# Studies on the Methodology for Hazard Identification and Mitigation of Cytokine Release Syndrome Caused by Monoclonal Antibody Pharmaceuticals

January 2020

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A Dissertation Submitted to
the Graduate School of Life and
Environmental Sciences, the University of
Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biological
Science (Doctoral Program in Biological Sciences)

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## Abstract

In this work, I focused on cytokine release syndrome (CRS), a type of immunotoxicity caused by monoclonal antibody (mAb) pharmaceuticals. Severe CRS can cause fatal or life-threatening reactions. The clinical signs of CRS correlate with immune cell activation following a cascade of systemic cytokine release. In the first-in-human trials to evaluate pharmaceutical candidates, the safety of clinical trial subjects is the priority. Identification of potential risk of CRS and appropriate strategies to mitigate the risk are critical aspects of the successful safety management of CRS. In 2006, a CD28 superagonist (CD28SA), TGN1412, caused severe CRS in a first-in-human trial. Preclinical studies using experimental animals failed to predict this CRS because of species differences in the immune system. The use of *in vitro* cytokine release assays with human cells are useful to identify potential risks of CRS. There are two major testing platforms for the detection of potential CRS risks: one composed of whole blood with aqueous-phase test articles (i.e. the whole blood cytokine assay [WBCA]), and the other composed of peripheral blood mononuclear cells (PBMCs) with solid-phase test articles (i.e. the PBMC assay). First, I determined an appropriate sample size and confirmed the suitability of the WBCA as a hazard identification tool for CRS. Next, I compared the cytokine producing cells after stimulation with the TGN1412 analogue, CD28SA, in the WBCA and the PBMC assay, and showed that different immune cells generate a positive response in the two *in vitro* assays, causing differences in the response to CD28SA. The results emphasize the need to understand the characteristics of these *in vitro* assays and to establish an optimal method that suits the mechanism of a therapeutic mAb candidate. It is currently possible to detect the potential risks of CRS, but it is still difficult to manage CRS. CD3 bispecific constructs show promising potential for cancer immune therapy, but they frequently induce CRS in clinical use. Finally, I investigated the effectiveness of an ascending dose regimen to mitigate CRS caused by a CD3 bispecific construct, ERY22, in cynomolgus

monkeys, and indicated that ascending doses can markedly mitigate CRS. The results from this study provide useful information for CRS hazard identification and CRS mitigation of mAb pharmaceuticals.

## Abbreviations

ADA anti-drug antibody

ADCC antibody-dependent cell-mediated cytotoxicity

ADCP antibody-dependent cellular phagocytosis

AF Alexa Fluor

APC antigen presenting cells

BCR B cell receptor

CD28SA anti-CD28 superagonistic mAb

CDC complement-dependent cytotoxicity

CRP C-reactive protein

CRS cytokine release syndrome

DAMPs damage-associated molecular pattern molecules

DC dendritic cell

DIC disseminated intravascular coagulation

ECLIA electrochemiluminescence immunoassay

EGFR epidermal growth factor receptor

EMA European Medicines Agency

GPC3 glypican 3

GVHD graft-versus-host disease

h hour

ICH International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IFN interferon

LYMPH lymphocyte

M mol/L

mAb monoclonal antibody

MABEL minimum anticipated biological effect level

MAC membrane attack complex

MHC major histocompatibility complex

min minute

MODS multiple organ dysfunction syndrome

NETs neutrophil extracellular traps

NHP nonhuman primate

NK cell natural killer cell

NOAEL no observable adverse effect level

PAD pharmacologically active dose

PAMPs pathogen-associated molecular pattern molecules

PBMC peripheral blood mononuclear cell

PRRs pattern recognition receptors

RESTORE assay resting T cells to original reactivity assay

RT room temperature

s second

SIRS systemic inflammatory response syndrome

TCR T cell receptor

TGF transforming growth factor

TNF tumor necrosis factor

Tc cytotoxic T cell

Teff effector T cell

Tfh follicular helper T cell

Th helper T cell

Treg regulatory T cell

WBCA whole blood cytokine assay

## General Introduction

*Immune toxicity and immune system*

Immunotoxicity is defined as adverse effects on the immune system caused by exposure to toxic substances, which modulate the immune system and may result in immunosuppression or immunostimulation. The immune system protects the host against foreign organisms and other substances (e.g. chemicals, pollens, potential food allergens and environmental agents). Since the immune system protects the host against invading agents, immunosuppressive substances make patients prone to infectious diseases and/or cancer. Immunostimulative agents can cause allergies, autoimmune diseases, or systemic inflammatory responses including cytokine release syndrome (CRS). Allergy occurs when the immune system reacts to allergens which are innocuous substances in the environment. In autoimmune diseases, healthy tissues are attacked by immune cells that fail to differentiate self-antigens from foreign-antigens (Gulati and Ray, 2009). The systemic inflammatory response can be triggered by various factors such as infections and certain drugs (Shimabukuro-Vornhagen *et al.*, 2018).

The immune system which is composed of immune cells, tissues, organs, and proteins protects the host against disease. There are traditional distinctions between adaptive and innate immunity (Litman *et al.*, 2005). The innate immune system, also called the nonspecific immune system, provides a general defense against pathogens and toxicants. Macrophages, mast cells, granulocytes (neutrophils, eosinophils, basophils), natural killer (NK) cells, and dendritic cells (DC) are leukocytes of the innate immune system, and some types of cells act as a bridge between innate and adaptive immunity (Table 1). Innate immune recognition is based on germline-encoded receptors called pattern recognition receptors (PRRs). PRRs are expressed in cells of the innate immune system and many epithelial cells,

which recognize pathogen-associated molecular pattern molecules (PAMPs) derived from microorganisms, and its signals trigger an immediate innate immune response and drive the adaptive immune response (Medzhitov, 2013). In contrast, damage-associated molecular pattern molecules (DAMPs) are released from host cells and alert the innate immune system to microbial invasion, unscheduled cell death, and in response to stress (Tang *et al.*, 2012). After activation of the innate immune system, immune cells are recruited to sites of infection and inflammation accompanied by cytokine production, which establishes a physical barrier and keeps pathogens from spreading and moving throughout the body (Gulati and Ray, 2009). Apart from cytokines, several chemical mediators including histamine, bradykinin, serotonin, leukotrienes, prostaglandins, oxygen- and nitrogen-derived free radicals are produced at the site of inflammation, which can contribute to, and alter inflammation (Abdulkhaleq *et al.*, 2018).

Table 1. Immune cells and functions based on Franks *et al.* (2015) and Abdulkhaleq *et al.* (2018)

| Cells | Function |
|---|---|
| Macrophages and monocytes | Phagocytosis, inflammatory induction, cytokine secretion, release of reactive oxygen species, tissue repair, and antigen presentation. |
| Mast cells and basophils | Allergic reaction, wound healing, defense against pathogens, and secretion of cytokines and chemical mediators such as histamine. |
| Neutrophils | Phagocytosis, secretion of cytokines and toxic reagents from granules, and neutrophil extracellular traps (NETs) formation. |
| Eosinophils | Defense against multicellular parasites, allergic reaction, secretion of toxic reagents, and free radicals. |
| NK cells | Cytokine secretion and cytotoxicity against infected host cells. |
| Dendritic cells | Antigen presentation, mediating key host defense to pathogens, and bridge between innate and adaptive immune response. |
| T cells | Cytokine secretion, cytotoxicity, and immune modulation. |
| B cells | Antibody production and antigen presentation |

The complement system is involved on the front line of the innate immune system where it defends the host from invading pathogens and clears potentially damaging debris (Cole and Morgan, 2003). The complement cascade is made up of a variety of proteins circulating in blood with nine major components designated C1 to C9. The complement system triggers the following immune responses: 1) opsonin (C3b) attaches to the surface of pathogens leading to phagocytosis, 2) anaphylatoxins (C3a and C5a) trigger degranulation of mast cells, increase vascular permeability and smooth muscle contraction (Drouin *et al.*, 2001), 3) chemotactic factors (C3a, C4a, C5a) attract granulocytes and monocytes to the site of infection and/or inflammation (Ward *et al.*, 1965; Piquette *et al.*, 1994), 4) the membrane attack complex (MAC), C5b-9, blasts holes in the cell membrane and mediates cell death, and 5) the complement is involved in the recognition and clearance of apoptotic cells (Cole and Morgan, 2003). In addition, the complement system can work as a part of the adaptive immune system, for example by enhancing response to antigens (Barrington *et al.*, 2001).

The adaptive immune system is based on somatically diversified and clonally expressed antigen receptors expressed on highly specialized, systemic cells such as T cells and B cells (Boehm, 2011). Both T cells and B cells carry antigen-specific receptors, the T cell receptor (TCR) or B cell receptor (BCR). T cells mediate the cell-mediated immune responses, whereas B cells are intimately involved in the humoral immune response (Fig. 1).

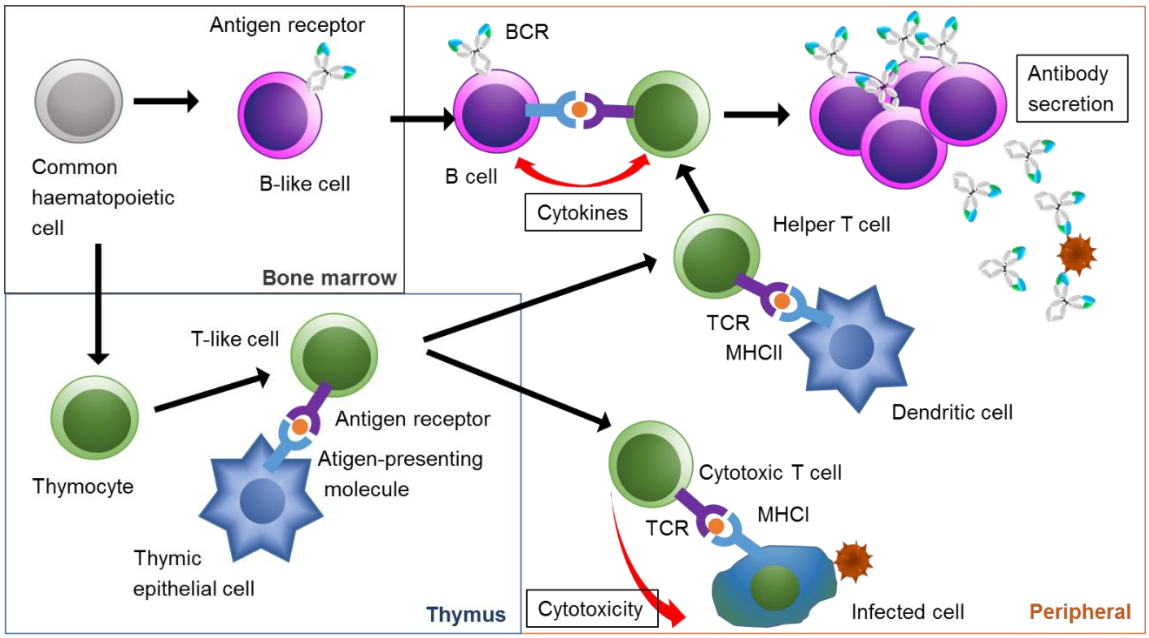


Figure 1. Adaptive immune system (Cambier *et al.*, 2007; Boehm, 2011; Radtke *et al.*, 2013).

There are two defined subpopulations of T cells: CD4+ helper T (Th) and CD8+ cytotoxic T (Tc) cells. TCRs bind to the major histocompatibility complex (MHC)/peptide complexes, which stimulate the adaptive immune system including formation of antibodies and Tc cells (Bachmann *et al.*, 2004). Antigen presenting cells (APC) such as DCs present antigen to naïve Th cells in conjunction with MHC class II, and the activation of naïve Th cells leads to differentiation into effector Th cells (Th1, Th2, Th9, regulatory T cells [Treg], Th17, follicular helper T cells [Tfh]). These Th-cell subsets have distinct effector functions with specific cytokine profiles (Table 2).

Table 2. Diversity of Th cells based on Stewart Leung *et al*. (2010) and Srivastava *et al.* (2018)

| T cell subsets | Cytokines for T cell differentiation | Cytokines produced by respective T cells | Functions |
|---|---|---|---|
| Th1 | Interleukin (IL)-12 | Interferon (IFN)-γ | Cell-mediated immunity, defense against intracellular pathogens, and activation of macrophages. |
| Th2 | IL-4 | IL-4 | Humoral immunity, defense against extracellular parasites, antibody production by B cells, allergy, and asthma. |
| Th9 | Transforming growth factor (TGF)-β, and IL-4 | IL-9 | Anti-tumor and prevention of autoimmunity. |
| Treg | TGF-β and IL-2 | IL-10 and TGF-β | Immune regulation, peripheral tolerance, and prevention of autoimmunity. |
| Th17 | TGF-β and IL-6 | IL-17, IL-12, and Tumor necrosis factor (TNF)-α | Cell-mediated inflammation, autoimmune diseases, defense against extracellular bacteria and fungi, and neutrophil activation. |
| Tfh | IL-6 and IL-21 | IL-6, IL-21, and TGF-β | Long term humoral immunity and defense against autoimmunity. |

The class I MHC presents endogenously synthesized antigens to Tc cells. Tc cells are essential for the control of some viruses (Bertoletti *et al.*, 1991) and are important for protection against tumor growth (Aerts and Hegmans, 2013).

B cells are essential for the production of antibodies that have an important role in humoral immunity, and recent studies have identified different B-cell subsets and its functions (Fig. 2). In addition, B cells mediate functions independent of antibody production, such as antigen presentation and the release of cytokines, including IL-10 (Tsiantoulas *et al.*, 2014).

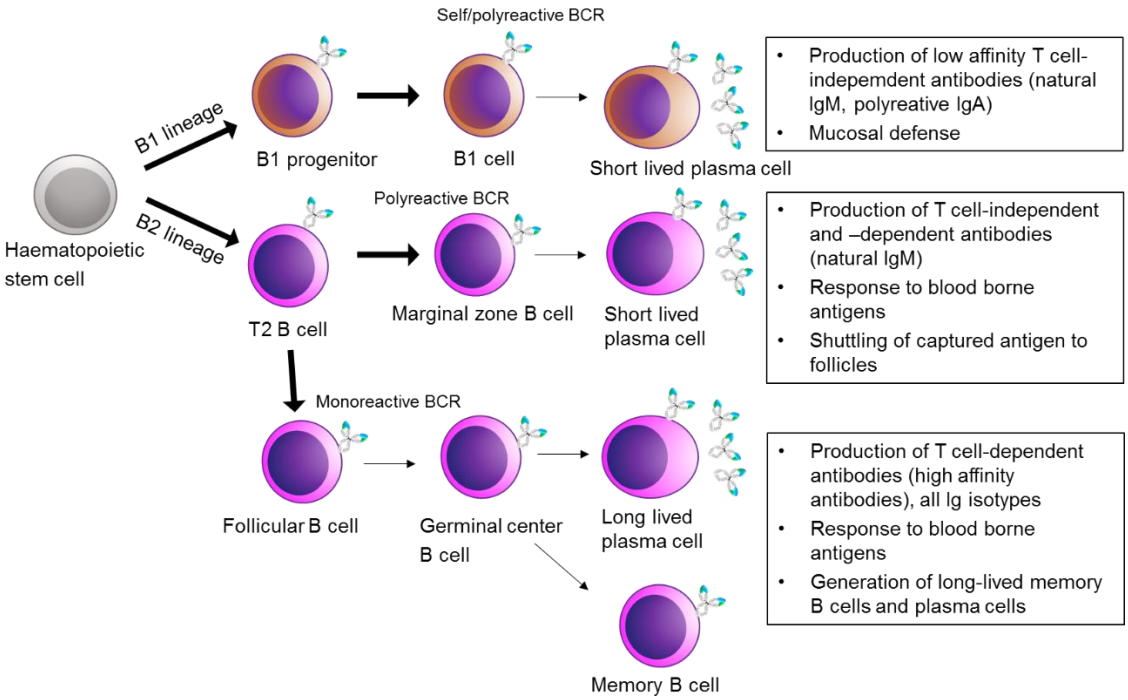


Figure 2. B cell lineage subsets and functions (Yam-Puc 2018 *et al.*; Schrezenmeier *et al.*, 2018).

*CRS and recombinant monoclonal antibody pharmaceuticals*

Cytokines are small polypeptides produced by various types of cells, including immune cells, vascular cells, and fibroblasts (Grignani and Maiolo, 2000; Van Linthout *et al.*, 2014). Cytokines act as cell signaling molecules that have important roles in inflammation, infection, differentiation, and maintenance of homeostasis in immune cells, (Wilson *et al.*, 1998; Boyman *et al.*, 2007; O'Shea and Murray, 2008) (Table 3). Once excessive amounts of cytokines are produced from systemically activated immune cells, the cytokines cause CRS. CRS induces an exaggerated systemic immune response, which involves the potential release of more than one hundred inflammatory mediators such as cytokines, chemokines, reactive oxygen species, complement factors, and coagulation factors (Bugelski *et al.*, 2009). CRS occurs in the event of graft-versus-host disease (GVHD) after transplantation, surgical infections, sepsis (Gerlach, 2016), influenza infection (Liu *et al.*, 2016), and

antibody-based therapies (Shimabukuro-Vornhagen *et al.*, 2018). Severe CRS is sometimes accompanied by systemic inflammatory response syndrome (SIRS), shock, disseminated intravascular coagulation (DIC), multiple organ dysfunction syndrome (MODS), which can all be life-threating (Suntharalingam *et al.*, 2006; Wang and Ma, 2008; Kulkarni and Kasi, 2012).

