Engineered Antibodies with Dual Inhibitory Activity against Toll-like Receptor Family Members

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Abstract

The immune system plays a crucial role in host defense in vertebrates. One of the major players in the immune system is the antibody, which recognizes antigens to eliminate pathogens. Antibodies induce cytotoxicity by activating natural killer cells, which destroy virus-infected cells; phagocytosis by neutrophils; and macrophages, which eliminate extrinsic antigens. After infection, the body generates an enormous variety of antibody sequences to recognize a wide variety of antigens. Additionally, antibodies can bind to a variety of proteins including both self-antigens and non-self-antigens. Due to their specificity for antigens and excellent stability in blood, antibodies are now widely used as research tools, in diagnostics and as therapeutics.

Toll-like receptors (TLRs) recognize extrinsic antigens in the vertebrate immune system in a different manner to antibodies. They respond to pathogen-associated molecular patterns derived from microorganisms and induce cytokine production for activating immune system processes that eliminate pathogens. There are 10 TLR family members, among which TLR4 has been reported to be associated with a variety of diseases. One of the most severe diseases with the highest mortality rate is sepsis, which arises through overactivation of the host immune system after infection. Despite the fact that a large number of patients develop sepsis every year, no therapeutic agents are available. Further, although TLR4 inhibitors have been trialed for the treatment of sepsis, none have shown sufficient efficacy. The reason for their lack of success in clinical trials is thought to be related to the complex mechanism underlying sepsis. Thus, the mechanism of sepsis, including its association with TLR4, should be investigated in more detail to enable the development of new therapeutic agents for treating sepsis.

The aim of my first study was to generate an anti-rat TLR4 antibody useful for rat models of sepsis. Such a tool antibody will be useful for clarifying the contribution of TLR4 to sepsis and investigating the dosage regimen of TLR4 inhibitors for clinical use. I first generated an anti-rat Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD2) antibody, called 52-1H4. The 52-1H4 antibody was obtained by immunizing TLR4 knockout (KO) mice with rat TLR4/MD2-expressing cells. 52-1H4 exhibited specific binding to rat TLR4/MD2 and completely neutralized lipopolysaccharide-mediated cytokine secretion. Moreover, complementary determining regions of 52-1H4 were grafted into rat framework regions to obtain the ratized antibody 52-1H4 e2 (e2) to reduce the immunogenicity in rats. The e2 antibody showed specific binding to rat TLR4/MD2 and comparable inhibitory activity to the parental antibody. Finally, pharmacokinetic and pharmacodynamic analysis of e2 showed that it could be a useful tool for rat *in vivo* studies.

The purpose of my second study was to generate an anti-TLR2 x anti-TLR4 bispecific antibody with the potential to become a therapeutic agent for sepsis. TLR2 has been reported to contribute to sepsis and dual blockade of TLR2 and TLR4 has been reported to show superior efficacy to monotherapy comprising a TLR2 or TLR4 inhibitor in mouse septic models. Thus, I hypothesized that a bispecific antibody directed at human TLR2 and human TLR4 would be more efficacious for treating sepsis. I generated an anti-human TLR2 (mAb-2) and anti-human TLR4 (mAb-4) antibody, both of which completely inhibited ligand-mediated cytokine production in an *in vitro* assay. Next, a bispecific antibody, called ICU-1, was obtained by genetically fusing mAb-2 with mAb-4. ICU-1 also completely inhibited ligand-mediated cytokine production in the same assay. Although ICU-1 showed comparable binding affinity for human

TLR2 to the parental antibody, it showed relatively lower binding affinity for human TLR4. Finally, the therapeutic potential of ICU-1 was evaluated using two bacterial species that have been detected in septic patients. ICU-1 completely suppressed bacterial stimulation-mediated cytokine production from human peripheral blood mononuclear cells. These findings suggest that ICU-1 has sufficient potential to be a therapeutic agent for treating sepsis.

Finally, the aim of my third study was to investigate the binding mode of the bispecific antibody to identify its mechanism of action. The binding affinity and functional properties of a bispecific antibody for two receptors have been reported to vary depending on the cells used in the assay. Specifically, the apparent binding affinity and functional properties of a bispecific antibody for the antigen with lower expression increases in cells with high relative expression of the two target antigens. In my study, the inhibitory activity of ICU-1 against TLR4 increased in the assay using bacteria. This was assumed to be caused by simultaneous binding of ICU-1 to TLR2 and TLR4. Subsequent evaluation of the inhibitory activity of ICU-1 against TLR4 in the presence of anti-TLR2 antibody mAb-2, which competes with ICU-1 for binding to TLR2, showed that blocking TLR2 with mAb-2 reduced the inhibitory activity of ICU-1 against TLR4. Further, the cells used in the assay had higher expression levels of TLR2 than TLR4. This condition is consistent with that reported previously in which a bispecific antibody showed increased apparent binding affinity, and suggests that ICU-1 simultaneously binds to TLR2 and TLR4. These findings will be useful for selecting suitable target molecules for bispecific antibodies.

In summary, I successfully generated the ratized anti-rat TLR4/MD2 antibody e2, which can be used to elucidate TLR4 function. I also generated the bispecific antibody ICU-1, which can completely

inhibit the functions of TLR2 and TLR4 and, consequently, has potential as a therapeutic agent for sepsis. Further, I elucidated the unique binding mode of ICU-1, which suggests that bispecific antibodies show enhanced affinity for the antigen with lower expression in cells with high relative expression of two target antigens. Therefore, the findings in my study suggest that a bispecific antibody targeting two antigens with comparable expression in normal tissues but different expression in abnormal tissues will have higher binding affinity and functional activity in abnormal tissues. My findings suggest that bispecific antibodies could be an innovative treatment solution with lower safety risk for patients with various diseases.

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Abbreviations

Ig	immunoglobulin
DC	dendritic cell
B cell	bone marrow-derived cell
T cell	thymus-derived cell
APC	antigen-presenting cell
MHC	major histocompatibility complex
CD4	cluster of differentiation 4
TCR	T cell receptor
CCR	C-C motif chemokine receptor
SHR	somatic hyper mutation
BCR	B cell receptor
Fab	antigen-binding fragment
Fc	fragment crystallizable
CDR	complementary determining region
FcR	Fc receptor
FWR	framework region
CH	constant region of heavy chain
CL	constant region of light chain
ADCC	antibody-dependent cellular cytotoxicity

CDC	complement-dependent cytotoxicity
RAG	recombination-activating gene
FcRn	neonatal Fc receptor
IgG4-RD	IgG4-related disease
OVA	ovalbumin
FcγR	Fc gamma receptor
ITAM	immune tyrosine-based activating motifs
C1q	complement component 1q
FDA	Food and Drug Administration
DNA	deoxyribonucleic acid
scFv	single chain fragment variable
PK	pharmacokinetic
PD	pharmacodynamic
K_D	equilibrium dissociation constant
SEC	size exclusion chromatography
HPLC	high performance liquid chromatography
CHO	Chinese hamster ovary
GS	glutamine synthase
MSX	methionine sulfoximine
DHFR	dihydrofolate reductase

MTX	methotrexate
ADA	anti-drug antibody
EGFR	epidermal growth factor receptor
MET	mesenchymal-epithelial transition factor
BiTE	bispecific T cell engager
PD-1	programmed cell death protein 1
dsFv	disulfide-stabilized Fv
DVD-Ig	dual variable domain immunoglobulin
KiH	knobs-into-holes
TLR	Toll-like receptor
SLE	systemic lupus erythematosus
IBD	inflammatory bowel disease
COPD	chronic obstructive pulmonary disease
RA	rheumatoid arthritis
PRR	pattern recognition receptor
PAMPs	pathogen-associated molecular patterns
NLR	nucleotide-binding oligomerization domain-like receptor
RLR	retinoic acid inducible gene-I-like receptor
LPS	lipopolysaccharide
MD-2	myeloid differentiation protein-2

MyD88	myeloid differentiation factor 88
TIR	Toll/interleukin-1 receptor
TRIF	TIR domain-containing adaptor inducing interferon-β
Mal	MyD88-adapter-like
IRAK	IL-1 receptor-associated kinase
NF-κB	nuclear factor kappa B
TNF-α	tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
IFN	interferon
IFNR	interferon receptor
ISGF3	IFN-stimulated gene factor 3
STAT	signal transducer and activator of transcription
IRF9	IFN regulatory factor 9
TNFR	TNF receptor
IL-6R	IL-6 receptor
gp130	glycoprotein 130
mIL-6R	membrane bound IL-6R
sIL-6R	soluble IL-6R
ADAM10	metalloproteinase domain-containing protein 10
JAK	Janus kinase

AKT	protein kinase B
MAPK	mitogen-activated protein kinase
SOCS	suppressor of cytokine signaling proteins
HMGB1	high-mobility group box-1
TGF-β	transforming growth factor beta
MDSC	myeloid-derived suppressor cell
EpCAM	epithelial cell adhesion molecule
HSP	heat shock protein
DAMPs	damage-associated molecular patterns
I/R	ischemic reperfusion
PCR	polymerase chain reaction
mAb	monoclonal antibody
ELISA	enzyme-linked immunosorbent assay
PBMC	peripheral blood mononuclear cell
SPR	surface plasmon resonance
KLH	keyhole limpet hemocyanin
MFI	mean fluorescence intensity

Chapter 1 General introduction

1.1 Roles of antibodies in the immune system

Antibodies are a group of immunoglobulins (Ig) which recognize and eliminate pathogens for host protection. Antibodies are major proteins in blood and are produced only by bone marrow-derived cells (B cells). In vertebrates, the reaction between antibodies and their antigens is a key process in the immune system, so much so that it forms the basis for the classical division of the immune system into innate and adaptive categories.

In the adaptive immune system, pathogens engulfed by dendritic cells (DC) are presented to thymus-derived (T) lymphocytes, and B cells are subsequently activated to produce antibodies. The recognition of pathogens by DC and their subsequent reaction form part of the innate immune system and will be described later [1]. DCs are a type of antigen-presenting cell (APC). Among the three types of APCs, namely DCs, macrophages and B cells, DCs are highly specialized to present antigens following loading onto major histocompatibility complex (MHC) molecules. After DCs engulf an antigen, the antigen is degraded by lysosomal proteases and antigen-derived peptide fragments are loaded on MHC to be presented to T cells (Figure 1.1). Recognition of the peptide-MHC complex by the T cell receptor (TCR) ultimately leads to activation of T cells. Such activation of T cells through antigen presentation by DCs is a key component of the adaptive immune response, in which some activated T cells migrate to sites of infection and kill virus-infected cells [1, 2]. These T cells, called cytotoxic killer T cells, play a crucial role in the adaptive immune system by preventing host species from viral infections and cancer. This antigen presentation process is common among APCs.

Another type of T cell, called helper T cells, help B cells become activated and produce antibodies

against pathogens through cognate interactions involving antigen presentation by B cells to T cells. B cells are also an important player in the adaptive immune response. First, as mentioned above, B cells engulf antigens and present the processed antigens to helper T cells. Next, an encounter with antigens increases expression of C-C motif chemokine receptor 7 (CCR7) on the surface of B cells. A chemokine gradient formed by CCR7 ligands CCL19 and CCL21 recruits B cells to T cell zones located next to germinal centers in secondary lymphoid tissues. The germinal center is a microstructure where the growth, selection and maturation of B cells have been observed. At the border of the T cell zone and B cell follicle, antigens on MHC-Class II are presented by B cells to cluster of differentiation 4 (CD4)-positive helper T cells. After interacting with T cells, B cells become activated and differentiate into short-lived plasma cells or germinal center B cells. The short-lived plasma cells produce antibodies in the early stage of the immune response, while germinal center B cells provide high-affinity antibodies by somatic hyper mutation (SHM) of the genes encoding the B cell receptor (BCR), which is the initial form of an antibody. Among the variants of BCRs, a small number of BCRs increase affinity for antigens. B cells with high affinity BCRs are selected and differentiate into long-lived plasma cells to produce high-affinity antibodies which specifically bind and efficiently eliminate antigens (Figure 1.1) [3].

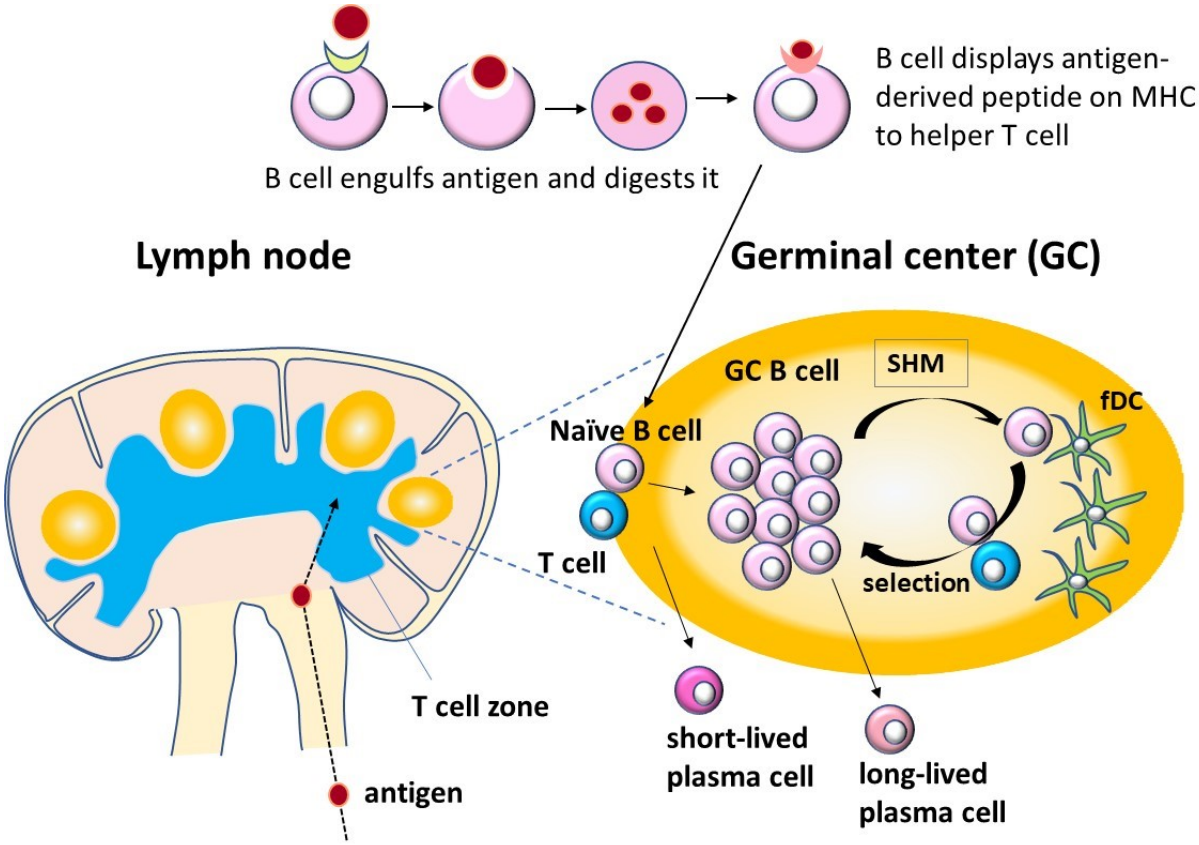


Figure 1.1. Germinal centers in lymph nodes.

1.2 Features of five classes of antibodies

There are five classes of Igs (IgA, IgD, IgE, IgG and IgM) in mammals [4]. The specific function of each class of Ig differs according to its structure in the regulation of immunological responses after antigen exposure. IgM is first produced by B cells as a BCR on the cell membrane and secreted in a decavalent state, which enables binding to antigens with high avidity (Figure 1.2). Short-lived plasma cells produce IgM until mature B cells are established by SHM and class switching occurs in the germinal center. The function of IgD, which remained unknown for a long period of time, was recently shown to be related to clonal deletion of B cells that react with self-antigens in bone marrow [4, 5]. Meanwhile, IgA is associated with the mucosal immune system and is present in saliva, colostrum, and the gastrointestinal mucosa to protect from the entry of pathogens [6].

IgA was the last antibody to be discovered, even though it is the most abundantly produced protein among the Ig subclasses. A unique feature of IgA is its association with the intestinal microbiota, which has been a recent topic of intense focus as the microbiota plays an important role in the response of immune-mediated therapy. IgA has the conventional antibody structure, which is composed of an antigen-binding fragment (Fab) and fragment crystallizable (Fc) domains. However, in contrast to IgG and IgA in serum, intestinal IgA is secreted as a dimer. Dimeric IgA has a joining (J) chain which connects two Ig molecules (Figure 1.2). This dimeric form of IgA is an essential feature of intestinal IgA, which differentiates it from IgA in other tissues. The most impressive feature of IgA is its noncanonical binding to antigens via glycans. This noncanonical binding requires non-complementary determining region (CDR) sequences while canonical binding is Fab-dependent and shows conventional binding capacity. IgA also has highly glycosylated regions, which potentially mediate its binding with microbiota [7].

IgE plays prominent roles in allergic immune responses such as asthma, rheumatoid arthritis and food allergies. Binding of IgE to mast cells and basophils with the Fc-Fc receptor (FcR) leads to secretion of histamine after antigen recognition. Leukocytes, antibodies and complement proteins infiltrate tissues and intensify the allergic reaction as histamine induces vasodilation and vascular hyperpermeability. Because of these rapid immune responses, production of IgE is strictly regulated and lowest among the Ig subclasses. IgE plasma cells are generated at germinal centers after the class switching of B cells. While IgG is produced from long-lived plasma cells, IgE plasma cells are short-lived and secrete low affinity antibodies. The concentration of IgE is 50-200 ng/mL in blood compared to the 10 mg/mL that other Igs reach. Moreover, the serum half-life of IgE is about 2 days in humans, which is the shortest of the Igs. These features of IgE minimize

undesirable anaphylactic reactions to maintain a steady state in the host. Conversely, detection of antigen-specific IgE is a useful diagnostic method for identifying antigens, especially in patients with unspecified allergic reaction [8]. Patients with allergic symptoms are typically treated with antihistamines, corticoids and anti-inflammatory therapeutic agents. However, a number of patients do not respond to these conventional treatments and require novel approaches to alleviate their allergic symptoms. The anti-IgE antibody omalizumab, which could be one such novel treatment, was approved for treating severe allergic asthma and is expected to be applicable to other allergic diseases [9].

IgG is the most abundant antibody in blood and is divided into four subclasses (IgG1, IgG2, IgG3 and IgG4) in humans [4]. The typical IgG antibody structure comprises a heterodimer of a heavy chain and a light chain that forms a homodimer through disulfide bonds in the hinge region of the heavy chains (Figure 1.3). The heavy chain variable region (VH) and the light chain variable region (VL) recognize antigens and comprise four types of framework regions (FWRs) and 3 types of CDRs. The FWRs, which are determined by germline sequences, dictate the antibody's stability, while the CDRs, which can have a wide variety of sequences randomly assembled by gene recombination, dictate the specificity. The heavy and light chains also contain constant regions located at the carboxy terminus (C-terminus) of each variable region. The heterodimer of the heavy and light chains is formed by a disulfide bond between heavy chain constant region 1 (CH1) and the light chain constant region (CL), and is designated the antigen-binding fragment (Fab). Heavy chain constant regions 2 and 3 (CH2 and CH3), which together are called the fragment crystallizable region (Fc region), mediate effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Antibodies produced by activated B cells eliminate pathogens using these

effector functions in the adaptive humoral immune system [4].

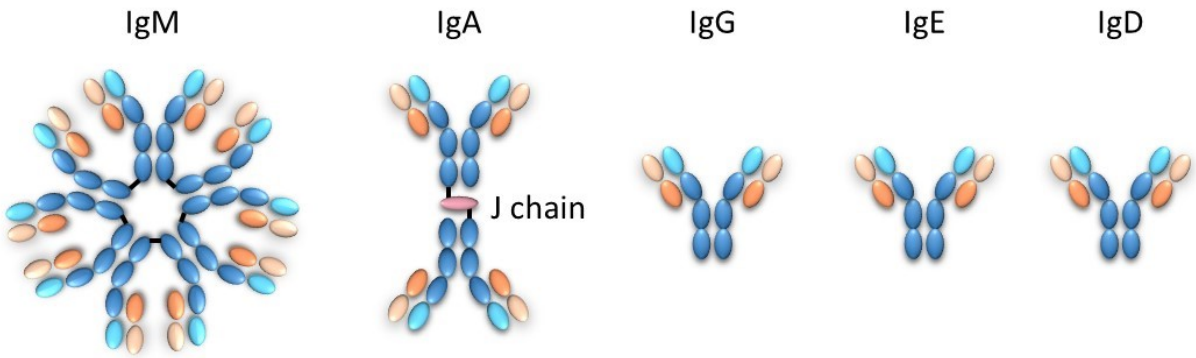


Figure 1.2. Structures of Ig subclasses. The structure of IgM is decavalent complex to bind antigens with high affinity. IgA has a J chain connecting two bivalent antibodies, resulting in a tetravalent structure. IgG, IgE, and IgD are bivalent antibodies.

Table 1.1. Features of each Ig subclass

	IgM	IgA	IgG	IgE	IgD
valency	decavalent	tetravalent	bivalent	bivalent	bivalent
% of antibodies	6%	13%	80%	0.002%	1%

The CDRs and FWRs in antibody sequences are defined using the antibody numbering scheme first reported in the 1970s by Kabat and Wu, who aligned immunoglobulin light chain sequences to study the amino acid variability at each position of the sequence. They defined three hyper variable regions in the light chain and three hyper variable regions in the heavy chain by analyzing the frequency of amino acids at each position. They found that clusters of these hyper variable regions form a surface that was responsible for binding an antigen and referred to the three hyper variable regions as complementarity determining regions (CDR)-1, 2 and 3. Although the Kabat numbering scheme is widely used and regarded as the standard method, it has some limitations. The scheme does not allow for unconventional insertions or deletions of amino acids in CDRs because it is based on the alignment of a limited number of sequences. Moreover, it does not accurately match

the 3D structure of antibodies, with discrepancies observed when numbering corresponding amino acid residues using the Kabat numbering scheme compared to the crystal structure. To address these limitations, in the 1980s, Chotia and Lesk suggested a novel numbering scheme based on the structure of antibody variable regions. They defined the structure of CDR loops and corrected the numbering scheme based on alignment of the crystal structure of antibodies. Moreover, Lefranc et al. reported a new numbering scheme based on amino acid alignment of germline V genes which has been registered in the international ImMunoGeneTics information system (IMGT). Now researchers are able to define CDRs and FWRs based on these numbering schemes [10].

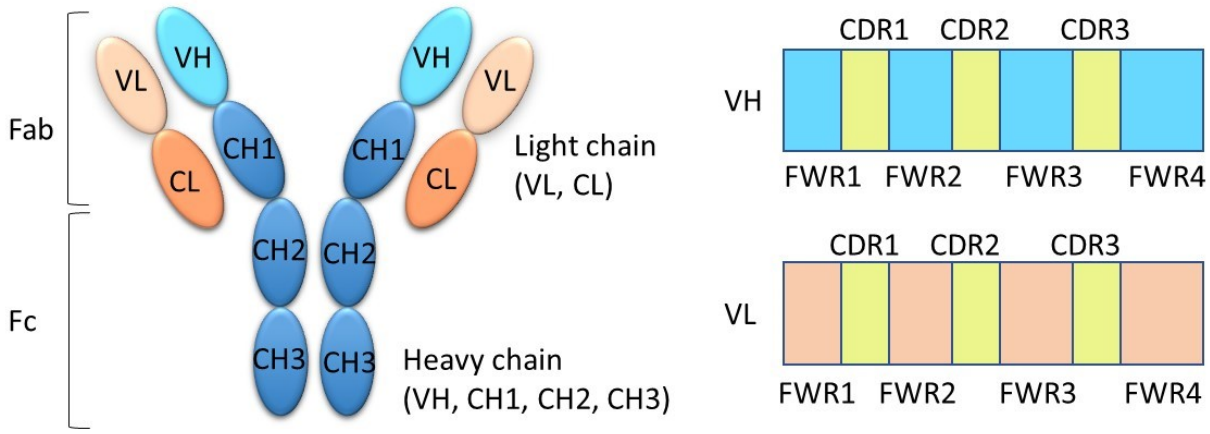


Figure 1.3. Antibody structure. The typical antibody structure comprises Fab and Fc domains. VH-CH1 and VL-CL are linked by disulfide bonds formed between CH1 and CL. The Fc domain is composed of two heavy chain CH2 and CH3 domains, which are linked by disulfide bonds in the hinge region of CH2. The combined structure is robustly stabilized by each disulfide bond and stability afforded by the amino acid sequence of each antibody.

Genes encoding Ig are composed of variable (V), diversity (D) and joining (J) gene segments. During the development of mature B cells in the germinal center, recombination-activating genes (RAG) assemble VDJ genes for class switching. Moreover, VDJ genes can be composed of many combinations of segments

and rearrangement by RAG protein produces a wide variety of antibodies with different heavy chains and light chains. The human IGH locus contains 50 VH segments, 6 JH segments and 27 DH (IMGT) while the human IGK locus is composed of 31-36 Vk and 5 Jk segments with no D gene segments (IMGT). Meanwhile, the human IGL locus comprises 29-33 VL and 4-5 JL segments (IMGT). Thus, about 2.5×10^6 different antibody sequences can be generated by combining these IGH and IGK or IGL gene segments (Figure 1.4). In addition to VDJ rearrangement, SHM also increases the variety of antibody sequences. Given that up to 20 amino acid mutations can be induced at each amino acid position in 2.5×10^6 different antibody sequences, the number of possible antibody sequences is astronomical [11-13]. However, not all of these antibodies are functional. Through rearrangement, functional Igs are only produced in their structurally complete form from one allele, while the non-functional allele is subject to allelic exclusion. This leads to monospecificity of BCRs, which means that each B cell clone produces only one type of antibody [14].

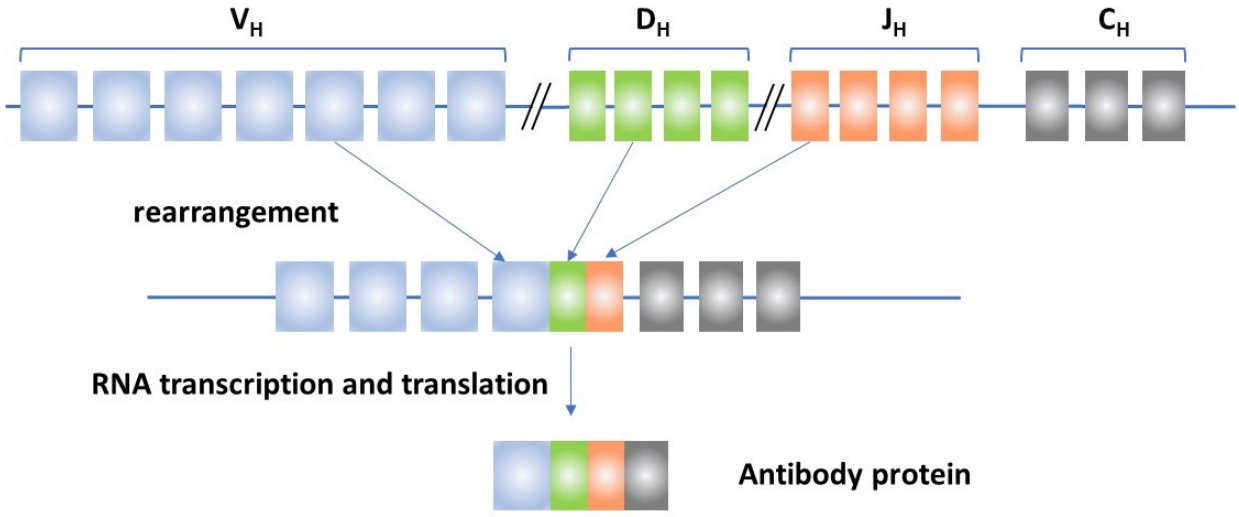


Figure 1.4. Rearrangement of antibody sequences in the IGH locus. Different combinations of gene segments selected from VH, DH, JH and CH can be assembled to generate a wide variety of antibodies.

