amplification process, which can be measured at each amplification cycle. Two different spectra are analyzed simultaneously in the StepOne plus™Real-Time PCR system, one for the gene of interest and the other one for internal standard control. Analyses were determined with the ddCT method. In this thesis, RNA was extracted from BM, gonadal fat, and liver which we believed to be affected by immune induction (paper I). In paper II, BM and spleen were utilized for mRNA analysis. The ribosomal RNA 18S was used as an internal control (paper I and II).

Statistics

All statistical calculations in this thesis were performed in GraphPad Prism 10 (Version 10.0.0 (131)). The P-value is the probability of difference between observations. A P-value of 0.05 is considered to be a significant difference between the groups. One-way analysis of variance (ANOVA) is used when considering three or more groups. Student T-test is used when comparing two groups together. In the thesis, Grubb’s test detected outliers and excluded them from the analysis. In papers I and II, OneWay ANOVA followed by the Dunnett multiple comparison tests were used when comparing with the vehicle groups as a reference, and Student T-tests were used when comparing two groups. In paper III, student T-tests were used to compare age groups within each mouse strain and between the WT and KO mice. The fold changes of antibody levels in response to infection were calculated based on the infection controls from respective groups of healthy control mice (paper III). Data are presented as

scattered bar graphs (papers I, and II), and box plots and whiskers (paper III). Data are presented as a mean ± standard error of the mean (SEM).

4. RESULTS

Paper I- Bazedoxifene does not share estrogen effects on IgG sialylation.

Paper I, utilized ovariectomized and OVA-immunized mouse models, followed by treatment with estrogen or a selective estrogen receptor modulator, Bazedoxifene (BZA). The mice models were characterized by looking at the estrogen- and BZA-sensitive organs. As expected, estrogen, but not BZA, increased uterus weight. Estrogen and BZA treatment considerably lowered the thymus and gonadal fat weight and increased tibial cortical bone mineral density. Total IgG was increased in estrogen-treated mice compared to vehicle mice, whereas BZA did not affect the total IgG. Neither of the treatments affected the OVA-specific IgG levels compared to the vehicle. Next, the effect of BZA on IgG pathogenicity was investigated and found that BZA did not have any alteration of the degree of IgG sialylation, while estrogen marginally increased the degree of sialic acid on IgG. Only estrogen displayed an impact on intracellular sialic acid levels but not BZA. No alteration in the sialylation of either total glycoprotein or glycolipids was displayed. Further estrogen and BZA demonstrated a trend in the downregulation of mRNA levels of glycosyltransferases in the bone marrow and gonadal fat of ovariectomized OVA-immunized mice.

Conclusion

This is the first study demonstrating the effects of BZA on IgG and total serum protein sialylation. The findings revealed that BZA lacks certain attributes, such as impact on IgG pathogenicity, and plasma cell induction, which is important for its potential therapeutic application in postmenopausal women with autoantibodies.

Paper II- Impact of Estrogen on IgG Glycosylation and Serum Protein Glycosylation in a Murine Model of Healthy Postmenopause.

In paper II, we studied whether estrogen affects IgG glycosylation and total serum protein glycosylation in healthy postmenopausal status. We utilized an ovariectomy-induced postmenopausal mouse model, followed by estrogen or vehicle treatment.

The highly sensitive LC-MS/MS glycoproteomic analysis demonstrated that E2 treatment significantly increased IgG-Fc sialylation while decreasing the A-galactosylated IgG level. In addition, IgG levels were reduced in mice with long-term estrogen deficiency, whereas the short-term treatment with E2 did not alter IgG levels in healthy postmenopausal conditions.

Furthermore, estrogen status did not affect total IgG sialylation or total serum glycoprotein sialylation as measured by ELISA. Additionally, flow cytometry analysis showed that estrogen status did not affect the total sialic acid levels in live cells, B cells, or plasma cells. Only sialyltransferase mRNA (*St6gal1*) levels, were significantly downregulated in total splenic tissue after estrogen treatment, whereas galactosyltransferase (*B4GalT2*) and fucosyltransferase (*Fut8*), were not affected. In the bone marrow, none of the glycosyltransferase mRNA levels was altered.