Table 3. Principal cytokines generated during CRS based on Grignani and Maiolo (2000), Bugelski *et al.* (2009), Schutte *et al.* (2009), and Tisoncik *et al.* (2012)

| Cytokine | Principal Source | Functions |
|---|---|---|
| IL-1 | Monocytes/macrophages and T cells | Proinflammatory, mediation of the host response to infection, fibrinolysis, coagulation, influence on endothelial cell function, costimulation, and proliferation and maturation on T, B, NK cells. |
| IL-2 | T cells | Expansion of B cells and activation of cytotoxic T cells. |
| IL-6 | Monocytes/macrophages and T cells | Proinflammatory, augment immune response, and coagulation. |
| IL-8 (CXCL8) | Macrophages and endothelial cells | Proinflammatory, chemoattractant factors for neutrophils. |
| IL-10 | Activated Th2 cells and macrophages | Anti-inflammatory, inhibition of the production of proinflammatory cytokines, and suppression of cellular immunity. |
| MCP-1 (CCL2) | A variety of cell types (macrophages, smooth muscle cells, endothelial cells, etc.) | Proinflammatory and chemoattractant factors for monocyte. |
| IFN | Activated Th1 cells and NK cells | Regulation of innate immunity, activation of antiviral properties, and antiproliferative effects. |
| TNF | Monocytes/macrophages and T cells | Proinflammatory, activates cytotoxic T lymphocytes, fibrinolysis, influence on endothelial cell function, vascular permeability, cell death, and cytokine-release cascade. |

Recombinant monoclonal antibody (mAb) pharmaceuticals are widely used and are being developed as therapies for cancer, transplant rejection, inflammatory/autoimmune diseases and antiviral prophylaxis, as well as a range of new indications (Bailey *et al.*, 2013). These mAb pharmaceuticals, mostly IgG subclasses, are stable in blood, and are highly specific to the target

molecules, which can reduce non-mechanism-based toxicity (Brekke and Sandlie, 2003; Brennan *et al.*, 2010). Antibodies produced by B cells distribute into extracellular spaces and protect hosts from microorganisms by the humoral immune response, while therapeutic mAbs are designed to interact with the immune system. The clinical efficacy of mAb pharmaceuticals is generally attributed to target-specific mechanisms including neutralization/blocking (e.g. neutralizing growth factors to inhibit cell proliferation), antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), signaling (e.g. agonistic receptor engagement to activate tumor immunity or affect tumor cell apoptosis) (Brekke and Sandlie, 2003; Strome *et al.*, 2007; Vogelpoel *et al.*, 2015; Beers *et al.*, 2016) (Fig. 3). As well as their promising therapeutic potential, mAb therapies also carry the risk of immune reactions such as serum sickness, the generation of anti-drug antibodies (ADAs), anaphylaxis, infusion reactions including CRS, and numerous adverse effects that are related to their specific targets (Hansel *et al.*, 2010).

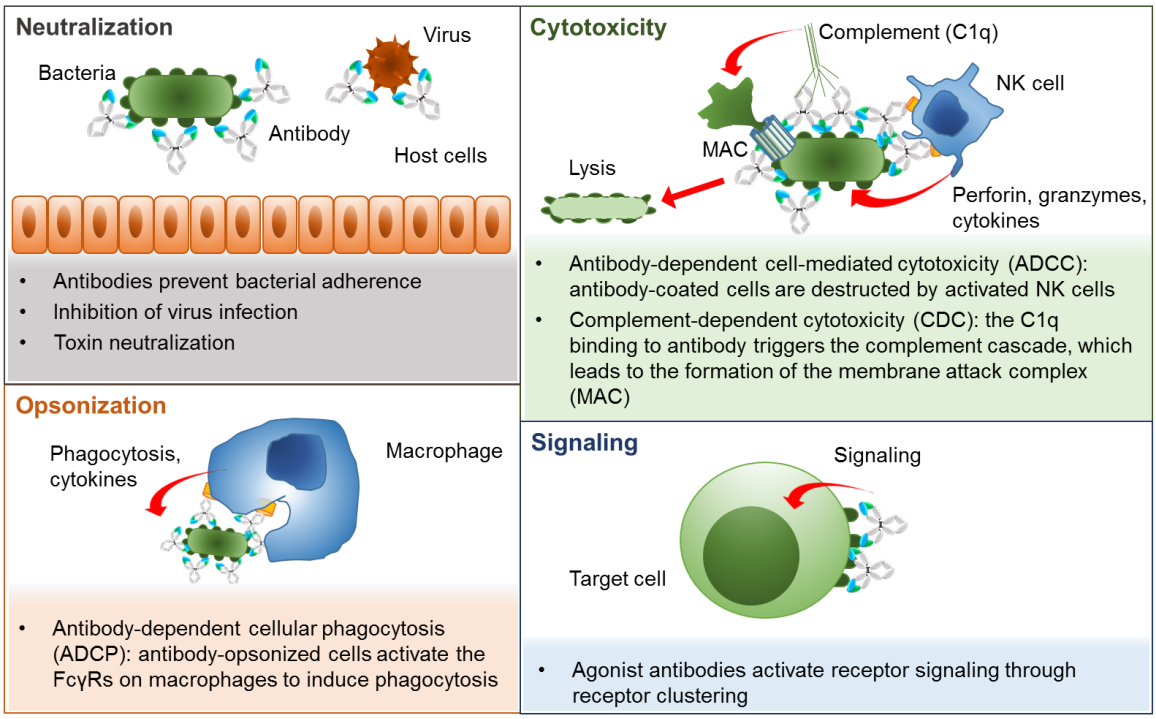


Figure 3. Mechanism of action of mAbs (Brekke and Sandlie, 2003; Strome *et al.*, 2007; Vogelpoel *et al.*, 2015; Beers *et al.*, 2016).

In this PhD thesis, I focus on an important adverse effect caused by mAb pharmaceuticals, immune cell activation following a cascade of systemic cytokine release termed CRS (Grimaldi *et al.*, 2016). CRS is characterized by a rise in cytokines (i.e. IL-2, IL-6, IL-8, IL-10, TNF-α, and IFN-γ), and is accompanied by numerous adverse events including fever, fatigue, headache, urticaria, pruritus, bronchospasm, dyspnea, rhinitis, nausea, vomiting, flushing, fever, chills, hypotension, sensation of the tongue or throat swelling, tachycardia, and asthenia at the sites of disease (Bugelski *et al.*, 2009).

*CRS hazard identification*

In 2006, life-threatening CRS occurred in the clinical study of TGN1412, a novel superagonist anti-CD28 mAb that directly stimulates T cells. Within 90 minutes of treatment with TGN1412, all six healthy volunteers had a systemic inflammatory response characterized by a rapid proinflammatory cytokine release and characteristic clinical symptoms, followed by disseminated intravascular coagulation and multiple organ failure (Suntharalingam *et al.*, 2006). TGN1412 had been developed for the treatment of autoimmune diseases through Treg activation by CD28 signaling (Beyersdorf *et al.*, 2005b). Tregs have a role in keeping autoreactive T cells from the initiation of autoimmunity (Beyersdorf *et al.*, 2005b). Tregs constitutively express the costimulatory receptor CD28 which is critical for Treg generation and homeostasis (Guo *et al.*, 2008). Treatment of CD28 superagonist leads to the expansion and activation of Tregs and ameliorates clinical symptoms of autoimmunity in animal models (Beyersdorf *et al.*, 2005a; Beyersdorf *et al.*, 2005b). Moreover, CRS was not observed after treatment with CD28 superagonist in animal studies (Duff, 2006).

The first human dose of TGN1412 had a 500-fold safety margin against the no observable adverse effect level (NOAEL) established in cynomolgus monkeys (50 mg/kg), but TGN1412 caused life-threatening CRS in all treated volunteers (Hanke, 2006; Suntharalingam *et al.*, 2006). Since preclinical

studies using animals failed to predict the CRS risk caused by TGN1412, a number of *in vitro* cytokine release assays using human cells have emerged to detect the potential risk of CRS (Stebbings *et al.*, 2007; Hansel *et al.*, 2010). There is no consensus on the optimal *in vitro* assay format that should be used to assess the potential risk of CRS (Grimaldi *et al.*, 2016). An *in vitro* assay format composed of human whole blood and aqueous mAbs, the whole blood cytokine assay (WBCA) (Wolf *et al.*, 2012; Bailey *et al.*, 2013), is a simple and convenient method which generates few false positives. However, the low sensitivity of the WBCA to TGN1412 raises the question of whether the WBCA is a practical tool to identify hazards (Thorpe *et al.*, 2013; Vessillier *et al.*, 2015). In their previous study, Vessillier *et al*. (2015) indicated that the sample size required for the WBCA to detect a positive response with TGN1412 is too large ($n$ = 52) because of the high background level of IL-8. The WBCA uses such a large sample size which is impractical, thus, improvements in the WBCA method are necessary. The objective of the first study (chapter 1) is to determine an appropriate sample size and confirm the suitability of the WBCA as a hazard identification tool for CRS.

There are two major testing platforms to detect the potential risk of CRS: one composed of whole blood with aqueous-phase test articles (WBCA) as mentioned above, and the other composed of peripheral blood mononuclear cells (PBMCs) with solid-phase test articles (PBMA assay) (Stebbings *et al.*, 2007; Wolf *et al.*, 2012). The WBCA is considered to be a generic assay with low false positive rates, but the sensitivity to TGN1412 is low (Findlay *et al.*, 2010; Wolf *et al.*, 2012). Meanwhile, the PBMC assay shows high sensitivity to TGN1412, but generates false positive results (Foreback *et al.*, 1997; Wing, 2008; Grimaldi *et al.*, 2016). The difference between the WBCA and the PBMC assay raises the question of how TGN1412 induces positive responses in the WBCA. The objective of the second study (chapter 2) is to understand how TGN1412 induces cytokines in the WBCA and whether the positive response is relevant to hazardous activity.

*CRS mitigation strategies following CRS hazard identification*

To date, it has been possible to detect the CRS hazard with a number of previous studies. In the case of TGN1412, cynomolgus monkeys were thought to be an adequate species for TGN1412 toxicological studies because TGN1412 bound to CD28 of humans and cynomolgus monkeys with very similar affinities (Hanke, 2006). However, the immune responses caused by TGN1412 showed large differences between human and cynomolgus monkeys, possibly due to species differences in CD28 expression patterns and signaling (Waibler *et al.*, 2008; Eastwood *et al.*, 2010). Recently, the appropriate *in vitro* assays using human cells allowed TGN1412 to rationally select a safe first-in-human dose for the clinical trial of TGN1412, rebranded TAB08 (Tkach and Writer, 2015). In this way, appropriate dose setting can avoid unexpected severe CRS. The next step after identifying the hazard of CRS is to manage clinical CRS. In case that a mAb has a large difference between the effective dose and the dose that causes CRS, appropriate dose setting is effective for the prevention of CRS. When CRS occurs with a narrow safety margin due to on-target effects related to therapeutic efficacy, it is necessary to mitigate CRS.

CD3 bispecific constructs targeting CD3 on T cells and tumor-associated antigens, have become an emerging part of cancer immunotherapy (Hoffmann *et al.*, 2005). Certain CD3 bispecific constructs exhibit remarkable therapeutic potency and efficacy for cancer cell lysis by eliciting T-cell activation, but they can also cause difficult-to-manage CRS in clinical use (Saber *et al.*, 2016; Saber *et al.*, 2017). Premedication with corticosteroids, antipyretics, and antihistamines is the gold standard for controlling CRS (Shimabukuro-Vornhagen *et al.*, 2018), but even the effect of premedication alone is limited (Luheshi and Rothwell, 1996; Maude *et al.*, 2014), suggesting that the dosage regimen might

be a more important factor in mitigating CRS. The objective of the last study (chapter 3) is to examine how effectively an intra-animal ascending dose regimen without premedication can mitigate CRS induced by the CD3 bispecific construct, ERY22, in cynomolgus monkeys. ERY 22 has two different Fab domains binding to CD3 and glypican 3 (GPC3), and it can bind to the CD3 and GPC3 of both human and cynomolgus monkeys.

In the studies described in this thesis, firstly, I proposed a practical design and an appropriate sample size for the WBCA as a hazard identification tool of CRS. Next, I clarified the mechanism of the difference in the sensitivity to TGN1412 between the WBCA and the PBMC assay. Finally, I investigated the effectiveness of ascending dose regimen for CRS mitigation in cynomolgus monkeys. This research is considered to provide useful information for CRS hazard identification and CRS mitigation of mAb pharmaceuticals.

## Chapter 1: Is an *in Vitro* Whole Blood Cytokine Assay Useful to Detect the Potential Risk of Severe Infusion Reaction of Monoclonal Antibody Pharmaceuticals?


### Abstract

After the life-threatening cytokine release syndrome (CRS) occurred in the clinical study of the anti-CD28 monoclonal antibody (mAb) TGN1412, *in vitro* cytokine release assays using human blood cells have been proposed for non-clinical evaluation of the potential risk of CRS. Two basic assay formats are frequently used: human peripheral blood mononuclear cells (PBMCs) with immobilized mAbs, and whole blood with aqueous mAbs. However, the suitability of the whole blood cytokine assay (WBCA) has been questioned, because an unrealistically large sample size would be required to detect the potential risk of CRS induced by TGN1412, which has low sensitivity. I performed the WBCA using peripheral blood obtained from 68 healthy volunteers to compare two high risk mAbs, the TGN1412 analogue anti-CD28 superagonistic mAb (CD28SA) and the FcγR-mediated alemutuzumab, with a low risk mAb, panitumumab. Based on the cytokine measurements in this study, the sample size required to detect a statistically significant increase in cytokines with 90% power and 5% significance was determined to be $n = 9$ for CD28SA and $n = 5$ for alemtuzumab. The most sensitive marker was IL-8. The results suggest that WBCA is a practical test design that can warn of the potential risk of FcγR-mediated alemtuzumab and T-cell activating CD28SA, but it cannot be used as a risk-ranking tool because there was apparently a lower response to CD28SA. The WBCA is suggested to be a helpful tool for identifying potential FcγR-mediated hazards, but further mechanistic understanding of the response to CD28SA is necessary before applying it to T cell–stimulating mAbs.


### Introduction

Cytokine release syndrome (CRS) is a serious adverse effect caused by some monoclonal antibody (mAb) pharmaceuticals. CRS is one of the causes of infusion reaction characterized by a rise in tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-6, IL-10, IL-2, and IL-8, and it leads to a complex of fatigue, headache, urticaria, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling, rhinitis, nausea, vomiting, flushing, fever, chills, tachycardia, asthenia, hypotension, and possibly shock (Bugelski *et al.*, 2009). After TGN1412, an anti-CD28 humanized mAb, caused life-threatening multiple organ failure in all volunteers due to CRS (Suntharalingam *et al.*, 2006), the importance of assessing cytokine release potential prior to entry into clinical studies was stated in the guideline of the European Medicines Agency (EMA, 2007).

However, the traditional battery of preclinical tests comprising *in vitro* and *in vivo* tests with relevant animal species failed to predict the potential risk of CRS in humans (Hansel *et al.*, 2010). Importantly, TGN1412 did not cause CRS in cynomolgus monkeys, even when the animals received a dose 500-fold higher than that given to human volunteers (Duff, 2006). Fundamental differences between human and nonhuman primate (NHP) species may explain the inconsistent responses. Differences were reported in the expression of the target molecule on effector memory T cells (Eastwood *et al.*, 2010), $Ca^{2+}$-signal strength (Waibler *et al.*, 2008), inhibitory Siglec-5 molecules (Nguyen *et al.*, 2006), FcγR expression patterns, and binding affinities (Warncke *et al.*, 2012). *In vitro* test systems using human blood cells have been proposed and are routinely applied non-clinically to evaluate the potential risk of CRS (Finco *et al.*, 2014). Two basic assay formats are frequently used: stimulation of human peripheral blood mononuclear cells (PBMCs) with immobilized test articles, and of whole blood cells with aqueous test articles. The assays have been generally developed to detect the potential of superagonistic CD28 monoclonal antibodies (anti-CD28 mAbs) using either an original TGN1412 or one reproduced from the published amino acid sequence.

However, to date, no consensus has been reached regarding assay format, analyzed factors, and statistical approach. Immobilized anti-CD28 mAb induced cytokines from human PBMCs *in vitro*, whereas aqueous anti-CD28 mAb did not cause cytokine release when simply incubated with diluted blood (Stebbings *et al.*, 2007). Use of PBMCs in the assay achieved higher sensitivity than whole blood cells, because red blood cells interfered with the interaction between immobilized TGN1412 and lymphocytes (Findlay *et al.*, 2010). A combination of PBMCs and immobilized mAbs provided the highest sensitivity, but immobilizing mAbs onto cell-culture-ware may increase the rate of false-positive results (Wing, 2008) due to non-physiological Fc receptor interaction with highly condensed mAbs (Foreback *et al.*, 1997).

An assay method composed of whole blood and aqueous mAbs, the WBCA (Wolf *et al.*, 2012; Bailey *et al.*, 2013), is much more convenient and generates less false positives than the assay with isolated cells and immobilized mAbs. Wolf *et al.* (2012, 2013) and Bailey *et al.* (2013) detected positive cytokine responses to anti-CD28 mAbs in the WBCA. However, the low sensitivity of the WBCA raises the question of how practical a tool it is to identify hazards (Thorpe *et al.*, 2013; Vessillier *et al.*, 2015) The biggest criticism is that the sample size required for the WBCA to detect a positive response with anti-CD28 mAb may be too big.

The aim of this study is to determine the appropriate sample size and to discuss the suitability of the WBCA as a hazard identification tool by using the accumulated data from 68 blood samples to compare two high-risk mAbs, TGN1412 analogue anti-CD28 superagonistic mAb (CD28SA) and alemtuzumab, with a low risk mAb, panitumumab. Alemtuzumab, which targets the CD52 antigen present on all lymphocytes and some monocytes, caused CRS in almost all patients when it was first given by intravenous infusion without premedication with steroids (Moreau *et al.*, 1996; Pangalis *et al.*, 2001). An Fc-mediated function is responsible for the CRS caused by alemtuzumab (Wing *et al.*, 1995; Brennan *et al.*, 2010; Hansel *et al.*, 2010). Panitumumab is an anti-epidermal growth factor

receptor (EGFR) mAb (Amgen Inc., 2015) that causes severe infusion reactions in less than 1% of patients, probably due to non-CRS mechanisms (Bugelski *et al.*, 2009).

## Materials and Methods

### *Human blood*

Peripheral blood samples were donated by 68 healthy Japanese volunteers after informed consent was obtained, and the samples were anonymized. The use of human-derived test materials was approved by the Research Ethics Committee of Chugai Pharmaceutical Co., Ltd. (No. BG0908, BG0915, BG1001). Blood was collected into Venous Blood Collection tubes (Becton, Dickinson and Company [BD], NJ, USA) containing lithium heparin. The blood was kept at room temperature until use and treated with test articles within 3 h after donation.

### *Monoclonal antibodies*

Alemtuzumab, an anti-CD52 humanized IgG1κ mAb, was purchased (MabCampath®, Bayer HealthCare Pharmaceuticals Inc., Berline, Germany). An anti-CD28 superagonist mAb (CD28SA) of a humanized IgG4 was generated internally according to the previously disclosed amino acid sequence of TGN1412 (Patent No. US2006/0286104 A1). CD28SA was prepared at 11.2 mg/mL in 20 mM histidine and 140 mM NaCl (pH6.0) and stored at −80ºC until use. CD28SA was previously used in an *in vitro* cytokine induction study (Bailey *et al.*, 2013). Panitumumab, an anti-human EGFR human IgG2κ mAb, was purchased (Vectibix®, Amgen Inc., CA, USA). Alemtuzumab and CD28SA were considered positive reference mAbs, and panitumumab a low risk reference mAb.