1.3 Features of IgG subclasses

IgG1 is mainly produced by mature B cells after exposure to soluble and membrane-bound antigens. Lower levels of IgG3 and IgG4 are also released during this process. Deficiency in IgG1 results in decreased levels of IgG proteins and hypogammaglobulinemia, or low levels of immunoglobulins, and increases the risk of opportunistic infection since IgG1 is the most affluent protein among the IgG subclasses [15].

IgG2 is characterized by its response to bacterial capsular polysaccharide and plays a central role in host defense against *Streptococcus pneumonia* [15]. Defects in IgG2 increase the risk of loss of anti-carbohydrate antibodies, although IgG1 and IgG3 can compensate for the deficiency in IgG2 by increasing protein production. In fact, IgG1 antibody responses to *Haemophilus influenza* b polysaccharide have been reported in humans [15].

IgG3 has the most potent effector functions due to its ability to bind to all types of FcRs. However, its strong pro-inflammatory immune reaction is limited by its shorter half-life, which is about half that of the other IgG subclasses [16]. The shorter half-life of IgG3 is due to its low binding capacity for the neonatal Fc receptor (FcRn), which salvages IgGs from lysosomal degradation after they are engulfed into endosomes [17]. While immune responses to viruses lead to both IgG1 and IgG3 production, IgG3 production occurs first.

Although the physiological function of IgG4 is unclear, there is evidence that it may be related to autoimmune diseases, in which elevated serum levels of IgG4 and infiltration of IgG4-positive plasma cells are observed in most of organs [15]. The mechanisms of IgG4-related disease (IgG4-RD) have not been clarified. However, in a recent study, mice expressing ovalbumin (OVA) in pancreatic islets injected with OT-1 mouse T cells, which recognize the OVA antigen, in conjunction with OVA-specific IgG4 antibody exhibited

IgG4-RD-like inflammatory responses. Because the researchers observed suppression of the pro-inflammatory response after blocking the interaction between IgG4 and FcγR, they suggested that IgG4 may have activated DCs, which initiated the immune response, and led to OT-1 T cell activation [18].

The effector functions ADCC and CDC are associated with the structural differences of the IgG subclasses. The mechanism of action of ADCC is based on engagement of effector and target cells through the Fc-FcγR interaction. FcγRIIc and FcγRIIIa are expressed on NK cells and bind to the Fc of IgGs. These FcγRs have immune tyrosine-based activating motifs (ITAM) in their cytoplasmic tails. After binding to the FcγRs on NK cells, the ITAM domains become phosphorylated, which leads to granulation and cytokine production to kill virus-infected cells and tumor cells [19]. Meanwhile, in CDC, first, the antibody binds antigens on the cell membrane and the Fc interacts with complement component 1q (C1q) to form the active C1q complex. After complement activation, target cells are opsonized by C3-drived opsonins and pro-inflammatory mediators such as C3a and C5a are secreted to recruit neutrophils and macrophages for efficient phagocytosis [20]. The binding affinity of FcγR or C1q to the Fc region of Igs influences the strength of these effector functions. As IgG1 and IgG3 bind to FcγRs with high affinity, they have strong effector functions. In contrast, as the Fc regions of IgG2 and IgG4 have low affinity for FcγRs, they have weak effector functions. However, the affinity of IgG subclasses for FcγRs differs in different species, as depicted in Table 1.2. Mouse IgG2a and mouse IgG2b show greater ADCC and CDC activity than mouse IgG1 and mouse IgG3. Further, while rat IgG2a induces greater NK-mediated cytotoxicity than IgG2b [21], rat IgG2b induces greater CDC than rat IgG1 and rat IgG2a [22]. In addition to Ig subclasses, the genotypes of FcγRIIc and FcγRIIIa, and glycosylation of the Fc region can influence the affinity of Fc for FcγRs. Polymorphisms in FcγRIIIa at amino acid position

158 have been reported to affect ADCC. Specifically, FcγRIIIA-158-V/V leads to better clinical outcomes by an ADCC-inducing antibody than FcγRIIIA-158-V/F or FcγRIIIA-158-F/F, in which affinity for FcγRIIIA is low [19]. In addition, deglycosylation of the Fc region at amino acid position N297 decreases binding activity to FcγRs and C1q. Moreover, galactosylation of IgG1 decreases ADCC while lack of fucose increases ADCC and binding activity to FcγRIIIA [23].

Table 1.2. ADCC and CDC by each Ig subclass

Human	IgG1	IgG2	IgG3	IgG4
ADCC	High	Low	High	Low
CDC	High	Low	High	Low

Mouse	IgG1	IgG2a	IgG2b	IgG3
ADCC	Low	High	High	Low
CDC	Low	High	High	Low

Rat	IgG1	IgG2a	IgG2b
ADCC		High	Low
CDC	Low	Low	High

1.4 History of antibody drug discovery

Since hybridoma technology was introduced to produce monoclonal antibodies in 1974, a number of IgG antibodies have been approved as therapeutic agents which induce cytotoxicity against tumor cells or neutralizing activity against pathogenic molecules. The high specificity of antibodies is one of the reasons why they have become popular biological therapeutic agents, promising a wider therapeutic window compared to small molecule compounds. However, the complex development process, including the hybridoma method and manufacturing, requires greater monetary costs than small molecule compounds. Since these costs are

reflected in the price of the drugs, antibody therapeutic agents have mainly been developed for serious diseases such as cancer.

Here, I will summarize the history and features of antibody therapeutics to provide a deeper understanding of the structural differences in the IgG subclasses. The first antibody therapeutic was Muromonab-CD3 (Orthoclone OKT3), an anti-CD3 monoclonal antibody, which was approved by the Food and Drug Administration (FDA) in 1986 for the treatment of renal, cardiac and hepatic allograft rejection [24]. As OKT3 was derived from mice and showed immunogenicity to humans, chimeric antibodies and humanized antibodies were subsequently developed to reduce the immunogenicity [24]. A chimeric antibody is an antibody in which the variable and constant regions are derived from different species and a humanized antibody is an antibody in which the FWR and constant regions are derived from humans while only the CDR is derived from a non-human animal (Figure 1.5). Rituximab, an anti-CD20 antibody for treating non-Hodgkin lymphoma, was the first approved chimeric antibody and daclizumab, an anti-CD25 antibody for preventing transplant rejection, was the first approved humanized antibody [25]. In 1990, a novel technology was developed to obtain a fully human antibody to further reduce the immunogenicity risk. This technology was based on the phage display system and involved expressing the single chain fragment variable (scFv) or Fab expressed on phages to isolate an antibody clone directed at a target molecule. This is one of the most commonly used antibody isolation techniques today, and is discussed further below. Although these approved antibody therapeutics all have different mechanisms of action, they all contain human IgG1 Fc, which induces high ADCC and CDC relative to the other IgG subclasses. Although the effector functions of human IgG3 are greater than those of human IgG1, most antibody therapeutics in which effector functions contribute to their

therapeutic effects contain IgG1 Fc for the following reasons: The half-life of IgG1 is longer than that of IgG3, the longer hinge region of IgG3 can be digested in the body, and protein A affinity purification cannot be performed on IgG3 [26].

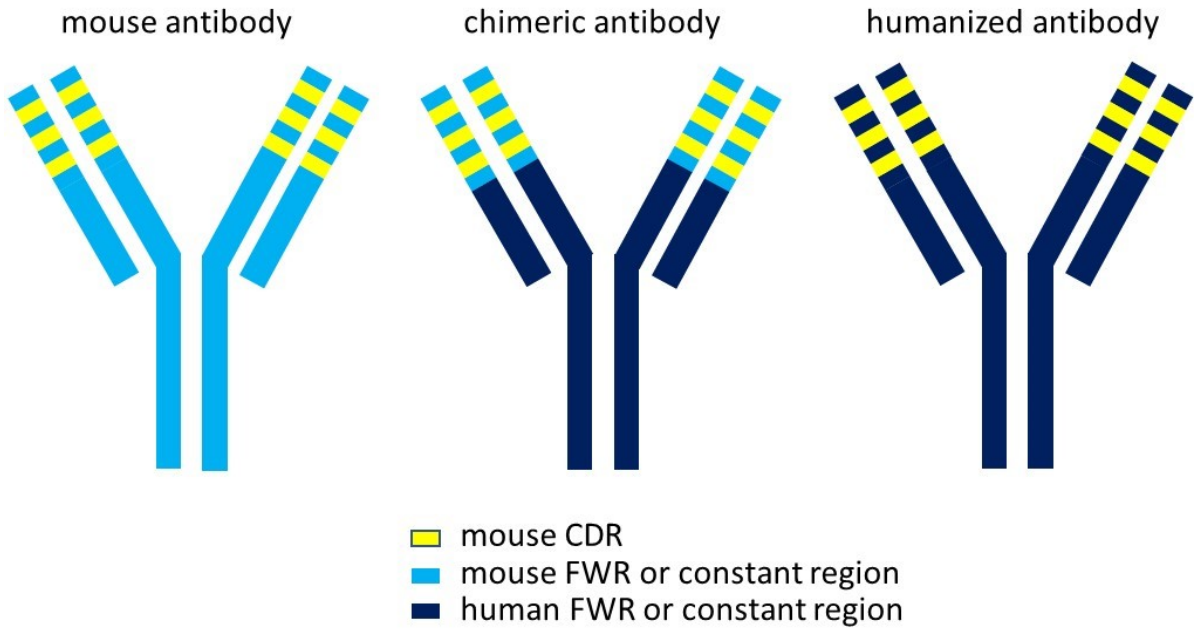


Figure 1.5. Structures of chimeric antibodies and humanized antibodies.

The Fc region in therapeutic antibodies is often modified to suit the intended application. An afucosylated IgG1 antibody was developed to treat cancer patients because its high ADCC had the potential to improve the therapeutic effect [27]. Conversely, IgG1 Fc containing substitutions such as L234A and L235A are widely used to treat cancer patients based on their immuno-oncology mechanism, since these mutations decrease affinity of the Fc for FcγRs and reduce toxicity to normal tissues. Other common substitutions in the IgG1 antibody are P331S and P331G, which attenuate binding to C1q to prevent unwanted adverse effects in normal tissues [28]. Further, IgG4 is commonly used to treat autoimmune disease patients to circumvent the adverse effects induced by effector functions. However, IgG4 is susceptible to half molecule exchange called

Fab-arm exchange, which is the exchange of a heavy and light chain unit of the therapeutic agent with the heavy and light chain unit of an endogenous IgG4 antibody. In IgG4, the amino acid sequence in the core hinge that forms the disulfide bond between the heavy chains is CPSCP, while the corresponding sequence in IgG1 and other subclasses is CPPCP. This difference in one amino acid residue prevents the formation of an intermolecular disulfide bond and promotes the formation of half antibodies consisting of one heavy chain and one light chain via intramolecular disulfide bonds. Half antibodies with different variable regions can subsequently form an intermolecular disulfide bond, leading to Fab-arm exchange. S228P substitution is commonly performed in the Fc region to inhibit Fab-arm exchange in therapeutic antibodies [29, 30]. Antibody therapeutic agents have become safer and more reliable due to these antibody engineering techniques.

1.5 Fragment antibodies

Not long after establishment of hybridoma technology, recombinant antibody production in *Escherichia coli* (*E. coli)* began to emerge. Advantages of using *E. coli* to express antibodies include: (1) better understanding of the physiology and metabolism of *E. coli* compared to other bacterial species; (2) faster growth speed compared to mammalian cells; (3) cheaper cost of growth medium [31]. However, the first study on antibody production in *E. coli* failed to obtain antibodies with any activity, a result that was thought to be due to improper protein folding in reducing intracellular conditions. Antibody heavy and light chains contain many intrachain and interchain disulfide bonds which do not form properly in reducing conditions. Thus, production of minimized antibodies such as scFv and Fab has since been studied in the periplasm space of *E. coli* which allows for the correct formation of disulfide bonds. This study of antibody production in *E. coli* contributed

greatly to the phage display system, which has enabled the production of several therapeutic agents. The typical scFv structure is depicted in Figure 1.6, in which VH and VL are combined by a flexible linker such as three repeats of GGGGS. Although the structure of scFv, an artificial sequence comprised of VH and VL, is unstable, this minimized domain antibody forms a key component of engineered antibodies and phage display technology [32].

Figure 1.6. Structure of scFv. VH and VL are genetically fused by a flexible linker (GGGGS). Generally, three or four repeats of GGGGS are used.

1.6 Methods for generating antibodies

Here, I will summarize the methods used to generate therapeutic antibodies (Figure 1.7). As mentioned above, hybridoma technology is commonly used to generate monoclonal antibodies. First, a purified protein of the target molecule is injected into an animal such as a mouse, rat, rabbit, camelid or chicken several times. This process is called immunization and is based on the fact that repeated exposure to antigens induces class switching of the antibody and SHM, and thus affords high affinity antibodies. Mature B cells produce IgG antibodies which recognize the injected antigen in the animal. Next peripheral or splenic B cells derived from the animals are fused to a myeloma cell line to generate hybridomas. Immortalized hybridomas secret IgG monoclonal antibodies into the cell culture supernatant. Finally, the hybridomas are isolated by limiting dilution or using an automatic cell picker and cultured for production of monoclonal antibodies, and the antibody sequences are analyzed [25].

In contrast, the phage display system, another commonly used method to generate monoclonal antibodies, does not require the immunization of animals but instead uses a library of antibodies displayed on phages. In the process of screening, also called panning, a purified antigen is immobilized on the solid surface of a polystyrene tube and mixed with phages displaying scFv or Fab antibodies. After repeated panning, phages displaying an antigen-specific antibody are infected into *E. coli* and sequenced to isolate monoclonal antibodies. Finally, the antibody sequence is cloned into an expression vector for *E. coli* or mammalian cells to produce antibodies. If a phage display library with sufficient diversity is available, the phage display system can be a quicker and easier strategy for generating monoclonal antibodies compared to the hybridoma method [25].

More recently, single B cell technology has emerged as a novel technique for antibody generation. After immunizing animals with the antigens of interest, antigen-specific B cells extracted from the spleen or lymph nodes are isolated using antigen-coated magnetic beads and fluorescence-conjugated antigens by flow cytometry [25]. A major advantage of this technology is the rapid isolation of monoclonal antibodies compared to the hybridoma method without the need for a fusion step [25].

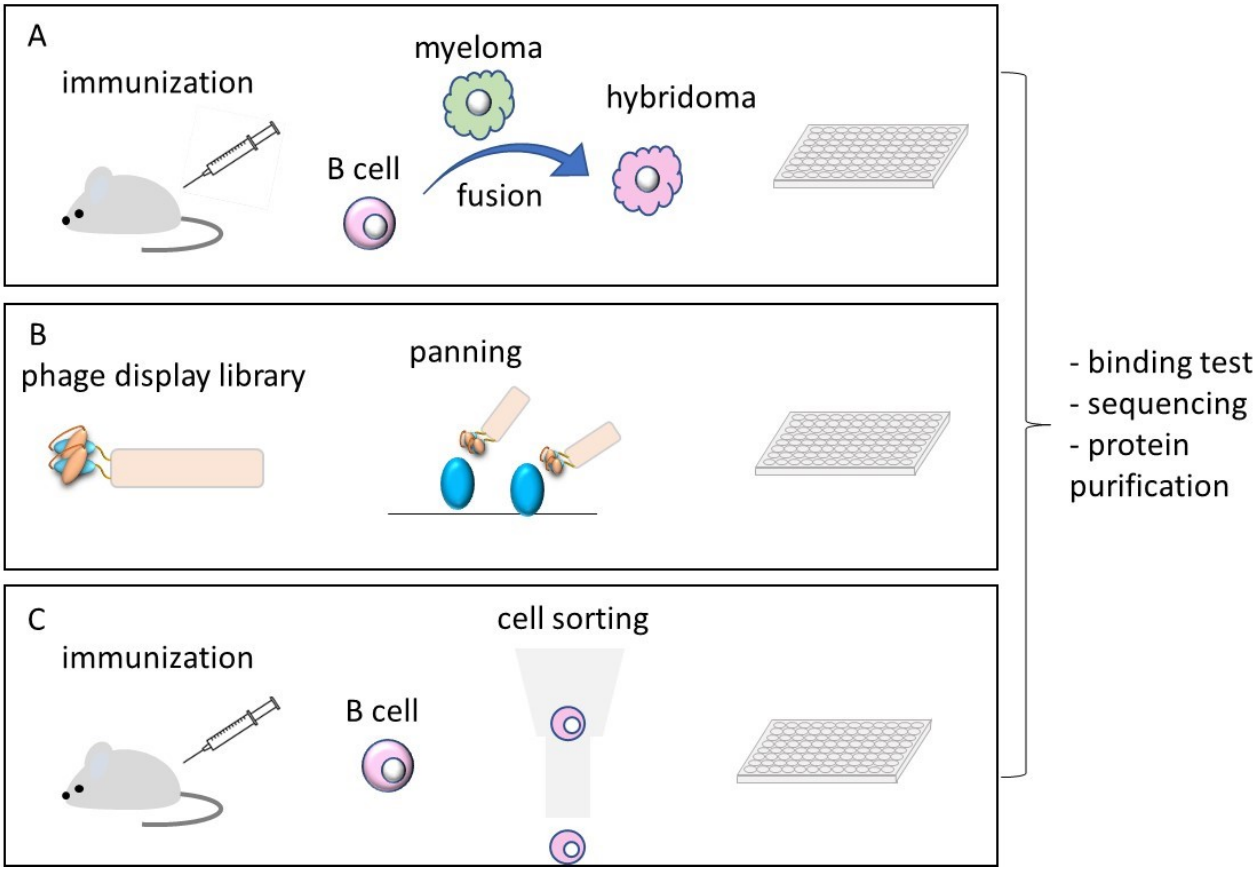


Figure 1.7. Methods for generating antibodies.

After isolating monoclonal antibody clones, the antibody sequence is analyzed and then inserted into an expression vector for recombinant antibody production in mammalian cells. Purified antibody clones are subjected to screening and characterization with the aim of identifying a lead antibody which is a representative clone with sufficient therapeutic potential. The antibody's agonistic or antagonistic function and binding specificity are then evaluated to identify potential features that may make it superior to existing therapeutic agents. Evaluation of cross-reactivity to homologues in other species is also crucial. This is because evaluation of drug efficacy is commonly performed in rodent models, while pharmacokinetic (PK), pharmacodynamic (PD) and toxicologic tests are performed using non-human primates. Finally, the physicochemical properties of the antibody are carefully checked to determine the best formulation, storage conditions and administration

route of the therapeutic agent. If there are amino acids which may reduce stability in the sequences of lead clones, the amino acid sequence of VH and VL should be modified by introducing amino acid substitutions. Several post-translational modifications have been shown to contribute to structural instability and affect the biological activity of an antibody. For example, asparagine deamidation has been reported to affect binding and PK. The amide in the side chain of asparagine and glutamine is removed in the deamidation chemical reaction under physiological conditions. The frequency of asparagine deamidation depends on the amino acid that follows asparagine, with more than 50% of peptides that include an asparagine-glycine sequence shown to undergo deamidation [33, 34]. Another example is that oxidation of methionine, tryptophan and histidine affects biological activity and PK. Tryptophan oxidation in CDRs reportedly influences binding activity and specificity. Additionally, methionine oxidation is frequently found in the Fc and affects its affinity for FcRs, but is less commonly observed in CDRs. High levels of methionine oxidation in the Fc may affect yields of the purified protein by lowering the affinity of Fc for protein A [33, 35, 36]. After optimizing the antibody sequence, only one lead clone with sufficient developability advances through to the large-scale recombinant antibody production step to generate sufficient amounts for clinical trials.

1.7 Generation of recombinant antibodies

Antibody purification is commonly performed by affinity chromatography using protein A or protein G. Protein A, which can be extracted from the cell wall of the gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*), was found to bind strongly to the Fc of IgG in the 1970s [37]. Protein G, which is also a bacterial protein, preferably binds to the Fc of IgG with an equilibrium dissociation constant (K_D) of about 10 nM, but

binds to Fab with much lower affinity. Protein A is more commonly used for antibody purification because it binds to IgG with the same affinity range as protein G and allows for elution in solutions with less acidic pH. In addition, protein L derived from *Peptostreptococcus magnus* is known to bind to the κ light chain with a K_D of about 100 nM, as well as Fab and scFv, and is commonly used to purify recombinant scFv or Fab antibodies without tag conjugation [37, 38].

After affinity chromatography, the purity of the purified antibody is often checked using size exclusion chromatography (SEC), in which proteins are separated by their physical size. Despite the development of many protein analysis methods, SEC is preferred for its speed and reproducibility. SEC uses the automated separation systems of high performance liquid chromatography (HLPC) while affinity chromatography can be manually performed for small-scale purification [39]. These chromatography methods produce antibodies of sufficient purity for functional evaluation [40].

In the screening process for lead antibodies, the yield of the purified antibody is a crucial parameter. Antibody drug products are commonly produced using mammalian cells such as Chinese hamster ovary (CHO) cells and NS0 murine myeloma cells that are transfected with an expression vector containing the antibody gene sequence. These host cells have been modified to obtain high yields of the purified antibody protein. The lack of endogenous glutamine synthase (GS) enzyme activity in NS0 and CHO-K1 cells enables them to secrete large amounts of antibodies by methionine sulfoximine (MSX) selection. DG44 and DUXB11, other lineages of CHO cells which do not have dihydrofolate reductase (DHFR) activity, are also suitable for methotrexate (MTX) selection [41, 42]. To establish stably-expressing cell lines, GS or DHFR genes are required in the antibody expression vector for MSX or MTX selection. Selection based on these genes

reportedly contributes to the establishment of cell lines with high yields of the antibody of interest (Figure 1.8). A stable transfection requires co-transfection of two expression vectors, one harboring an antibody heavy chain gene and the other a light chain gene, or a bicistronic vector harboring both the heavy and light chain genes [43]. The protein yield is also affected by expression levels of the genes encoding the antibody heavy and light chains. Further, the expression of recombinant antibodies can be affected by a difference in codon bias between the immunized host and the host used for expression. This issue can be resolved by performing codon optimization on the genes encoding the heavy and light chains by selecting suitable codons for the host used for expression. Today, codon optimization is commonly performed through a commercial service at the time of gene synthesis to modify the antibody sequence in the humanization process described later. These ingenuities improve the protein yield of antibody therapeutics and ultimately reduce the cost of antibody generation.

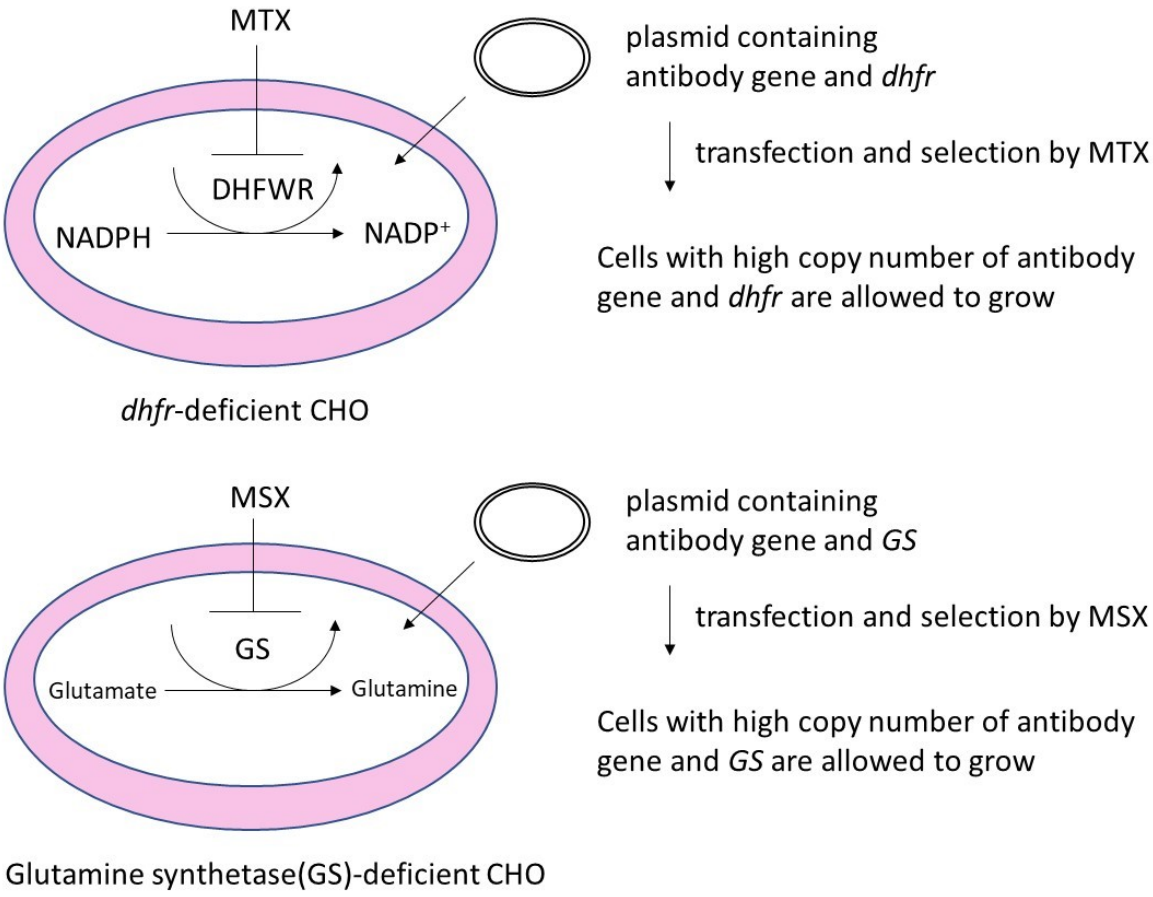


Figure 1.8. Gene expression systems.

1.8 Humanization of antibodies for reducing immunogenicity

Humanization is an indispensable technique for reducing the immunogenicity of antibody drug agents derived from non-human hosts in the human body. Once antibody therapeutics are recognized as extrinsic antigens, anti-drug antibodies (ADA) are produced by B cells to eliminate the drug agents. Immunogenicity is a common issue for therapeutic antibodies because ADAs neutralize the effects of the therapeutic antibodies and might even induce adverse effects in patients. Various factors can induce ADA production, including therapeutic antibodies with low purity, features of the target molecule and disease state. ADA production is thought to arise from an immune reaction involving CD4-positive T cells presented exogenous antigens displayed by class II

MHC on APCs, although details of the mechanism remain unclear. Additionally, T cell-independent ADA formation has also been reported, which suggests that aggregates of an antibody may induce ADA by cross-linking B cell receptors for B cell activation. Another crucial factor in ADA formation is the target molecule of the therapeutic antibody. Alemtuzumab, which targets CD52, induces ADA in about 85% of multiple sclerosis patients. Since CD52 is expressed on APCs including B cells, DCs and monocytes, alemtuzumab, despite being a humanized antibody, might be taken into these cells and be recognized as a non-self antigen. Moreover, the disease state of patients also affects ADA formation. High frequency ADA formation has been reported in patients with autoimmune disease as a hyperactive immune status can enhance antigen presentation [44].