Conclusion

Paper II concluded that estrogen treatment increases IgG-Fc glycosylation, including sialylation, and decreases A-galactosylation, implying that estrogen plays a regulatory role for IgG sialylation also in healthy postmenopausal mice and could thus be one mechanism by which estrogen influences immunity.

Paper III- The impact of TLR2 and aging on the humoral immune response to *Staphylococcus aureus* bacteremia in mice.

In paper III, we studied the impact of TLR2 and aging on the humoral immune response in an *S.aureus*-induced bacteremia mouse model. Paper III demonstrated in healthy conditions, there was an increase in the levels of IgM and IgG levels in the aged mice group compared to the young mice. We found that TLR2 was required for the increased level of IgG in elderly mice. Following bacteremia, aged mice wild-type (WT) and TLR2$^{-/-}$, showed insufficient IgM response compared to young. When calculating the fold change, we found that the total IgG response was reduced in both aged WT mice and in young TLR2$^{-/-}$ mice compared to young WT mice. We also measured IgG Isotypes and found they were dependent on both aging and TLR2 distinctly. Both aged and TLR2$^{-/-}$ increased the levels of anti-*staphylococcal* IgM response but no alteration in anti-*staphylococcal* IgG. The levels of sialylated IgG in WT mice were higher in aged WT in both healthy and bacteremia. Young TLR2$^{-/-}$ animals had higher levels of sialylated IgG than young WT mice in healthy. Following infection, unlike WT mice, no age-dependent increase in IgG sialylation was observed in TLR2$^{-/-}$ mice. Fold change in response to infection did not affect the level of IgG sialylation in either of the mice groups.

Conclusion

To summarise paper III, aging regulates immunoglobulin levels, including IgM, IgG, and IgG subtypes IgG2a, and IgG2b production except for the IgG1 isotype, thereby reducing the humoral immune response against bacteremia in aged mice. TLR2 is also critical in the regulation of IgG levels but not IgM response.

5. DISCUSSION

In this thesis, we have explored the potential physiological and pathological implications on the humoral immune response and IgG glycosylation using murine models. We have shown, in alignment with numerous studies using murine models, that the sex hormone estrogen (papers I and II), as well as bacteremia TLR2, and aging (paper III), have a significant impact by altering the antibody levels and its immunomodulatory effect, in the context of IgG glycosylation.

Estrogen-driven changes in IgG-Glycosylation

The interaction between sex hormones and the immune system is complex. Estrogen has been found to modulate various immunological diseases as well as enhance the induction of systemic humoral immune response [53, 91, 94, 97, 101, 104, 107, 109]. The influence of sex steroids on immunological function is critical to understand since it varies depending on gender and life stages, such as puberty, pregnancy, and menopause in women [107]. Estrogen has been shown to increase IgG sialylation in both inflammation-induced animals and postmenopausal RA patients [101]. The reduction of estrogen during menopause decreases IgG sialylation, potentially exacerbating autoimmune diseases. In Paper I, we hypothesized that not only estradiol (E2) but also the SERM Bazedoxifene (BZA) might affect IgG sialylation in OVA-immunized postmenopausal mice, and in Paper II, we hypothesized that estrogen could also act as a regulatory factor that influences antibody pathogenicity in healthy postmenopausal.

We demonstrated in paper I [153], that estrogen treatment increased serum IgG levels consistent with previous studies [101, 104, 154]. However, BZA did not alter the level of serum IgG. In paper II [155], in healthy conditions, we could not observe the effect on IgG levels following estrogen treatment as observed in paper I, suggesting that estrogen treatment has only a limited impact on antibody production in a healthy condition Instead, the influence of estrogen on IgG production appears to be more reliant on an activated immune system. In contrast to a previous study in mice where no alteration was observed in the levels of different subsets of IgG as well as IgM within five months of ovariectomy [156]. We demonstrated that prolonged estrogen deficiency in mice results in decreased IgG and a trend in the reduction of IgG subclasses (IgG1, IgG2a, IgG2b, and IgM) in healthy conditions (paper II).