### *Treatment*

The WBCA was performed as previously described (Wolf *et al.*, 2012; Bailey *et al.*, 2013). Briefly, an aliquot of blood (193 μL/well) from each donor was plated onto 96-well culture plates (Corning Inc., 3799, NY, USA) containing 6.6 μL/well of the test article appropriately diluted with sterile PBS to final treatment concentrations of 0.1, 1, 10, and 100 μg/mL. After incubation for 24 h at 37°C in a humidified atmosphere with 5% $CO_2$, culture supernatant was harvested by centrifugation at 24°C and stored at −80°C. Triplicated wells were prepared for each treatment.

*Measurement of cytokines*

IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 were measured in the plasma samples using a Human Inflammatory Cytokine CBA Kit (BD) and BD FACSArray, according to the manufacturer's instructions. FCAP Array Version 1.0.1 software (Soft Flow Hungary, Ltd., Pecs, Hungary) was used for data analysis.

*Statistical analysis*

The mean concentration of the cytokines from each treatment was obtained by measuring the triplicated culture wells. Assuming that the distribution of each dataset follows a log-normal distribution, statistical significance was examined with parametric Dunnett's Multiple Comparison Test ($p < 0.05$) between the positive reference mAbs and the low risk mAb at comparable concentrations. Dose-response analysis of data (transformed by $\log_{10}$) on IL-8 after treatment with panitumumab was calculated by Williams' test ($p < 0.05$).

The required sample sizes for 90% power of a positive response for alemtuzumab or CD28SA with $p < 0.05$ statistical significance compared to panitumumab were calculated from a paired *t*-test that compared each treatment against a comparable concentration of panitumumab. Statistical analysis was carried out using SAS Version 8.2 (SAS Institute Japan Ltd., Tokyo, Japan).

## Results

*Measurements of cytokines*

The cytokines induced after treatment with test articles are shown in Fig. 4. Cytokine values after panitumumab treatment were compared with those from PBS. After treatment with 0.1–100 μg/mL of panitumumab, cytokine levels were similar to PBS, except for IL-8. Panitumumab showed a dose-dependent increase of IL-8 that was significantly higher than that of the PBS control at 10 and 100 μg/mL (Williams test, $p < 0.05$). The statistical significance of cytokine levels after treatment with high risk mAbs was tested by comparing them to those for the corresponding doses of panitumumab.

CD28SA significantly elevated IL-8 and IL-10 at doses of 1 μg/mL and higher when compared with panitumumab (Fig. 4A and D). The median of IL-8 release increased from 314, 484, and 608 pg/mL after panitumumab treatment to 1270, 1074, and 2196 pg/mL after CD28SA treatment at 1, 10 and 100 μg/mL, respectively. CD28SA significantly elevated IL-6 at doses of 1 μg/mL and 100 μg/mL when compared with panitumumab (Fig. 4C); medians of IL-6 levels were, respectively, 607 and 584 pg/mL compared to 305 and 271 pg/mL at 1 and 100 μg/mL with panitumumab treatment. No significant increase was seen in IL-1β, TNF, or IL-12p70 after treatment with CD28SA.

Alemtuzumab induced significantly higher levels of IL-8, IL-1β, IL-6, and TNF compared with panitumumab at all the treatment concentrations ($p < 0.05$, Fig. 4A, B, C and E). The highest median of IL-8 after treatment with alemtuzumab was observed at 100 μg/mL. Medians of IL-1β, IL-6, and TNF were highest at 1 μg/mL of the treatment. No significant increase was seen in IL-10 or IL-12p70 (Fig. 4D, F).

*Frequency distributions of cytokine values*

Frequency distributions (transformed by $\log_{10}$) of the cytokine concentrations of IL-8, IL-1β, IL-6, IL-10, and TNF from 68 donors at the dose where the treatment provided the highest median of each cytokine are shown in Fig. 5. The shapes of the histograms of the log-transformed values show that normal distribution can be assumed. Compared with panitumumab, histograms of IL-8, IL-1β, IL-6, and TNF were shifted to a higher concentration by treatment with alemtuzumab. Compared with panitumumab, measurements of IL-8 and IL-10 were shifted to a higher concentration by the treatment with CD28SA. CD28SA caused a very slight elevation of the IL-6 peak frequency. No clear difference was seen in IL-1β and TNF between CD28SA and panitumumab.

*Estimating the sample size required to detect cytokine release*

The results of the statistical power analysis are shown in Table 4. The most sensitive concentration of mAb was 100 μg/mL for IL-8 and 1 μg/mL for IL-1β, IL-6, IL-10, and TNF. The estimated sample sizes required for 90% power, as calculated by paired *t*-test, were $n = 5$ for IL-8, $n = 11$ for IL-1β, $n = 6$ for IL-6, and $n = 5$ for TNF when the responses to alemtuzumab were compared with panitumumab, and $n = 9$ for IL-8, $n = 28$ for IL-6, and $n = 14$ for IL-10 when comparing CD28SA and panitumumab. The most sensitive markers to detect cytokine induction potential were IL-8 and TNF for alemtuzumab, and IL-8 for CD28SA.

**Discussion**

The results in this study demonstrated that the WBCA elicited a positive response to either CD28SA or alemtuzumab. Data from all the 68 donors were integrated into the statistical analysis in this study in order to know the shape of distribution of cytokine measurements. Assuming that the distribution of each dataset follows a log-normal distribution, I compared the cytokine measurements between the high-risk mAbs and panitumumab. The WBCA exhibited significant increases of IL-8,

IL-6, and IL-10 with CD28SA, and of IL-8, IL-1β, IL-6, and TNF with alemtuzumab (Fig. 4). Except for IL-8, the elevation of cytokines with CD28SA in the WBCA was much lower than those previously reported for immobilized assays.

Measuring IL-8 as a biomarker and using a range of treatment concentrations up to 100 μg/mL were suggested to be important to detect activity of CD28SA in the WBCA. IL-8 was found to be the most sensitive marker for CD28SA in the WBCA in this study. A significant increase of IL-8, IL-10 and IL-6 can be detected with $n$ = 9, 14, and 28 sample sizes at 90% statistical power after treatment with CD28SA. This was inconsistent with the previous finding by Vessiller *et al*. (2015) that a sample size of at least $n$ = 52 was needed to detect a positive response in whole blood and that IL-8 was not significantly increased by TGN1412. They reported that the background level of IL-8 in the WBCA was 2538 pg/mL with the IgG4 control and 2474 pg/mL with TGN1412 at 5 μg/mL of treatment (Vessillier *et al.*, 2015). In the present study, medians of IL-8 measurements were 608 pg/mL when treated with panitumumab at 100 μg/mL and 375 pg/mL with PBS, which significantly increased to 1270, 1074, and 2196 pg/mL when concentrations of CD28SA were 1, 10 and 100 μg/mL, respectively (Fig. 4). Wolf *et al.* (2012) observed a significant increase in IL-8 from less than 100 pg/mL with a low risk control to nearly 1000 pg/mL with 1 μg/mL of anti-CD28 mAb. Bailey *et al*. (2013) also reported that the background level of IL-8 was 4362 pg/mL and increased significantly to 17,467 pg/mL at only 100 μg/mL of anti-CD28 mAb. Taken together, it is likely that having a higher background IL-8 makes it more difficult to detect the potential risk of TGN1412, but the reasons for the wide variability of IL-8 background measurements between studies remain unclear. PBMCs adherent to plastic culture plates produce IL-8 (Kasahara *et al.*, 1991), so the material and surface coating of a culture plate might affect the background levels of IL-8. In addition, the longer culture time used by Vessillier *et al*. (48 h compared to 24 h) may contribute to higher baseline IL-8 values.

In this study, induction of cytokines after treatment with CD28SA was seen at ≥ 1 μg/mL of treatment concentration, which is similar to the estimated blood concentration of 2 μg/mL that caused CRS in the clinical trial with TGN1412 (Stebbings *et al.*, 2007). However, the profile of induced cytokines was very different from those of the clinical trial. TGN1412 dramatically induced TNF, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12p70 in all the volunteers in the clinical trial (Suntharalingam *et al.*, 2006), whereas CD28SA significantly induced IL-8, IL-6 and IL-10 but did not induce TNF, IL-1β and IL-12p70 in this WBCA. This difference was also reported in other studies; the WBCA by Bailey *et al.* (2013) reported low sensitivity of IL-1β, TNF-α, and IFN-γ to anti-CD28 mAb, and in the Wolf's study (2012) only slight increases of roughly 10 pg/mL of IL-2 in 60% of donors and IL-4 in 25% were seen in the WBCA after treatment with the anti-CD28 mAb. Because stimulation of CD28 is expected to result in T cell activation accompanied with elevation of IL-2 and IFN-γ, T cells may not be the major source of IL-8 after treatment with CD28SA, which suggests that the WBCA can only detect a partial response to CD28SA.

The assay format using immobilized mAb and PBMC provided much higher cytokine responses from anti-CD28 mAb (Stebbings *et al.*, 2007). In the WBCA, alemtuzumab induced a more potent cytokine response than CD28SA, whereas TGN1412 caused the most dramatic CRS. In other words, the results of the WBCA did not correspond to clinical severity. Because clinical severity of CRS largely depends on dose and injection rate, it is quite difficult for any *in vitro* assay to compare the risk to patients from mAbs given in different regimens, against different targets, or with different modes of action; therefore, the usefulness of *in vitro* risk ranking may be limited to a comparison of very similar mAbs, such as that needed for clinical candidate selection in a project. However, although the WBCA cannot be a comprehensive ranking tool, an alert from the assay can act as a trigger for further mechanistic investigation and encourage careful setting of the clinical regimen.

Contrary to CD28SA, alemtuzumab provided a cytokine response that mimicked the result expected from clinical experience. I detected cytokine increase after treatment with alemtuzumab at 0.1 to 100 μg/mL in the WBCA (Fig. 4), in which 450 clinical samples ranged in blood concentration from 0.1 to 9.5 μg/mL (Jilani *et al.*, 2004). The kinetics of cytokines after treatment with alemtuzumab in whole blood culture was similar to that seen in clinical samples, and ligation of FcγRIII on NK cells is considered responsible for CRS caused by alemtuzumab (Wing *et al.*, 1996). The results in this study further support the sensitivity of the WBCA to cytokine release activity with FcγR-related mechanisms.

Modifying the binding affinity of mAbs to FcγRs is a recently emerging technology that is expected to enhance the efficacy of various mAb therapeutics (Horton *et al.*, 2011; Li and Ravetch, 2012; Mimoto *et al.*, 2013). Increased binding affinity to an FcγR may increase activity that stimulates immune cells. Therefore, identifying potential risks caused by FcγR may be important for assessing therapeutic candidates of a mAb equipped with this technology. The WBCA could be a helpful tool in identifying the hazards of the new type mAbs.

Interlaboratory validation of the method and alignment of the experiment details should be considered. In this regard, the initiative by the cytokine release assay working group of the Immunotoxicology Technical Committee to produce and cross-validate standard Ab controls will be valuable. It was considered preferable to include a low risk reference mAb in the assay for comparison with the test articles. In this study, I used panitumumab, which induced infusion reactions in less than 5% of patients and severe ones in less than 1% (Chung, 2008; Amgen Inc., 2015), both of which values are considered irrelevant to CRS (Bugelski *et al.*, 2009). Panitumumab caused marginal but statistically significant dose-related elevation of IL-8 in the WBCA when compared to that of the PBS control. The serum concentration of panitumumab was around 180 μg/mL when patients received panitumumab at 6 mg/kg (Stephenson *et al.*, 2009); therefore, the slight elevation of IL-8 after

treatment with panitumumab in this study was not due to a no clinically relevant high concentration. Because natalizumab was reported to induce infusion reactions in 1–4% of patients (Maggi *et al.*, 2011), the antibody is also recognized as a low-risk antibody. In the previous WBCA study (Wolf *et al.*, 2012), mean values of IL-8 after treatment with natalizumab were slightly higher than with the control, though the statistical significance was not examined. Slight elevation of IL-8 in this assay was not considered significant for predicting potential CRS risk; therefore, including a low risk reference mAb is helpful to interpret IL-8 data. For a positive control, I consider that CD28SA is not suitable. Although TGN1412 is a generally recognized benchmark for cytokine assays, TGN1412 itself cannot be purchased, and the substitute agonist mAbs available are of varying quality. In my view, marketed pharmaceutical mAbs like alemtuzumab are ideal as positive controls because of their strictly controlled and standardized quality and the wide experience of using them in clinic provides reliable information on risk.

The results in this study demonstrated that the WBCA was sufficiently predictive of the FcγR-mediated potential risk of alemtuzumab and could provide a positive response to T cell activation mediated by CD28SA using a practical test design that included measuring IL-8 in a $n \geq 9$ sample size with up to 100 μg/mL treatment, 24-hour culture, and a low risk control mAb. However, the WBCA could only partially reveal the activity of CD28SA, and the positive response in the WBCA may not be induced by the T cell activation that is a major biological activity of CD28SA. The WBCA is useful for identifying FcγR-mediated hazards, but the mechanism by which CD28SA induces IL-8 needs to be further investigated before the assay can be used to assess the risk of T cell–activating mAbs. In addition, the WBCA may not be available for mAbs whose target antigens are not included in the assay system.

The CRS caused by TGN1412 was more severe than that caused by alemtuzumab, yet the opposite would be inferred from the results shown in this study. The response to CD28SA in the

WBCA may not be caused by T cell stimulation through CD28, but by other side effects. However, the WBCA was able to detect the CRS risk potential of CD28SA. The WBCA is therefore not considered to be predictive of the clinical severity of adverse effects of mAb therapeutics and not suitable for ranking the risk, but is rather a tool for hazard identification. The severity of CRS could be decreased by an appropriate dosage regimen (Hale *et al.*, 2004; Tabares *et al.*, 2014) or by medication with anti-inflammatory agents (Lee *et al.*, 2014). I considered that the WBCA is able to detect the potential of severe CRS occurring with high frequency in the worst case. I need to consider the dosage regimen to prevent severe infusion reaction after an alert from the WBCA.

**Figures and Tables**

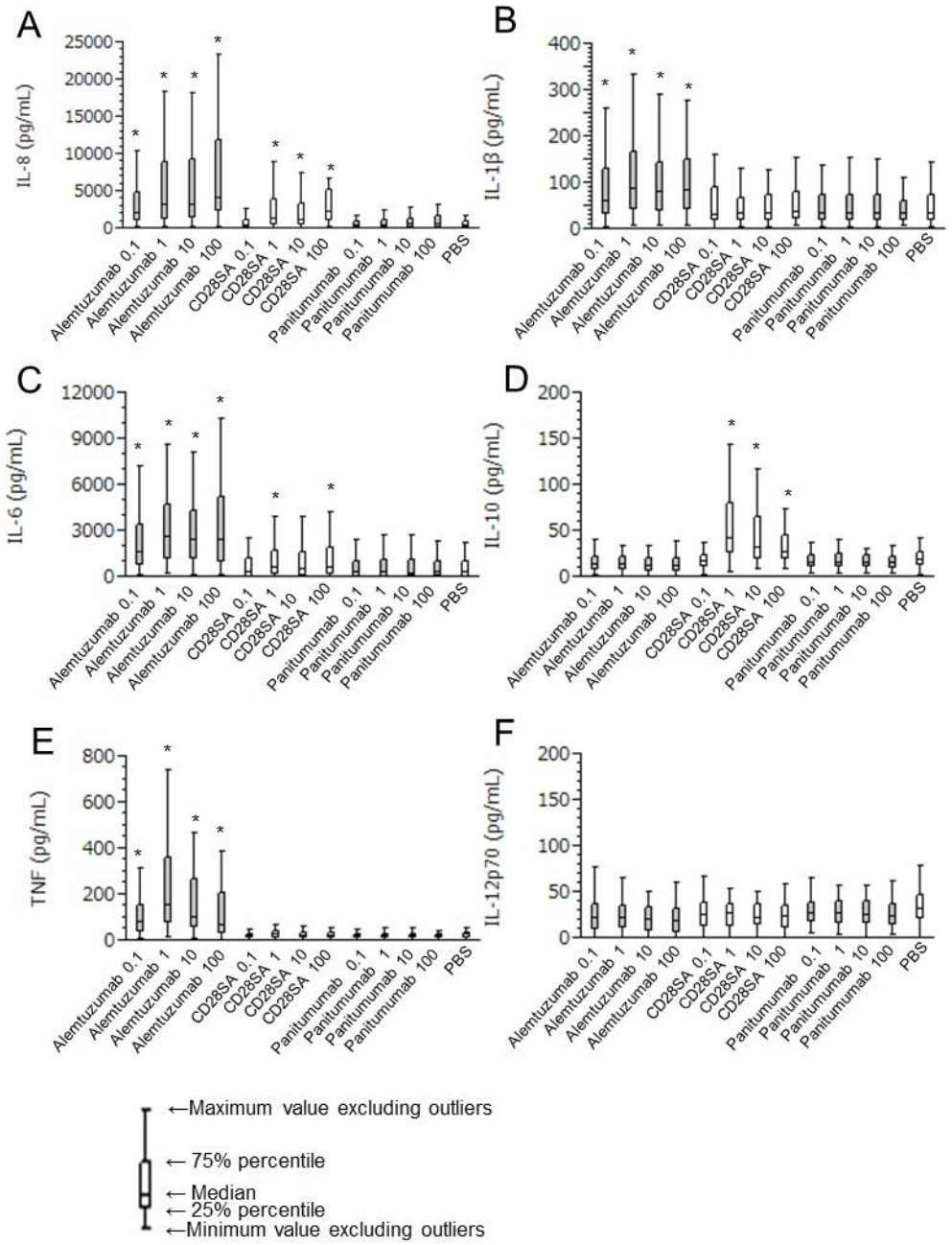


Figure 4. Alemtuzumab and CD28SA significantly elevated cytokines when compared with panitumumab. Released IL-8 (A), IL-1β (B), IL-6 (C), IL-10 (D) TNF (E), and IL-12p70 (F) in the WBCA from 68 donor blood samples after treatment with alemtuzumab, CD28SA, panitumumab, and PBS. Whole blood was treated with mAbs ranging from 0.1 to 100 μg/mL or with PBS, and incubated for 24 h. Medians and ranges of data distribution from 25% to 75% percentile were indicated. Statistical significance of elevation was examined on $\log_{10}$-transformed data set between mAbs and panitumumab at corresponding concentrations with Dunnett's test (*: $p < 0.05$).