Antibody humanization was made possible by the development of chimerization technology, which combines the variable domain of murine antibodies with the human Fc domain to reduce immunogenicity, introduced in the first half of the 1980s. Initially, although chimeric antibodies retained the specificity of their parental murine antibody, immunogenicity was a problem. In the second half of the 1980s, Winter *et al*. used CDR grafting to increase the similarity with a human antibody. CDR grafting, which is based on the technique of transplanting the CDRs of the parental antibody into the FWRs of the human antibody, needs a standardized numbering scheme to precisely identify the CDRs in the donor antibody [45] (Figure 1.9).

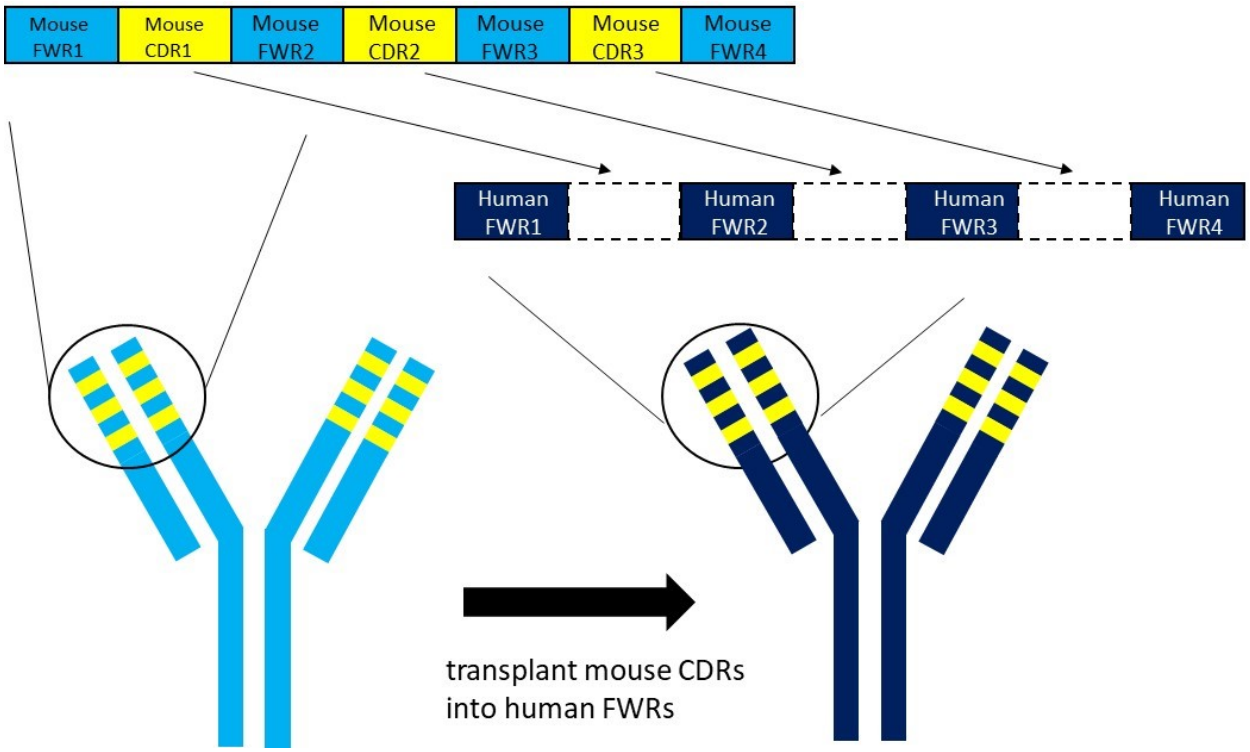


Figure 1.9. Illustration of the CDR grafting process.

After CDR grafting, back-mutation is required to prevent loss of affinity that is often induced by incompatibility between the CDRs in the donor and FWRs in the recipient antibody sequences. As some amino acids in the FWRs act to stabilize the structure of the CDR loops, these amino acids should be retained after CDR grafting to preserve the affinity of the humanized antibody. The process of reverting the amino acids in the FWRs that stabilize the CDR structure back to those in the parent sequence after CDR grafting is referred to as back-mutation. Studies on humanization have shown that the optimal position and specific amino acids to target in back-mutation vary depending on the case. Hence, back-mutation should be carefully designed by modeling the structure of the parental antibody and identifying amino acids in the FWRs responsible for stabilizing the structure of CDR loops [10]. Today, the occurrence of ADAs following treatment with antibody drugs has decreased thanks to this humanization technique [44].

Another approach for decreasing immunogenicity is to use a fully human antibody, which can be generated using transgenic animals such as XenoMouse (Abgenix), HuMabMouse (Medarex), OmniRat (Ligand), KyMouse (Kymab), VelocImmune mice (Regeneron). In 1994, HuMabMouse, the first humanized transgenic mouse, was generated based on a mouse that lacked the heavy and kappa chain genes. The human heavy and light chain genes in HuMabMouse are less than 80 kb in size, even though in the genome they are 1.29 Mb and 1.39 Mb, respectively. Since antibody diversity is dependent on the recombination of germline VDJ genes, introducing larger size heavy chain and light chain genes into a mouse is expected to lead to greater antibody diversity. XenoMouse was also generated by introducing the human heavy chain and light chain into a mouse lacking the heavy and light chain genes. In contrast to HuMabMouse, yeast artificial chromosome (YAC) vectors were used to introduce larger size human genes into XenoMouse (heavy: 700 kb, light: 1 Mb) for greater antibody diversity. Although greater diversity was expected in XenoMouse, this was not observed because Ig class switching and SHM, both of which affect the efficiency of antibody generation, were abnormal due to the lack of a mouse constant region, which acts to reduce interactions of the Fc and facilitate an efficient immune response in the adaptive immune system. OmniRat, in which human VH and VL genes are linked to rat constant regions, was generated in 2013 by transfecting fertilized rat oocytes with bacterial artificial chromosome (BAC) and YAC vectors [46]. KyMouse and VelocImmune mice were generated by transfecting mouse embryonic stem cells with BAC vectors harboring human VH and VL genes so that the human antibody variable regions are linked to the upstream region of mouse constant region genes [47]. These animals efficiently produce chimeric antibodies with high affinity for their target molecules through Ig class switching and SHM. The constant region of chimeric antibodies produced by these animals is swapped with that of

human antibodies when used as a therapeutic agent to reduce immunogenicity [25]. These humanized mice and humanization techniques have accelerated the antibody generation process needed to develop antibody therapeutics for clinical use.

Table 1.3. Humanized mouse technologies

Name of technology	Size of introduced genes	Constant region	Year
HuMabMouse	heavy and light <80Kb	human	1994
XenoMouse	heavy: 0.7Mb, light: 1Mb	human	1997
OmniRat	heavy: 0.4Mb, light: 0.6Mb	rat	2013
KyMouse	heavy: 1Mb, light: 1.7Mb	mouse	2014
VelocImmune mouse	heavy: 1Mb, light: 0.5Mb	mouse	2014

1.9 Bispecific antibodies

Bispecific antibodies, which have become a major research focus, have been developed for serious diseases that do not respond to a single therapeutic agent because they have multiple causes that induce complex reactions in the human body. A bispecific antibody is an antibody composed of two variable domains that target different molecules (Figure 1.10). For example, multiple cytokine inhibitors such as interleukin (IL)-4 x IL-13 bispecific antibodies have been developed by Sanofi and Glaxo Smith Kline for the treatment of idiopathic pulmonary fibrosis, and Jansen R&D is developing an epidermal growth factor receptor (EGFR) x c-mesenchymal-epithelial transition factor (c-MET) bispecific antibody which inhibits cross-talk of the signaling pathways of these receptor for the treatment of cancer [25].

Another unique type of bispecific antibody is the bispecific T cell engager, which recognizes CD3 on T cells and tumor-associated antigens on cancer cells to redirect T cells to tumor cells. Blinatumomab, the first bispecific T cell engager (BiTE) to receive approval (approved in 2014), recognizes CD3 and CD19 to

engage T cells and cancerous B cells. This engagement of T and tumor cells promotes anti-tumor T-cell-mediated killing. While T cells recognize cancer-associated antigens displayed on MHC using their T cell receptor (TCR), tumor cells often lose the MHC on their cell membrane to circumvent the host immune system. Introduction of this T cell bispecific engager, and subsequently anti-programmed cell death protein 1 (PD-1) antibodies, improved treatment prospects in immuno-oncology. In fact, blinatumomab was designated as breakthrough therapy by the FDA.

As blinatumomab is composed of two different scFv, this bivalent scFv antibody has been studied to produce bispecific antibodies with the simplest structure possible. Studies of scFv have been performed since 1988 and have reported undesirable physicochemical properties such as a propensity for aggregation and low thermostability compared to the parental monoclonal IgG antibody [48]. To stabilize the structure of scFv, researchers have introduced an interchain disulfide bond by substituting VH44-VL100 residues with Cys residues [49].

The disulfide-stabilized Fv (dsFv) has been leveraged for generating tetravalent bispecific antibodies (IgG-dsFv), in which dsFv is genetically fused to the heavy or light chain [50]. This tetravalent antibody was derived from the IgG-scFv first reported in 1997 [51] (Figure 1.10). Many studies have reported that the location of scFv affects the feasibility of obtaining a stable and functional IgG-scFv [50, 52-54]. A study of a dual variable domain immunoglobulin (DVD-Ig) reported that fusing an additional variable domain to the amino terminus (N-terminus) of IgG reduced the affinity of the inner variable domain [55] (Figure 1.10). Additionally, lower yields have been reported for IgG-scFv in which scFv is fused to the N-terminus of a heavy or light chain. These studies suggest that the fusion position of scFv should be validated for novel IgG-scFv

antibodies.

DVD-Ig is a type of tetravalent bispecific antibody developed by AbbVie. In DVD-Ig, an additional heavy and light variable domain is fused to the N-terminus of an IgG in which a short peptide linker connects each heavy variable domain with each light variable domain (Figure 1.10). Although this dual variable domain structure is stable, steric hindrance is an issue. While IL-12 x IL-18 DVD-Ig has been shown to have comparable inhibitory activity to its parental antibodies [56], the binding affinity and inhibitory activity of some types of IL-1α x IL-1β DVD-Ig are less potent than that of the parental antibodies [57]. The inhibitory activity of the DVD-Ig reportedly changes depending on the length of the linker between the two variable regions in the heavy and light chains. The flexibility and orientation of VH and VL affect inhibitory activity especially in the inner variable domains [57].

Most protein engineers avoid including tandem repeats in the variable domains because of this issue and prefer IgG-like bivalent bispecific antibodies [58]. Such IgG-like bivalent bispecific antibodies are generated by co-expressing two different heavy chains and light chains. However, correct pairing of bispecific antibodies leads to a rational yield of only 12.5% using this method [59]. For more efficient antibody production, the knobs-into-holes (KiH) method was proposed in 1952 to join the amino acids of adjacent alpha-helices (Figure 1.10). This technology was leveraged to generate a CD3 x CD4 bispecific antibody in 1996 [60]. A small amino acid in the CH3 of an anti-CD4 IgG was replaced with a larger one to generate a 'knob' (T366Y), while a large amino acid in the CH3 of an anti-CD3 IgG was substituted for a smaller one to generate a 'hole' (Y407T). Using KiH increased yields of the CD3 x CD4 IgG heterodimer to up to 92%, while co-expression of anti-CD3 and anti-CD4 IgG heavy chains harboring wild-type CH3 yielded just 57% CD3 x

CD4 IgG heterodimer [60]. However, although the KiH method markedly improved yields of heterodimeric heavy chains, mispairing of heavy and light chains continued to lead to low yields of bispecific antibodies. The simplest way to prevent the mispairing is to use a common light chain, which can be obtained using phage display technology. Currently, other technologies such as KiH used in the light chain and CrossMab are leveraged to avoid the mispairing of heavy and light chains [61]. These formats are well-established and leveraged to develop bispecific antibodies used in clinical trials. A limitation of these formats, however, is that they produce bispecific antibodies with lower affinity for their antigen because they bind to the antigen with their monovalent arm. Tetravalent bispecific antibodies should have high affinity when used as antagonistic antibodies to ensure complete inhibition of target functions.

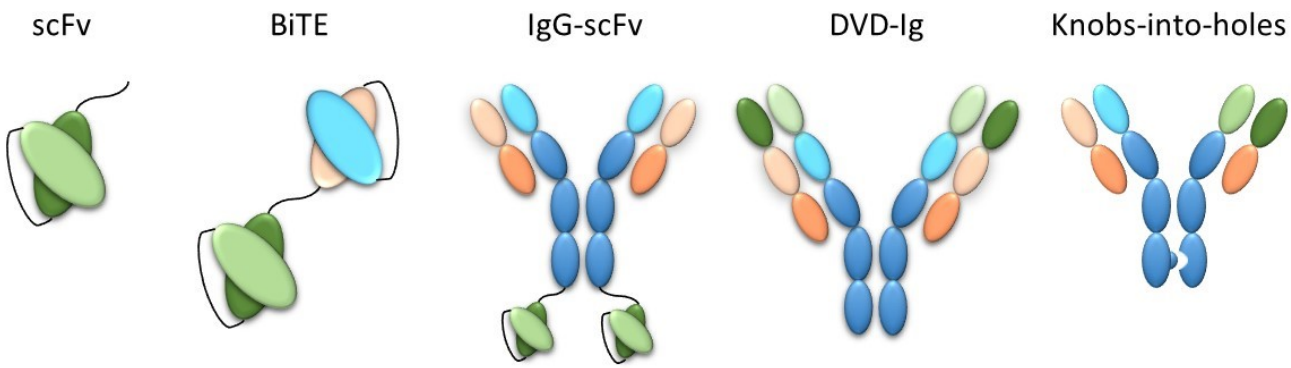


Figure 1.10. Structure of bispecific antibodies. A scFv consists of only VH and VL connected by a short peptide linker. BiTE is a bivalent bispecific antibody comprising two scFvs connected by a peptide linker. IgG-scFv is a tetravalent bispecific antibody of which scFv is fused to N or C-terminus of heavy or light chain. DVD-Ig possesses additional heavy and light variable domains linked in tandem by short peptide linkers to the N-terminus of IgG. Asymmetric bivalent bispecific antibody is generated by Knobs-into-holes which induce heterodimerization.

1.10 Inflammatory diseases

As mentioned above, bispecific antibodies are an effective approach for treating complex diseases as they

target multiple molecules of interest. To date, bispecific antibodies have mainly been developed to treat inflammatory diseases and cancer. Among inflammatory diseases, sepsis is a serious disease that affects a large number of patients, for whom no effective treatment has yet been approved. Sepsis is a life-threatening condition which results in organ dysfunction and death. It is caused by an overreaction of the innate immune system which normally acts to protect the host from infection. Since sepsis has been reported to be associated with Toll-like receptor 4 (TLR4), TLR4 inhibitors have been developed to treat the condition to date. However, all trialed TLR4 inhibitors have failed. The complex mechanism of sepsis, which involves multiple molecules, may explain why all TLR4 inhibitors have failed to show sufficient efficacy in clinical trials. Although a bispecific antibody which inhibits multiple signaling pathways contributing sepsis may be effective for treating sepsis, no bispecific antibodies have been tested in clinical trials.

1.11 The TLR family and its signaling pathways

TLR family members have established roles in several inflammatory diseases, including systemic lupus erythematosus (SLE), sepsis, asthma, inflammatory bowel disease (IBD) and chronic obstructive pulmonary disease (COPD) [62]. Agonists of TLR7/9 such as IMO-3100 and IMO-8400 have been developed for patients with psoriasis [63]. NI-0101, a monoclonal antibody against TLR4, has been developed for patients with rheumatoid arthritis (RA) [64]. Since TLR family members are thought to be attractive targets for treating various diseases, I expect that successful generation of a monoclonal antibody or bispecific antibody that targets TLRs will be a great contribution to society and the life sciences field.

TLR is a homologue of the *Drosophila melanogaster* Toll receptor. Ten human TLRs have been

reported. TLR1/2/4/5/6 exist on the cell membrane where they recognize lipids or proteins, while TLR3/7/8/9 are found on the membranes of the endoplasmic reticulum (ER) and endosomes and respond to signals in the nucleus. TLR is a type of pattern recognition receptor (PRR), which recognizes pathogens such as viruses, bacteria and fungi. PRRs are expressed on macrophages and neutrophils and initiate an immune reaction in the innate immune system, which is the body's first line of defense against infection. Macrophages exist in all tissues and are the first to recognize a microorganism. In contrast, neutrophils are not found in healthy tissues, but are recruited to infected tissues by macrophages. PRRs respond to common patterns found in bacterial cell walls called pathogen-associated molecular patterns (PAMPs). TLR, nucleotide-binding oligomerization domain-like receptor (NLR), and retinoic acid inducible gene-I like receptor (RLR) are all PRRs [65, 66]. When these receptors are activated, macrophages and neutrophils begin to release pro-inflammatory cytokines and perform phagocytosis, a type of receptor-mediated endocytosis in which cells eat microorganisms, cell debris and apoptotic cells. After endocytosis by phagosomes, the microorganisms are transported to lysosomes to be digested. These initial reactions of the innate immune system rapidly regulate early-stage infection, which is in contrast to the adaptive immune system, where DCs, T cells and B cells play a central role in controlling the long-term immune reaction, as described above.

TLR4 is one of the most studied receptors and is a key sensor of lipopolysaccharide (LPS). Pro-inflammatory cytokines are released downstream of the TLR4 signaling pathway. TLR4 and its co-receptor myeloid differentiation protein-2 (MD2) form a heterodimer before LPS binds to a large hydrophobic pocket of MD2 [67]. The dimer of TLR4-MD2-LPS complexes that forms after LPS recognition leads to the activation of two downstream signaling pathways (Figure 1.11). TLR4 signaling is mediated by the adaptor proteins

myeloid differentiation factor 88 (MyD88) and Toll/Interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF), which interact with the intracellular domain of TLR4. Both TLR4 and MyD88 have a TIR domain which they use to interact with each other in association with another adapter protein, MyD88-adapter-like (Mal). This interaction is a pivotal step for transmission of the TLR4 signal into the cytosol [68]. The MyD88-dependent signal is transmitted to IL-1 receptor-associated kinase (IRAK) by the interaction of MyD88 with IRAK at the death domains of the two molecules and activates transcription factors of nuclear factor kappa B (NF-κB), which leads to the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6. The MyD88-dependent pathway is involved in the signaling of all TLRs except for TLR3. Mal is required for sufficient signal transduction only for TLR2 and TLR4 in the MyD88-dependent pathway. In the TRIF pathway, the TLR4-LPS signal is transmitted to TRIF-related adaptor molecule (TRAM) and activates TRAF3 together with TRIF. The TRIF signal ultimately activates IFR3 and leads to secretion of type I interferons (IFN) [67] (Figure 1.11). Type I IFNs (IFN-α, -β, -ω, -κ and -ε) were first reported in 1957 to interfere with viral replication. Type I IFNs activate a heterodimer of IFN receptors (IFNRA1 and IFNRA2) and lead to the activation of IFN-stimulated gene factor 3 (ISGF3), which is composed of signal transducer and activator of transcription 1 (STAT1), STAT2 and IFN regulatory factor 9 (IRF9). ISGF3 translocates to the nucleus and affects the transcription of hundreds of IFN-stimulated genes, the protein products of which play key roles in the anti-virus response [69].

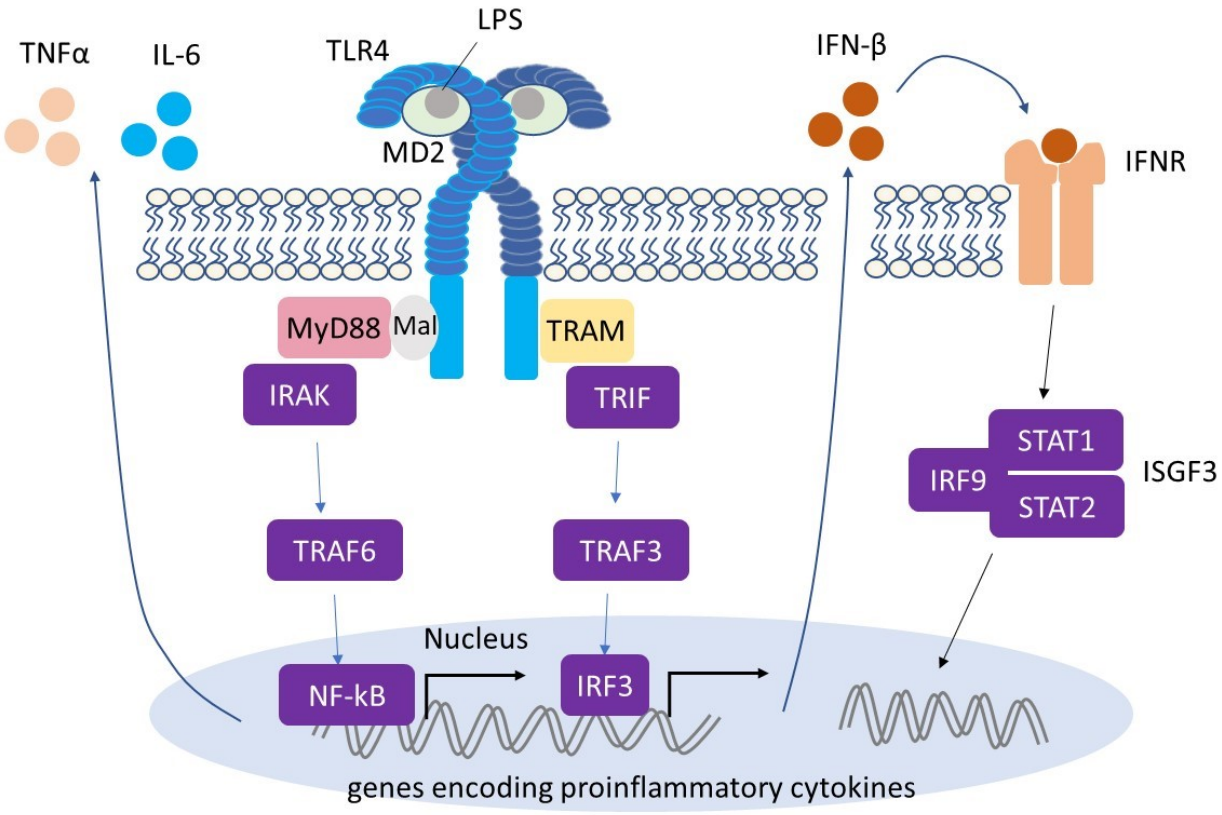


Figure 1.11. TLR4 signaling pathway.

Other crucial pro-inflammatory cytokines related to TLR4 are TNF-α and IL-6, which are produced downstream of the Myd88-dependent pathway. TNF-α was discovered in studies of necrosis in human tumors induced by bacterial endotoxins. Researchers speculated that the endotoxin-mediated tumor necrosis was indirect and TNF was subsequently found in the serum of mice that had been infected with bacillus Calmette-Guérin and treated with endotoxin. After confirming that serum containing TNF had the same tumor necrotic effect on several types of tumor, researchers cloned the cDNA of human TNF-α. Today, TNF-α is a well-known member of the TNF/TNF receptor (TNFR) super family, which includes more than 40 molecules [70]. TNF-α is thought to have a pivotal function as a master regulator of the pro-inflammatory cytokine cascade, because it is immediately released after exposure to bacteria-derived LPS, at early stages of infection and in autoimmune diseases. TNF-α also promotes the production of lipid signal transduction mediators such as

prostaglandins and platelet activating factor. Therefore, TNF-α has been proposed to play important roles in cell activation and recruitment in acute and chronic inflammatory diseases [70, 71].

TNF-α is mainly produced from macrophages downstream of TLR4 activation. Treatment of a mouse macrophage cell line with LPS has been showed to lead to production of IFN-γ. Macrophages are activated by TNF-α in the presence of IFN-γ, and this activation enhances NF-κB activation. Activated macrophages migrate to sites of infection and phagocytose pathogens, which are subsequently lysed via lysosomes. In addition to its pro-inflammatory function, TNF-α also contributes to the homeostasis of macrophages. Autocrine signaling of TNF-α facilitates long-term survival of macrophages, which is related to septic shock. Moreover, TNF-α is thought to play a role in the differentiation of macrophages because gene transcripts of TNF-α have been observed during bone marrow-derived macrophage differentiation. Based on these results, TNF-α may be essential for homeostatic regulation of macrophages in the pro-inflammatory state.

IL-6 is another essential and pleiotropic cytokine related to maturing B cells and proliferation of thymic and peripheral T cells which plays crucial roles in the immune system. Several studies have reported distinct functions for IL-6 under different names, such as B-cell stimulator factor, which induces differentiation of activated B cells, hepatocyte-stimulating factor, which enhances growth of hybridoma, and IFN-β2, which has antiviral activity. In 1989, these factors were renamed IL-6 [72].

IL-6 binds to a heterodimer of the IL-6 receptor (IL-6R) and glycoprotein 130 (gp130) that forms a hexameric complex that includes IL-6 itself. IL-6R exists as membrane-bound IL-6R (mIL-6R) or soluble IL-6R (sIL-6R), each of which plays different roles (pro-inflammatory or anti-inflammatory function). mIL-6R is expressed in hepatocytes, monocytes, neutrophils and T cells where it performs normal physiological functions

via a classical signaling pathway which results in anti-inflammatory response (Figure 1.12). sIL-6R is shed from mIL-6R by proteolytic cleavage by metalloproteinases such as disintegrin, metalloproteinase domain-containing protein 10 (ADAM10) and ADMA17 or by alternative splicing of mIL-6R mRNA. Trans-signaling derived from the sIL-6R and gp130 complex can be observed in all gp130-expressing cells and leads to pro-inflammatory responses. Both classical and trans-signaling activate Janus kinase (JAK)-STAT, protein kinase B (AKT) and the mitogen-activated protein kinase (MAPK) pathways, which induce cell proliferation, differentiation and apoptosis. While the release of cytokines in the classical signaling pathway leads to feedback inhibition via suppressor of cytokine signaling proteins (SOCS) to regulate overactivation, increased transcription of cytokines is induced in the trans-signaling pathway due to suppression of SOCS. The versatile effects of IL-6 are associated with various diseases, such as cancer, rheumatoid arthritis, asthma, multiple sclerosis, Alzheimer's disease and IBD [72].

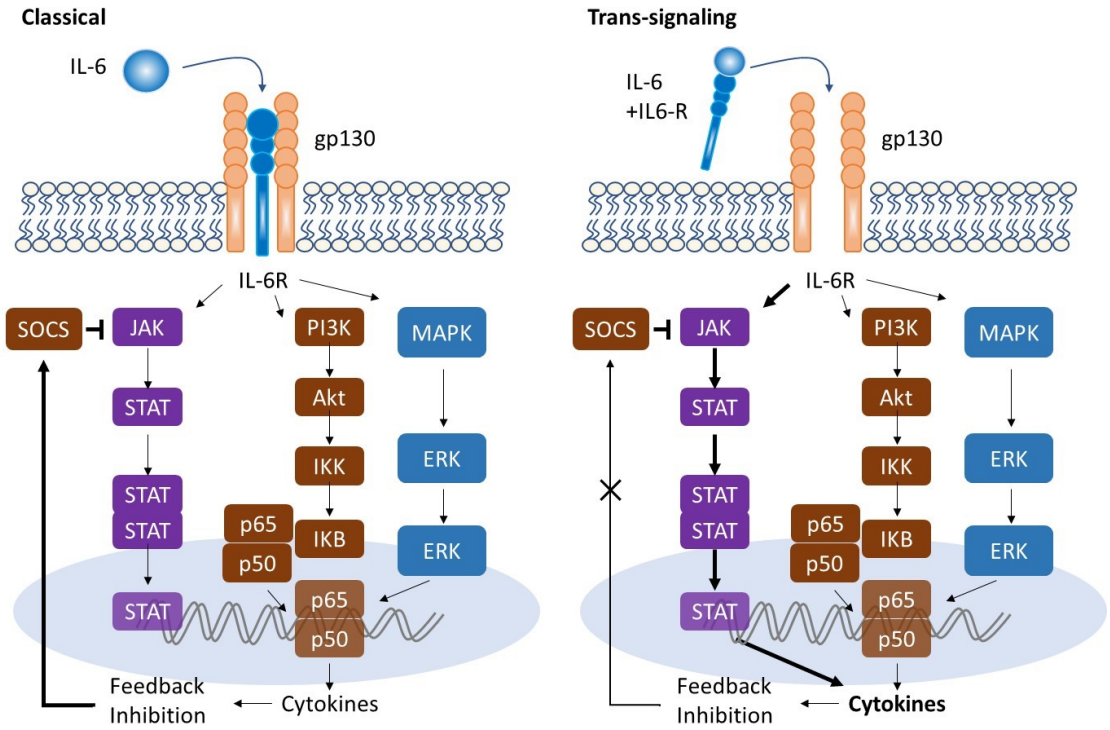


Figure 1.12. IL-6 signaling pathways.