Estrogen is well-known for its role in immunological response and ability to suppress B-cell lymphopoiesis [98, 102, 104, 122]. In paper I, estrogen treatment reduced B-cell frequency without affecting plasma cell frequency. Previous pre-clinical research suggested that BZA, similar to estrogen, could decrease B-cell frequency [98, 102, 122] whereas we could not confirm this effect with BZA treatment in the paper I. Additionally, the limited effect of BZA on B-cells and plasma cells might be dose dependency. In contrast to estrogen treatment in paper I, paper II did not show any estrogen-mediated effect on sialic acid levels either in total live cells, B-cells, or plasma cells. This suggests that the response observed in paper I, was likely due to an active immune system, while paper II involved healthy postmenopausal conditions. Moreover, in both paper I and paper II, there were no changes observed in sialic acid levels in total serum protein under either immune-induced conditions or in healthy circumstances, regardless of whether influenced by estrogen or estrogen-analog BZA treatment.

Further, in paper I, we demonstrated that BZA could not alter the IgG pathogenicity by altering the degree of sialylation in immune-induced conditions. However, we noticed a trend in the E2-treated group. When comparing E2 groups only to the vehicle and not considering BZA, a significant increase in IgG sialylation was shown, which aligned with the previous study of postmenopausal immune-induced mice [101]. This suggests that reduced estrogen levels during menopause may enhance antibody pathogenicity via a decrease in IgG-glycosylation, gaining greater importance and potentially playing a critical role in estrogen-deficient conditions.

In paper II of normal postmenopausal circumstances, we demonstrated that estrogen treatment increased IgG-Fc sialylation (specifically the G1S/G2S form), decreased A-galactosylation (G0 form), and displayed a strong tendency toward increased galactosylation (G1/G2 form) on IgG [155]. These changes suggest an anti-inflammatory effect of estrogen on IgG pathogenicity in healthy postmenopausal circumstances. Importantly, increased levels of IgG-Fc sialylation have been found to limit interaction with FcγRs while increasing pathogen engulfment, destruction, and complement activation, providing a balance between pro- and anti-inflammatory immune responses [53].

Increased sialylation of IgG, on the other hand, decreases its affinity for FcγRIIIa while increasing the affinity with FcγRIIb. Large population studies indicated that A-galactosylated IgG increases after menopause [157]. Still, in younger females, the A-galactosylated IgG

decreases during puberty [108, 145] and in pregnancy [118, 146], implying that the sex hormone estrogen regulates the alteration of IgG glycosylation and influences the interaction affinity with FcγRs. Moreover, estrogen treatment increases IgG-Fc glycosylation in normal healthy women [53, 158].

We found (paper I) that neither estrogen nor BZA treatment in an active immune system affects the mRNA expression of glycosyltransferase. In contrast to paper I, paper II, showed that E2 treatment reduced the *St6gal1* mRNA expression in normal postmenopausal spleen tissue, indicating an upregulation or no effect in *St6gal1* mRNA at the systemic level is due to the differential expression of *St6gal1* mRNA on different types of immune cells. Nonetheless, there appears to be a correlation between glycosyltransferase expression and glycan levels, suggesting that regulatory mechanisms may be more complex than a simple alteration in glycosyltransferase expression [158, 159] in different environment.

Inflammaging and TLR2-driven alteration in humoral immune response and IgG- sialylation.