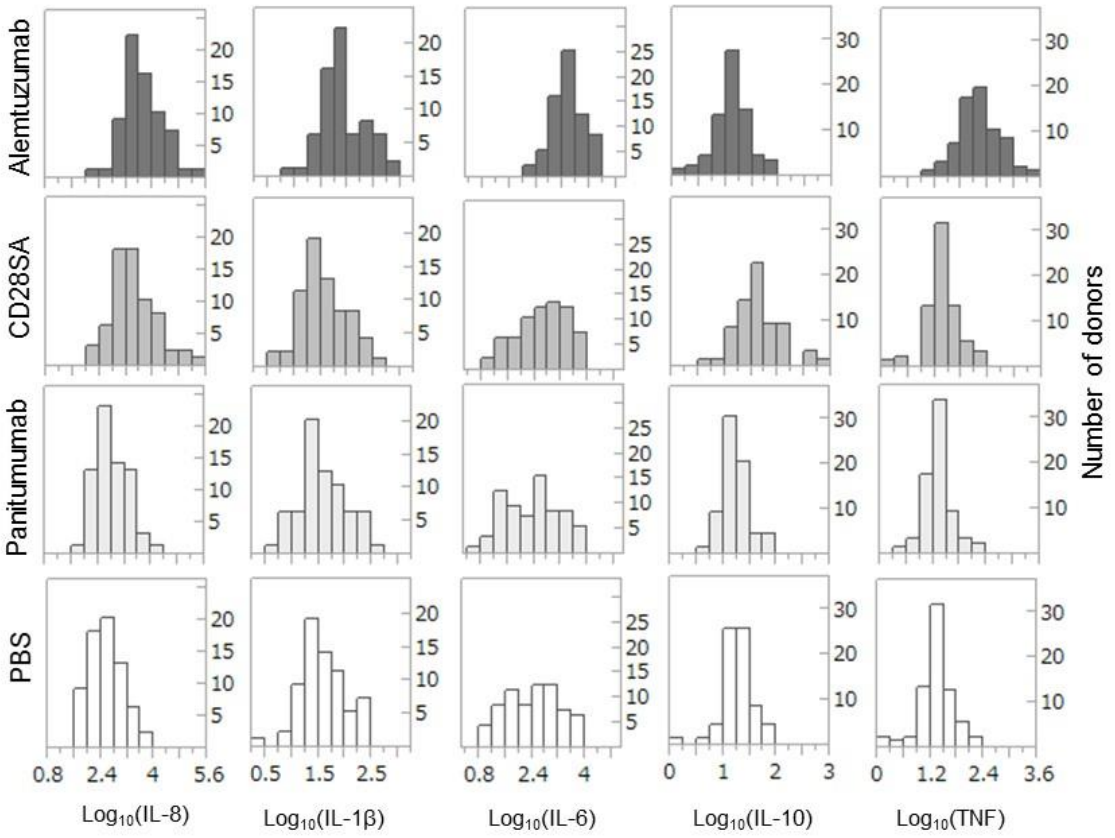


Figure 5. Distributions of $log_{10}$-transformed cytokine concentration were changed after treatment with reference mAbs. Distribution of $log_{10}$-transformed values of IL-8, IL-1β, IL-6, IL-10, and TNF after treatment with alemtuzumab, CD28SA, panitumumab, and PBS. Representative histograms were made from the treatment concentrations providing the highest medians with alemtuzumab or CD28SA, 100 μg/mL for IL-8 and 1 μg/mL for IL-1β, IL-6, IL-10, and TNF.

Table 4. Estimated sample sizes for positive responses with 90% statistical power

| | Sample size[1)] | | | | |
|---|---|---|---|---|---|
| Test article | IL-8 | IL-1β | IL-6 | IL-10 | TNF |
| Alemtuzumab | 5[2)] | 11[3)] | 6[3)] | ns[3)] | 5[3)] |
| CD28SA | 9[2)] | ns[3)] | 28[3)] | 14[3)] | ns[3)] |

ns: no significant increase with $n = 68$

1) calculated based on the data from highest median group for paired *t*-test on $\log_{10}$-transformed data compared with panitumumab.

2) from 100 μg/mL group data

3) from 1 μg/mL group data

## Chapter 2: Different Players Generate Positive Responses in Two *in Vitro* Cytokine Assay Formats with Aqueous and Immobilized TGN1412 Analogue


### Abstract

To detect potential risk of severe cytokine release syndrome, *in vitro* assay formats with human cells have been developed. The two major testing platforms are a combination of whole blood with aqueous-phase test articles (whole blood cytokine assay, WBCA) and peripheral blood mononuclear cells with solid-phase articles (PBMC assay). Significant induction of cytokines was seen in both assays after treatment with a widely used control agent, TGN1412 or its analogue CD28SA, but the WBCA cytokine profile differed from what was expected from clinical experience. In the WBCA, potential risk of CD28SA was detected by elevation of IL-8 whereas IL-2, a key cytokine after stimulation of CD28, was not induced in approximately 40% of donor samples. Therefore, further mechanistic understanding of the different responses in the *in vitro* assay was needed. In this study of donor samples treated with CD28SA, I compared the induction of cytokines and identified the cytokine-producing cells in the two assays. IL-2 was markedly elevated in all the donors in the PBMC assay but only in 1 of 3 donors in the WBCA. IL-8, the most sensitive biomarker in the WBCA, was produced by monocytes and granulocytes. T cells, the most relevant player in the PBMC assay with CD28SA, did not contribute to the positive response seen in two donors in the WBCA, which suggests that different players caused the positive cytokine responses to CD28SA in the two assays.


### Introduction

Because of the failure of animal studies to identify the almost lethal effect of a superagonistic anti-CD28 antibody, TGN1412, *in vitro* assay formats with human cells are being developed and proposed to test monoclonal antibody (mAb) drug candidates for potential activity that causes severe

cytokine release syndrome (CRS) (Duff, 2006). There are two major testing platforms: one combines whole blood cells with aqueous-phase test articles and the other combines peripheral blood mononuclear cells (PBMCs) with solid-phase test articles (Stebbings *et al.*, 2007; Wolf *et al.*, 2012). The PBMC assay with immobilized mAbs shows high sensitivity to TGN1412, but the interaction of non-physiological Fc receptor with highly condensed immobilized mAbs causes false positive results (Foreback *et al.*, 1997; Wing, 2008; Grimaldi *et al.*, 2016). In general, the whole blood cytokine assay (WBCA) is a convenient tool that presents test articles in aqueous form and thus can reduce false positives. The WBCA elicited positive responses to a TGN1412 analogueue, a superagonistic anti-CD28 mAb (CD28SA), and some high-risk mAb therapeutics with an FcγR-mediated mode of action (Bailey *et al.*, 2013; Chapter 1 of this thesis). However, previous studies showed that the WBCA was less sensitive to TGN1412 or CD28SA than the PBMC assay (Findlay *et al.*, 2010; Wolf *et al.*, 2012). TGN1412 substantially induced TNF-α, followed by IFN-γ and IL-10, IL-8, IL-6, IL-4, IL-2, IL-1β, and IL-12p70 in the clinical trial (Suntharalingam *et al.*, 2006). IL-2 and IFN-γ are in line with the expected pharmacological activity of TGN1412 to stimulate T cells (Eastwood *et al.*, 2010; Eastwood *et al.*, 2013; Stebbings *et al.*, 2013). Induction of these cytokines after treatment with CD28SA in the WBCA was much weaker than in the PBMC assay (Wolf *et al.*, 2012; Bailey *et al.*, 2013; Thorpe *et al.*, 2013). When CD28SA was tested in the WBCA, the most apparent elevation was seen with IL-8 (Thorpe *et al.*, 2013; Chapter 1 of this thesis). In the WBCA, IL-2, a key cytokine relevant to CD28 stimulation, was not induced in approximately 40% of donor samples (Wolf *et al.*, 2012). Because it is not likely that T cells are the major source of elevation of IL-8, the question has been raised how CD28SA induced positive responses in the WBCA. The objective of this study is to understand how CD28SA induces cytokines in the WBCA and whether the positive response is relevant to hazardous activity.

To understand the difference in the two assay formats, in this study I compared the cells that CD28SA binds to, the cytokine levels induced, and the cells that produced cytokines in each assay after treatment with CD28SA.

## Materials and Methods

### *Monoclonal antibodies*

Humanized anti-CD28 superagonist IgG4 (CD28SA) was used as a positive control. CD28SA was internally prepared based on the amino acid sequence of TGN1412 (Patent No. US2006/0286104 A1) as previously described (Bailey *et al.*, 2013; Chapter 1 of this thesis). The human IgG4 mAb natalizumab (Tysabri, Biogen, Cambridge, MA, USA) was used as a negative control. Natalizumab recognizes human α4-integrin and in clinical use there is no concern of it causing CRS.

### *Human blood*

Peripheral blood samples were donated by three healthy volunteers after informed consent had been obtained. The samples were anonymized and used for the WBCA, the PBMC assay, and flow cytometry. The use of human-derived test materials was approved by the Research Ethics Committee of Chugai Pharmaceutical Co., Ltd. (No. BG1305). The samples of heparinized blood (10 U/mL blood, Heparin sodium; AY Pharmaceuticals, Tokyo, Japan) were kept at room temperature and employed for the following procedures within 3 hours from collection.

### *PBMC isolation*

A fraction of each heparinized blood sample was centrifuged to obtain autologous plasma. PBMCs were isolated from the remaining blood samples by density gradient separation (Ficoll-Paque

PLUS; GE Healthcare Japan, Tokyo, Japan). PBMCs were re-suspended in RPMI1640 media (NACALAI TESQUE, Kyoto, Japan) with 2% autologous plasma at a concentration of $1\times10^6$ cells/mL.

*Whole Blood Cytokine Assay (WBCA)*

Detailed procedures of the WBCA have been previously described in the first chapter. Briefly, mAbs were appropriately diluted with sterile PBS, plated into 96-well culture plates (Corning 3799, Kaiserslautern, Germany) at final treatment concentrations of 0.1, 1, 10, and 100 μg/mL, then an aliquot of blood from each donor was added to each well. After incubation for 6 h or 24 h at 37°C in a humidified atmosphere with 5% $CO_2$, culture supernatant was harvested by centrifugation at room temperature (RT) and stored at −80°C in a deep freezer. Cytokine concentrations were measured using a Human ProInflammatory 9-Plex Ultra-Sensitive Kit (MSD, MD, USA). Duplicated wells were prepared for each treatment.

*PBMC assay*

The basic assay procedures have been previously described (Findlay *et al.*, 2010). Test article mAbs were diluted with sterile PBS, and added at 50 μL/well into the 96-well U-bottomed polypropylene microplates (Corning 3879), resulting in plating amounts of 0.02, 0.2, 2, and 20 μg/well. The plates were left overnight at RT, air-dried in a laminar flow cabinet and washed twice with sterile PBS. The amount of immobilized mAb was measured on another plate with the same air-dry treatment using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). PBMCs ($2\times10^5$ cells/200 μL/well) were added to the air-dried plate. After incubation for 6 h or 24 h at 37°C in a humidified atmosphere with 5% $CO_2$, culture supernatant was harvested by centrifugation at RT and stored at −80°C in a deep freezer. Cytokine concentrations were measured using a Human ProInflammatory 9-Plex Ultra-Sensitive Kit (MSD). Duplicated wells were prepared for each treatment.

*Flow cytometry*

The following anti-human protein antibodies were used to analyze subsets: CD14-APC, CD16-Pacific Blue, IFN-γ-FITC, TNF-α-PE, IL-8-FITC, IL-6-PE, IL-2-FITC, 7AAD (all from Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA); CD3-Brilliant Violet 510 (BioLegend, San Diego, CA, USA); and CD19-PE-Cy7 (Beckman Coulter, Brea, CA, USA). Monocytes, T cells, NK cells, or B cells were defined with immunofluorescence staining using anti-CD14, anti-CD3, anti-CD16, or anti-CD19, respectively. Granulocytes were gated based on the forward- and side-scatter signals.

For binding cell analysis, CD28SA and natalizumab were labeled with Alexa Fluor (AF) 488 Monoclonal Antibody Labeling Kit (Thermo Fisher Scientific). After incubation with labeled CD28SA or natalizumab for 1 h, blood was stained with marker antibodies for 30 min at 4°C, then red blood cells were lysed with 10% BD FACS Lysing Solution. PBMCs were stained with marker antibodies for 30 min at 4°C, then fixed with Cytofix/Cytoperm (BD).

Intracellular cytokine staining was done in both blood and PBMCs at early (0–6 h) and late (9–21 h) phases of the incubation with CD28SA and natalizumab. Brefeldin A (BFA, BD) solution was added to the culture well (2 μL/well) at 0 h and 9 h of the incubation in order to reduce extracellular secretion of cytokines.

At the end of the incubation, blood was stained with surface markers, hemolyzed in FACS Lysing Solution (BD), permeabilized with permeabilizing solution 2 (BD), stained for intracellular cytokines, and fixed with 1% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan). At the end of the incubation, PBMCs were stained for surface markers, permeabilized in Cytofix/Cytoperm (BD), and stained for intracellular cytokines using Perm/Wash (BD).

FACS analysis was performed using a FACSCanto II (BD) and Flow Jo Version 7.6.5 software (Tomy Digital Biology, Tokyo, Japan).

**Results**

*Binding of CD28SA to leucocytes*

The results of the binding assay are shown in Fig. 6. The binding of CD28SA to leucocytes was observed using AF488-labeled CD28SA. CD28SA bound to T cells at concentrations of 1 μg/mL and higher both in blood and PBMCs. The binding was saturated at 1 μg/mL in 2 out of 3 donors and at 10 μg/mL in 1 out of 3 donors. The maximum percentage of CD28SA-positive T cells was approximately 70%. The dose response curves of binding in each donor sample were similar between blood and PBMCs.

Significant binding of CD28SA with monocytes was seen at 100 μg/mL. Almost all the monocytes were positive at 100 μg/mL in PBMCs, while approximately 30–80% of the monocytes were positive in blood. A lower level of binding was seen in granulocytes and B cells in blood. Binding to NK cells was ambiguous. Approximately 10–80% of B cells were CD28SA positive at 100 μg/mL in PBMCs and 2–20% in blood. Natalizumab bound to almost all monocytes and lymphocytes at ≥ 1 μg/mL both in blood and PBMCs. A lower level of binding by natalizumab to granulocytes was seen in blood.

*Cytokine measurements in the WBCA*

Cytokine levels in the WBCA using blood from 3 healthy donors were measured after incubation for 6 h and 24 h. The induced cytokine profiles at 24 h (Fig. 7A) were similar to those at 6 h in each donor. The cytokine levels at 24 h were relatively higher than those at 6 h in the WBCA. There was a significant individual difference in cytokine responses after treatment with CD28SA between donor 2

and other donor samples. At the highest dose of 100 μg/mL, donor 2 showed a substantial release of the cytokines IL-2 (899 pg/mL), TNF-α (171 pg/mL) and IFN-γ (2456 pg/mL) at 24 h, whereas in the other two donors a clear elevation was seen in only IL-8 after treatment with CD28SA. In the blood from donor 2, all the cytokines were elevated from background levels at doses ≥ 1 μg/mL. CD28SA increased IL-8 (8200, 21,752, and 438 pg/mL in donors 1, 2, and 3, respectively) to much higher than background levels with PBS (3904, 167, and 39.6 pg/mL) in all donors' blood at 24 h. Although there were wide individual differences in the cytokine profiles induced in the WBCA, IL-8 was induced in a dose-dependent manner in the three donors after treatment with CD28SA. Natalizumab did not show clear induction of any cytokines.

*Cytokine measurements in the PBMC assay*

Based on the approximation of immobilized CD28 after air-drying a serial dilution of CD28SA, amounts of treated test articles in the PBMC assay were calculated as 0, 0.2, 0.5, and 2 μg/well. Cytokine levels in the PBMC assay with CD28SA were measured after treatment for 6 h and 24 h (Fig. 7B). The induced cytokine profiles at 24 h were similar to those at 6 h, and the concentrations of the cytokines were relatively higher at 24 h than at 6 h. CD28SA induced levels of IFN-γ (5376, 3594, 683 pg/mL), IL-2 (504, 311, 318 pg/mL), IL-6 (4892, 5248, 1590 pg/mL), and TNF-α (4608, 3771, 3722 pg/mL) that were more than 7 times as much as the levels of cytokines observed in all donors after 24-h treatment at the top concentration of natalizumab in all donors (IFN-γ at 23, 160, 28 pg/mL, IL-2 at 71, 21, 37 pg/mL, IL-6 at 38, 44, 50 pg/mL, and TNF-α at 121, 143, 349 pg/mL). IL-8 was increased after treatment with CD28SA (16,701, 12,841, 18,699 pg/mL) at 24 h. In the PBS control of the PBMC assay, the basal levels of IL-8 at 24 h were very high at 4496, 4822, and 7263 pg/mL. Immobilized natalizumab did not induce any cytokines.

*Intracellular cytokine production*

After treatment with CD28SA, I identified the cell sources of IL-2, IFN-γ, TNF-α, IL-8, and IL-6 in the WBCA or the PBMC assay using flow cytometry after staining intracellular cytokines and cell-surface markers.

In the WBCA, IL-8 was produced by both monocytes and granulocytes (Fig. 8A, B). Increases in the percentage of IL-8-positive monocytes at 9–21 h after treatment with CD28SA compared with the background level were seen in donors 1 (0.4% to 5.6%) and 3 (2.0% to 11.3%). IL-8-positive granulocytes were increased in all three donors at 0–6 h (0.5% to 7.7%, 0.5% to 3.8%, 0.9% to 3.7%). Though concentration of IL-8 in the culture supernatant of the sample from donor 2 (21,752 pg/mL) was higher than in that of the other donors (8200, 438 pg/mL), IL-8-positive monocytes did not increase. IL-6-positive or TNF-α-positive monocytes did not increase in the WBCA.

In the PBMC assay, the percentages of IL-8-, IL-6-, and TNF-α-positive monocytes increased after treatment with immobilized CD28SA in all the donors (Fig. 8C–E). After incubation with PBS for 9–21 h in the PBMC assay, the basal level of IL-8-positive monocytes was > 50% (Fig. 8C), which is much higher than in the WBCA (≤ 2%) (Fig. 8A) and is consistent with IL-8 levels seen in the culture supernatants (Fig. 7). IL-8, IL-6, and TNF-α were produced by monocytes after treatment with immobilized CD28SA in the PBMC assay.

The result of intracellular staining of cytokines in T cells is shown in Fig. 9. The results were similar among the three donors in the PBMC assay, but there were individual differences between donor 2 and donors 1, 3 in the WBCA. Production of IL-2 and TNF-α from T cells after treatment with CD28SA was observed in donor 2 in the WBCA and in all the donors in the PBMC assay. In donors 1 and 3, no increase of the IL-2- or TNF-α-positive T cells was observed in the WBCA. Though IFN-γ was elevated in donor 2 in the WBCA and in all three donors in the PBMC assay (Fig. 7), increase of

IFN-γ-positive T cells was not observed in either assay (Fig. 9C, F). No notable increase of IL-2-, TNF-α-, or IFN-γ-positive T cells was seen in donors 1 and 3 in the WBCA.