Since pro-inflammatory cytokines secreted downstream of TLR4 such as TNF-α and IL-6 affect the proliferation and activation of multiple cell types, TLR4 is associated with several diseases. Sepsis is one of the most severe diseases with high mortality rate related to TLR4 [73]. Although sepsis is reportedly related to TNF-α and IL-6, gene knockout (KO) and inhibitors of these cytokines only produce a partial beneficial effect [74, 75]. As a result, despite the conduct of numerous clinical trials, no drugs have been approved for the treatment of sepsis, including anti-TNF-α antibodies [76]. Therefore, further research and development of therapeutic agents for sepsis should take into account the disease mechanism. Given that several cytokines (IL-8, IL-12, IL-18, and IFN-γ as well as TNF-α and IL-6) are upregulated in patients with sepsis, the ideal therapeutic agent should inhibit all of these pro-inflammatory responses to regulate systemic cytokine release syndrome in the treatment of sepsis [77]. Additionally, levels of high-mobility group box-1 (HMGB1), a type of damage-associated pattern, reportedly increase in the serum and tissues of septic patients [77]. Since HMGB1 responds to TLR4 in association with LPS and induces a pro-inflammatory response, it should also be targeted in treatments for sepsis [78]. Based on previous studies and clinical trials on treatments for sepsis, inhibiting cytokines produced downstream of inflammatory responses may have limited efficacy for treating sepsis. Thus, to design a robust treatment strategy, the mechanisms and pathophysiology sepsis should be thoroughly investigated in animal disease models.

1.13 Mechanism of sepsis

Here, I will summarize the physiology and mechanism of sepsis to identify potential target molecules which play central roles in sepsis. Individuals who develop systemic inflammatory response syndrome through

infection are diagnosed with sepsis. After infection, the innate immune system initiates an early immune response in which immune cells engulf pathogens after antigen recognition. APCs such as macrophages, B cells and DCs recognize components of bacterial cell walls such as LPS through TLR family members. This early immune response induces the production of pro-inflammatory cytokines for further activation of immune cells such as macrophages at sites of infection. DCs activate T cells, and helper T cells support B cell maturation for class-switching and antibody production [79].

In addition to the pro-inflammatory immune response, an anti-inflammatory immune response is concomitantly induced after infection (Figure 1.13). Myeloid-derived suppressor cells (MDSCs), which are known to induce immune suppression, secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β). Once activated, T cells express programmed death protein 1 (PD-1) to inhibit overactivation, which leads to apoptosis of the T cells themselves. Although controlled apoptosis of T cells is beneficial for host defense once the pathogens have been eliminated, inhibition of the pro-inflammatory response in patients with sepsis aggravates the disease state [79].

Given these complex mechanisms of sepsis, balancing pro-inflammatory and anti-inflammatory responses is thought to be the best strategy for treating sepsis. For example, combining a TLR4 inhibitor with a PD-1 inhibitor may improve the survival of sepsis patients. Although a clinical trial of an anti-PD-1 antibody for sepsis patients has been reported [80], dual blockade of TLR4 and PD-1 has not. Given that the timing of the activation of TLR4 differs from that of PD-1, a TLR4 inhibitor should be administered in the early stages of sepsis, while a PD-1 inhibitor should be administered after the pathogen has been eliminated. However, it would be difficult to design the optimal dosing time for these two inhibitors because of the rapid progression

of the septic immune response.

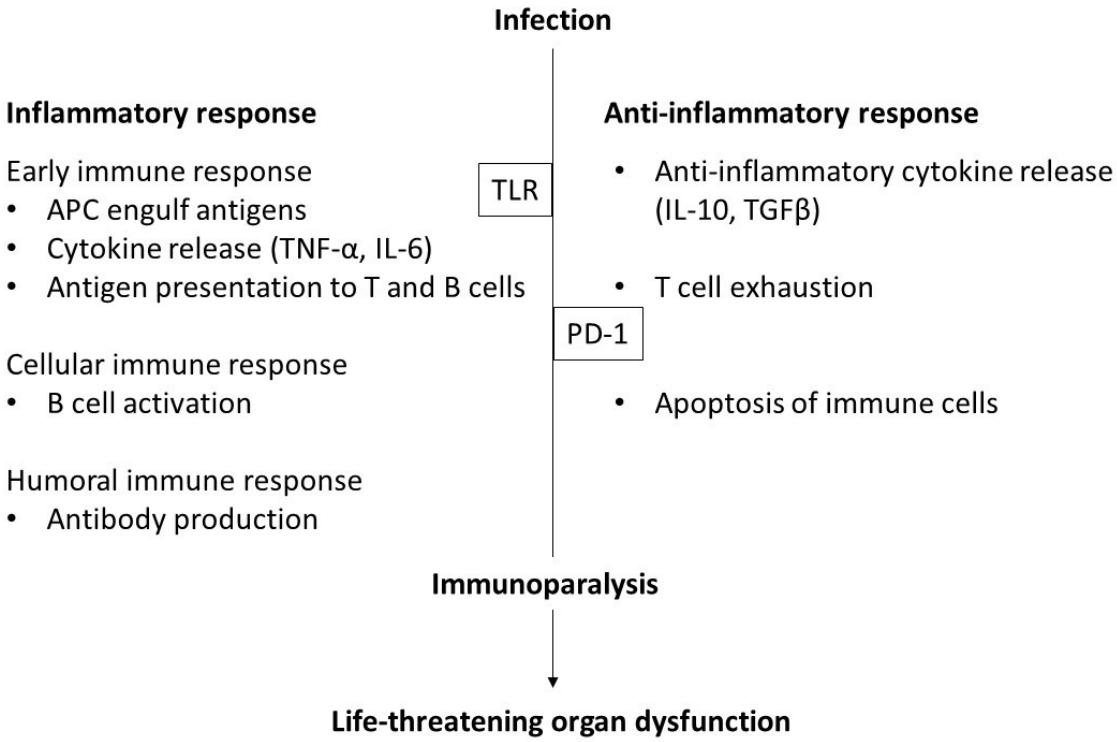


Figure 1.13. Changes in the immune response with the progression of sepsis.

1.14 Research outline

I designed the research to generate a novel therapeutic agent for treating sepsis as follows: (1) Generation of an anti-rat TLR4 antibody to clarify the contribution of TLR4 to sepsis, (2) Generation of anti-human TLR2 and anti-human TLR4 antibodies for engineering a bispecific antibody that targets human TLR2 and human TLR4 and (3) Analysis of the binding mode of a bispecific antibody targeting TLR2 and TLR4. First, an anti-rat TLR4 antibody was generated. Since inhibitors of TLR4 and gene KO of TLR4 lead to improved survival of septic rodent models [81-83], a TLR4 inhibitor may be effective for treating sepsis. However, because the efficacy of TLR4 inhibition varies among septic models, the contribution of TLR4 remains unclear.

Additionally, the optimal dosage timing for a TLR4 inhibitor has not been determined. The time of sepsis onset is uncertain in most patients because they are often hospitalized after they notice unusual symptoms. Thus, as therapeutic agents can only be administered after the onset of sepsis, TLR4 inhibitors should be effective once sepsis has been initiated. A novel TLR4 blocker that completely inhibits TLR4 would be a useful tool for investigating the contribution of TLR4 to sepsis and the optimal dosing regimen for TLR4 inhibitors.

Next, a bispecific antibody that targets human TLR2 and human TLR4 was developed. Dual blockade of TLR2 and TLR4 has been reported to show greater efficacy in a septic mouse model than inhibition with either TLR2 or TLR4 alone [82, 83]. Given that both TLR2 and TLR4 signaling cascades share the adaptor protein Myd88, down-regulating both signaling pathways is expected to lead to a marked decrease in cytokine secretion. To my knowledge, there were no reports of a dual inhibitor of TLR2 and TLR4 or treatment with a combination of TLR2 and TLR4 inhibitors in clinical trials at the start of this study. As many patients with sepsis constantly receive a variety of treatments in hospital intensive care units, such as antibiotics, ventilator support and dialysis [82], bispecific antibodies that can be administered in a single dose can speed up treatment and are more clinically convenient than administering a combination of anti-TLR2 and anti-TLR4 antibodies. Such a bispecific antibody would be a novel potential therapeutic agent for treating sepsis.

Finally, the binding mode of a bispecific antibody targeting TLR2 and TLR4 was analyzed. The bispecific antibody, MM-131, which targets c-MET and epithelial cell adhesion molecule (EpCAM), has been reported to have a unique binding mode depending on the expression ratio of these receptors. Specifically, while the inhibitory activity of MM-131 was comparable to that of the parental anti-c-MET antibody in cells with equivalent expression of EpCAM and cMET, it was higher in cells with a higher EpCAM/c-MET ratio

[84]. Since TLR2 and TLR4 are expressed on the same immune cells, a bispecific antibody targeting TLR2 and TLR4 is expected to have similar mechanism of action to MM-131. Thus, analysis of the binding mode of a bispecific antibody will provide hints to the mechanism of action of bispecific antibodies in the clinic.

Chapter 2 Generation and engineering of an anti-rat TLR4 antibody for *in vivo* models

2.1 Introduction

In the innate immune system of vertebrates, the Toll-like receptor (TLR) family is known to play a prominent role in host defense by recognizing conserved structures in bacteria. The Toll receptor was first identified in *Drosophila melanogaster* as an essential molecule for embryonic development and immunity. Nine Toll genes were identified, and all Toll receptors had a similar extracellular domain structure composed by leucin-rich repeats, which is very similar to mammalian TLRs [85]. Among the TLR family members, TLR4 is the best characterized and responds to LPS and damage-associated molecular patterns (DAMPs) such as HMGB1 and heat shock protein (HSP). Pro-inflammatory cytokines are produced downstream of the TLR4 signaling pathway and are associated with several diseases.

In infectious diseases caused by gram-negative bacteria, overactivation of TLR4 leads to overproduction of cytokines, one of the causes of a cytokine storm, and ultimately results in sepsis. Tissue ischemic reperfusion (I/R) injury was also reported to be associated with TLR4 as DAMPs are secreted from damaged cells after I/R [86]. Several rodent models in which TLR4 is inhibited by TLR4 inhibitors or genetically deleted have been used to clarify the roles of TLR4 [87-89].

Rodent disease models are commonly leveraged to investigate the roles of target molecules or efficacy of therapeutic agents when the functions of the target molecule are conserved between humans and rodents. Although the sequences and number of members of the TLR family show some divergence, basic functions and signaling pathways are conserved among species [90]. Hence, the association between TLRs and diseases elucidated in rodent models may be translatable to other species such as humans.

TLR4 KO mice have been used for septic mouse models to investigate the contribution of TLR4 to the disease. Compared to using inhibitors, which may not completely inhibit the target molecule, an advantage of KO mice is that function of the target molecule is fully suppressed. In contrast, the results can vary depending on the efficacy of inhibitors. The advantage of inhibitors in rodent disease models is that they can be administered prophylactically and therapeutically. In contrast, only a prophylactic effect can be investigated in KO mice. Given that it is not feasible to prevent TLR4-related diseases in humans because patients are hospitalized after disease onset, TLR4 inhibitors are more appropriate for determining the potential of treatment comprising the inhibitors and contribution of TLR4 to the diseases.

While the commercially available anti-mouse TLR4 antibody MTS510 is the only one reported to ameliorate pathological conditions in rat disease models, details of its antagonistic activity were unclear [91, 92]. In my previous study, I found that MTS510 did not show antagonistic activity against LPS in the rat macrophage cell line NR8383. As sensitivity to LPS varies depending on the type of bacteria and cells used in an assay, I speculated that MTS510 was unable to neutralize TLR4 which had been potently stimulated. Partial or weak inhibition of TLR4 in disease models would obscure contribution of the TLR4 signaling pathway to the disease mechanism. Therefore, a potent anti-rat TLR4 antagonistic antibody would be most desirable for elucidating TLR4 function and its contribution in rat disease models.

The purpose of this study is to generate an anti-rat TLR4 antagonistic antibody useful for rat disease models. First, an anti-rat TLR4/MD2 antibody was generated by immunizing TLR4 KO mice with rat TLR4/MD2-stably-expressing cells. By screening for hit clones using the hybridoma method, 52-1H4 was found to bind to TLR4/MD2-stably-expressing cells and inhibit LPS-mediated IL-6 secretion from a

macrophage cell line. The sequence of 52-1H4 was modified, or ratized, so that it was more similar to a rat antibody sequence, to reduce immunogenicity in rat *in vivo* studies. Thereafter, a PK and PD study using Wistar rats showed the potential usefulness of the novel ratized antibody as a pharmacological tool for elucidating the biology of TLR4 in rat models.

2.2 Materials and Methods

2.2.1 Generation of rat TLR4/MD2-stably-expressing cells

Rat liver Kupffer cells were isolated from Wister rats (Charles River Laboratory, MA, USA) using a previously described method [93]. Subsequently, a rat subtractive cDNA library was constructed from the Kupffer cells according to a conventional reverse transcription polymerase chain reaction (RT-PCR) method to clone the rat TLR4 gene (NM_019178.1) [94]. PCR was performed to amplify the rat TLR4 gene as follows. To combine the Flag epitope tag with rat TLR4, a DNA fragment of the signal sequence of rat TLR4 and part of the Flag epitope tag was amplified using forward primer 1 and reverse primer 1. Another DNA fragment of part of the Flag epitope tag and rat TLR4 gene was amplified by PCR using forward primer 2 and reverse primer 2. The oligo DNA sequences used to amplify the genes are shown in Table 2.2.1. Subsequently, PCR using forward primer 1 and reverse primer 2 was performed to fuse the two amplified DNA fragments. Finally, the amplified PCR product was introduced into a pEB6CAG vector (kindly provided by Dr. Miwa of the University of Tsukuba) between PstI and NotI sites. The rat MD2 gene (NM_001024279.1) was also amplified by PCR using the cDNA of rat Kupffer cells as a template. The c-myc epitope tag sequence was combined with the rat MD2 gene using forward primer 3 and reverse primer 3. In the same way as that performed for rat TLR4-FLAG, the

PCR product was introduced into a different pEB6CAG vector between KpnI and NotI sites. Next, these plasmid vectors were transfected into CHO-K1 cells to establish overexpressing cell clines. Cells expressing both genes can be selected by the antibiotics 1 mg/mL G418 and 200 μg/mL zeocin, and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS). Protein expression levels of rat TLR4 and rat MD2 were confirmed by FACSVerse (BD Biosciences, CA, USA) using an anti-Flag M2 antibody (Sigma Aldrich, St. Louis, USA) and an anti-c-myc 9E10 antibody (Santa Cruz, CA, USA). The anti-Flag M2 antibody and anti-c-myc antibody were detected using an anti-mouse IgG FITC antibody.

Table 2.2.1. Sequences of oligo DNAs used for PCR

Primer	Sequence
Forward primer 1	5′-GGTACC GCC ACC ATG ATG CCT CTC TTG CAT CTG-3′
Reverse primer 2	5′-CTT GTC ATC ATC GTC CTT GTA GTC TGG TCT CAG GCA GGA AAG G-3′
Forward primer 1	5′-GAC TAC AAG GAC GAT GAT GAC AAG GGA AGC TTG AAT CCC TGC ATA GAG-3′
Reverse primer 2	5′-GCGGCCGC CTA GGT CAA AGT TGT TGC TTC TTG TTC-3′
Forward primer 3	5′-GGTACC GCC ACC ATG TTG CCA TTT TTT CTC TTT TCG ACG-3′
Reverse primer 3	5′-GCGGCCGC CTA CAG ATC TTC TTC AGA AAT AAG TTT TTG TTC ATT AAC ATT ATG GTG GTG AAT GAT GG-3′

2.2.2 Generation of an anti-rat TLR4 antagonistic antibody

TLR4 KO mice (Oriental Bio Service, Kyoto, Japan) were used to generate anti-rat TLR4/MD2 antibody. The mice were immunized with the rat TLR4/MD2-stably-expressing cells (5×10^6 cells/head) obtained above. AbISCO-100 (Funakoshi, Tokyo, Japan), used as an adjuvant for efficient vaccination, was mixed with the stably-expressing cells and injected into the hind footpads of mice. After 4-8 immunizations in two months, inguinal lymph nodes were collected from the mice. B lymphocytes were then isolated and fused with a myeloma cell line to generate hybridomas. Each hybridoma was carefully handled to ensure they produced a

single clone without contamination by other clones to generate monoclonal antibodies. After a few weeks of incubation, supernatants from the hybridomas were collected to screen hit clones which show specific binding to and antagonistic activity against rat TLR4.

2.2.3 Ratization of 52-1H4

To reduce immunogenicity of the mouse antibody in rats, the antibody sequence of 52-1H4 was analyzed and ratized for *in vivo* use. First, total RNA was extracted from the hybridoma of 52-1H4 using an RNeasy kit (QIAGEN, Hilden, Germany) and then first-strand cDNA was synthesized by reverse transcription reaction and PCR using a SMARTer RACE cDNA Amplification kit (Takara Bio, Shiga, Japan). Agarose gel electrophoresis using 1% agarose gel was performed to isolate the PCR products. The DNA fragments were purified using a Gel Extraction kit (QIAGEN). The heavy chain and light chain antibody sequences were analyzed using a previously described method [95]. The nucleotide sequences of VH and VL of 52-1H4 were registered in GenBank (GenBank accession no. MN201583 and MN218725, respectively).

CDRs of the VH and VL of the 52-1H4 antibody were assigned according to the Kabat or Chothia definition [96, 97]. Ratization of 52-1H4 was performed by CDR grafting according to the method described by Almagro and Fransson for humanization of mouse antibodies [45, 97]. CDRs of 52-1H4 were grafted onto the FWRs of IGHVS285, IGHJ-1, IGVK147, and IGJK4 (IMGT: http://www.imgt.org/) to generate ratized VH and VL sequences. The ratized antibody was designated 52-1H4 e2 (e2).

2.2.4 Expression and purification of 52-1H4 and e2

The VH of 52-1H4 was inserted into the glutamine synthetase (GS) vector pEE6.4 (Lonza, Basel, Switzerland) between HindIII and EcoRI sites, and the VL of 52-1H4 was inserted into the GS vector pEE12.4 (Lonza) between HindIII and EcoRI sites. The recombinant pEE6.4 and pEE12.4 vectors were subsequently digested at their PvuI and NotI sites, and the DNA fragments were ligated to generate a dual gene vector encoding the gamma chain and kappa chain of 52-1H4.

The amino acid sequence of the e2 antibody was artificially synthesized (Thermo Fisher Scientific, MA, USA) to generate an expression vector. The VH of e2 (GenBank accession no. MN231086) was inserted into the pEE6.4 vector with the constant region of rat gamma chain-1. The VL of e2 (GenBank accession no. MN231087) was also inserted into the pEE12.4 vector with the constant region of the rat kappa chain. The recombinant pEE6.4 and pEE12.4 vectors of e2 were digested at their PvuI and NotI sites and ligated in the same way as that performed for 52-1H4.

The dual gene vectors were electroporated into CHO-K1SV (Lonza) cells to produce recombinant 52-1H4 or e2 antibody proteins. The cell culture supernatant was collected after culturing for about one week. The recombinant antibody protein in the collected cell culture supernatant was subjected to purification by affinity chromatography using protein A.

2.2.5 Size exclusion chromatography (SEC) analysis

SEC analysis was performed using ACQUITY UPLC H-Class (Waters, MA, USA) in a BEH200 SEC column, 4.6 mm x 300 mm (Waters). Purified antibody samples were diluted with PBS (-) containing 0.2 M arginine. The diluted samples were loaded into the column and separated at 0.5 mL/min. The column was maintained at

30°C during the separation.

2.2.6 Cell enzyme-linked immunosorbent assay (ELISA)

Rat TLR4/MD2-stably-expressing cells were seeded onto BioCoat™ Poly-D-Lysine 384-well plates (Corning, NY, USA) at 1.25×10^4 cells per well or 96-well plates (Becton Dickinson, NJ, USA) at 6×10^4 cells per well and incubated at 37°C overnight. The medium was removed from the plates and test articles, namely the purified 52-1H4 antibody, e2 antibody or rat plasma samples collected from rats injected with e2 antibody, were added to the wells. The plates were incubated for 1 h at 37°C. Then the plates were washed twice with Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific), which includes 10 mM HEPES (Sigma Aldrich) and 0.1–1% bovine serum albumin (BSA; Sigma Aldrich). To detect antibody binding to the TLR4/MD2-stably-expressing cells, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Southern Biotech, AL, USA) or HRP-conjugated anti-rat IgG antibody (Southern Biotech) was used as a secondary antibody and added to the wells. After incubating at 37°C for 1 h, the plates were washed twice with the HBSS buffer mentioned above. TMB substrate-chromogen (Dako, CA, USA) was added to the wells and the plates were incubated for 5-30 min at room temperature. Subsequently, 1 M H_2SO_4 (Wako, Tokyo, Japan) was added to the wells to stop the reaction. Absorbance at 450 nm and 540 nm was read by Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland). Readings obtained at 540 nm were subtracted from readings obtained at 450 nm. EC50 was calculated from a sigmoidal dose-response curve and analyzed using GraphPad Prism ver. 8.0.2 for Windows (GraphPad Software, CA USA). Apparent equilibrium dissociation constant (K_D) values were determined from the observed EC50 values and molecular weight (MW) of the antibodies

according to following formula:

$$\text{apparent } K_D = \frac{\text{EC50}}{\text{MW}}$$

2.2.7 Neutralization assay using rat macrophage cell line NR8383

The rat macrophage cell line NR8383 (ATCC CRL2192) was cultured in F12 (Thermo Fisher Scientific) medium with 15% FBS at 37°C, 5% CO_2. Approximately 5×10^4 NR8383 cells were seeded onto 96-well plates (AGC Techno Glass, Tokyo, Japan) and incubated at 37°C overnight. Purified 52-1H4 or e2 antibodies were added to the wells and incubated for 30 min at 37°C. LPS (lipopolysaccharide from *E. coli*, Serotype O55:B5 S-form; Enzo Life Sciences, NY, USA) was added to the wells at a final concentration of 1-100 ng/mL. The plates were incubated at 37°C overnight. The cell culture supernatants were collected to measure secreted IL-6 in the cell culture supernatants using a Rat IL-6 Quantikine ELISA kit (R&D Systems, MN, USA). The rate of inhibition of IL-6 secretion by the antibody was determined by measuring that absorbance at 450 nm compared with that in untreated wells.

2.2.8 Pharmacokinetic analysis of e2 in Wistar rats

A single dose *in vivo* study was performed in normal rats to analyze the PK and PD of the e2 antibody. Wistar rats (Japan SLC, Shizuoka, Japan) were intravenously injected with 8.5 mg/kg of e2 antibody (the day of injection was designated day 0). Rat blood samples were collected on day 0, 1, 8, 15, 22, 26 and 28 using heparin as an anticoagulant and centrifuged to obtain rat plasma. The plasma concentration of e2 was measured

by cell ELISA as described above. The unknown plasma e2 concentration was calculated using the standard curve of the purified e2 antibody. Non-compartmental analysis was performed to determine the terminal half-life ($t_{1/2}$) of the e2 antibody using Phoenix WinNonLin 7.0 (Certara, CA, USA).

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Further, Astellas Pharma Inc., Tsukuba Research Center has been awarded Accreditation Status by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All efforts were made to minimize the number of animals used and their suffering.

2.2.9 Pharmacodynamic analysis of e2

Pharmacodynamic (PD) analysis was performed to determine the availability of e2 antibody for *in vivo* study. A 1:1 dilution of the rat blood samples collected on day 28 described above and D-PBS (-) (Nikken Bio Medical Laboratory, Kyoto, Japan) was plated. Subsequently, 1 μg/mL of e2 antibody was added to some wells to fully occupy TLR4. Next, 30 ng/mL of LPS was added to the wells. The plates were incubated at 37°C for 3.5 h. After centrifugation, the supernatants were collected to measure TNF-α concentrations using a rat TNF-α ELISA kit (Thermo Fisher Scientific). Plasma TNF-α concentration was calculated according to the kit protocol.

2.3 Results and Discussion

2.3.1 Generation of an anti-rat TLR4 antibody

Among the methods used to generate antibodies (described in Chapter1), the hybridoma method is the most

commonly used by laboratory researchers who do not have a specialized phage library or sorting machines to screen for single B cells. We immunized TLR4 KO mice with rat TLR4/MD2-stably-expressing cells. Mice deficient in the target antigen are useful for immunization when the antigen show high homology in the host and donor species. In my previous study, I failed to obtain anti-rat TLR4 antibodies when using wild-type mice. Moreover, selection of the appropriate antigen is crucial for immunization and should be determined based on the structure of the target molecules. Leveraging the extracellular domain of target molecules is a common and effective way to generate antibodies against proteins expressed on the cell membrane through immunization, although loss of binding activity is often observed in hit clones identified through cell-based screening. This is thought to be caused by incorrect protein folding of the recombinant proteins used for immunization. Therefore, immunization with rat TLR4/MD2-stably-expressing cells reduced concern for efficient screening.

After immunization, hybridomas were generated using a standard method. We then screened monoclonal antibodies that were secreted by the hybridomas into the supernatant in a binding assay using the rat TLR4/MD2-stably-expressing cells. Hit clones were evaluated for their antagonistic activity in a neutralization assay using the rat macrophage cell line NR8383. This led to the identification of the mouse IgG2a antibody 52-1H4 as an antagonistic antibody to rat TLR4/MD2. To evaluate the potency and efficacy of 52-1H4, we collected and then purified the 52-1H4 hybridoma cell culture supernatant by affinity chromatography using protein A. Purified 52-1H4 proteins exhibited potent binding activity to the rat TLR4/MD2-expressing cells (Figure 2.1). To examine the neutralizing activity of 52-1H4, we performed a neutralization assay using the rat monocyte cell line NR8383, which produces inflammatory cytokines in the presence of LPS. 52-1H4 completely inhibited IL-6 secretion from NR8383 even following stimulation with

a high concentration of LPS (Figure 2.2). These results suggest that 52-1H4 is a novel anti-rat TLR4/MD2 antibody with specific binding activity and potent neutralizing activity.