Inflammaging is characterized by elevated levels of pro-inflammatory cytokines generated by low-grade chronic inflammation in the absence of acute infection. It is now well recognized that the disruption in the IgG glycosylation is not only an important factor during aging, but it also shifts with the inflammation. With aging, adaptive immunity drops, and innate immunity undergoes a minor alteration that may result in low immune activity in older individuals as described in [46, 133, 160]. In paper III, we studied the effect of systemic inflammation induced by *S.aureus-induced* bacteremia, on humoral immune response and IgG-sialylation using a young/aged, WT, TLR2$^{-/-}$ mouse model and age-related changes [161].

Natural antibodies, including IgM, are spontaneously formed in the body, with IgM being present before encountering infections [18]. Moreover, when infections are extensive, plasma cells secrete IgM in a secretory form. Several studies have demonstrated that the number of total IgM varies inconsistently with age [139, 162] but at the same time, other findings have indicated an increase in antigen-specific antibodies, as observed in mice with early aging of mucosal immunity [163]. In paper III, we observed elevated levels of IgM in both aged healthy and bacteremia mice of both genotypes. The increase was more limited in bacteremia, and interestingly, upon calculating the fold change, we found that IgM showed a significant

increase, approximately 2.5 times, in young mice of both genotypes. Notably, there was no discernible change observed in IgM levels in old mice, both WT and TLR2$^{-/-}$, suggesting a potential saturation of the immune response in aged mice. TLR2 deficiency did not affect the levels of IgM. The IgG was significantly higher in both healthy WT old mice and young TLR2$^{-/-}$ compared to young WT healthy controls. There is some evidence that age modulates IgG response through induction in the levels [131, 164, 165]. Neither age nor TLR2$^{-/-}$ altered the IgG levels in bacteremia, suggesting that both TLR2 and aging control the IgG levels. Previous studies in TLR2$^{-/-}$ mice and immunization against salmonella typhi did not increase the IgG, indicating the importance of TLR2 in antibody production and the shaping of antibodies [33].

Mice have been observed to generate all four subclasses of IgG in response to diverse antigens, such as bacterial adhesins and infection [166, 167] , and a range of viruses [168-170]. The levels vary based on the type of antigen as IgG1 responds more with protein antigens, while IgG2 levels are more associated with polysaccharide antigens [170, 171]. These isotypes show different binding affinity with FcγRs and mediate the effector function (Figure 4). In paper III, we measured only three IgG isotypes, IgG1, IgG2a, and IgG2b. We found that in both WT and TLR2$^{-/-}$ mice under healthy conditions, age led to increased IgG2a levels with no impact on IgG1 levels from either TLR2 deficiency or age. When comparing elderly WT mice to their younger counterparts, IgG2b levels significantly increased, while TLR2$^{-/-}$ mice showed no change in IgG2b levels. Additionally, a study involving polymicrobial abdominal sepsis in mice showed that serum IgG1 and IgG2b levels increased during sepsis [28]. Post-immunization studies on bacterial infection and the ischemia model revealed a slight rise in IgG1 and IgG2b titers, but a significant decrease in IgG2a titers in TLR2$^{-/-}$ mice [32, 33]. We found that following bacteremia, elderly WT mice had lower IgG1 and IgG2a levels than their younger counterparts. However, IgG2b levels significantly increased. The fold change calculations showed a dramatic increase in IgG1 and IgG2a levels in both young and old mice of both phenotypes. This suggests that the subclass of IgG is controlled by age and in a different manner with TLR2.

The IgG glycosylation holds significant importance in inflammation and aging, which is characterized by high levels of proinflammatory response in the bloodstream and stands as a potent risk factor for various diseases. It is also hypothesized that the disruption in IgG glycosylation in aging and inflammation contributes to the shift in anti- to pro-inflammatory response [172]. In paper III, we analyzed the total sialic acid content on total IgG using lectin