**Discussion**

The results of the present study suggested that different players and different mechanisms generate the positive responses in the WBCA and the PBMC assay after treatment with CD28SA. In the samples from three donors in the WBCA, there was a T cell responder (donor 2) and two non-responders (donors 1, 3) (Fig. 9A, B), which was consistent with a previous report that blood from approximately 40% of donors did not produce IL-2 after treatment with CD28SA (Wolf *et al.*, 2012). In the T cell non-responders, CD28SA caused dose-dependent increase of IL-8, but not of other cytokines in the WBCA (Fig. 7A). Production of IL-8 from monocytes and granulocytes (Fig. 8A, B) was therefore the main reason for the positive outcome in the WBCA that used blood from non-responders. In the PBMC assay, T cell activation accompanied with multiple cytokine induction caused positive results in all the donors (Fig. 9D, E). The response in PBMC was similar to that of the responders in the WBCA (Fig. 9).

In the non-responders in the WBCA, it is unlikely that the elevation of IL-8 is related to CRS via the CD28 signaling pathway, because IL-8 was increased without IL-2 induction (Fig. 7A). Also, although TNF-α from monocytes was suggested to be an initial response of CRS with TGN1412 (Sandilands *et al.*, 2010), TNF-α-positive monocytes were not increased in the WBCA. The most relevant mechanism involved in the life-threatening CRS caused by TGN1412 is activation of T cells via cross-linking of CD28 molecules (Wing, 2008; Eastwood *et al.*, 2010). IL-2 levels from activated T cells in an *in vitro* assay with PBMCs and immobilized CD28SA are recognized as the best predictive marker for clinical severity of anti-CD28-mAb-mediated cytokine release (Eastwood *et al.*, 2013;

Stebbings *et al.*, 2013). Taken together, the induction of IL-8 in the WBCA was irrelevant to stimulation of CD28.

Cytokine induced by TGN1412 was thought to be triggered by its binding to FcγRI on the surface of immune cells (Sandilands *et al.*, 2010). Previous *in vitro* studies showed that when FcγRs are cross-linked with human IgG (Marsh *et al.*, 1995) or IgG4 (Foreback *et al.*, 1997), monocytes are the principal IL-8 producer. In the WBCA, when IL-8 was induced at 100 μg/mL in the non-responders, CD28SA binding to monocytes or granulocytes was observed at 100 μg/mL, whereas in the responder IL-8 was induced at ≥ 1 μg/mL (Fig. 7A). Serum-derived IgG in the WBCA is estimated at 10 mg/mL, based on the normal blood IgG level (Cassidy *et al.*, 1974). Such a high treatment concentration as 100 μg/mL of CD28SA might be needed to bind FcγRs in the assay condition in this study because IgG4, the subclass of CD28SA, has weaker binding affinity to FcγRs than IgG1, the most predominant IgG subclass in blood (Ball *et al.*, 2012).

It is still unclear whether FcγRs play a pivotal role in the induction of IL-8 in the non-responders in this study, because only a small part of monocytes and granulocytes were IL-8 positive (Fig. 8A, B). Natalizumab has the same IgG4 Fc region as CD28SA and also bound to blood cells without inducing IL-8. Hussain *et al.* (2009) described that IgG4 barely stimulated IL-8 in granulocytes because of a lack of binding with FcγRs on granulocytes. In contrast, Holland *et al.*, (2004) reported that IgG4 has the capacity to activate granulocytes.

Inclusion of an IL-8 biomarker into the WBCA greatly improved the assay sensitivity to CD28SA as shown in Chapter 1. The present study showed that IL-8, which is the most sensitive biomarker in the WBCA, was produced from monocytes and granulocytes that are not relevant to the main mechanism of the severe CRS caused by TGN1412. This suggests that the WBCA may not be an appropriate tool to assess risk for T cell receptor superagonistic mAbs. On the other hand, the WBCA is sufficiently sensitive to FcγR-triggering responses (Wing *et al.*, 1996; Wing, 2008; Chapter 1 of this

thesis). Since better pharmacokinetic profiles and improved efficacy in mAb pharmaceuticals are staring to be achieved by modifying their binding affinity to the Fc receptor (Horton *et al.*, 2011; Li and Ravetch, 2012; Mimoto *et al.*, 2013; Herter *et al.*, 2013), the WBCA could be better suited for assessing Fc-modified mAbs rather than T cell-stimulating mAbs.

**Tables and Figures**

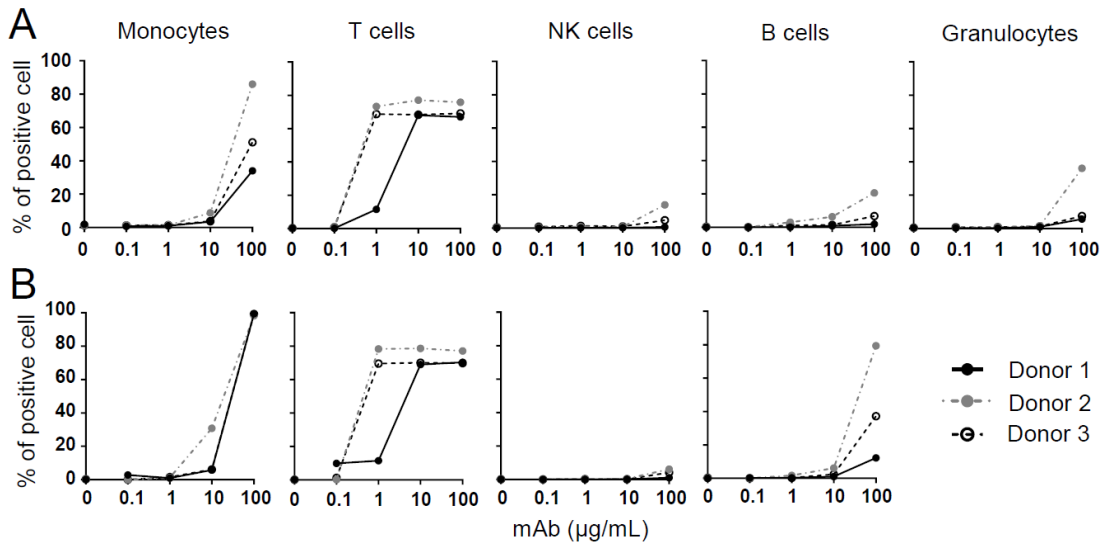


Figure 6. Percentages of CD28SA-positive cells in each population of immune cells after treatment with AF488-labeled CD28SA in blood (A) or in PBMCs (B).

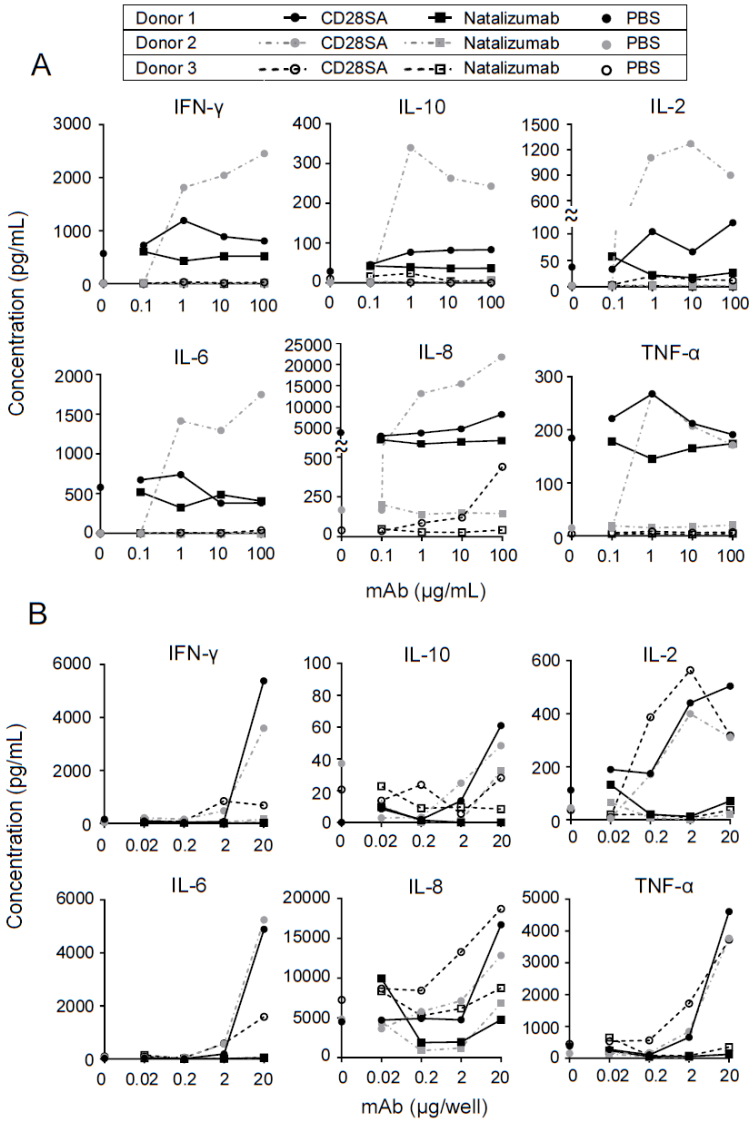


Figure 7. Induction of cytokines in 3 donors in the WBCA (A) or the PBMC assay (B) after treatment with CD28SA or natalizumab for 24 h. Cytokine in culture media at each treatment concentration were measured. Levels of cytokines at 24-h treatment with CD28SA (circles) or natalizumab (squares) or PBS (circles on the y-axis) are indicated. Concentrations of mAbs were 0.1, 1, 10, 100 μg/mL in the WBCA. Amounts of immobilized mAbs on the culture plates were 0.02, 0.2, 2, 20 μg/well in the PBMC assay.

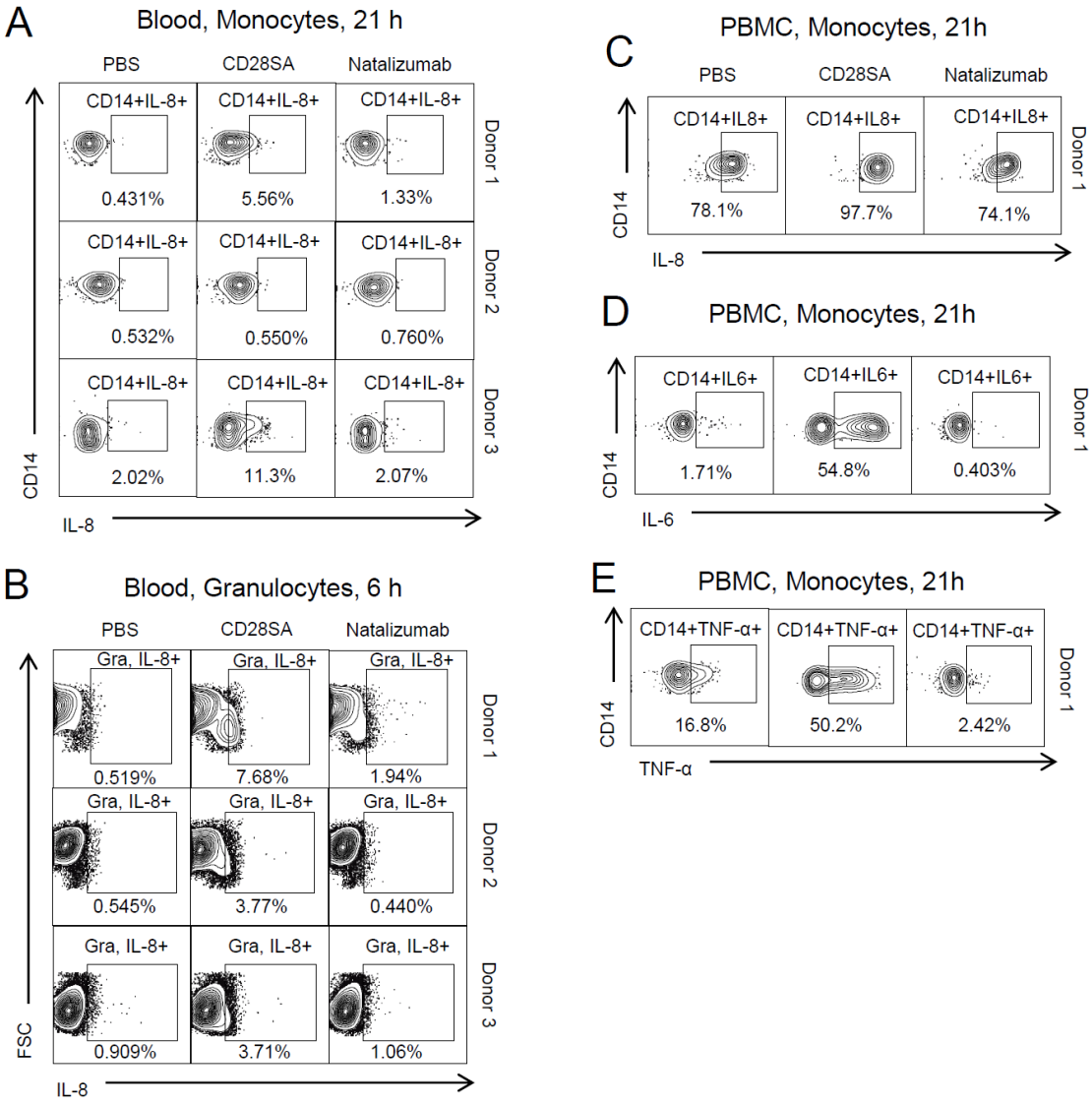


Figure 8. Intracellular staining of IL-8 in monocytes (A) and granulocytes (B) in the WBCA, and that of IL-8 (C), IL-6 (D), and TNF-α (E) in monocytes in the PBMC assay. Cells from 3 donors were treated with PBS, CD28SA, or natalizumab at a concentration of 100 μg/mL. One of three individual experiments with similar results is shown in the PBMC assay. Cytokines were stained after 6 or 21 h incubation. Brefeldin A was used to accumulate cytokines in the cells incubated for 0–6 h or 9–21 h.

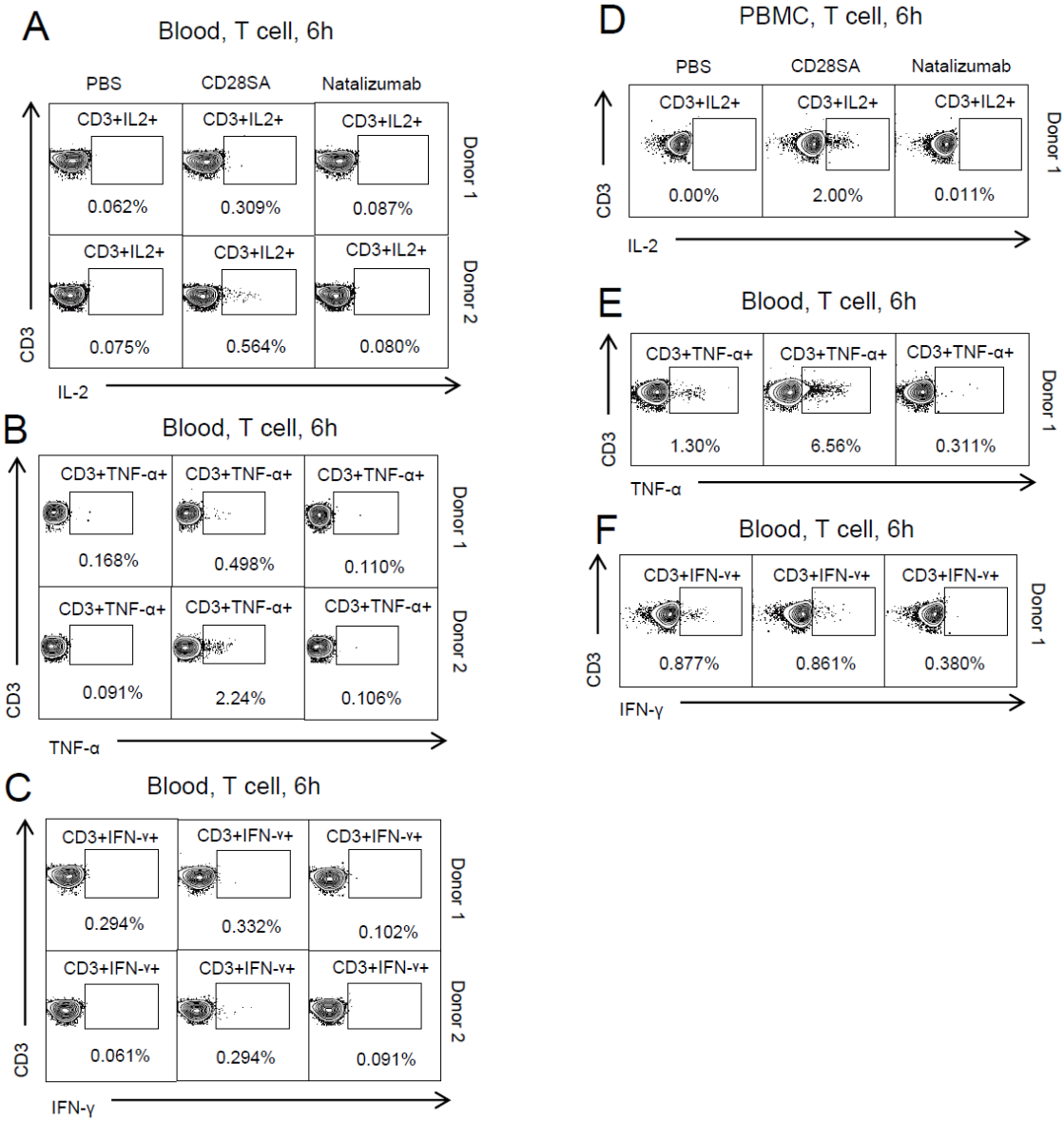


Figure 9. Intracellular staining of IL-2 (A), TNF-α (B) and IFN-γ (C) in T-cells in the WBCA, and that of IL-2 (D), TNF-α (E), and IFN-γ (F) in T-cells in the PBMC assay. Cells from 3 donors were treated with PBS, CD28SA, or natalizumab at a concentration of 100 μg/mL in the WBCA (A–C) or at 20 μg/well of immobilized mAbs in the PBMC assay (D–F). T cell responder (donor 2) and one (donor 1) of two non-responders in the WBCA were shown. One of three individual experiments with similar results is shown in the PBMC assay. Cytokines were stained after 6 or 21 h incubation. Brefeldin A was used to accumulate cytokines in the cells incubated for 0–6 h or 9–21 h.