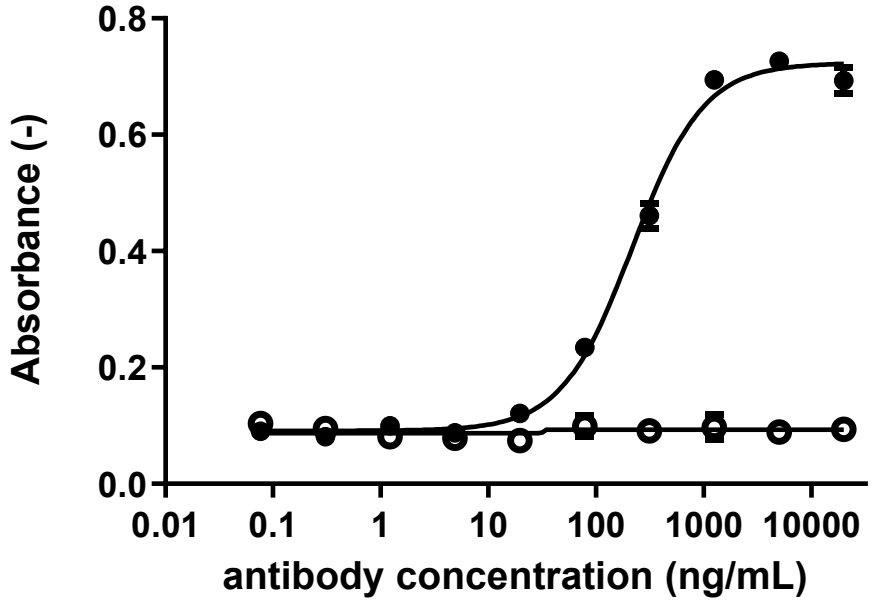


Figure 2.1. Specific binding of 52-1H4 to rat TLR4/MD2. Binding to rat TLR4/MD2-stably-expressing cells was examined. 52-1H4 (closed circles) and mouse IgG2a isotype control (open circles) were detected using an HRP-conjugated anti-mouse antibody. The graph shows the absorbance at 450 nm (after subtracting the absorbance at 540 nm) on the Y-axis versus the 52-1H4 concentration on the X-axis. Data (mean ± SD) are representative of two independent experiments performed in triplicate.

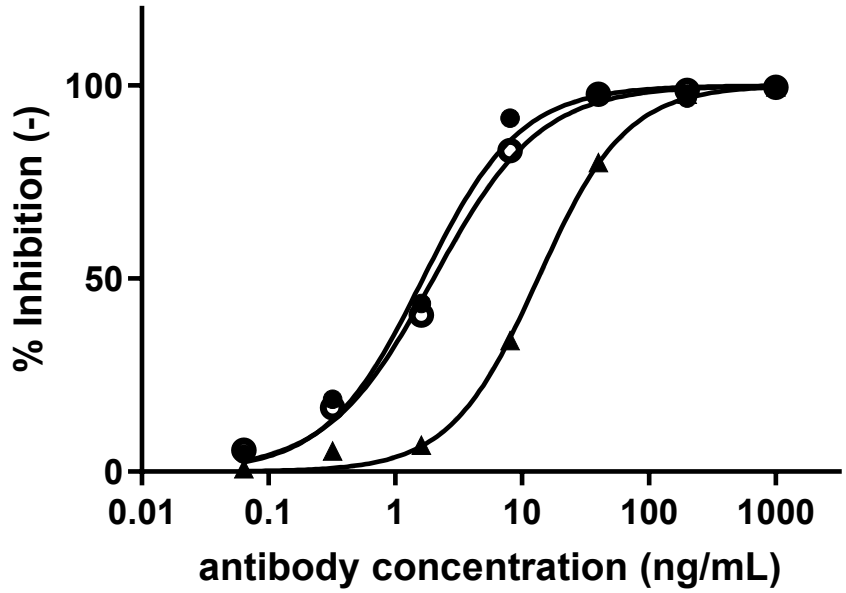


Figure 2.2. Inhibition of the rat TLR4/MD2 response to LPS. NR8383 cells were stimulated with LPS at 1 ng/mL (closed circles), 10ng/mL (open circles) or 100 ng/mL (triangles) in the presence of 52-1H4. Rate of inhibition by 52-1H4 was calculated as a percentage of IL-6 production. The mean value in LPS-untreated wells was defined as 100% inhibition and the mean value in antibody-untreated wells was defined as 0% and used as control. Data (individual points and mean line) are representative of two independent experiments performed in duplicate.

2.3.2 Ratization of 52-1H4

The immunogenicity of antibody therapeutic agents remains an issue in the clinic, with ADAs having been reported to inhibit the function of therapeutic antibodies derived from mice. To minimize the immunogenicity of mouse antibodies, several humanization techniques can be applied. While humanization focuses on therapeutic agents, immunogenicity of mouse antibodies in rats is also an issue, as ADAs are induced in rats injected with a mouse antibody and vice versa [98, 99]. Although mouse and rat genes generally have high homology, I hypothesized that 52-1H4 would show immunogenicity and pose a risk of inducing ADAs in rats. Thus, I attempted to ratize 52-1H4 using CDR grafting and back-mutation (described in the General

Introduction).

To identify the CDRs of 52-1H4, the amino acid sequences of heavy and light chain variable regions were numbered based on the numbering schemes reported by Kabat and Chotia. The homology between the variable regions of 52-1H4 and the corresponding rat germline sequences was taken into account to select the rat FWR for CDR grafting. FWRs of IGHV2S85, IGHJ-1, IGVK147 and IGJK4 were combined with CDRs of 52-1H4, and back mutations were introduced into the ratized sequences (Figure 2.3A). The ratized 52-1H4 was designated 52-1H4 e2 (e2) and its sequence was registered in the genetic sequence database GenBank. Subsequently, the e2 antibody sequence was synthesized and expressed in CHOK1-SV cells. After protein A purification, the ratized e2 antibody comprised >99% of the monomer (Figure 2.3B).

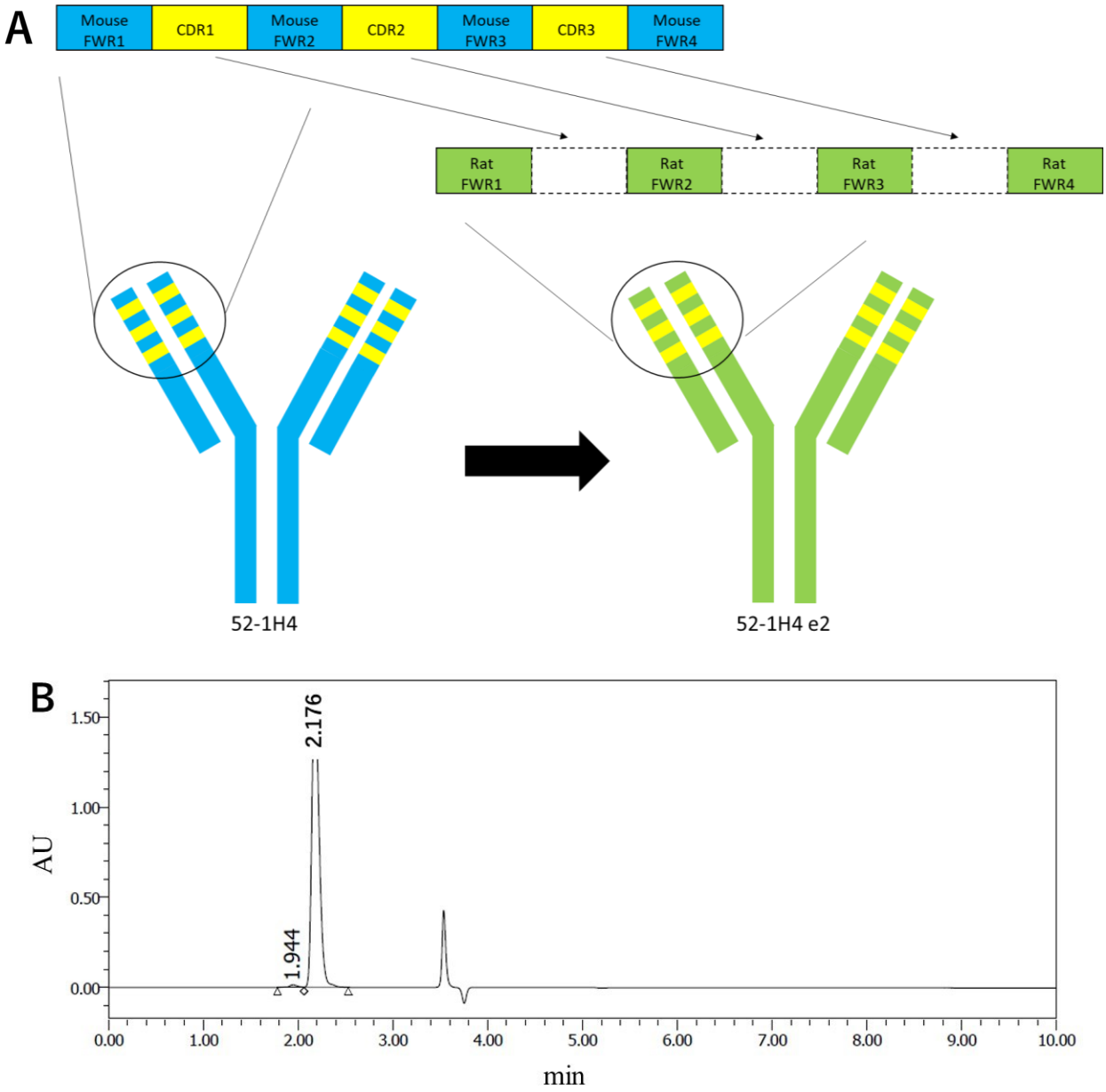
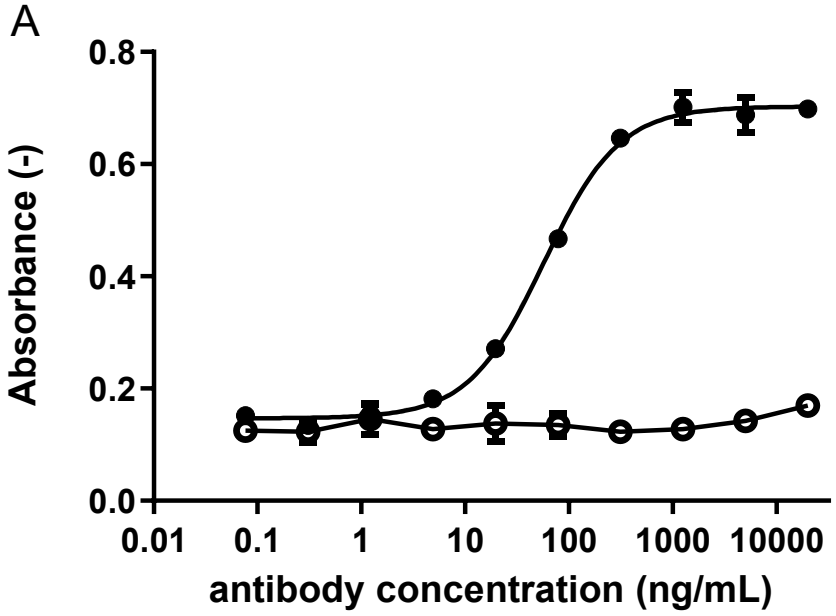


Figure 2.3. Generation of ratized antibody e2. (A) CDRs of 52-1H4 were grafted into rat FWRs. The ratized e2 antibody contains mouse CDRs, rat FWRs, and rat constant regions. (B) Analytical size exclusion chromatography (SEC) was conducted after protein A purification.

2.3.3 Characterization of e2

FWRs contribute to the structural stability of an antibody and VH-VL orientation, which is crucial for antigen recognition. Hence, ratization of 52-1H4 can potentially reduce specificity and affinity for rat TLR4/MD2. To determine the binding and antagonistic activity of e2, we assessed its binding and functional capacity. Potent

binding activity was observed between e2 and rat TLR4/MD2-stably-expressing cells, but not between e2 and host CHO cells (Figure 2.4A and 2.4B). The apparent K_D value, calculated from the EC50 of the sigmoidal dose-response curve of e2, was 0.40 nM. Furthermore, the antagonistic activity of e2 was confirmed using the NR8383 assay. e2 completely inhibited LPS-mediated IL-6 production (Figure 2.4C). These data suggest that e2 retained specific binding and antagonistic activity for rat TLR4/MD2. In contrast, the commercially available anti-TLR4/MD2 antibody MTS510 did not exhibit antagonistic activity in the NR8383 assay, despite MTS510 having been reported to partially inhibit LPS-induced IL-6 secretion in rat cardiac microvascular endothelial cells [91]. This result is inconsistent with that of previous reports. These data suggest that e2 is the only anti-TLR4/MD2 antibody which completely inhibits LPS-mediated cytokine production *in vitro*.



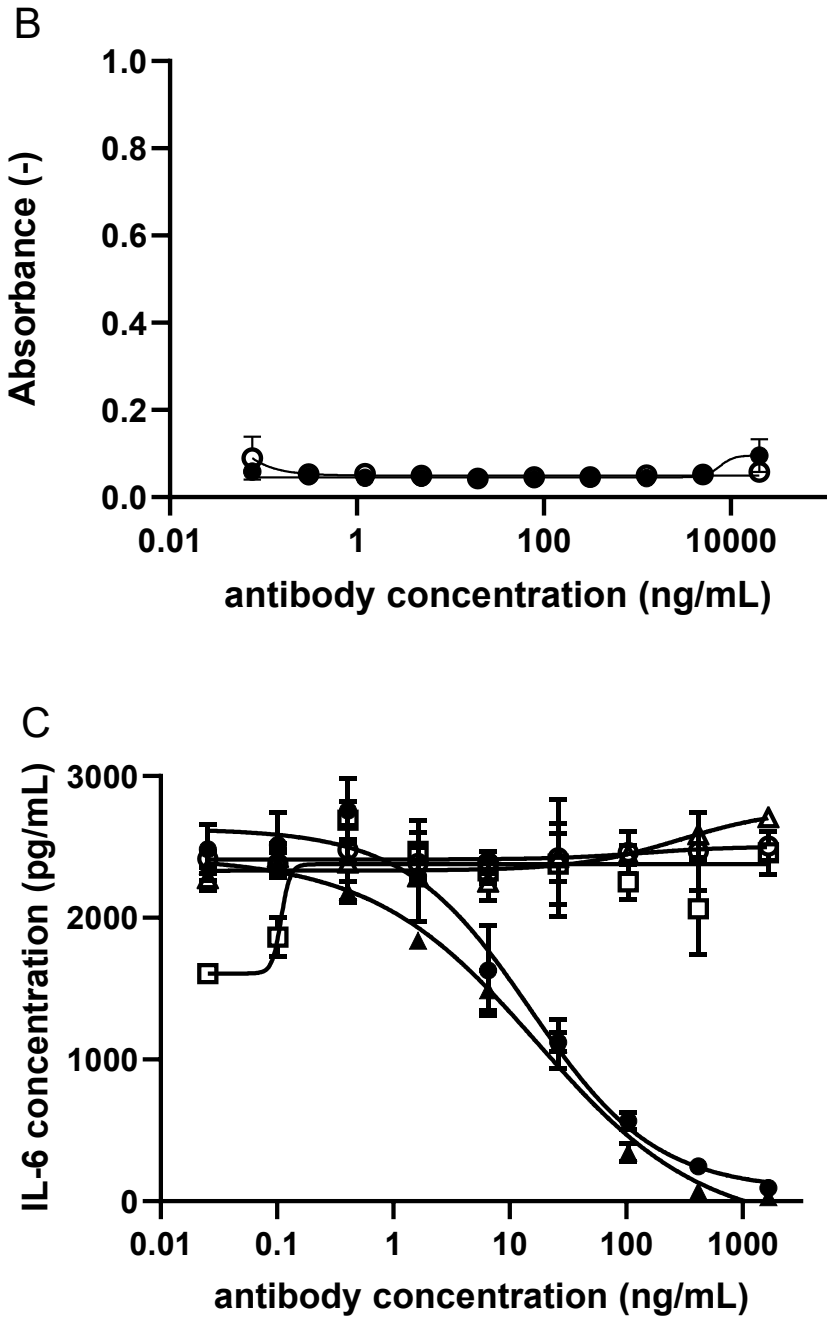


Figure 2.4. Binding and functional activity of e2. (A) Serial dilutions of e2 antibody and control antibody were detected by cell ELISA using rat TLR4/MD2-stably-expressing CHO cells and (B) parental CHO cells. Closed circles: e2, open circles: rat IgG1 isotype control. (C) The rate of inhibition by mAbs was calculated as a percentage of IL-6 production. Inhibitory activity of 52-1H4 and e2 antibodies were analyzed in the assay using NR8383 cells stimulated with 100 ng/mL LPS. Closed triangles: 52-1H4, open triangles: mouse IgG2a isotype control, closed circles: e2, open circles: rat IgG1 isotype control, open squares: MTS510. All data (mean ± SD) are representative of two independent experiments performed in triplicate.

2.3.4 PK and PD of e2 in Wistar rats

To determine the availability of e2 in an *in vivo* study using rats, PK and PD analyses were performed by observing the circulating antibody concentration of e2 for 28 days after a single intravenous dose to rats. After injection of e2 at a dose of 8.5 mg/kg, rat blood samples were collected and the concentrations of e2 were determined by cell ELISA using rat TLR4/MD2-expressing cells. Plasma e2 concentrations were above 10 μg/mL in all collected blood samples (Figure 2.5A). The terminal half-life of e2 in the Wistar rats was 9 days, which is mostly comparable to that of general IgG.

On day 28 of the *in vivo* study, the PD of e2 was analyzed if the concentration of e2 was high enough to inhibit TLR4/MD2 on lymphocytes. Blood samples collected on day 28 were stimulated with LPS and secretion of TNF-α was measured. TNF-α production was inhibited in samples taken from rats injected with e2 compared to samples taken from rats injected with vehicle. Adding e2 to samples taken from rats injected with e2 did not change TNF-α levels compared to not adding extra e2 (Figure 2.5B). These results suggest that TLR4/MD2 was functionally regulated by e2 on 28 day and probably all other days since high e2 concentration was maintained throughout the test period.

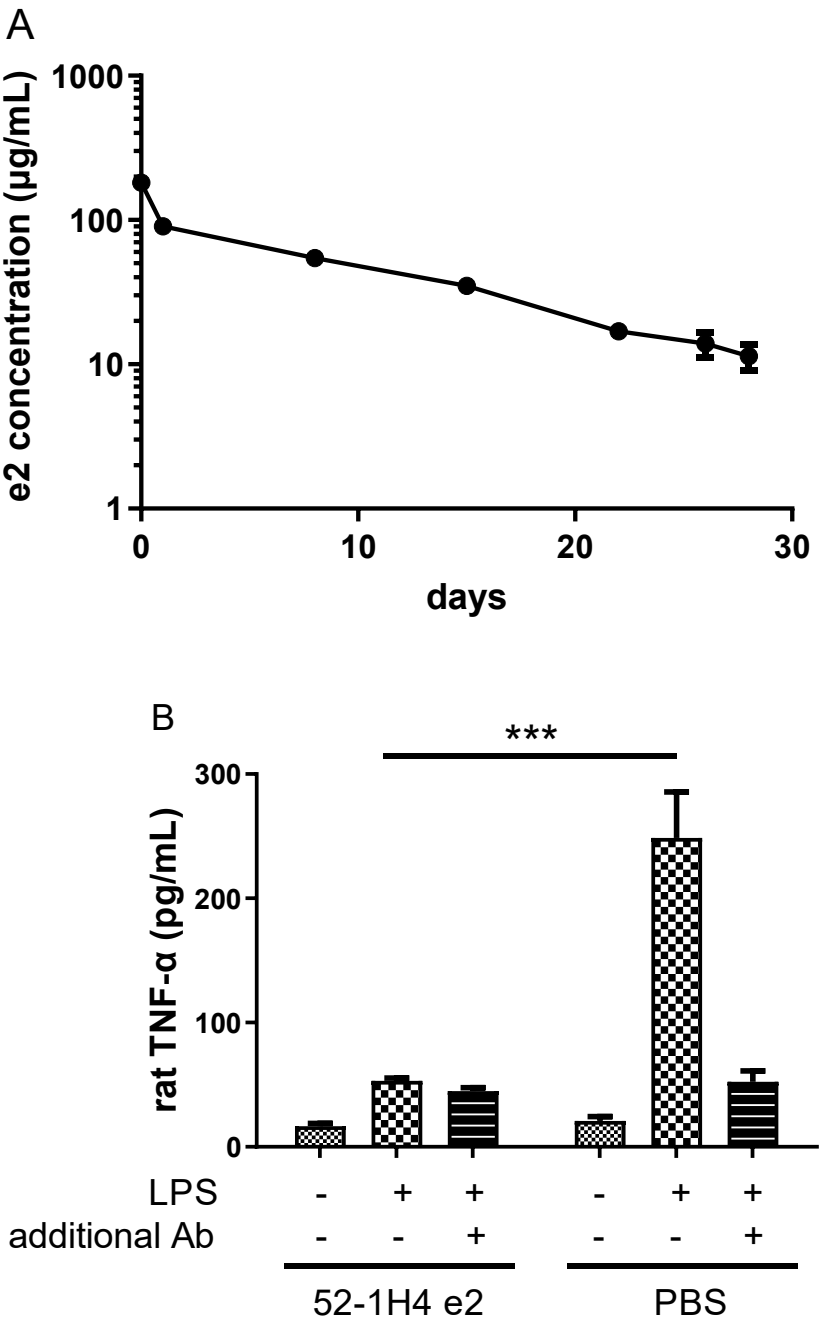


Figure 2.5. PK and PD analysis of e2 antibody. (A) After injection of e2 antibody at a dose of 8.5 mg/kg into Wistar rats, mean plasma concentrations of e2 were measured on day 0, 1, 8, 15, 22, 26 and 28; n = 3. (B) PD analysis was performed using an ex vivo assay in which rat blood samples were stimulated with LPS. On day 28, the collected rat blood samples were diluted with PBS (1:1) and stimulated with 30 ng/mL LPS. Additional e2 antibody (Ab) was added to some samples before LPS stimulation to fully occupy TLR4. ***p < 0.001, unpaired Student's two-tailed t test. Each column shows the concentration of TNF-α (mean ± SEM) obtained in triplicate.

Since testing of mouse antibodies began in clinical trials, immunogenicity and ADA formation have become emerging issues. Humanization of mouse antibodies by CDR grafting was developed to address this issue, and has been shown to successfully minimize immunogenicity and decrease ADA [100]. Recently, a variety of host species including mice, rats, rabbits and camelids have been used to generate therapeutic and *in vivo* experimental antibodies [101, 102]. Immunogenicity issues have also been reported in experimental antibodies. ADA is reportedly induced in rats injected with a mouse antibody and vice versa [98, 99]. These reports suggest that mouse antibodies are potentially immunogenic in rats and could be eliminated by ADA. Similar to the case for therapeutic antibodies, CDR grafting is thought to be useful for decreasing the immunogenicity of experimental antibodies that are derived from species different from that of the host animal in *in vivo* experiments.

While the CDR grafting technique is effective for decreasing the immunogenicity of antibodies in donor animals, it presents several hurdles for preserving neutralizing activity. Maintaining the orientation of VH and VL is important for preserving antigen-binding affinity [103]. I therefore designed various CDR-grafted VH and VL *in silico.* Although there are many reports on humanization, no reports on ratization were available at the initiation of this study. Thus, I had to test several rat FWR sequences to identify the most appropriate FWR sequences for CDR sequences of 52-1H4. I speculated that the affinity of CDR-grafted antibodies would decrease because of incompatibility between mouse CDRs and rat FWRs. Hence, as is commonly conducted for humanization, back mutations were introduced into each FWR to restore affinity. Finally, I confirmed the neutralization activity of each newly designed antibody *in vitro*. This method allowed me to determine the most suitable VH-VL combination.

Theoretically, e2 poses minimal immunogenicity risk. While I could not estimate its immunogenicity, the results of our PK and PD study strongly support my hypothesis. The e2 antibody likely escapes detection by the rat immune system for 28 days in Wistar rats. If e2 was eliminated by the rat immune system, plasma e2 levels would have rapidly decreased.

A limitation of this study is that the only ligand used in the neutralization assays was LPS. While LPS is commonly used as a TLR4 ligand, other PAMPs and DAMPs such as HMGB1, which is secreted from damaged cells, have been reported to stimulate TLR4 [104]. LPS and HMGB1 form a complex and synergistically activate human peripheral blood mononuclear cells (PMBCs) [105, 106]. If the e2 antibody is found to inhibit HMGB1, this will further clarify the mechanisms of TLR4.

2.4 Conclusion

The novel anti-rat TLR4/MD2 antibody 52-1H4 completely inhibited activation of rat TLR4. Moreover, the ratized antibody 52-1H4 e2 was generated from 52-1H4 using CDR grafting. The PK and PD study using Wistar rats demonstrated potential availability of e2 for in vivo studies. Therefore, the e2 antibody is expected to be a useful pharmacological tool for elucidating the biology of TLR4 in rat models.

Chapter 3 Generation of an IgG-dsFv bispecific antibody directed at TLR2 and TLR4

3.1 Introduction

TLRs play a prominent role in host defense in the innate immune system. Given that pro-inflammatory cytokines are produced downstream of TLR signaling pathways, TLRs are thought to be related to several diseases. Among the TLR family members, TLR4 is the best characterized. It responds to LPS and endogenous soluble factors such as HMGB1 and HSP. Several polymorphisms of TLR4 have been identified and are associated with inflammatory bowel disease, in which TLR4 expression levels are elevated. TLR4 signaling is thought to lead to the production of pro-inflammatory cytokines, which induces gut inflammation [107]. Upregulation of the TLR4 gene has also been observed in patients with Alzheimer's disease and Parkinson's disease. Microglial cells express TLR4 and act as a sensor in the central nervous system [86]. Among the TLR4-related diseases, sepsis is the most severe, with the mortality rate of patients with septic shock reaching 40% [108]. Septic patients need to be managed in the intensive care unit for treatment with fluids and antibiotics. The causal treatment for sepsis is elimination of the pathogen that is causing an immune imbalance in early-stage infection. Although administration of antibiotics is recommended within one hour of diagnosis, it can take time to identify the causative pathogens to inform which antibiotic therapy to administer because patients may be infected with various types of bacteria [109].

Early treatment of sepsis is important for increasing the chance patient survival. A delay in antimicrobial therapy increases the chance of treatment failure. However, uncertainty of the timing of disease onset in patients with community-acquired infections makes initiation of early intervention difficult and leads to delays in microbe identification. Currently, the standard therapy for sepsis focuses on empirical antibiotic

therapy and additional intensive care, including vasopressor administration, renal replacement therapy and mechanical ventilation. Thus, current treatment approaches focus on using adjunctive therapy to control the immune balance. Although the early stages of sepsis are characterized by pro-inflammatory responses, recent evidence suggests that concomitant anti-inflammatory responses become initiated in the middle stages of the disease. Concomitant immunosuppression subsequently arises through downregulation of cell surface molecules involved in the immune response, T cell exhaustion and an increase in apoptotic lymphocytes. This ultimately leads to paralysis of immune cells and makes patients vulnerable to bacterial infection. Therefore, administration of powerful anti-inflammatory therapy before progression of the immune imbalance and initiation of the concomitant anti-inflammatory response in the early stages is needed for successful sepsis treatment [79].

One way to treat sepsis using immunomodulation is to inhibit factors upstream of multiple signaling cascades. TLR2 and TLR4 have been reported to be associated with sepsis and knocking out either gene in septic mice improves survival [82, 83]. Both TLR2 and TLR4 recognize the cell wall components of gram-positive or gram-negative bacteria and share the adaptor protein Myd88 in their signaling pathway. Given the similarity between TLR2 and TLR4, the former is thought to compensate for the other and vice versa in response to pathogens. Thus, dual blockade of the two receptors may be more effective than blocking either receptor for treating sepsis. In fact, septic mice with a double KO of TLR2 and TLR4 or deletion of Myd88 showed greater improvements in sepsis than a single KO of TLR2 or TLR4 [110, 111]. Based on these studies, anti-TLR2 and anti-TLR4 antibodies may be effective for treating sepsis and a TLR2 x TLR4 bispecific antibody could be a powerful therapeutic agent.