ELISA, we found that the degree of IgG sialylation was increased in old WT mice compared to young WT mice of both healthy and infected conditions but no change in the TLR$^{-/-}$ mice which align with many population studies as reviewed in [46], that aging [55, 108, 141] and inflammation decrease the IgG sialylation [173]. In paper III, we demonstrated that aging, bacteremia, and TLR2 play an important role in the regulation of humoral immune response as well as IgG sialylation. Elucidating the mechanism of altered humoral immune response in the elderly needed further exploration in the functional assay including in vivo and in vitro approaches.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The impact of hormones on IgG glycosylation patterns emphasizes the complex ways in which hormones influence the shape of immune responses. This insight increases our understanding of immune function and opens the possibility for therapeutic interventions and mechanisms, particularly in postmenopause-related diseases where hormonal fluctuations play a significant role.
The synergistic effects of aging and inflammation in mice's humoral immune response and on IgG sialylation highlight the changes in immune function over time. The influence of aging, TLR2 deficiency, and systemic immune stimulation on antibody levels is of special interest to better understand the early or late phases of disease etiology, as well as the molecular mechanisms of immunosenescence and inflammaging. This will enable the therapeutic approaches to manage altered immunological responses in the elderly. The integration of these factors into this thesis increased our understanding of immune regulation in mice which holds great promise for advancing both basic immunology and therapeutic approaches in the context of immunological disorders.

This thesis focused mostly on N-linked glycosylation in murine models, although other areas required further investigation. Estrogen's effect on O-linked glycosylation, testosterone's effect on IgG-Fc glycans, and the role of hormones such as progesterone, menarche, menopause, and pregnancy are all areas that require additional investigation. The optimal timing and duration of estrogen and SERM treatment require additional investigation. Ongoing studies aimed at correlating IgG glycan with illnesses, inflammation, and immunological deficiencies appear to be primarily focused on population studies, but functional models are needed as well to improve efficacy, appropriate doses, and so on. Interactions between environmental variables and genetic susceptibility, contribute to disease manifestation (for example, autoimmune disease), necessitating the use of analytical techniques such as glycomics and glycoproteomics to understand the IgG glycosylation mechanisms.

7. Related publications not included in the thesis.

1. Yiwen Jiang, Karin Horkeby, Petra Henning, Jianyao Wu, Lina Lawenius, Cecilia Engdahl, **Priti Gupta**, Sofia Movérare-Skrtic, Karin Nilsson, Ellis Levin, Claes Ohlsson, and Marie Lagerquist. **Loss of membrane estrogen receptor α initiated signaling modulates the sensitivity to estradiol treatment in a dose- and tissue-dependent manner.** *Sci Rep. 2023 Jun 3;13(1):9046.*
2. Zhicheng Hu, Pradeep Kumar Kopparapu, Meghshree Deshmukh, Anders Jarneborn, **Priti Gupta,** Abukar Ali, Ying Fei, Cecilia Engdahl, Rille Pullerits, Majd Mohammad, Tao Jin. **The impact of aging and TLR2 deficiency on the clinical outcomes of Staphylococcus aureus bacteremia.** *J Infect Dis. 2023 Aug 11;228(3):332-342.*
3. Marie K. Lagerquist, **Priti Gupta**, Edina Sehic, Karin L. Horkeby, Julia M. Scheffler, Jauquline Nordqvist, Lina Lawenius, Ulrika Islander, Carmen Corciulo, Petra Henning, Hans Carlsten, Cecilia Engdahl**. Reduction of mature B cells and immunoglobulins results in increased trabecular bone reduction of mature B cells and immunoglobulins results in increased trabecular bone.** *JBMR Plus. 2022 Aug 30;6(9):e10670.*
4. Jauquline Nordqvist, Cecilia Engdahl, Julia M. Scheffler, **Priti Gupta**, Karin L. Gustafsson, Marie K. Lagerquist, Hans Carlsten, Ulrika Islander. **A tissue-selective estrogen complex as treatment of osteoporosis in experimental lupus A tissue-selective estrogen complex as treatment of osteoporosis in experimental lupus.** *Lupus. 2022 Feb;31(2):143-154.*

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