## Chapter 3: Daily Ascending Dosing in Cynomolgus Monkeys to Mitigate Cytokine Release Syndrome Induced by ERY22, Surrogate for T-cell Redirecting Bispecific Antibody ERY974 for Cancer Immunotherapy


### Abstract

CD3 bispecific constructs show promising therapeutic potential as anti-tumor antibodies, but it has concurrently been difficult to manage cytokine release syndrome (CRS) in clinical use. Currently, the most effective measure for reducing CRS is considered a combination of intra-patient/animal dose escalation and corticosteroid premedication. To examine how effectively an intra-animal ascending dose regimen without premedication would mitigate CRS, I compared plasma cytokine levels in two groups of cynomolgus monkeys; one group was given a single dose, and the other a three-fold daily ascending dose of a CD3 bispecific construct that targets and cross-reacts with both glypican 3 and CD3 (ERY22). Ascending doses up to 1000 μg/kg of ERY22 dramatically reduced the peak cytokine levels of IL-6, TNF-α, and IFN-γ, IL-2 as well the clinical severity of CRS compared with a single dose of 1000 μg/kg. Peak cytokine levels following the single and ascending doses were 60,095 pg/mL and 1221 pg/mL for IL-6; 353 pg/mL and 14 pg/mL for TNF-α; 123 pg/mL and 16 pg/mL for IFN-γ; and 2219 pg/mL and 42 pg/mL for IL-2. The tolerance acquired with daily ascending doses up to 1000 μg/kg remained in effect for the following weekly doses of 1000 μg/kg.


### Introduction

The use of CD3 bispecific constructs as anti-tumor treatments has become an emerging part of cancer immunotherapy. A CD3 bispecific construct physically links CD3 on the surface of T cells to a target tumor antigen, thus activating the T cell to kill the tumor cell (Van Spriel *et al.*, 2000; Bargou *et al.*, 2008). Catumaxomab, the first CD3 bispecific construct targeting EpCAM and CD3, was approved

for malignant ascites in 2009 (Seimetz *et al.*, 2010). Blinatumomab, a CD3 bispecific construct targeting CD19 and CD3, has shown remarkable anti-tumor activity in CD19-positive lymphoma or leukemia (Bargou *et al.*, 2008; Topp *et al.*, 2011). Recently, various CD3 bispecific constructs have entered the clinical phase (Kontermann and Brinkmann, 2015).

CD3 bispecific constructs show promising therapeutic potential as anti-tumor antibodies, but they can also cause difficult-to-manage adverse effects in clinical use (Saber *et al.*, 2016; Saber *et al.*, 2017). Cytokine release syndrome (CRS), a potentially life-threatening systemic inflammatory response associated with elevated levels of circulating cytokines (Lee *et al.*, 2014), is the most common adverse effect associated with the use of CD3 bispecific constructs in cancer immunotherapy (Klinger *et al.*, 2012; Lee *et al.*, 2014; Saber *et al.*, 2017). Serious CRS has been reported with the clinical use of catumaxomab (Seimetz *et al.*, 2010) or blinatumomab (Shimabukuro-Vornhagen *et al.*, 2018). The potential risk of CRS is also a major disadvantage for cancer patients joining phase I/II studies because a conservative multi-step dose escalation from a minimum anticipated biological effect level (MABEL) to a pharmacologically effective level takes a long time. In addition, protocol amendments are sometimes required due to CRS (Saber *et al.*, 2016; Saber *et al.*, 2017), which further delays dose escalation.

There is still much to be understood to successfully control CRS. Premedication with corticosteroids, antihistamines and antipyretics is the gold standard (Shimabukuro-Vornhagen *et al.*, 2018). A combination of intra-patient/animal dose escalation and premedication with corticosteroid was reported to prevent severe CRS (Hoffman and Gore, 2014; Wu *et al.*, 2015; Saber *et al.*, 2017), and this is at present considered the most effective measure for reducing CRS, but even this effect is limited with premedication alone (Luheshi and Rothwell, 1996; Maude *et al.*, 2014), suggesting that the dosage regimen itself might be a more important factor in reducing CRS.

The purpose of this study is to examine how effectively an intra-animal ascending dose regimen without premedication can mitigate CRS induced by the CD3 bispecific construct ERY22 in cynomolgus monkeys. ERY22 is comprised of two different Fab domains binding to glypican 3 (GPC3) and CD3. It is a surrogate antibody for ERY974, a promising treatment for GPC3-positive solid tumors (Ishiguro *et al.*, 2017; Shiraiwa *et al.*, 2019). Though most CD3 bispecific constructs bind poorly to non-human targets (Saber *et al.*, 2016; Saber *et al.*, 2017), ERY22 can bind to the GPC3 and CD3 of both humans and cynomolgus monkeys. Ishiguro *et al*. (2017) reported that the ascending dosage of ERY974 decreased the induced IL-6 level in cynomolgus monkeys; the peak IL-6 blood level of approximately 2500 pg/mL after a single injection was only 500 pg/mL after ascending doses. The surrogate ERY22 as a single injection induced severe CRS in cynomolgus monkeys, with the peak IL-6 value > 60,000 pg/mL and a marked increase in TNF-α, IFN-γ and IL-2. In this study, I examined the suppressive effect of ascending doses with ERY22.

**Materials and Methods**

*Animals*

All procedures associated with this study were reviewed and approved by the Institutional Animal Care and Use Committee at Chugai Pharmaceutical Co., Ltd. (No. 11-068, 12-047). All animals were under the care of a laboratory animal veterinarian during the entirety of the study. The test facility has been accredited by AAALAC international. Male cynomolgus monkeys (Macaca fascicularis; Vietnam), between 3 and 4 years old were obtained from Hamri (Ibaraki, Japan). Animals were housed in climate controlled rooms given with free access to water and were given enrichment such as bananas, raisins or fruit gummy candies (Meiji, Tokyo, Japan) in addition to their diet (certified primate diet 5048; PMI Nutrition International). A Solid natural rubber toy (Kong; Bio-serve, NJ) and a stainless steel mirror were supplied as environmental enrichment in each cage. Animals were maintained in a

room with a 12 h light/dark cycle, an ambient temperature of 18–28°C, and relative humidity of 35–75%. Only one animal per group was enrolled in the middle and high dose groups due to the potential for adverse toxic effects. Other experimental groups included two animals. The high dose was set to correspond to the estimated medicinal dose of ERY974.

*Monoclonal antibody*

ERY22 is a humanized T-cell redirecting bispecific antibody produced by Chugai pharmaceutical Co., Ltd., which recognizes GPC3 and CD3 in humans and cynomolgus monkeys. The Fc region was modified to reduce binding to FcγRs (Shiraiwa *et al*., 2019). For injection formulations, ERY22 was appropriately diluted with 20 mM histidine-HCl buffer containing 150 mM NaCl and 0.05% (w/v) Polysorbate 80.

*Single dose administration*

The first dosing day was defined as Day 1, and the previous day as Day −1. ERY22 was intravenously administrated to the animals at doses of 10 ($n$ = 2), 100 ($n$ = 1), or 1000 μg/kg ($n$ = 1). The dose volume was 3 mL/kg, and the dosing speed was 2 mL/min. Saline was intravenously administrated to all animals as training during the acclimation period to reduce the stress of administration. General condition and food consumption was examined every day. Lymphocyte count was examined on a day in the acclimation period (Pre 1) and Pre 2 (24 h after saline injection), and on Days 1 (8 h after ERY22 injection), 2, 3, 8, 15, and 22. C-reactive protein (CRP) concentration was examined on Pre 1, Pre 2 (24 h), Days 2, 3, 8, 15, and 22. Body temperature was examined before dosing (pre), at 30 min, 1, 2, 4, 8, 24, 32, 48, 54, and 72 hours after dosing. Serum cytokine concentrations were examined on Pre 1, Pre 2 (pre, 2, 8, 24 h), Days 1 (pre, 2, 8 h), 2, and 3. Animals were sacrificed on Day 3 after 1000 μg/kg, on Day 10 after 10 μg/kg, and on Day 24 after 100 μg/kg.

*Ascending dose administration*

ERY22 was intravenously administrated to 2 male animals with daily ascending doses of 1, 3, 10, 30, 100, 300 and 1000 μg/kg from Days 1–7, followed by two injections of 1000 μg/kg after one week intervals on Days 14 and 21. The dose volume was 3 mL/kg, and the dosing speed was reduced to 0.3 mL/min based on clinical signs observed after single dose administrations. Animals were sacrificed at the end of the study (Day 28). Saline was intravenously administered to all animals on Day −8 of the acclimation period. General condition and food consumption was examined every day. Lymphocyte count was examined on Days −15, −7 (24 h), 1 (8 h), 3 (8 h), 5 (8 h), 7 (8 h), 8 (24 h), 10, 14 (pre, 8 h), 21 (pre, 8 h), and 27. CRP concentration was examined on Days −15, −7 (24 h), 2 (pre), 3 (pre), 4 (pre), 5 (pre, 8 h), 6 (pre), 7 (pre), 8 (24 h), 10, 14 (pre), 15 (24 h), 21 (pre), 22 (24 h), and 27. Body temperature was examined on Days 1 (pre, 30 min, 1, 2, 4, 8 h), 2 (pre, 2, 8 h), 3 (pre, 2, 8 h), 4 (pre, 2, 8 h), 5 (pre, 2, 8 h), 6 (pre, 2, 8 h), 7 (pre, 30 min, 1, 2, 4, 8 h), 8 (24, 32 h), 14 (pre, 30 min, 1, 2, 4, 8 h), 15 (24, 33 h), 21 (pre, 30 min, 1, 2, 4, 8 h), and 22 (24 h). Serum cytokine concentrations were examined on Days −15, −8 (pre, 2, 8 h), −7 (24 h), 1 (pre, 8 h), 2 (pre, 8 h), 3 (pre, 8 h), 4 (pre, 8 h), 5 (pre, 8 h), 6 (pre, 8 h), 7 (pre, 2, 8 h), 8 (24 h), 14 (pre, 2 , 8 h), 15 (24 h), 21 (pre, 2, 8 h), 22 (24 h), and 27. Percentages of activation marker (CD69) positive cells within CD4+ or CD8+ T cell (CD2+CD16−) populations were examined on Days −7 (24 h), 7 (8 h), 14 (pre), 21 (8 h), and 27. The plasma concentrations of ERY22 were measured on Days 1 (15 min), 5 (15 min), 7 (15 min), 14 (pre, 15 min), 21 (pre, 15 min), 22 (24 h), 24, 28. Anti-ERY22 antibodies (ADA) were measured on Days −15, 14 (pre), 21 (pre), 24, and 28.

*Toxicological examination*

General condition, body temperature, food consumption, hematology, blood chemistry, cytokines, percentages of activation marker positive T cells, and the serum concentration of the test article was examined. Increase in body temperature was calculated by subtracting the body temperature before dosing from that after dosing. Cytokines (TNF-α, IFN-γ, IL-6, IL-2) were measured using CBA non-human primate Th1/Th2 cytokine kit (BD, Franklin Lakes, NJ). Serum CRP was measured with a latex agglutination immunoassay using TBA-120FR (Canon Medical Systems Corporation, Tochigi, Japan). Hematological parameters were scored with ADVIA 120 (Siemens Healthcare Diagnostics, Tokyo, Japan). Percentages of CD69 positive cells in CD4+ or CD8+ T cell populations were detected using FACS Canto II (BD) using anti-CD69, anti-CD2, anti-CD16, anti-CD4, and anti-CD8 antibodies (all from BD).

*Toxicokinetics*

The plasma concentration of ERY22 was determined by an electrochemiluminescence immunoassay (ECLIA) method in the ascending dose experiment. Briefly, GPC3 protein (Chugai pharmaceutical) was immobilized on the surface of ECLIA plates (Meso Scale Diagnostics, Rockville, MD). Appropriately diluted plasma samples were added to the plate, then GPC3-captured ERY22 was detected by biotin-labeled anti-human Kapper Light Chain Goat IgG (Immuno-Biological Laboratories, Gunma, Japan) and SULFO-TAG labeled Streptavidin (Meso Scale Diagnostics).

ADA was measured by ECLIA using biotin-labeled ERY22 and SULFO-TAG labeled ERY22.

*Quantification of GPC3 mRNA*

The GPC3 mRNA expression was examined using a frozen stock of total RNA taken from the various organs of two healthy animals. Each snap-frozen organ was homogenized and mixed with RLT buffer supplied with RNeasy Mini Kit (Qiagen, Valencia, CA), Trizol LS (Invitrogen, Carlsbad, CA),

Trizol (Invitrogen) and/or QIAzol (Qiagen), and the total RNA was extracted and purified with RNeasy Mini Kit. Whole blood was collected in PAXgene Blood RNA Tube and the total RNA was extracted with PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's instructions. Expression of GPC3 mRNA was quantified by real-time reverse transcription-PCR using a SuperScript III Reverse Transcriptase (Invitrogen), StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA), and FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany). PCR conditions included incubation at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min using primers for GPC3 mRNA, forward 5'-GATGAAGATGAGTGCATTGGAG-3', reverse 5'-CATCCAGATCATAGGCCAGTT-3', and fluorescent Universal ProbeLibrary probe #9 (Roche Diagnostics). SK-pca13a and SK-pca31a, human GPC3 transfected liver tumor cell lines, were added as reference. Calculated GPC3 protein expression levels were $1.1\times10^3$ molecules/cell for SK-pca31a, and $4.8\times10^4$ molecules/cell for SK-pca13a.

**Results**

*Single dose administration*

The observed general conditions of the animals are shown in Table 5. After administration of ERY22, facial flushing and crouched posturing was observed in all dosing groups (10–1000 μg/kg).

Reduced food consumption was observed after 10 μg/kg in 1/2 animals and 100 μg/kg in 1/1 animal. A transient increase in body temperature (> 1°C) was observed after the 100 or 1000 μg/kg dose, and a transient increase in body temperature (0.9°C) was observed in 1/2 animals after 10 μg/kg dosing (Fig. 12C). In the animal that received 1000 μg/kg, vomiting, scratching, decrease of locomotor activity, and poor food consumption were observed. Increased veterinary monitoring was provided, and additional fruit enrichment was given to the animal after consulting with the attending veterinarian. The animal ate the fruit enrichment on Days 1 and 2, but not on Day 3. Due to clinical presentation of

severe adverse effects, the animal was sacrificed on Day 3 under the supervision and care of the attending veterinarian.

The results of concentration of serum cytokines are shown in Fig. 10. The high dose, 1000 μg/kg, substantially elevated plasma TNF-α to 353 pg/mL, IFN-γ to 123 pg/mL, IL-6 to 60,095 pg/mL, and IL-2 to 2219 pg/mL within 24 hours of administration. The highest values during the acclimation period for each cytokine was 19 pg/mL for TNF-α, 34 pg/mL for IFN-γ, 43 pg/mL for IL-6, and 38 pg/mL for IL-2. In the animal administered with 100 μg/kg of ERY22, the peak value of each cytokine within 24 hours after dosing was 63 pg/mL for TNF-α, 38 pg/mL for IFN-γ, 23,502 pg/mL for IL-6, and 468 pg/mL for IL-2. The highest value during the acclimation period for each cytokine was 29 pg/mL for TNF-α, 48 pg/mL for IFN-γ, 75 pg/mL for IL-6, and 45 pg/mL for IL-2. In the two animals administered with 10 μg/kg of ERY22, the peak values of each cytokine within 24 hours after dosing were 6 and 8 pg/mL for TNF-α, 13 and 19 pg/mL for IFN-γ, 399 and 2065 pg/mL for IL-6, and 0 and 53 pg/mL for IL-2. The highest value during the acclimation period for each cytokine was 0 pg/mL for TNF-α in both animals, 15 and 14 pg/mL for IFN-γ, 78 and 87 pg/mL for IL-6, and 46 and 35 pg/mL for IL-2.

CRP concentration is shown in Fig. 12B. CRP peaked at Day 2 or Day 3 after 10–1000 μg/kg. The highest CRP concentration in each animal was 14.5 mg/dL in the 1000 μg/kg dose group, 13.0 mg/dL in the 100 μg/kg dose group, and 9.0 or 12.8 mg/dL in the 10 μg/kg dose group. The values of CRP during acclimation period were ≤ 1.3 mg/dL in all animals.

A decrease in lymphocytes (Fig. 12A) was observed in all animals in dose groups of ≥ 10 μg/kg. The change in lymphocytes after 10 μg/kg dosing was much slighter than changes after 100 or 1000 μg/kg. The lymphocyte count 8 h after dosing in each animal was $0.3\times10^3$/μL in the 1000 μg/kg dose group, $0.9\times10^3$/μL in the 100 μg/kg dose group, 2.0 or 1.8 $\times10^3$/μL in the 10 μg/kg dose group, and

the lowest values for lymphocytes during the acclimation period in each animal were $3.8\times10^3/\mu L$, $11.5\times10^3/\mu L$, 4.7 or $5.2\times10^3/\mu L$, respectively.

*Ascending dose administration*

The observed general conditions of the animals are shown in Table 5. In one of the two animals (animal No. 1), facial flush was observed after a dose of 10 μg/kg and 30 μg/kg, and vomiting and diarrhea were observed at 24 and 48 h after the second 1000 μg/kg dose on Day 15. Reduced food consumption was also observed after the 300 μg/kg dose, and after the first and second 1000 μg/kg dose. The other animal (animal No. 2) showed no test article-related abnormalities in clinical observations. In both animals, a transient increase in body temperature was seen after the ≥ 30 μg/kg dose. The highest elevation in animal No. 1 was 2.5°C at 8 h after the 30 μg/kg dose, and that of animal No. 2 was 1.7°C at 8 h after the 100 μg/kg dose (Fig. 13C).

Serum concentration of cytokines are shown in Fig. 11. Much higher cytokine concentrations were observed 8 hours after a single dose than after ascending doses (Table 6). The highest cytokine concentrations in the two animals within 24 hours after dosing were 13 pg/mL in animal No. 1 and 14 pg/mL in animal No. 2 for TNF-α, 14 and 16 pg/mL for IFN-γ, 1221 and 306 pg/mL for IL-6, and 33 and 42 pg/mL for IL-2. The highest values during acclimation period were 10 and 25 pg/mL for TNF-α, 13 and 39 pg/mL for IFN-γ, 43 and 49 pg/mL for IL-6, and 23 and 74 pg/mL for IL-2.

Serum CRP concentrations are shown in Fig. 11. An increase in CRP could already be observed after the 1 μg/kg dose. The values increased dose-dependently from Day 2 to 5, and thereafter reached a plateau. The elevated CRP after the 1000 μg/kg dose decreased to a normal level after 7 days. The highest CRP concentration was 18 and 9 mg/dL in animal No. 1 and 2, and the values of CRP during the acclimation period were ≤ 1.4 mg/dL.

A decrease in lymphocytes (Fig. 13A) was seen after ≥ 10 μg/kg dosing in both animals. The lymphocyte count 8 h after the first 1000 μg/kg dose was $2.9\times10^3$/μL and $2.8\times10^3$/μL in animal No. 1 and 2, and these changes were much slighter than those following the single 1000 μg/kg dose ($0.3\times10^3$/μL). The lowest value for lymphocytes was $2.5\times10^3$/μL in both animals after the 100 μg/kg dose on Day 5, and the lowest value for lymphocytes during the acclimation period was $5.3\times10^3$/μL and $7.5\times10^3$/μL.