Several bispecific antibody formats have been studied and many types of antibodies have been reported in the 60 years since the concept of a bispecific antibody was first introduced in 1960. The first reported bispecific antibody was generated by simply combining multiple pepsin-digested antibody fragments, which resulted in a hybrid F(ab')2 (Figure 3.1) [112]. This simple structure could be easily produced in eukaryotic and prokaryotic cells, allowing bispecific antibodies to be generated with high yields. However, the plasma half-life of this simple bispecific antibody was too short for *in vivo* use due to the lack of an Fc region. In contrast, conventional IgG antibodies have much longer half-lives due to neonatal Fc receptor (FcRn)-mediated recycling, which prevents IgG from undergoing intracellular degradation. However, the bispecific T cell engager, blinatumomab, is a simple bispecific antibody composed of two scFv that lacks an Fc region and has been approved as a therapeutic agent by the FDA. Although it has proven to be an effective treatment for cancer, cancer patients still have unmet medical needs as they have to carry an intravenous (IV) bag for continuous 24-h administration [113]. A bispecific antibody harboring an Fc or trispecific antibody with an additional antibody fragment that binds to human serum albumin (HSA) could solve the issue of a short half-life since HSA is reportedly recycled with FcRn [114]

To design an Fc region that enables a longer half-life, studies have reported an asymmetric bivalent bispecific antibody and a symmetric bispecific antibody (IgG-scFv). An asymmetric bivalent bispecific antibody is obtained by co-transfecting plasmid vectors that code the sequence of two different heavy chains and two different light chains. Although this enables the potential production of 10 different bivalent antibodies and bispecific antibodies, correct pairing leads to rational yields of only 12.5%, a problem called the chain-association issue (Figure 3.2) [59].

In contrast, in IgG-scFv, IgG and scFv are fused to the N or C-terminus of the heavy or light chain (General Introduction Figure 1.10). This symmetric structure addresses the chain-association issue. Furthermore, the symmetric structure allows for simple co-expression of two polypeptides coding the heavy or light chain. This leads to high purity and yield using the standard purification technique of protein A affinity chromatography. However, generation of IgG-scFv requires the identification of the fusion position of scFv that will preserve binding affinity and stabilize the structure.

In this study, I attempted to identify the best IgG-scFv format for designing a bispecific antibody directed at TLR2 and TLR4.

Figure 3.1. Hybrid F(ab')2.

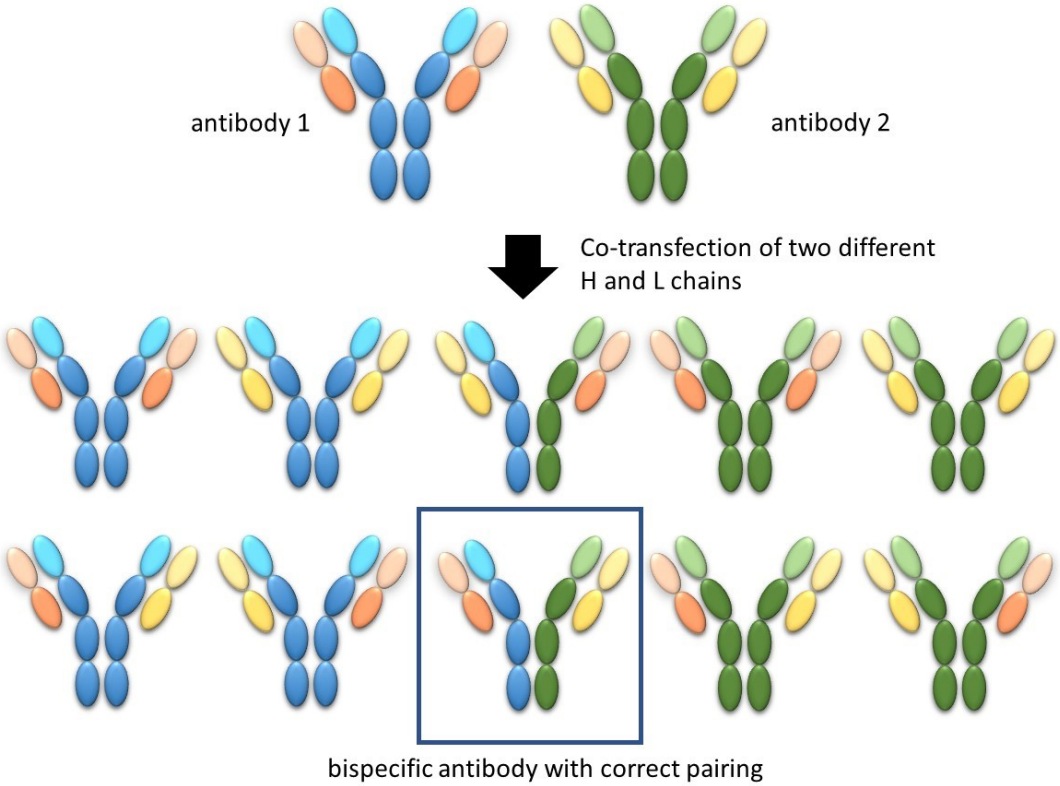


Figure 3.2. Chain-association issue of bispecific antibody generation.

3.2 Materials and Methods

3.2.1 Generation and screening of anti-TLR2 and anti-TLR4 antibodies

VelocImmune mice (Regeneron, NY, USA), which produce human antibodies, were immunized with recombinant human TLR2 (R&D Systems, MN, USA). The human TLR2 recombinant protein was emulsified with TiterMax Gold (Titer Max, GA, USA) and injected into the hind footpads of mice. TiterMax Gold is an adjuvant used to enhance the immune response to immunized antigens. After multiple immunizations, the antibody titer was measured. Subsequently, inguinal lymph nodes were collected from mice with high titers of antibody bound to TLR2 recombinant protein. B cells from the lymph nodes were fused to a myeloma cell line, and a conventional method was used to generate hybridomas [115]. Neutralizing activity against TLR2 was tested using the hybridoma supernatants. Screening the hybridoma led to identification of #31 as an anti-TLR2 antagonistic antibody. To generate anti-TLR4 antibodies, VelocImmune mice were immunized with human TLR4 recombinant protein and hybridomas were screened as described above. Finally, #48 was identified as an anti-TLR4 antagonistic antibody.

The CDR sequences of #31 and #48 were determined using the numbering scheme reported by Kabat and Chotia and subjected to CDR grafting to generate physicochemically stable antibodies. Human VH subgroup III and VL-kappa subgroup I consensus sequences were empirically selected as stable frameworks upon which to graft CDRs [116-118]. The antibody generated by CDR grafting from #31 was designated mAb-2 and that from #48 was designated mAb-4.

The Institutional Animal Care and Use Committee of Astellas Pharma Inc. approved all experimental procedures using animals. Furthermore, Astellas Pharma Inc. Tsukuba Research Center has been awarded

Accreditation Status by AAALAC International. All efforts were made to minimize the number of animals used and their suffering.

3.2.2 Linkage of the single chain Fv (scFv) to IgG

Next, scFv antibody fragments were designed and constructed. The VH of the obtained anti-TLR2 or anti-TLR4 antibody was designed to be conjugated to VL through the flexible linker $(GGGGS)_3$, which is commonly used in scFv antibodies. To stabilize the scFv structure using a covalent bond between VH and VL, the amino acid at VH44 and VL100 has been substituted with cysteine in some bispecific antibodies [49]. The scFv sequence was combined with the amino (N) or carboxyl (C)-terminus of the Igγ1 chain or light chain using a $(GGGGS)_{4\text{-}12}$ linker. The designed heavy chain and light chain sequences were artificially synthesized for protein expression. The artificially synthesized DNA was inserted into the glutamine synthetase (GS) expression vector pEE6.4 (Lonza) or pEE12.4 (Lonza) using restriction enzymes HindIII and EcoRI. The recombinant pEE6.4 and pEE12.4 vectors were digested at their PvuI and NotI sites. The DNA fragments were ligated to generate a dual gene vector encoding the designed heavy and light chains.

3.2.3 Expression and purification of antibodies

To express monoclonal or bispecific antibodies, dual gene vectors were electroporated into CHOK1SV® (Lonza) cells. After culturing at 37°C for approximately 1 week, the culture supernatant was collected. Protein-A affinity chromatography was performed to purify antibodies from the supernatant.

3.2.4 Size-exclusion chromatography (SEC)

To measure the monomer content of the purified antibodies, SEC analysis was performed using an LC1100 (Agilent, CA, USA) in a TSK gel Super SW3000 SEC column (Tosoh, Tokyo, Japan). The purified antibody proteins were loaded onto the column and separated at 0.075 mL/min. The column was maintained at 30°C during the separation.

3.2.5 Neutralization assay using U937 cells

The human monocyte-like cell line U937 (ATCC CRL1593.2) was cultured in RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) with 10% FBS. The cells were seeded into the 96-well cell culture plates (AGC Techno Glass, Tokyo, Japan) at 7.5×10^4 per well. Subsequently, 100 nM PMA (Sigma–Aldrich, St. Louis, USA) was added to the wells to induce the differentiation of monocytes into macrophages and increase expression levels of TLR2 and TLR4. After overnight culture, purified antibodies and TLR ligands were added to the wells. Pam2CSK4 (InvivoGen, CA, USA) is a synthetic ligand for TLR2 and was used at a final concentration of 10 ng/mL. *E. coli* Serotype O55:B5 S-form LPS (Enzo Life Sciences, NY, USA) was used as the TLR4 ligand at a final concentration of 10-100 ng/mL. The plates were incubated overnight at 37°C and cell culture supernatants were collected the next day. The IL-6 concentration in the cell culture supernatant was measured using an AlphaLISA human IL-6 Immunoassay Kit (PerkinElmer, MA, USA). The luminescence was measured using EnVision 2105 Multimode Plate Reader (PerkinElmer) according to the AlphLISA protocol. Inhibitory activities of each antibody were expressed as % IL-6 secretion, which was calculated based on the IL-6 levels in each treated well compared with that in untreated wells.

3.2.6 Neutralization assay using human peripheral blood mononuclear cells (PBMCs)

Human PBMCs (Lonza) were seeded into a 384-well cell culture plate at 1.875×10^4 cells per well. The cells were maintained in RPMI 1640 medium with 10% human serum (Lonza). Purified recombinant antibodies were added to the plate. *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO-1 (provided by Dr. Naomasa Goto, Kyoto Pharmaceutical University) and *E. coli* 21006 (provided by BML, Tokyo, Japan) were boiled in boiling water to prepare heat-killed bacteria. Heat-killed *P. aeruginosa* or heat-killed *E.coli* was added to the wells at a final concentration of 1×10^6 colony formation units (CFU)/ml. The next day, TNF-α concentrations in the cell culture supernatant were quantified using an AlphaLISA human TNF-α Immunoassay Kit (PerkinElmer). The fluorescence intensity was measured using an EnVision 2105 Multimode Plate Reader (PerkinElmer) according to the AlphaLISA protocol.

3.2.7 Binding affinity analysis by surface plasmon resonance (SPR)

The binding kinetics and affinity of each antibody were analyzed using a Biacore T200 (GE Healthcare UK Ltd, England). A recombinant anti-human IgG antibody was immobilized onto a CM5 biosensor chip using a Human Antibody Capture Kit (GE Healthcare). SPR was performed at flow rate of 5 μL/min in HEB-EP+ buffer. The monoclonal or bispecific antibodies were diluted to 0.25 μg/ml or 0.5 μg/ml and captured in the flow cells. Serial dilution of recombinant human TLR2 or recombinant human TLR4 was performed using HBS-EP+ buffer (GE Healthcare) and were injected at flow rate of 50 μL/min. K_D was calculated using the formula of K_d/K_a, where K_d and K_a were measured in the binding kinetics analysis using Biacore T200 software (GE Healthcare) with a 1:1 binding model.

3.3 Results and Discussion

3.3.1 Generation and characterization of anti-TLR2 and anti-TLR4 antibodies

Humanization of monoclonal antibodies was conducted to minimize the immunogenicity risk of treating patients; however, fully human antibodies are rationally less immunogenic in the human body. Velocimmune mice, which produce chimera antibodies, were leveraged to generate anti-human TLR2 and human TLR4 antibodies. After immunizing VelocImmune mice with recombinant human TLR2 or TLR4, hybridomas were generated to screen for monoclonal antibodies that bound to and neutralized human TLR2 or TLR4. We identified an anti-human TLR2 antibody which we designated #31 and an anti-human TLR4 antibody which we designated #48. The CDR sequences of both antibodies were analyzed according to the numbering schemes described above and grafted into a human FWR to improve their physicochemical properties. CDR grafting can be used to increase structural stability even when an antibody's sequences are derived from human genes. After CDR grafting, #31 and #48 were redesignated as mAb-2 and mAb-4, respectively.

The antagonistic activities of mAb-2 and mAb-4 were evaluated using a neutralization assay employing the U937 human monocyte cell line. Both mAbs inhibited ligand-mediated IL-6 secretion by U937 cells that were exposed to the synthetic TLR2-specific ligand, Pam2CSK4, or the TLR4-specific ligand, LPS (Fig. 3.3A and B).

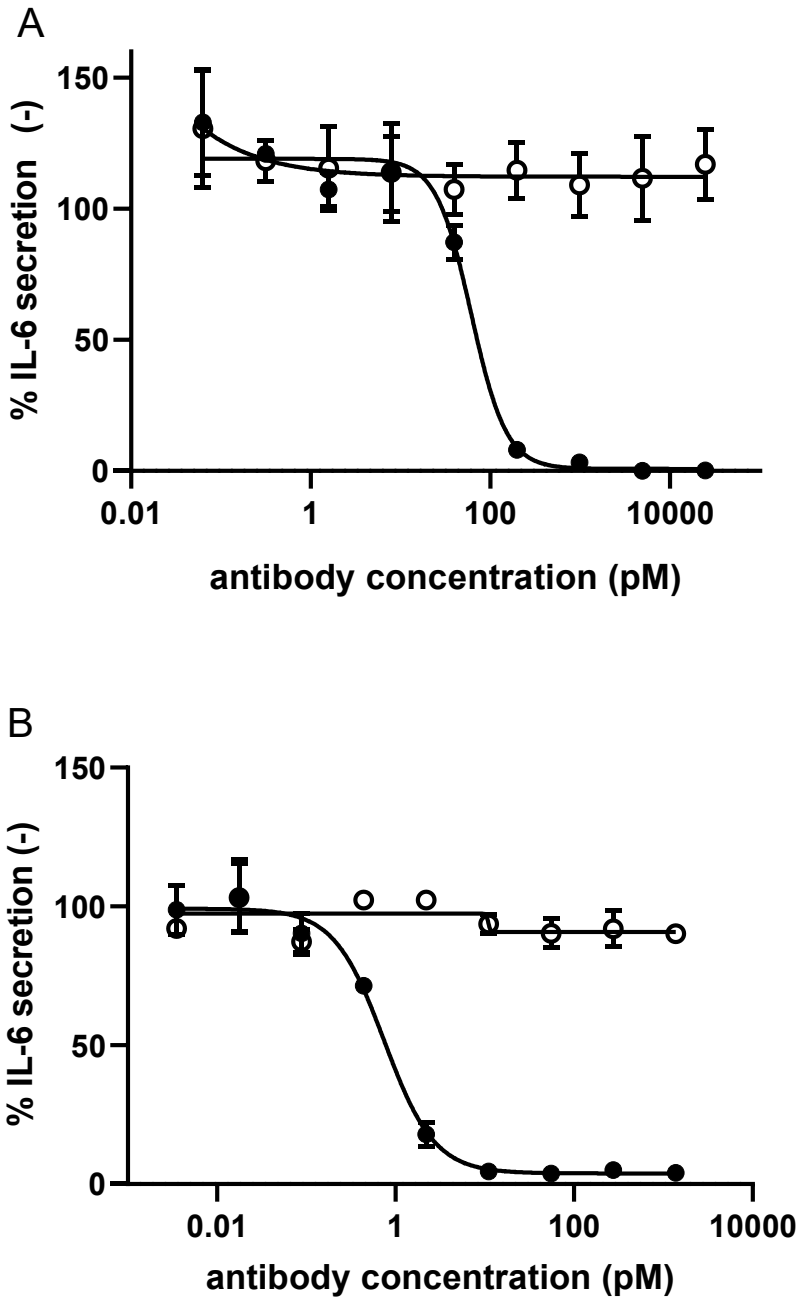


Figure 3.3. Neutralization assay of TLRs. (A) The neutralizing activity of mAb-2 was determined using U937 cells. The cells were treated with Pam2CSK4, an agonist of TLR2, in the presence of mAb-2. Closed circles: mAb-2, open circles: isotype control. The mean value in antibody-untreated wells was defined as 100% IL-6 secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Data (mean ± SD) represent two independent experiments performed in triplicate. (B) The neutralizing activity of mAb-4 was determined using U937 cells treated with LPS. Closed circles: mAb-4, open circles: isotype control. The mean value in antibody-untreated wells was defined as 100% IL-6 secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Data (mean ± SD) represent two independent experiments performed in triplicate.

3.3.2 Design of four types of IgG-scFv

Among the many types of bispecific antibodies, I speculated that tetravalent bispecific antibodies such as IgG-scFv and DVD-Ig would exhibit potent binding affinity for target proteins because multivalency should increase apparent affinity. Moreover, binding affinity is commonly correlated with antagonistic activity. Potent neutralizing activity is preferable for targeting TLR2 and TLR4 because it would enable rapid improvement in over-reactive immune responses in the early stage of sepsis. Therefore, to achieve the functional properties required to treat sepsis, I sought to identify a multivalent bispecific antibody with powerful antagonistic activity. However, DVD-Ig is known to induce steric hindrance between the inner and outer variable domains [55]. Although this issue was addressed in the report on IL-1α x IL-1β DVD-Ig, it was unclear whether DVD-Ig directed at proteins expressed on the cell membrane would also circumvent steric hindrance. Hence, I tried to generate IgG-scFv directed against TLR2 and TLR4.

I generated four types of IgG-scFv according to the four fusion positions identified for scFv (Figure 3.4). Structural stability and production yields differed among the four IgG-scFv [52]. Due to the structural instability of scFv, I speculated that IgG-scFv would have limitations related to protein expression or long-term storage. Therefore, a stable or stabilized scFv is crucial for generating IgG-scFv. Artificially introducing a disulfide bond between the VH and VL of scFv is known to be an effective way to stabilize scFv. Thus, I generated four types of IgG-scFv using the anti-TLR2 antibody designated mAb-1 and anti-TLR4 antibody designated mAb-4 to select the best format of IgG-scFv for a bispecific antibody directed at TLR2 and TLR4. Although mAb-1 had similar pharmacological profiles to mAb-2, it was used only in investigational studies to identify the best format for the bispecific antibody. Each variable sequence of mAb-1 or mAb-4 was genetically

modified to scFv and fused to the C-terminus or N-terminus of the heavy chain or light chain. Some formats had a disulfide-stabilized Fv (dsFv). After expressing and purifying the IgG-scFv or IgG-dsFv, a neutralization assay was performed to evaluate their antagonistic activity against TLR2 and TLR4 (Table 3.1 and 3.2).

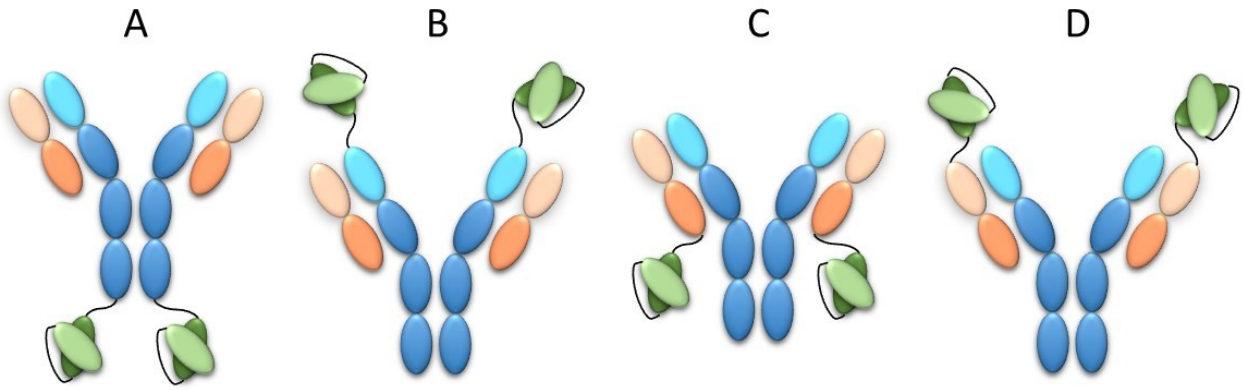


Figure 3.4. Four types of Ig-scFv. The structures of the IgG-scFv are divided into four types according to the fusion position of scFv. (A) scFv is fused to the C-terminus of the heavy chain. (B) scFv is fused to the N-terminus of the heavy chain. (C) scFv is fused to the C-terminus of the light chain. (D) scFv is fused to the N-terminus of the light chain.

Table 3.1. Type A

					EC50 (ng/mL)	
	IgG	scFv	Linker	Disulfide	TLR2	TLR4
1	mAb-1	mAb-4	(G4S)4	-	12	0.24
2	mAb-1	mAb-4	(G4S)8	-	8.2	0.10
3	mAb-1	mAb-4	(G4S)8	+	9.2	0.18
4	mAb-1	mAb-4	(G4S)12	-	12	0.24
5	mAb-4	mAb-1	(G4S)4	-	77	1.1
6	mAb-4	mAb-1	(G4S)8	-	49	0.93
7	mAb-4	mAb-1	(G4S)8	+	86	0.96
8	mAb-4	mAb-1	(G4S)12	-	partial inhibition	2.43
mAb-1	-	-	-	-	4.9	-
mAb-4	-	-	-	-		0.14

Table 3.2 Type B

					EC50 (ng/mL)	
	IgG	scFv	Linker	Disulfide	TLR2	TLR4
9	mAb-1	mAb-4	(G4S)4	-	1.5	0.52
10	mAb-1	mAb-4	(G4S)8	-	3.8	0.10
11	mAb-4	mAb-1	(G4S)4	-	4.3	0.65
12	mAb-4	mAb-1	(G4S)8	-	5.4	0.36
mAb-1	-	-	-	-	0.65	
mAb-4	-	-	-	-		0.13

In type A, scFv was fused to the C-terminus of the heavy chain. While the antagonistic activity of mAb-4 against TLR2 and TLR4 was comparable when it was converted to scFv, the potency of mAb-1 decreased when it was transformed to scFv. This is consistent with the results obtained for type B, where scFv was fused to the N-terminus of the heavy chain. Thus, I concluded that antibody mAb-1 was not suitable for forming scFv. Although the potency of mAb-4 remained unchanged when it was converted to scFv, the potency of mAb-1 decreased in the type B format. This was thought to be caused by steric hindrance of the mAb-4 scFv close to the variable domain of mAb-1. Further, using the long (G4S)8 linker did not improve the reduced potency. Similar to observations in type A, linker length did not have a major impact on inhibitory activity in type B. In type C, scFv was fused to the C-terminus of the light chain. No expression was observed in the culture supernatant when either mAb-1 or mAb-4 was converted to scFv. In type D, scFv was fused to the N-terminus of the light chain. Antibodies of this format exhibited decreased potency in the IgG domain (data not shown). Moreover, the disulfide bond in scFv did not affect the potency of mAb-4, which I speculate may contribute to the improvement in stability of mAb-4 scFv without reducing inhibitory activity. From these exploratory tests of bispecific antibody generation, I concluded that mAb-4 was suitable for conversion to scFv

to generate an IgG-scFv directed against TLR2 and TLR4. Furthermore, the dsFv of mAb-4 exhibited comparable potency to that of mAb-4. These data suggest that an IgG-dsFv in which the mAb-4 dsFv is fused to the C-terminus of the mAb-1 heavy chain is the optimal format for a TLR2 x TLR4 bispecific antibody.

3.3.3 Generation of the novel TLR2 x TLR4 bispecific antibody ICU-1

Based on the exploratory tests of IgG-scFv generation described above, I attempted to generate a TLR2 x TLR4 bispecific antibody with the type A format, in which mAb-4 is converted to dsFv and fused to the C-terminus of the heavy chain of mAb-2. I generated this tetravalent bispecific antibody and designated it ICU-1. Expression and purification of ICU-1 were performed using standard techniques. To assess the physicochemical properties of ICU-1, changes in the monomer content and aggregation were evaluated using SEC after storage at 5°C for 13 weeks. The monomer content or aggregation of ICU-1 did not change after storage compared to before (Figure 3.5). This data suggests that the IgG-dsFv format had sufficient stability when mAb-4 was converted to dsFv and fused to the heavy chain of mAb-2. Moreover, ICU-1 was thought to exhibit good developability even when not stored in optimized formulation buffer.

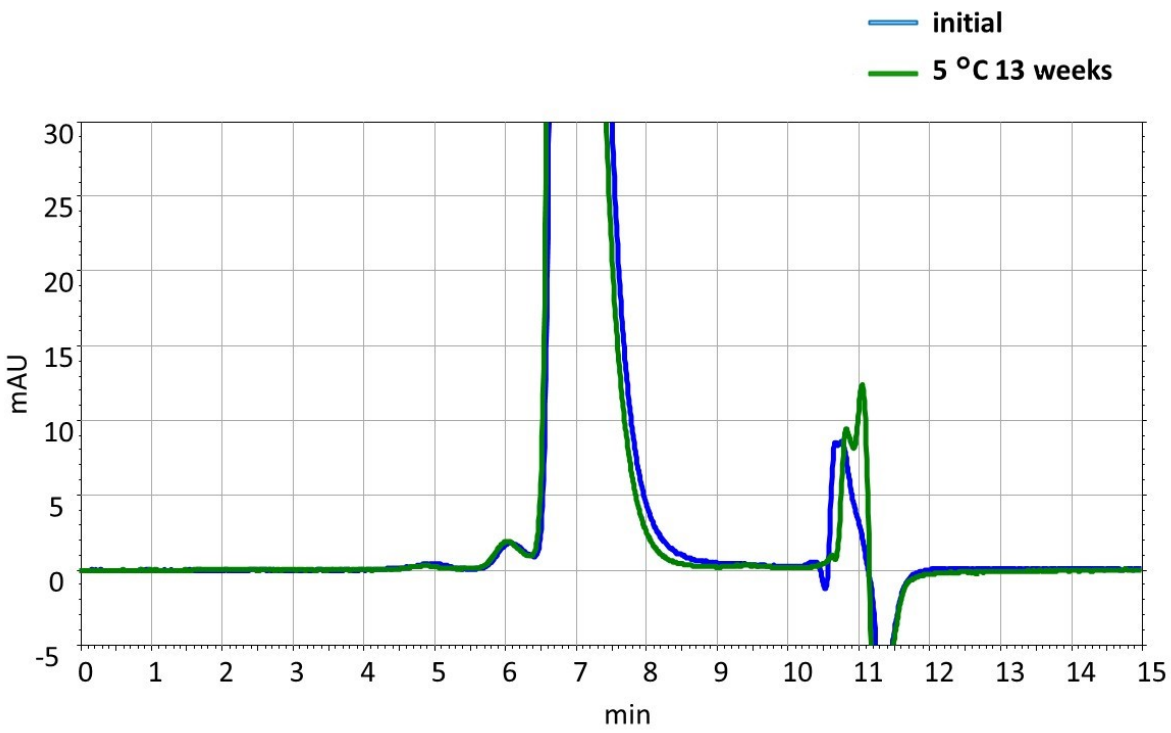


Figure 3.5. SEC analysis of ICU-1 after incubation at 5°C for 13 weeks. The purified ICU-1 was analyzed before (blue) and after (green) incubation.

3.3.4 Characterization of ICU-1

One of the major concerns when generating a bispecific antibody is that the antibody will show decreased binding affinity and specificity due to loss of the original VH-VL orientation. Although I had already confirmed that the type A IgG-scFv of mAb-1 and mAb-4 exhibited comparable functional activity to the parental antibodies in Table 3.1, I compared the binding and neutralization activity of ICU-1 with those of the parental antibodies again. The binding affinity of ICU-1 for TLR2 was comparable to that of mAb-2, while affinity for TLR4 was three-fold weaker than that of mAb-4 (Table 3.3).

Table 3.3. K_D values (nM) analyzed by SPR

	TLR2	TLR4
mAb-2	1.71	
mAb-4		1.51
ICU-1	1.00	4.66

Next, the antagonistic activity of ICU-1 was assessed in a neutralization assay using the U937 cell line. ICU-1 demonstrated complete inhibition of ligand-induced IL-6 secretion and comparable antagonistic activity to the parental antibodies (Figure 3.6). These data suggest that ICU-1 has sustained antagonistic activity against TLR2 and TLR4.

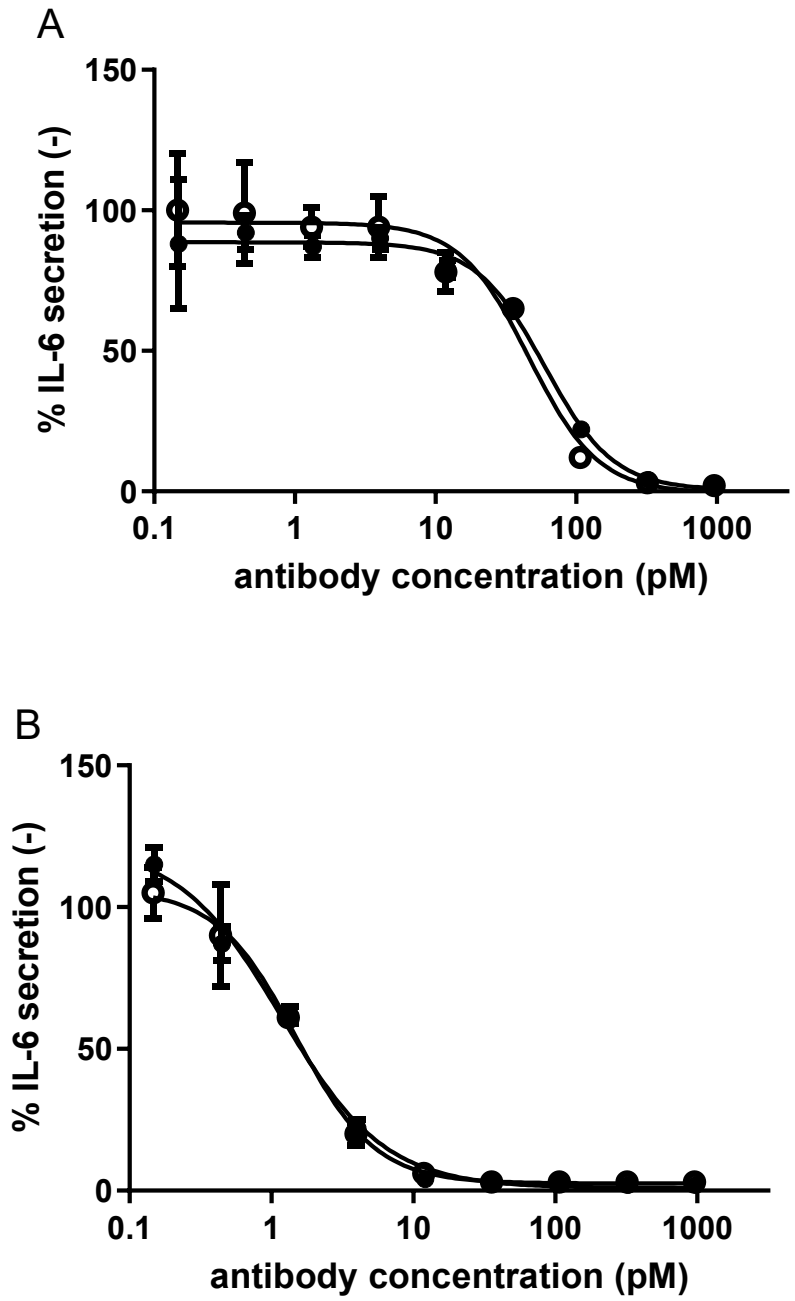
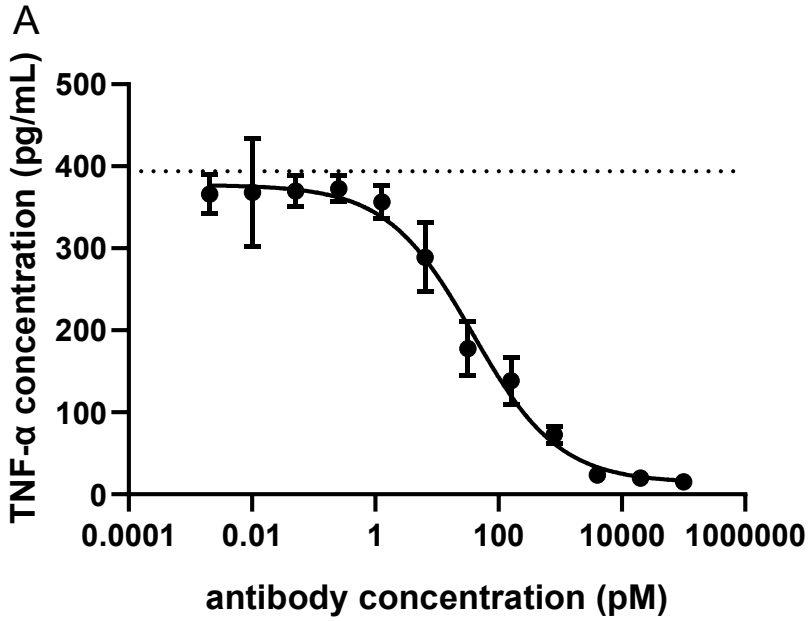


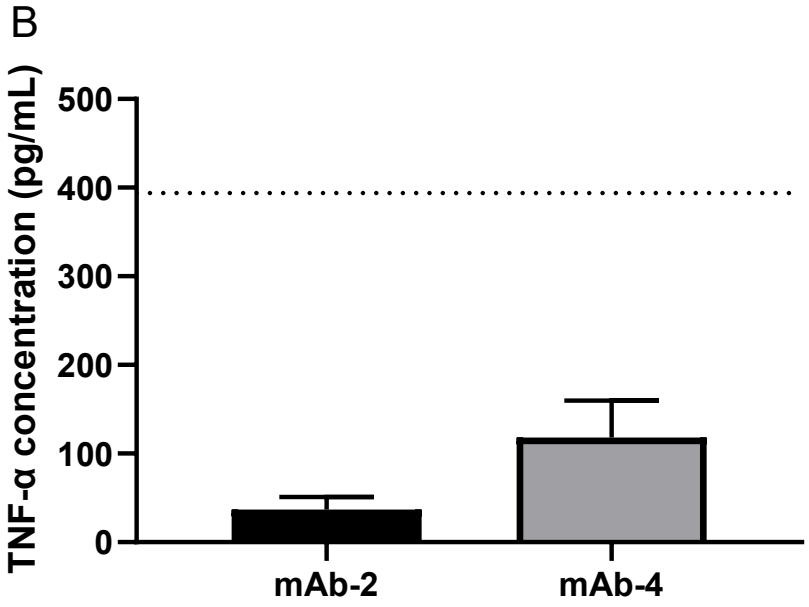
Figure 3.6. Inhibitory activity of ICU-1. Inhibitory activity of ICU-1 was measured using U937 cells treated with (A) Pam2CSK4 and (B) LPS. Closed circles: ICU-1, open circles: mAb-2 + mAb-4. The mean value in antibody-untreated wells was defined as 100% IL-6 secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Data (mean ± SD) represent two independent experiments performed in triplicate.

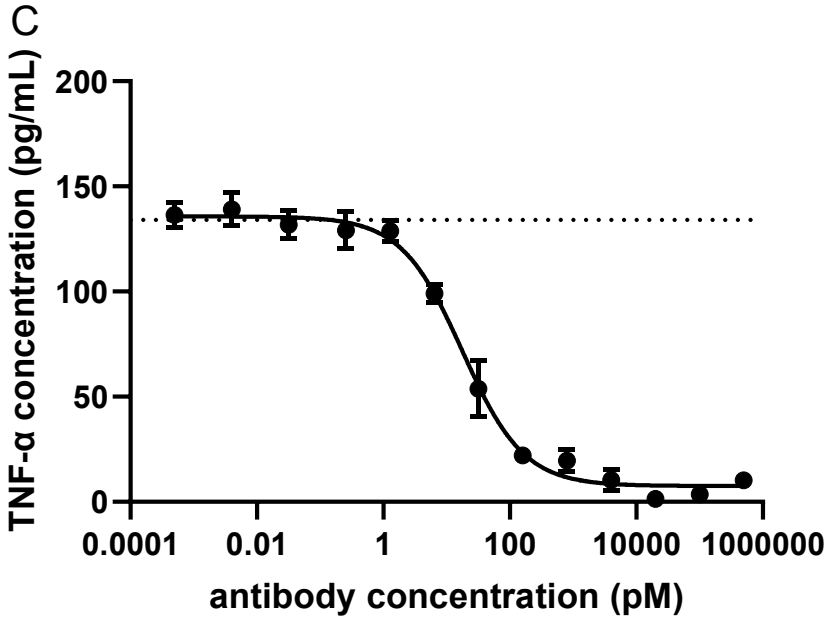
3.3.5 Therapeutic potential of ICU-1

Bacterial infection is the most common cause of sepsis and about 15% of sepsis patients reportedly harbor

multiple pathogens such as *E. coli* (21.5%) and *P. aeruginosa* (4.1%) [119]. Polymicrobial infection by gram-positive or gram-negative bacteria may simultaneously stimulate TLR2 and TLR4 to trigger an inflammatory response. Hence, understanding how these pathogens regulate TLR2 and TLR4 signaling would inform better treatments for sepsis in the clinic. To clarify the therapeutic availability of ICU-1, I assessed the inhibitory activity of ICU-1 against TLR2 and TLR4 in the presence of heat-killed bacteria. Human PBMC secreted TNF-α into the culture supernatant in the presence of heat-killed *E. coli* or *P. aeruginosa*. ICU-1 completely inhibited the bacteria-induced TNF-α production, while mAb-2 and mAb-4 showed only partial inhibition (Figure 3.7A-D). These data suggest that the bacterial components of a single pathogen stimulate both TLR2 and TLR4, which supports the hypothesis that dual blockade of TLR2 and TLR4 would be an effective therapeutic approach for sepsis.




B
TNF-α concentration (pg/mL)
500
400
300
200
100
0
mAb-2
mAb-4


C
TNF-α concentration (pg/mL)
200
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antibody concentration (pM)

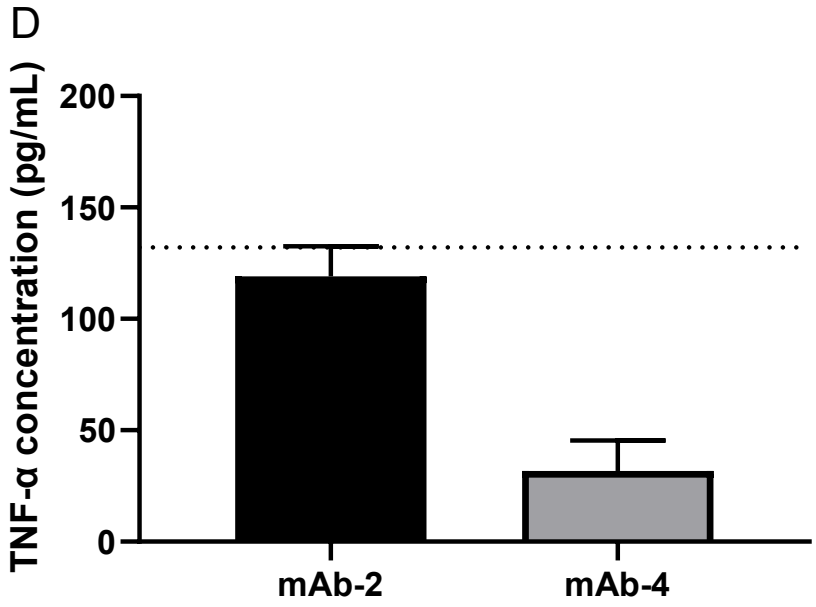


Figure 3.7. ICU-1 inhibits bacteria-induced immune response. Human PBMCs were treated with *P. aeruginosa*, a clinically relevant bacterium in sepsis, in the presence of (A) ICU-1 or (B) 1×10^5 pM of mAb-2 or mAb-4. Human PBMCs were treated with *E. coli*, a clinically relevant bacterium in sepsis, in the presence of (C) ICU-1 or (D) 5×10^5 pM of mAb-2 or mAb-4. Dotted lines show the mean value in antibody-untreated wells. Data (mean ± SD) represent two independent experiments performed in triplicate.

Although I succeeded in generating an IgG-scFv directed against TLR2 and TLR4, the bispecific antibody showed decreased binding affinity for TLR4. This may be because conversion of mAb-4 IgG to scFv did not completely preserve the VH-VL orientation for antigen binding. In contrast, the inhibitory activity of ICU-1 against TLR4 was surprisingly comparable to that of mAb-4. The mechanism underlying the binding affinity and inhibitory activity of ICU-1 should be clarified in further studies to provide a deeper understanding the binding mode of bispecific antibodies.

3.4 Conclusion

Although dual blockade of TLR2 and TLR4 has been reported to be more effective than single inhibition of either TLR2 or TLR4 in septic mouse models, no dual blocker of TLR2 and TLR4 has been reported to date. I generated an IgG-dsFv directed against TLR2 and TLR4 which completely inhibited bacterial stimulation. Additionally, the developability of ICU-1 was also confirmed through analysis of its physicochemical properties. Thus, ICU-1 is a potential therapeutic agent for treating sepsis.

Chapter 4 Binding mode analysis of a bispecific antibody directed at TLR2 and TLR4

4.1 Introduction

Advances in antibody engineering technology have led to the launch of engineered antibodies to the market and a wide variety of modified antibodies continue to be studied. Among the engineered antibodies, the number of bispecific antibodies being developed in clinical trials is increasing because their unique mechanism of action offers the potential for greater efficacy. The mechanism of bispecific antibodies is varied and complicated due to multivalency, in which multiple binding patterns can arise between the bispecific antibody and two target antigens. Despite the rise in the number of bispecific antibodies, few studies have reported the binding mode of bispecific antibodies, leaving their binding mechanism largely a mystery.

The bispecific antibody ICU-1 reported in Chapter 3 completely retained inhibitory activity against TLR4, despite its binding affinity for TLR4 being weaker than that of the parental antibody mAb-4. Since binding affinity and biological potency are thought to be correlated [120], this finding suggests that there is a discrepancy between the binding affinity determined by SPR and the antagonistic activity determined in the neutralization assay using U937 cells. I hypothesized that other factors compensated for the reduction in binding activity under physiological conditions. To confirm this theory, I again compared the inhibitory activity of ICU-1 and the combination of parental antibodies, this time in an assay using human PBCM in the presence of human serum, which was added to better mimic physiological conditions. Surprisingly, unlike the U937 assay in the previous study (Chapter 3), ICU-1 showed significantly greater antagonistic activity than the combination of parental antibodies (Figure 4.1). I speculate that the Fc-FcγR interaction influenced the binding affinity of ICU-1 in the U937 assay because the IgG antibodies in human serum used in the PBMC assay

blocked the Fc-FcγR interaction. From these results, I hypothesized that the cell-based assay induced an avidity effect on ICU-1 that compensated for the decrease in binding affinity measured by SPR. This data also implies that the actual potency of ICU-1 was hidden by the FcγR-mediated avidity effect since human serum contains high concentrations of human immunoglobulin which could inhibit the Fc-FcγR interaction. Enhancement of the antagonistic activity of an anti-TLR4 antibody has in fact been reported to occur via FcγR [121].

In addition, another study reported that the binding potency of a bispecific antibody changed depending on the expression ratio of the two antigens recognized by the bispecific antibody on the cell membrane. In particular, the study suggested that the antibody had high avidity for the antigen with lower expression [122]. Based on these reports, I hypothesized that there could be two main reasons why ICU-1 has stronger antagonistic activity than the combination of the parental antibodies. The first is the FcγR-mediated avidity effect and the second is the avidity effect mediated by simultaneous binding to TLR2 and TLR4. The aim of this study was to identify the binding mode of bispecific antibodies which affects their antagonistic activity.

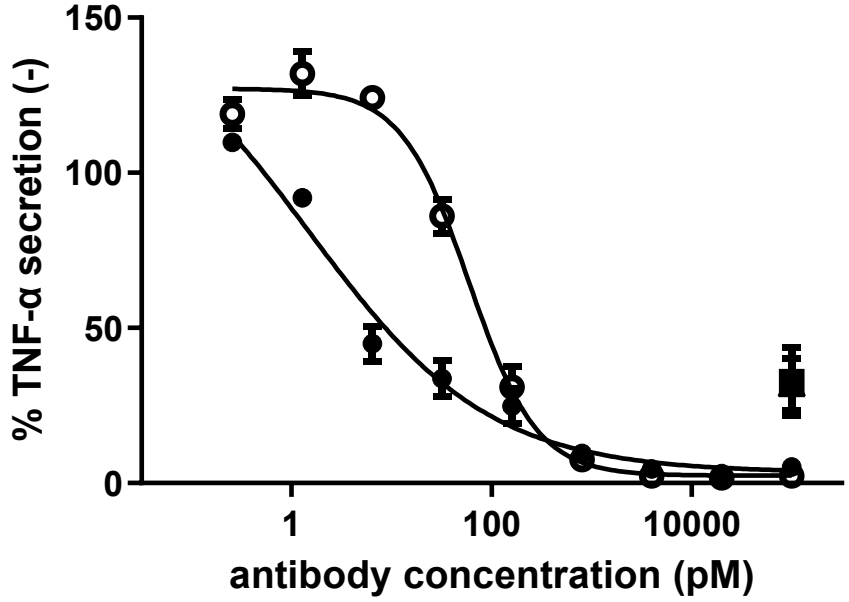


Figure 4.1. Comparison of the inhibitory activity of ICU-1 and mAb-2 + mAb-4. Human PBMCs were treated with *P. aeruginosa*, a clinically relevant bacterium in sepsis, in the presence of ICU-1, mAb-2 and mAb-4 combined, or 1×10^5 pM of mAb-2 or mAb-4. The mean value in the antibody-untreated wells was defined as 100% TNF-α secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Closed circles: ICU-1, open circles: mAb-2 + mAb-4, open squares: mAb-2, open triangles: mAb-4. Data (mean ± SD) were obtained from duplicate experiments.

4.2 Materials and Methods

4.2.1 Neutralization assay using U937 cells

The human monocyte-like cell line U937 (ATCC CRL1593.2) was maintained in RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) with 10% FBS. The cells were seeded into 96-well cell culture plates (AGC Techno Glass, Tokyo, Japan) at 7.5×10^4 per well. Subsequently, 100 nM PMA (Sigma–Aldrich, St. Louis, USA) was added to the wells to induce the differentiation of monocytes into macrophages and increase expression levels of TLR2 and TLR4. After overnight culture, purified mAb-2, mAb-4 or ICU-1 antibodies were added to the wells and incubated at 37°C for 30 min. Pam2CSK4 (InvivoGen, CA, USA), a synthetic

ligand for TLR2, was added to the wells at a final concentration of 10 ng/mL. *E. coli* Serotype 0111:B4 LPS (InvivoGen, CA, USA), a TLR4 ligand, was added to the wells at a final concentration of 100 ng/ml. The plates were incubated overnight at 37°C. The cell culture supernatants were collected the next day. The IL-6 concentrations in the cell culture supernatant were quantified using an AlphaLISA human IL-6 Immunoassay Kit (PerkinElmer, MA, USA). The fluorescence signal was read by EnVision 2105 Multimode Plate Reader (PerkinElmer) according to the AlphaLISA protocol. Inhibitory activities of each antibody were expressed as % IL-6 secretion, which was calculated based on the IL-6 levels in each treated well compared with that in untreated wells.

4.2.2 Neutralization assay using human PBMCs

Human PBMCs (Lonza) were seeded into a 384-well cell culture plate at 5×10^4 cells per well. The cells were maintained in RPMI 1640 medium with 10% human serum (Lonza). Purified mAb-2, mAb-4 and ICU-1 antibodies were added to the wells. *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO-1 (provided by Dr. Naomasa Goto, Kyoto Pharmaceutical University) and *E. coli* 21006 (provided by BML, Tokyo, Japan) were boiled in boiling water to prepare heat-killed bacteria. Heat-killed *P. aeruginosa* PAO-1 (provided by Dr. Naomasa Goto, Kyoto Pharmaceutical University) was added to the wells at a final concentration of 1×10^6 CFU/ml. The next day, TNF-α concentrations in the cell culture supernatant were quantified using an AlphaLISA human TNF-α Immunoassay Kit (PerkinElmer). The fluorescence intensity was measured by an EnVision 2105 Multimode Plate Reader (PerkinElmer) according to the AlphaLISA protocol. Inhibitory activities of each antibody were expressed as % TNF-α secretion, which was calculated based on the levels of

TNF-α in each treated well compared with that in untreated wells.

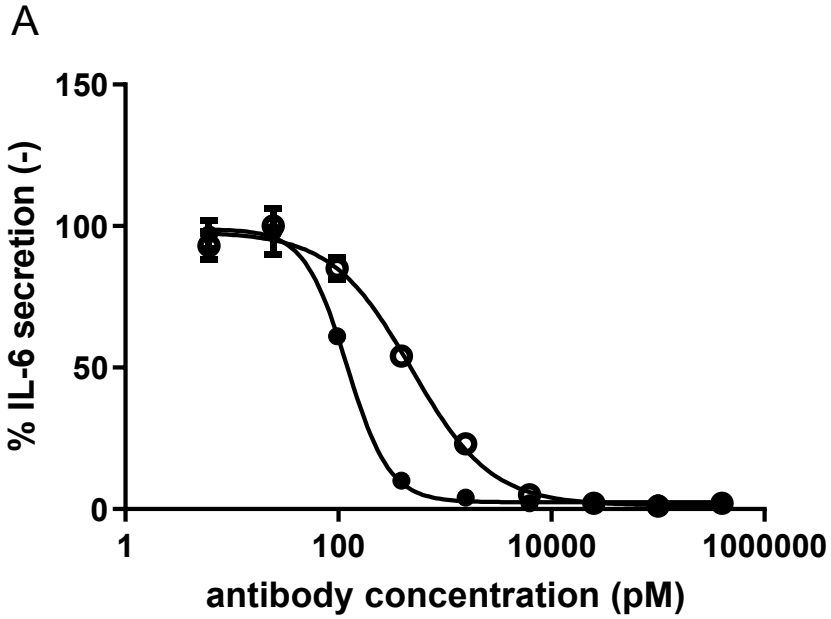
4.2.3 Flow cytometry analysis

Expression levels of TLR2, TLR4 and FcγRI (CD64) were determined using flow cytometry. The following primary antibodies were used: anti-TLR2 (in-house mAb-2 with mouse IgG2a Fc), anti-TLR4 (in-house mAb-4 with mouse IgG2a Fc), anti-CD64 (R&D Systems) and anti-keyhole limpet hemocyanin (KLH) (in-house mouse IgG1 Fc, used as isotype control). U937 (ATCC CRL1593.2) cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) containing 10% FBS as described above. Collected U937 cells were plated on a 96-well U-bottom plate (Corning) at 2×10^5 cells/well and incubated with 10 μg/mL of primary antibody for 30 min on ice. The samples were then washed twice with 200 μL of FACS buffer composed of PBS (-), 5% FBS and 0.1% sodium azide. Next, 2 μg/mL of Alexa488-labeled anti-mouse IgG secondary antibody (Thermo Fisher Scientific, MA, USA) was added. After washing the samples twice with FACS buffer, the expression levels of each receptor were analyzed using a Guava easy Cyte HT Flow Cytometer (Luminex, TX, USA).

4.3 Results and Discussion

TLR4 is a key player in inflammatory diseases and anti-TLR4 antibodies have been examined in clinical trials [12]. One anti-human TLR4 antibody was reported to have enhanced antagonistic activity via FcγR [121]. Since ICU-1 harbors a human IgG1 Fc which binds strongly to FcγRs, I hypothesized that binding to FcγRs induces an avidity effect and compensates for the decreased affinity of ICU-1 to TLR4. To confirm this hypothesis, the U937 assay was repeated in the presence of excess amounts of anti-KLH human IgG antibody

(hIgG), which bind to FcγRs. Antagonistic activity of mAb-2 and ICU-1 against TLR2 decreased in the presence of hIgG (Figure 4.2A and B). As there have been no reports that FcγR contributes to the antagonistic activity of anti-TLR2 antibodies, this could be the first report to suggest a relationship between TLR2 and FcγR. While the anti-TLR4 antagonistic activity of mAb-4 markedly decreased in the presence of hIgG, as expected, that of ICU-1 did not decrease at all (Figure 4.2C and D). These data suggest that binding to FcγR enhances the antagonistic activity of mAb-2 and ICU-1 against TLR2, and that of mAb-4 against TLR4, but, surprisingly, not that of ICU-1 against TLR4. Therefore, the results of this experiment do not support my hypothesis that FcγR contributes to the enhanced binding affinity of ICU-1 against TLR4.