The flow cytometry results for activation marker (CD69) expression in T cells are shown in Fig. 13B. Activated T cell percentage increased 8 h after the first (Day 7) and the third 1000 μg/kg dose (Day 21). CD69+CD4+ T cell percentages after the first 1000 μg/kg dose were 3% and 4% in animal No. 1 and 2, and those after the third 1000 μg/kg dose were 39% and 2%, whereas those in the acclimation period were 1% for both animals. Percentages of CD69+CD8+ T cell after the first 1000 μg/kg dose were 29% and 33% in animal No. 1 and 2, and those after the third 1000 μg/kg dose were 39% and 15%, and those in the acclimation period were 16% and 5%.

The toxicokinetics results are shown in Fig. 14. In animal No. 1 and 2, plasma concentrations 15 minutes ($C_{15min}$) after administration of ERY22 were 0.022 and 0.007 μg/mL at 1 μg/kg, 2.4 and 3.5 μg/mL at 100 μg/kg, and 16 and 15 μg/mL at 1000 μg/kg. ADA was detected on Day 14 and later in both animals. $C_{15min}$ after the second and the third dose of 1000 μg/kg on Days 14 and 21 were similar to that of Day 7.

*mRNA expression for GPC3 in normal cynomolgus monkey tissues*

GPC3 mRNA was expressed in some normal tissues in cynomolgus monkeys (Fig. 15). The sciatic nerve, kidney, lung, epididymis, pituitary gland, and trachea expressed GPC3 mRNA at higher levels than SK-pca31a, one of the GPC3 transfected cell lines.

**Discussion**

A daily ascending dose up to 1000 μg/kg, corresponding to a dose with expected clinical efficacy, markedly reduced CRS whereas a single dose of 1000 μg/kg induced severe CRS (Table 5, 6). The ascending regimen reduced peak cytokine levels within 24 hours after dosing in animal No. 1 or 2 compared to a single dose of 1000 μg/kg, from 60,095 pg/mL to 1221 or 306 pg/mL for IL-6, from 353 pg/mL to 13 or 14 pg/mL for TNF-α, from 123 pg/mL to 14 or 16 pg/mL for IFN-γ, and from 2219 pg/mL to 33 or 42 pg/mL for IL-2 (Fig. 10, 11). Serum cytokine levels, except for IL-6, after the ascending doses were similar to those in the acclimation period. A transient lymphocyte decrease is commonly observed after treatment with CD3 bispecific constructs due to the adhesion of lymphocytes to endothelial cells (Molema *et al.*, 2000; Dettmar *et al.*, 2012). Lymphocytes decreased to 2.9 or $2.8\times10^3$/μL after an ascending dose of 1000 μg/kg which was much more moderate than the decrease to $0.3\times10^3$/μL after single dose of 1000 μg/kg (Fig. 12A, 13A). The lowest lymphocyte count during the acclimation period in all animals was $3.8\times10^3$/μL. Thus, the elevation of serum cytokines and decrease in lymphocytes were much more severe after a single dose than an ascending dose.

There were two main differences between the ascending and the single dose regimens. One was in the daily ascension from 1 μg/kg to 1000 μg/kg compared to the single administration of 1000 μg/kg. The other was in injection speed, 0.3 mL/min for the ascending dose and 2 mL/min for the single dose. Previous literature suggests that the continuous intravenous infusion of blinatumomab is better tolerated than bolus infusion (Friberg and Reese, 2017), but that a 2 h infusion of OKT3 did not mitigate CRS (Buysmann *et al.*, 1997). Therefore, I concluded that daily ascending dosage was the most important factor in reducing CRS in this study.

The general condition and cytokine levels appeared to improve with ascending dosing, but elevated body temperature and CRP remained high (Fig. 11, 13C). The higher body temperature and CRP may be caused by the moderate increase of IL-6 following the ascending dose because IL-6 is an

important mediator of fever (Luheshi and Rothwell, 1996) and CRP production (Libby, 2002). CRP levels were higher in the animal with higher IL-6 levels (Fig. 11).

Although the ascending dose prevented excessive CRS, the peak IL-6 level during the ascending period (1221 and 306 pg/mL in animal No. 1 and 2) was still high enough to induce fever or CRP elevation, which raises a question: where did the IL-6 come from? An elevation in activated T cells was also observed after the ascending 1000 μg/kg dose (Fig. 13B). As a first step in immune stimulation, ERY22 stimulates CD3 signaling and activates T cells, but it is implausible that the T cells would produce a high level of IL-6 without also inducing other cytokines. It was reported that IL-6 is produced from myeloid cells, including monocytes and macrophages, after T cell stimulation (Barrett *et al.*, 2014; Giavridis *et al.*, 2018). Direct cellular contact between T cells and myeloid cells promotes IL-6 production (Giavridis *et al.*, 2018). It is therefore likely that while the ascending dose regimen effectively reduced the production of T-cell derived cytokines, it may still be possible for the direct interaction of T cells and myeloid cells to induce IL-6.

The results in this study suggested that tolerance formed in the ascending phase was still effective for the following weekly doses, which is consistent with previous studies (Chung, 2008; Klinger *et al.*, 2012; Nagele *et al.*, 2017). With no repetition of the ascending phase, the following 1000 μg/kg weekly doses on Days 14 and 21 did not induce severe CRS in animal No. 1 and 2, even though plasma concentrations of ERY22 before administration were 49 and 292 ng/mL on Day 14, and 13 and 221 ng/mL on Day 21 (Fig. 14). ADA was detected on Day 14 and later in both animals on the ascending regimen. I think that ERY22 was still effective after induction of ADA because of the three reasons; 1) $C_{15min}$ following the 1000 μg/kg dose was similar both before and after ADA induction (Fig. 14), 2) the increase of CRP (Fig. 11), and 3) decrease in lymphocytes (Fig. 13A) were seen alongside ADA.

The frequency of GPC3 expression in cynomolgus monkeys was sufficient for ERY22 to induce CRS. In humans, GPC3 is highly expressed in mesodermal-derived tissues such as lung, liver and

kidney in fetuses (Pilia *et al.*, 1996). This expression was barely detected in adult tissues in one study (Zhu *et al.*, 2001), but low level expression of GPC3 has been reported in normal adult tissues in humans more recently (Andisheh-Tadbir *et al.*, 2019). In cynomolgus monkeys, the mRNA expression levels of GPC3 in the sciatic nerve, kidney, lung, epididymis, pituitary gland, and trachea were higher than in SK-pca31a cells expressing GPC3 at $1.1\times10^3$ molecules/cell (Fig. 15).

The present study demonstrated that an optimal ascending regimen can markedly reduce CRS induced by ERY22 in cynomolgus monkeys. My results also show that tolerance achieved with daily ascending doses up to 1000 μg/kg remained in effect for the following weekly doses of 1000 μg/kg. Further animal studies are needed to understand the mechanism behind the induction and maintenance of the tolerance, which will provide useful information to be extrapolated for human and will support future applications for CD3 bispecific constructs.

**Figures and Tables**

Table 5. Comparison of general conditions between single and ascending dosing

| | Single (μg/kg) | | | | Ascending (μg/kg) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 10 | 100 | 1,000 | 1 | 3 | 10 | 30 | 100 | 300 | 1,000 | 1,000 | 1,000 | 1 | 3 | 10 | 30 | 100 | 300 | 1,000 | 1,000 | 1,000 |
| Facial flush | + | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Crouched posturing | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Vomiting | - | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| Scratching | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Decreased locomotor activity | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Diarrhea | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| Increase in body temperature > 1°C | - | - | + | + | - | - | + | + | + | + | + | - | - | - | - | - | + | + | + | + | - | - |
| Decreased food consumption | - | + | + | + | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - | - | - | - |
| Moribund | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Animal No. | 1 | 2 | 3 | 4 | 1 | | | | | | | | | 2 | | | | | | | | |

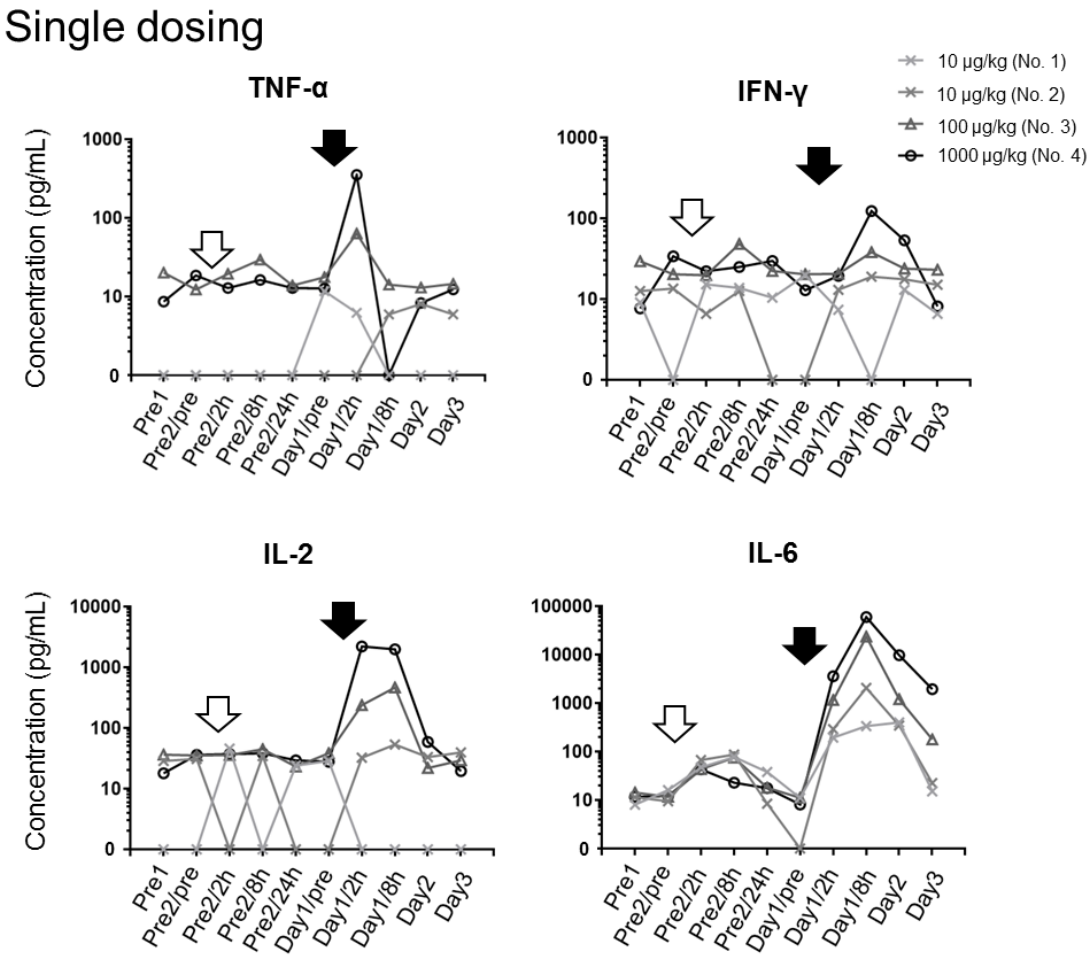


Figure 10. Serum cytokine concentrations in 4 cynomolgus monkeys. ERY22 was intravenously administered at 10 (*n* = 2), 100 (*n* = 1), 1000 μg/kg (*n* = 1) on Day 1 (black arrows). Physiological saline was administered in the acclimation period (Pre, white arrows).

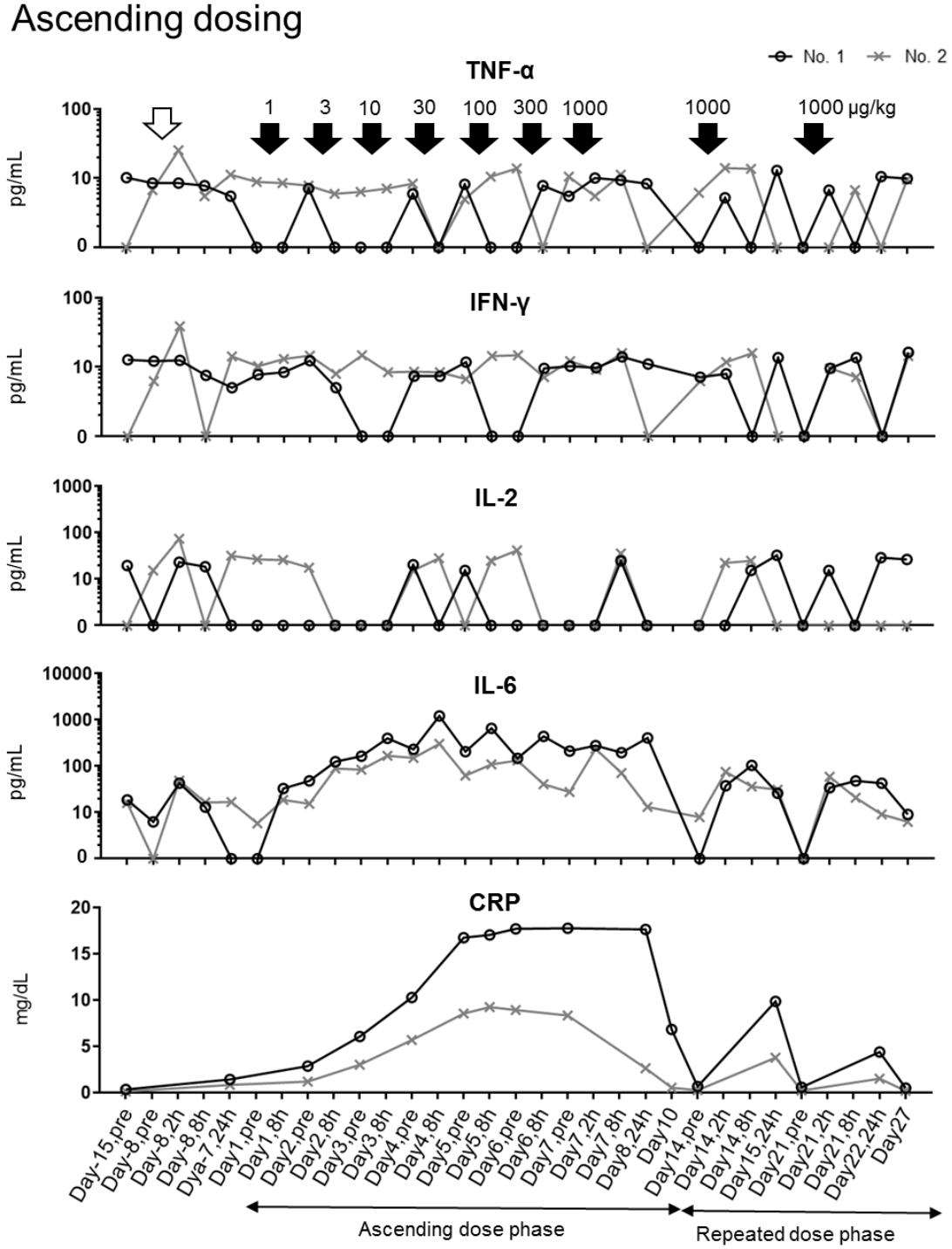


Figure 11. Serum cytokine concentrations (TNF-α, IFN-γ, IL-2, IL-6) and serum CRP concentrations in 2 cynomolgus monkeys (animal No. 1 and 2). ERY22 was repeatedly intravenously administered daily ascending doses of 1, 3, 10, 30, 100, 300 and 1000 μg/kg over 7 days, followed by weekly dosing at 1000 μg/kg for 2 weeks (black arrows). Physiological saline was administered in the acclimation period (Day −8, white arrows).

Table 6. Comparison of cytokine levels at 8 h after administration

| | Cytokine levels (pg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | TNF-α | | IFN-γ | | IL-6 | | IL-2 | |
| Dosing | Single | Ascending | Single | Ascending | Single | Ascending | Single | Ascending |
| 10 μg/kg | 6, 0 | 0, 7 | 19, 0 | 0, 8 | 336, 2065 | 403, 168 | 0, 53 | 0, 0 |
| 100 μg/kg | 14[1)] | 0, 11 | 38[1)] | 0, 14 | 23502[1)] | 663, 110 | 468[1)] | 0, 25 |
| 1000 μg/kg[2)] | 0[1)] | 9, 11 | 123[1)] | 14, 16 | 60095[1)] | 198, 71 | 1991[1)] | 25, 36 |

[1)] Cytokine level from one animal

[2)] The first 1000 μg/kg dosing in the ascending study

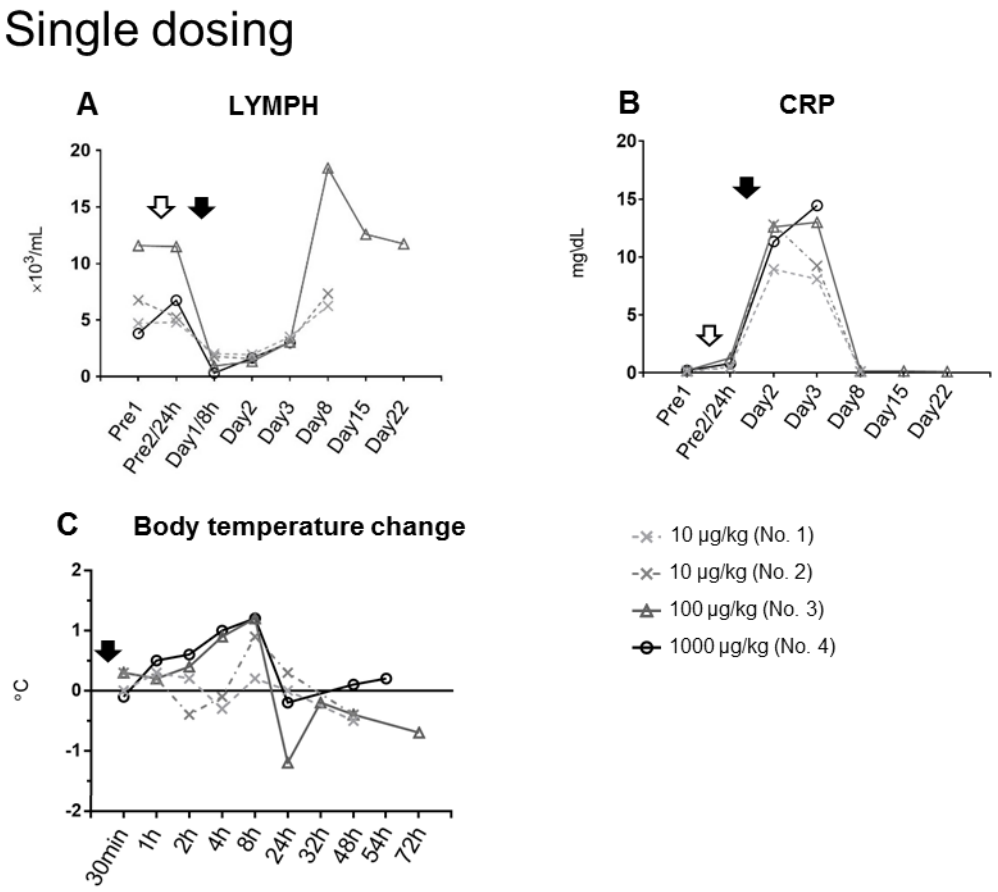


Figure 12. Blood lymphocyte (LYMPH) counts (A), serum CRP concentrations (B), and body temperature change from baseline after administration (C) in 4 cynomolgus monkeys. ERY22 was intravenously administered at 10 ($n$ = 2), 100 ($n$ = 1), 1000 μg/kg ($n$ = 1) on Day 1 (black arrows). Physiological saline was administered in the acclimation period (Pre, white arrows).