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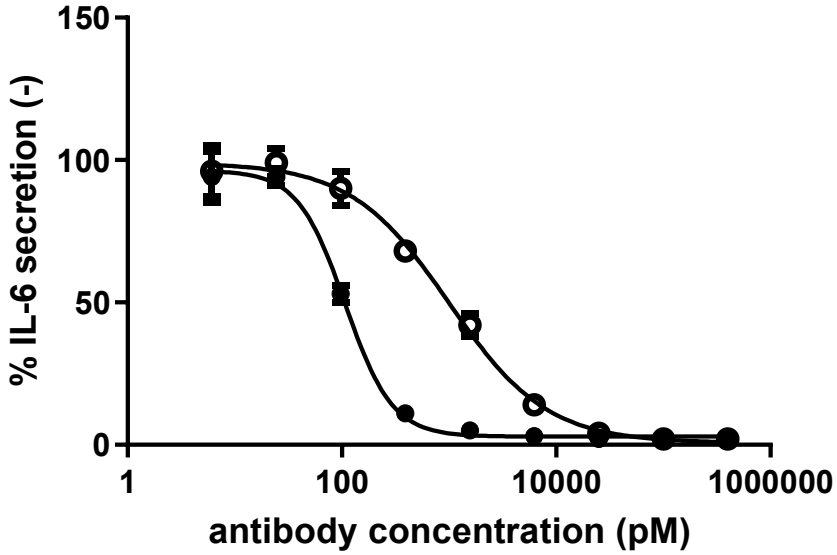
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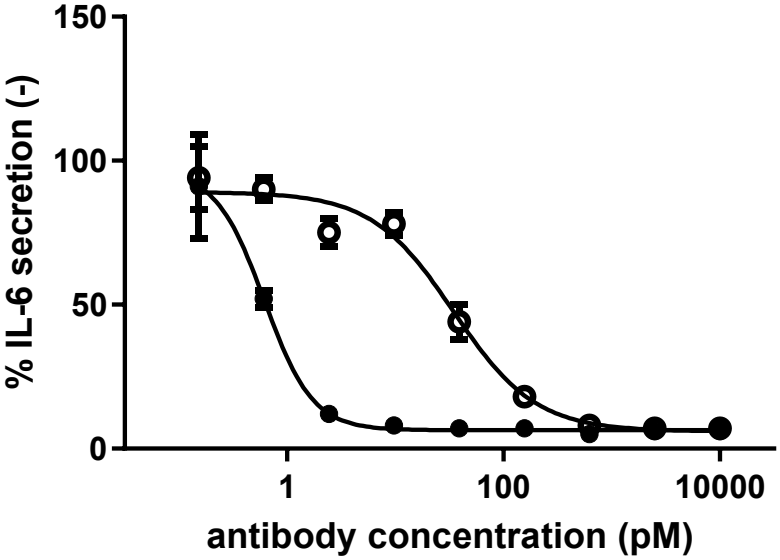
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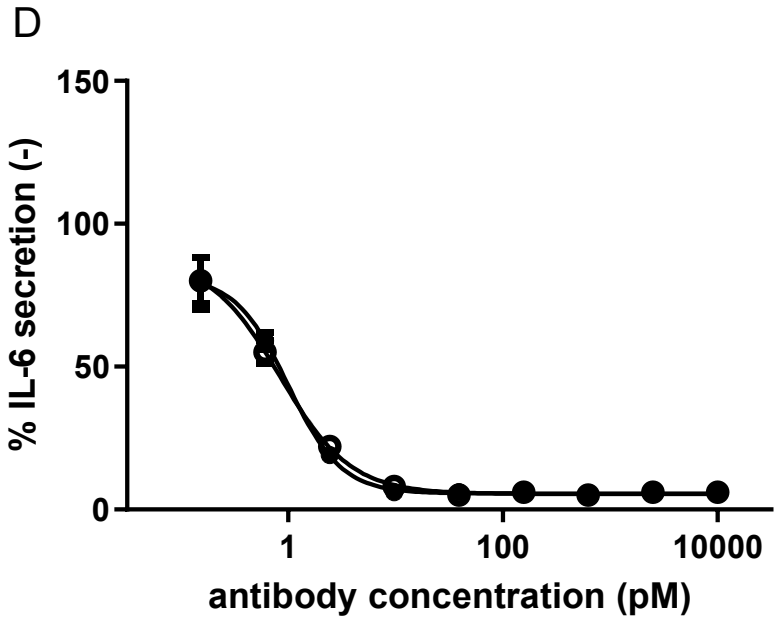

Figure 4.2. U937 assay in the presence of hIgG. U937 cells were treated with 100 ng/mL of Pam2CSK4 (A and B) or 100 ng/mL of LPS (C and D) for TLR2 or TLR4 stimulation, respectively. The cells were also treated with mAb-2, mAb-4 or ICU-1 before the stimulation in the presence or absence of 100 μg/mL of hIgG. (A) Closed circles: mAb-2, open circles: mAb-2 + hIgG. (B) Closed circles: ICU-1, open circles: ICU-1 + hIgG. (C) Closed circles: mAb-4, open circles: mAb-4 + hIgG. (D) Closed circles: ICU-1, open circles: ICU-1 + hIgG. The mean value in antibody-untreated wells was defined as 100% IL-6 secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Data (mean ± SD) represent two independent experiments performed in triplicate.

Next, I hypothesized that the binding mode of ICU-1 contributed to its antagonistic activity against TLR4. In a previous study, a bispecific antibody was reported to have greater avidity when the expression ratio of its two antigens was high. Specifically, the antibody's binding affinity for the antigen with lower expression seemed to increase, as the bispecific antibody bound to the antigen with lower concentration [122]. From this report, I expected the expression levels of TLR2 and TLR4 to differ such that the antigen expression ratio was high. To determine the expression levels of each receptor, flow cytometry analysis was performed. Indeed, the expression level of TLR2 was higher than that of TLR4 on U937 cells. Moreover, the expression level of CD64 (FcγRI) was highest among the receptors (Figure 4.3A and B). These data suggest that FcγR could enhance

the apparent binding affinity of monoclonal antibodies (mAb-2 and mAb-4) or ICU-1 for the antigen with lower expression (TLR2 and TLR4, respectively). This finding supports the results of the blocking assay in which the presence of hIgG decreased inhibitory activity. Moreover, I predicted that TLR2 may also contribute to the enhanced binding affinity of ICU-1 for the antigen with lower expression (TLR4).

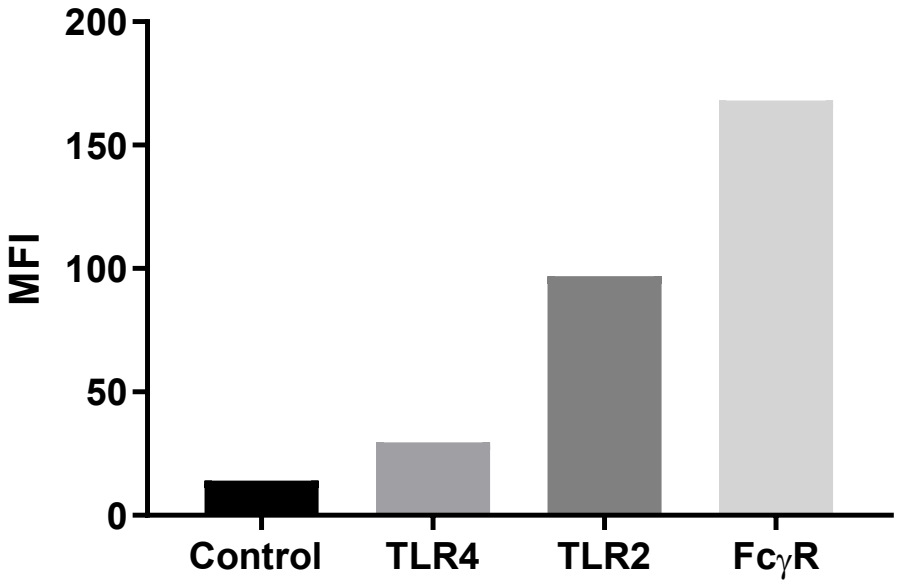

Figure 4.3. Expression levels of TLR2, TLR4 and FcγR on U937 cells. (A) Expression of each receptor on U937 cells was detected by mAb-2 (TLR2), mAb-4 (TLR4) and an anti-CD64 (FcγR) antibody using flow cytometry. The mean fluorescence intensity (MFI) of each receptor was analyzed.

To investigate the hypothesis that the apparent enhancement in binding affinity of ICU-1 against TLR4 was due to binding to TLR2, a competitive assay was performed. In the U937 assay, excess amounts of mAb-2 were added to block binding between ICU-1 and TLR2 in the presence of excess amounts of hIgG to mask FcγR. Blocking TLR2 in the presence of hIgG markedly decreased the antagonistic activity of ICU-1 against TLR4 (Figure 4.4). Given that antagonistic activity is correlated with binding affinity, this data suggests that simultaneous binding to TLR2 caused ICU-1 to exhibit enhanced binding affinity for TLR4.

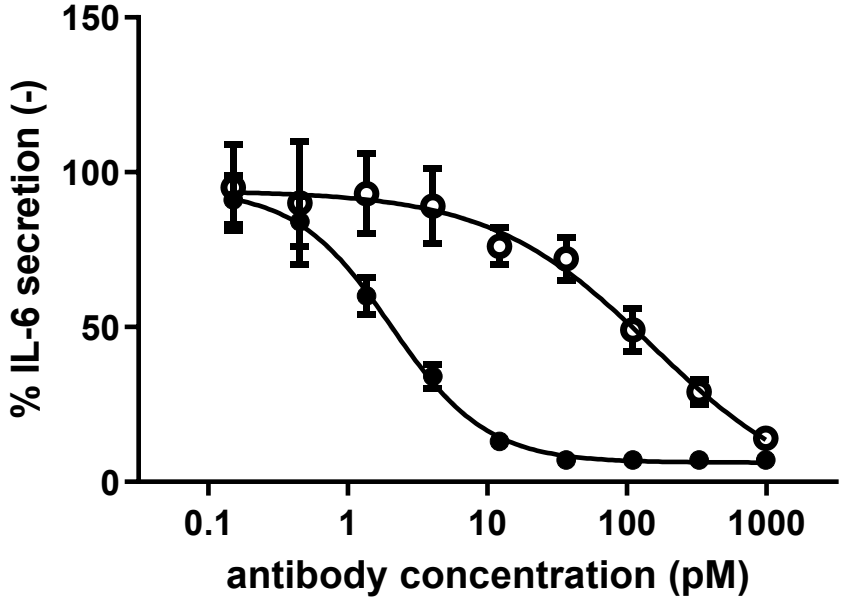

Figure 4.4. U937 assay with TLR2 blocker. U937 cells were stimulated with LPS in the presence of ICU-1 (closed circles) or ICU-1 with mAb-2 and hIgG (open circles). The mean value in antibody-untreated wells was defined as 100% IL-6 secretion and the mean value in ligand-untreated wells was defined as 0% and used as control. Data (mean ± SD) represent two independent experiments performed in triplicate.

I assumed that the inhibitory activity of ICU-1 against TLR4 would decrease because the KD value of ICU-1 was lower than that of mAb-4 (Chapter 3 Table 3.3). However, ICU-1 exhibited comparable antagonistic activity against LPS-mediated IL-6 secretion to the parental antibodies (Figure 3.6). Despite prior evidence indicating that the antigen expression ratio influences the binding affinity of a bispecific antibody, I could not assume the presence of an avidity effect of ICU-1 in the U937 assay until increased antagonistic activity of ICU-1 could be demonstrated in an assay using primary human PBMC (Figure 4.1). The PBMC assay included human serum to better mimic physiological conditions, where there are high concentrations of human immunoglobulin. Since human blood contains 10 mg/mL of immunoglobulin, 10% of human serum was thought to contain about 1 mg/mL of immunoglobulin, which was enough to block the Fc-FcγR interaction.

This data implies that the actual potency of ICU-1 was hidden by the FcγR-mediated avidity effect. As expected, in the presence of excess amounts of hIgG in the U937 assay, binding to FcγR enhanced antagonistic activity, except in the case of inhibition of TLR4 by ICU-1 (Figure 4.2). This data implies that the enhanced TLR4 neutralizing activity of ICU-1 contributes to more potent bacterial regulation compared to the combination of parental antibodies in the primary human PBMC assay when FcγR blocking is enhanced (Figure 4.1).

The avidity effect of a bispecific antibody was reported to be associated with the antigen expression ratio [84]. Since binding to the antigen with higher expression increases the apparent binding affinity for the antigen with lower expression, higher expression levels of TLR2 than TLR4 may have led to increased TLR4 neutralizing activity. The results of flow cytometry analysis supported this hypothesis, where, in addition to TLR2, FcγR was also more highly expressed compared to TLR4. Blocking FcγR with hIgG caused the inhibitory activity of mAb-2 and mAb-4 to decrease (Figure 4.2A and C). These data were reasonable and suggest that the FcγR-mediated avidity effect enhanced inhibitory activity against TLR2 and TLR4 because FcγR had the highest expression. One aspect of the relationship between FcγR expression and FcγR-mediated inhibitory activity that was unexpected was that ICU-1 showed comparable inhibitory activity against TLR4 in the presence and absence of hIgG. Considering that the difference in expression levels between FcγR and TLR4 was greater than that between TLR2 and TLR4, FcγR was more likely to exhibit greater neutralizing activity against TLR4 than TLR2. I assume that the binding affinity between Fc and FcγR was also crucial. The affinity constant (K_A) of hIgG for FcγRI has been reported to be $6.5 \times 10^7\ M^{-1}$, from which the K_D can be calculated from the formula ($K_D = 1/ K_A$) as 1.5×10^{-8} M [123]. Among the FcγRs, the K_A of FcγRI was highest and FcγRI was therefore most likely to exhibit increased neutralizing activity against TLR2 and TLR4.

However, it was inferior to the affinity of ICU-1 for TLR2 (Chapter 3 Table 3.3). Hence, the binding propensity of ICU-1 must be assessed based on both affinity and expression level.

The advantage of a bispecific antibody has been discussed in terms of developability. The U.S. food and drug administration (FDA) states that the safety of each investigational drug alone should be assessed in a Phase I clinical trial as well as in combination if two or more new investigational drugs are to be administered in combination to humans (https://www.fda.gov/media/80100/download). This requirement means that sponsors of clinical trials must carefully test the combination of new investigational drugs for the first-in-human dose. In contrast, a bispecific antibody does not need to be tested on multiple cohorts in a Phase I study, but rather, requires a standard dose escalation study of the bispecific antibody alone. While most sponsors develop bispecific antibodies for this reason, this advantage may not necessarily relate to the effect of the new investigational drug. In fact, the efficacy of a bispecific antibody has been thought to be the same as that of the combination of its parental antibodies. My findings in the present study, where I compared a bispecific antibody to its parental antibodies, addresses this long-standing issue. The observed potent inhibitory activity of the bispecific antibody in the *in vitro* assay has potential to accelerate the onset of action of the drugs. This is extremely important for treating sepsis because the immune reaction in patients with sepsis advances rapidly. Inflammatory cytokines such as TNF and IL-6 have been reported to increase within a few hours in patients with sepsis from its onset [124]. The rapid reaction of TLRs and subsequent cytokine production may not be fully inhibited by the combination of parental antibodies, the inhibitory activities of which I showed actually decreased in the presence of human serum. Thus, I propose that the ICU-1 bispecific antibody is superior to the combination of its parental antibodies not only in terms of developability but also efficacy in patients.

4.4 Conclusion

Binding mode analysis studies of the ICU-1 bispecific antibody clarified its mechanism of action. My binding mode analysis of ICU-1 examined the influence of Fc-FcγR-mediated binding and simultaneous binding to TLR2 and TLR4. While binding to FcγR increased the inhibitory activity of ICU-1 against TLR2, binding to TLR2, but not FcγR, increased inhibitory activity against TLR4. Thus, this study showed that simultaneous binding to two target antigens and the Fc-FcγR interaction can both affect the binding affinity of a bispecific antibody.

Chapter 5 General discussion

Antibody binding kinetics and K_D are typically determined using SPR. To accurately measure k_{on} and k_{off} using SPR, rebinding of an antibody molecule to the immobilized antigen should be avoided [125, 126]. This is because rebinding of an antibody to an antigen induces an avidity effect that can influence the accuracy of calculated K_D values. Rebinding occurs when there is a high density of antigens around an antibody molecule for it to rebind to after dissociation, which can result in higher K_D values [126-128]. The same situation is thought to be occurred in cell-based assays and in the human body where antigens are expressed at high density on the cell surface. Given that a therapeutic antibody works in such a situation to treat disease, pharmacological assays that reflect the physiological condition are important for accurate assessment of an antibody's abilities. Since the affinity and avidity of an antibody to its antigens have been reported to be associated with its functional capacity [129-132], SPR analysis using the 1:1 binding model is insufficient to evaluate the function of a therapeutic antibody.

As IgG antibodies have an Fc region which binds to FcγRs, an avidity effect can be induced to enhance agonistic or antagonistic potency. The results of the U937 assay were insufficient to conclude the functional capacity of the anti-TLR2 or anti-TLR4 antibody (Figure 3.6). Although FBS, which should be an abundant immunoglobulin, was added to the U937 assay, it was heat inactivated to remove CDC activity. Heat inactivation is generally performed by incubating at 56°C for 30 min, which may cause protein denaturation of the Fc of IgG. I speculate that this was the reason why excess amounts of hIgG reduced the inhibitory activity in the U937 assay despite inclusion of 10% FBS (Figure 4.2). To rectify this issue, human serum should be used in *in vitro* assays for therapeutic antibodies. Further, performing an *in vivo* study using rodent models

can be effective if the antibody cross-reacts with the target in rodents. However, ICU-1 did not bind to mouse TLR2 and mouse TLR4 (data not shown). Thus, I evaluated the inhibitory activity of ICU-1 in physiological conditions using human PBMCs and human serum (Figure 4.1).

Another essential consideration when assessing bispecific antibodies is the antigen expression ratio on the cell surface. A higher antigen ratio is associated with greater apparent affinity of a bispecific antibody for the antigen with lower expression [122]. A conceptual illustration of the binding mode of ICU-1 is depicted in Figure 1A-C. The anti-TLR2 antibody mAb-2 likely to binds to TLR2 with one of its two Fab arms and to TLR2 with the remaining Fab arm, which induces an avidity effect on mAb-2. This bivalency results when there is sufficient expression of TLR2 on the cell membrane (Figure 5.1A). While the anti-TLR4 antibody mAb-4 also likely to binds to TLR4 with one of its two Fab arms, it may find it difficult to bind to TLR4 with its remaining Fab arm on cells with lower TLR4 expression. No avidity effect is induced on mAb-4 in this case (Figure 5.1B). The bispecific antibody ICU-1 likely to binds to TLR2 because expression levels of TLR2 are higher than those of TLR4, which provides more chances of binding to TLR2. Next, ICU-1 can bind to TLR2 with its second Fab arm or to TLR4 with one of its two scFv arms (Figure 5.1C). When ICU-1 binds to TLR2 with its second Fab arm, an avidity effect is induced on ICU-1, as is observed in the case of mAb-2. When ICU-1 simultaneously binds to TLR2 and TLR4, an avidity effect is also induced on ICU-1. In this case, the apparent affinity of ICU-1 for TLR2 might be comparable to that in which ICU-1 binds to two TLR2 molecules through its two Fab arms, given that the K_D values of ICU-1 for TL2 and TLR4 are within several nM of each other. Thus, the apparent affinity and inhibitory activity of ICU-1 to TLR2 is likely the same as that of mAb-2. In contrast, the apparent affinity of ICU-1 for TLR4 is likely higher than that of mAb-4 because the avidity

effect on mAb-4 was rarely induced on cells with low TLR4 expression. Although these mechanisms are speculative based on the observed binding affinity and inhibitory activity of ICU-1, other studies offer support to my theory.

A prior study reported a similar model of antibody binding in which bivalent antibodies quickly bind to a second target with its remaining arm after binding to the first target. Moreover, a tetravalent bispecific antibody directed at MET and EGFR showed enhanced apparent binding affinity for the antigen with lower expression when the antigen expression ratio was high. This was thought to be associated with the occupancy of the two receptors. The bispecific antibody was more likely to occupy the antigen with lower expression at lower concentrations than the antigen with higher expression [122]. Moreover, another study reported that a bispecific antibody targeting MET and EpCAM had increased inhibitory activity on cells with a high antigen ratio. A computational simulation further demonstrated increased avidity of bispecific antibody when EpCAM expression was 10-fold higher than MET expression [84].

Given these reports, the high antigen ratio of TLR4 and TLR2 on U937 cells suggests that ICU-1 could have an avidity effect (Figure 4.3). Further, both receptors must be densely expressed for simultaneous binding by ICU-1. Reports of localization of TLR2 and TLR4 on the cell membrane suggest that this may be the case. Both receptors have been reported to accumulate on lipid rafts, although this accumulation is limited after ligand stimulation [133, 134]. Further, an anti-TLR4 antibody has been shown to have avidity effects via cross-linking with FcγR on the lipid rafts [135]. In the cellular assays performed here, antibodies were added before ligand stimulation. Therefore, I hypothesize that ICU-1 induced an avidity effect by simultaneously binding to TLR2 and TLR4 even in the unstimulated state. However, in clinical conditions, the treatment effect

of ICU-1 would be more potent than that observed in the in vitro assay because bacterial stimulation can induce accumulation of TLR2 and TLR4 on lipid rafts.

Although a range of bispecific antibodies have been produced since the concept of bispecific antibodies was first proposed in 1960 (General introduction), the binding mode of bispecific antibodies has remained unclear. Prior reports and my findings suggest that bispecific antibodies have enhanced inhibitory effects on cells with a high antigen ratio by binding to two receptors simultaneously and increasing avidity.

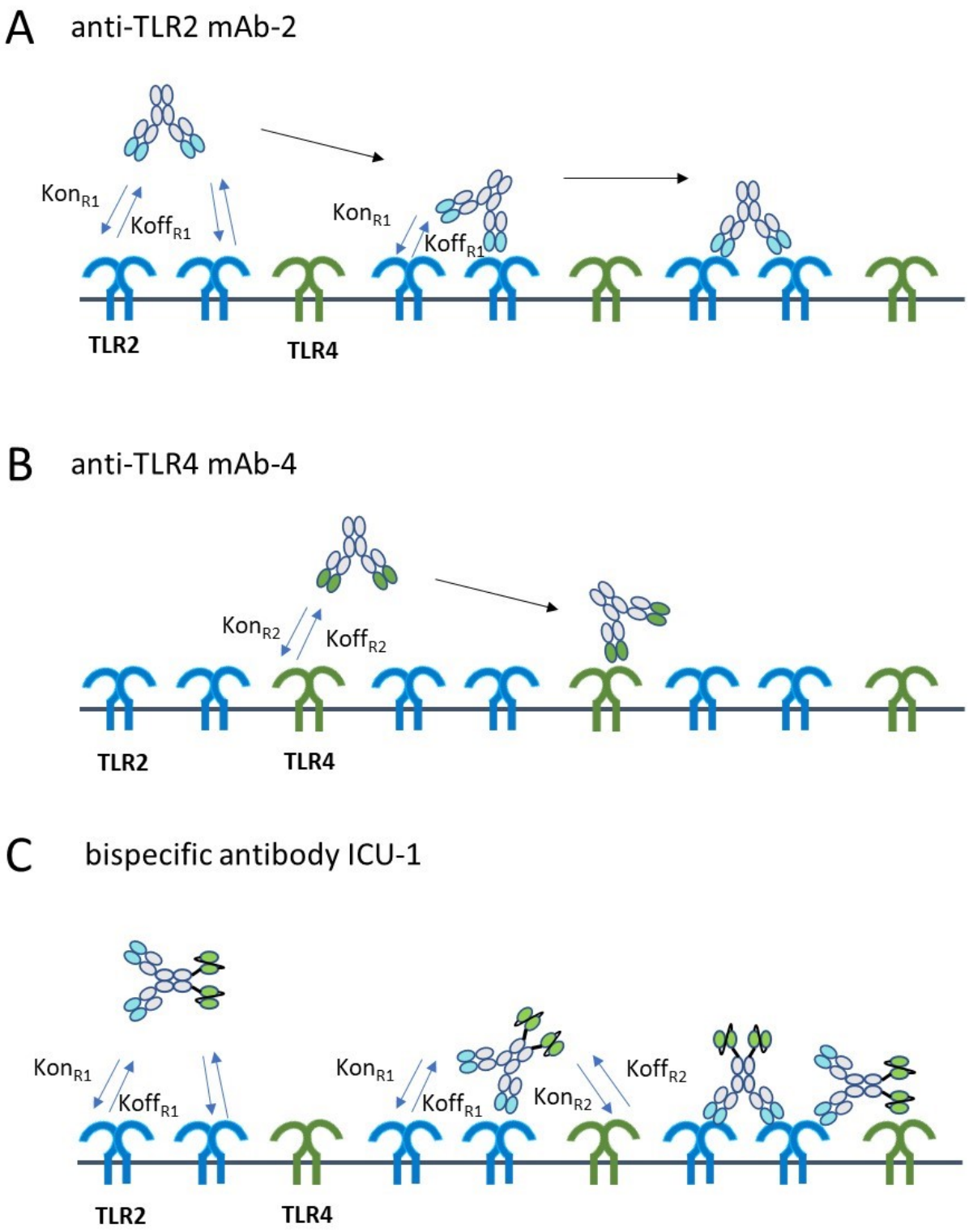

Figure 5.1. Illustration of the theorized binding mode of ICU-1.

Chapter 6 Overall conclusion and perspectives

My research on the binding mode of the bispecific antibody ICU-1 demonstrates the important finding that binding affinity changes according to antigen expression levels. A bispecific antibody potentially has higher functional activity on cells that differentially express its target antigens. This mechanism can be leveraged to regulate the activity of bispecific antibodies in different cells with different expression levels and to reduce the adverse effects of antibody therapeutic agents. A novel way to reduce the risk of adverse effects could be to first develop a bispecific antibody that binds to target 1 with high affinity and to target 2 with low affinity in healthy tissue. This bispecific antibody would have agonistic or antagonistic activity against target 2, which should be adjusted so that it is too weak to stimulate or block targets in healthy tissue. Next, in abnormal tissue, the expression level of target 1 should be higher than that of target 2. Cells with high expression of target 1 will induce an avidity effect on the bispecific antibody and facilitate agonistic or antagonistic activity against target 2. This strategy leads to site-specific activation in abnormal tissue and reduces adverse effects in healthy tissue. The most important part of this strategy is to select antigens that meet the following criteria: the antigen with lower expression in abnormal tissue should play a crucial role in improving the disease and the antigen with higher expression should be locally expressed at the site of the lesion. Today, antigen expression on the cell surface can by predicted using single-cell RNA sequence (scRNA seq) analysis or the Genotype-Tissue Expression (GTEx) database (https://gtexportal.org/home/singleCellOverviewPage), which can be used for antigen selection. Specifically, expression data of tumor-associated antigens is provided by The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The appropriate antigens to generate a bispecific antibody for treating cancer can be screened

based on the expression data. Selection of antigens based on my study and a published database is expected to aid in the generation of bispecific antibodies with a wide therapeutic index.

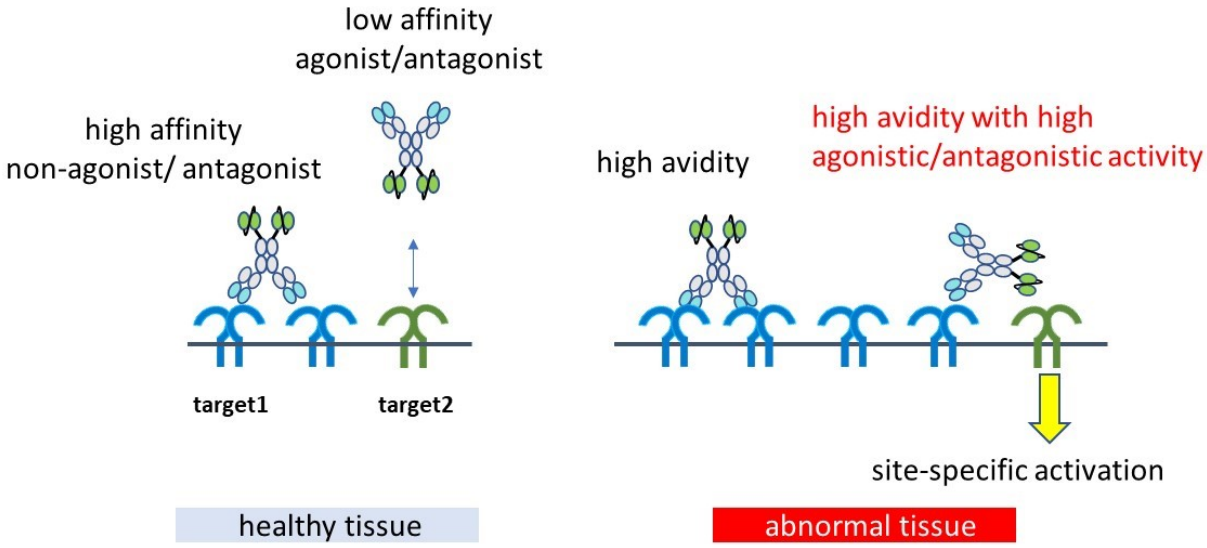

Figure 6.1 Illustration of site-specific activation by a bispecific antibody

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