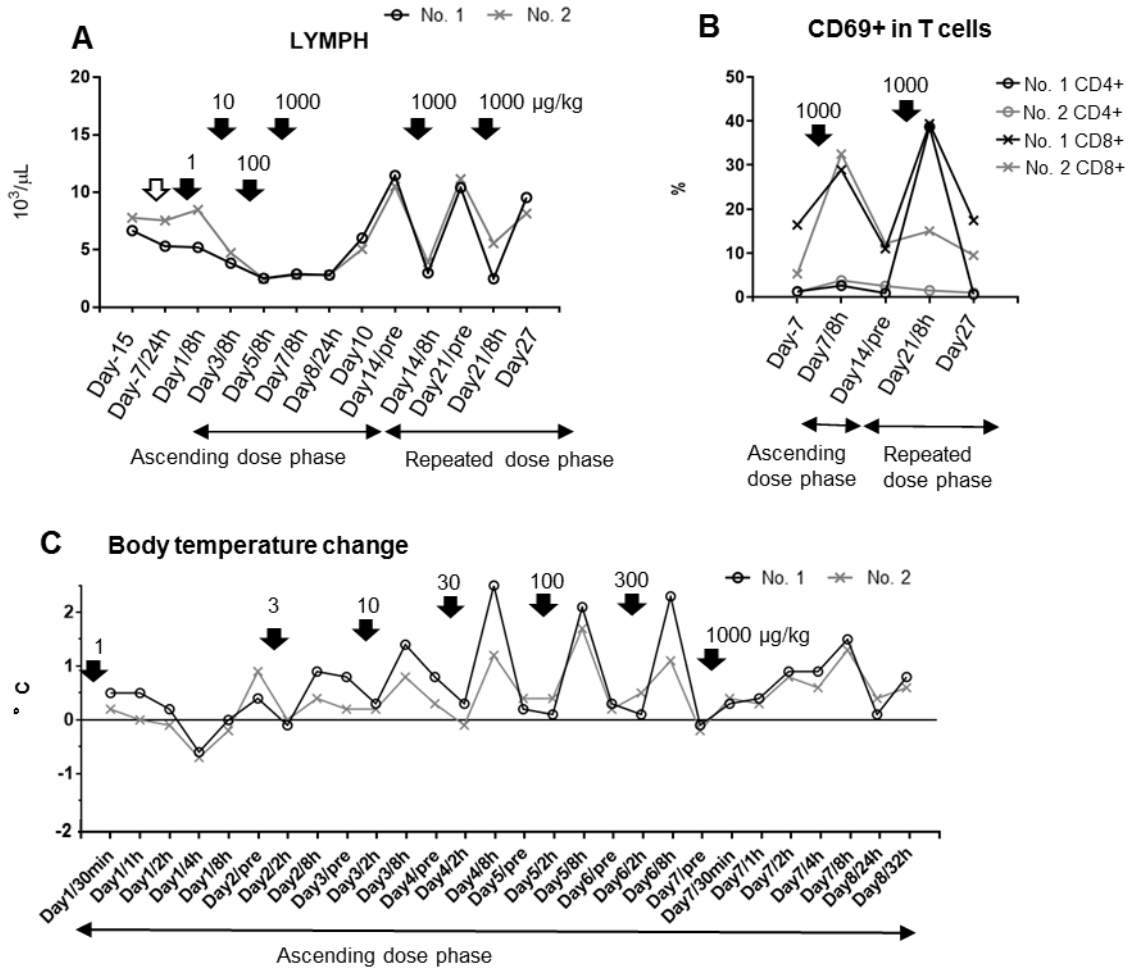


Figure 13. Blood lymphocyte counts (A), percentages of activation marker (CD69) positive in CD4+ or CD8+ T cells (B) body temperature change from baseline (C), and in 2 cynomolgus monkeys (animal No. 1 and 2). ERY22 was repeatedly intravenously administered daily ascending doses of 1, 3, 10, 30, 100, 300 and 1000 μg/kg over 7 days, followed by weekly dosing at 1000 μg/kg for 2 weeks (black arrows). Physiological saline was administered in the acclimation period (Day −8, white arrows).

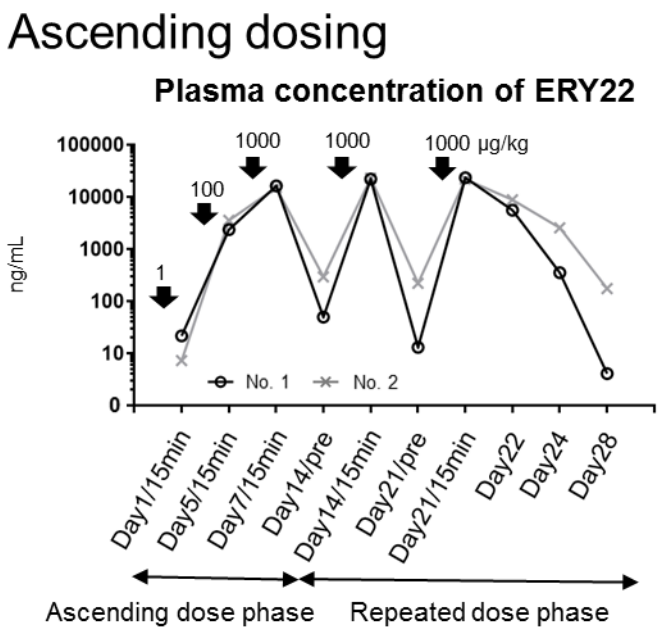


Figure 14. Plasma concentrations of ERY22 after ascending dosing in cynomolgus monkeys (animal No. 1 and 2). ERY22 was repeatedly intravenously administered daily ascending doses of 1, 3, 10, 30, 100, 300 and 1000 μg/kg over 7 days, followed by weekly dosing at 1000 μg/kg for 2 weeks (black arrows).

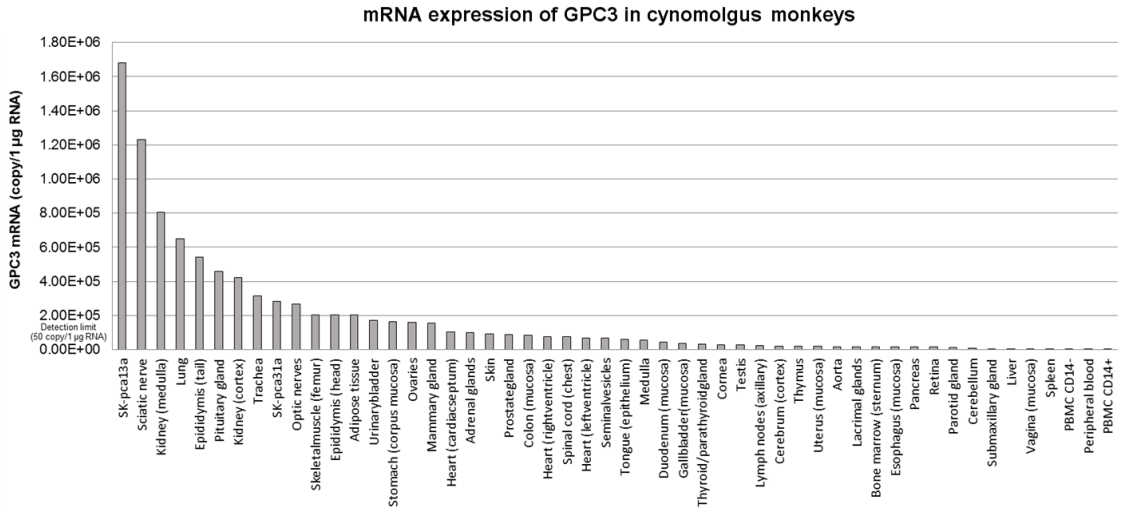


Figure 15. GPC3 mRNA expression was quantified by real-time reverse transcription-PCR in cynomolgus monkeys. Means of two animals are shown. SK-pca13a and SK-pca31a are human GPC3 transfected liver tumor cell lines.

**General Discussion**

In first-in-human trials to evaluate pharmaceutical candidates, the safety of clinical trial subjects should be the priority. Hazard identification and appropriate strategies to minimize risk are critical aspects of successful safety management (Van Gerven and Bonelli, 2018). Traditional preclinical studies were not able to predict the risk of severe cytokine release syndrome (CRS) caused by TGN1412 due to a failure of interpretation of preclinical studies and a lack of sufficient *in vitro* studies using human cells (Attarwala, 2010). After the TGN1412 CRS incident, *in vitro* cytokine release assays using human cells have become commonly used as hazard identification tools for therapeutic mAb candidates (Grimaldi *et al.*, 2016). In the first chapter of this research, I focused on the WBCA, one of the *in vitro* cytokine release assays using human blood cells, in order to propose an appropriate assay design to detect potential risks of CRS. The results of the first study suggest that the WBCA is sufficiently predictive for the potential risk of CRS induced by alemutuzumab and TGN1412-analogue (CD28SA) using a practical test that included measuring IL-8 in an $n \geq 9$ sample size. As similar as previous studies about WBCA, cytokine profile induced by CD28SA in the WBCA was different from that of the clinical trial (Suntharalingam *et al.*, 2006), and a high inter-donor variability in cytokine response was observed (Wolf *et al.*, 2012). The WBCA is a practical *in vitro* cytokine release assay that can warn of the potential risk of ADCC-related alemtuzumab and T-cell activating CD28SA, but the WBCA cannot be used as a risk-ranking tool for CRS because there was a lower response to CD28SA than alemtuzumab. Further mechanistic knowledge of the response to CD28SA is useful when the WBCA is applied to T cell-stimulating mAbs.

There are various *in vitro* assays to detect the potential risk of CRS, and each has its own advantages and limitations. In investigating the underlying mechanism of cytokine response in each assay, it is important to establish a method which accurately mimics *in vivo* responses and prevents a failure of hazard identification. The two major testing platforms to detect the potential risk of CRS are a combination of whole blood with aqueous-phase mAbs (the WBCA) and peripheral blood mononuclear cells (PBMCs) with solid-phase mAbs (the PBMC assay). In the study described in the second chapter, I investigated the mechanism by which two *in vitro* cytokine release assay formats generate positive responses to CD28SA. I compared the induction of cytokines and identified the cytokine-producing cells in the two assays. In the PBMC assay, IL-2 was markedly produced in all donor T cells after treatment with CD28SA, but in only 1 T-cell responder of the 3 donors in the WBCA. There was a significant individual difference in cytokine responses after treatment with CD28SA in the WBCA, which can cause the low sensitivity to CD28SA. Monocytes and granulocytes produced IL-8 which was the most sensitive biomarker in the WBCA, and IL-8 was elevated in the clinical trial of TGN1412 (Suntharalingam *et al.*, 2006). T cells, the most relevant player in the PBMC assay with CD28SA, did not contribute to the positive response in 2 out of the 3 donors (T cell non-responders) in the WBCA, suggesting that different immune cells and different mechanisms generate the positive cytokine responses to CD28SA in the two assays. In the PBMC assay, high-density immobilized TGN1412 onto culture plates enables cross-linking of CD28 receptors and activation of T cells (Findlay *et al.*, 2010), but high-density immobilized mAbs may cause false positive results (Foreback *et al.*, 1997; Wing, 2008; Grimaldi *et al.*, 2016). Romer *et al.* (2011) reported that lymph node cells had much higher TGN1412 reactivity than PBMCs and cellular interactions within the tissues were important to retain T-cell responsiveness. Moreover, peripheral blood contains less than 10% of all lymphocytes (Eastwood *et al.*, 2010). Thus, low sensitivity of the WBCA to CD28SA is possibly due to lower T-cell responsiveness in peripheral blood than that in lymphoid tissues. The

induction of IL-8 in the WBCA was not caused by T cells that were directly activated by CD28SA through CD28 stimulation, which is not relevant to the main mechanism of the severe CRS cause by TGN1412. The WBCA can detect the hazardous activity of CD28SA, but the WBCA may not be an appropriate *in vitro* assay to assess the risk for agonist mAbs to T-cell surface molecules. In contrast, the WBCA is thought to be sufficiently sensitive to FcγR-mediated responses (Holland *et al.*, 2004; Wing, 2008). When using *in vitro* cytokine release assays, it is necessary to understand the characteristics of *in vitro* assays and choose/establish an optimal method which suits the mode of action of a therapeutic mAb candidate.

Consideration of species differences is essential for safety evaluation of potentially adverse events of target modulation, and studies in non-relevant animal species may lead to misinterpretations (ICH, 2011). A number of factors should be taken into consideration when determining species relevancy including comparisons of target sequence homology, followed by *in vitro* assays to compare target binding, and assessments of functional activity demonstrated in *in vitro* and/or *in vivo* pharmacology or toxicology studies. Cynomolgus monkeys were considered to be an adequate species for TGN1412 because of the 100% amino acid sequence homology of the extracellular domain of CD28 and very similar binding affinities of TGN1412 to human and cynomolgus monkey CD28 (Hanke, 2006). The first human dose of TGN1412 was set at 0.1 mg/kg, which was 500-fold lower than the no observable adverse effect level (NOAEL, 50 mg/kg) established in cynomolgus monkeys. However, the first human dose was high enough to activate not only Tregs but also effector T (Teff) cells, which caused life-threatening CRS (Fig. 16). Stebbings *et al.* (2007) showed that PBMCs from cynomolgus monkeys did not respond to immobilized TGN1412 in the same way as human PBMCs, and importantly, TGN1412 was a CD28 superagonist in humans but not in cynomolgus monkeys. The

studies following the TGN1412 incident have revealed that the pharmacological and physiological properties of TGN1412 differs among species. TGN1412 shows similar binding affinities to CD28 of human and cynomolgus monkeys (Hanke, 2006), but the expression pattern of CD28 differs between humans and cynomolgus monkeys. Both Tregs and Teff cells express CD28 in humans (Dilek *et al.*, 2013), but in Teff cells, the key source of proinflammatory cytokines by TGN1412 stimulation, does not express CD28 in cynomolgus monkeys (Eastwood *et al.*, 2010). Appropriate preclinical models which can efficiently predict the potential CRS caused by mAb therapeutic candidates prior to first-in-human trials are essential. Current International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines discourage the use of non-relevant animal species, and encourages the use of *in silico* tools and (humanized) *in vitro* assays (Van Gerven and Bonelli, 2018).

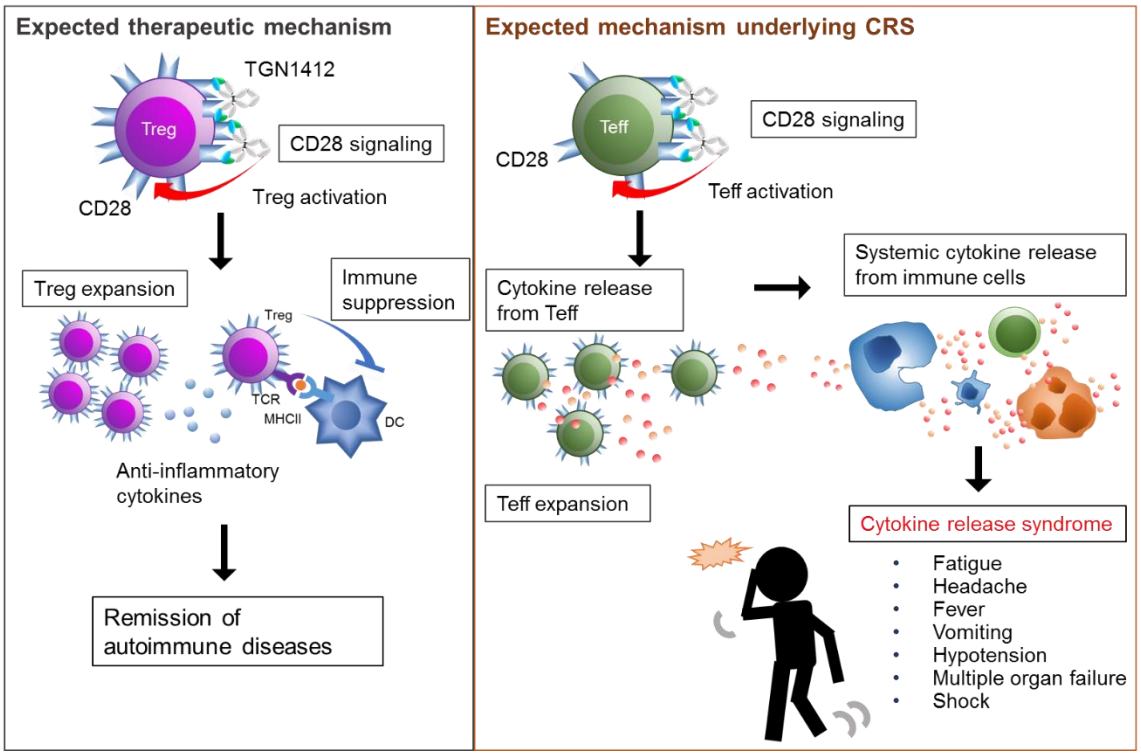


Figure 16. Expected therapeutic mechanism and the mechanism underlying CRS caused by TGN1412.

Careful dosing selection for first-in-human clinical trials is a vital element to safeguard the clinical trial subjects (Van Gerven and Bonelli, 2018). According to the European Medicines Agency (EMA) guidelines published in 2017, the starting dose for healthy volunteers should not exceed the pharmacologically active dose (PAD) or MABEL, the dose or exposure required at the bottom end of the dose response curve in humans, unless a robust scientific rationale can be provided for a higher dose (EMA, 2017). The MABEL of TGN1412 was considered to be 0.5 mg/kg for rat models using the rat CD28 surrogate mAb JJ316, and the safe starting dose was calculated to be 5 μg/kg which is 10,000-fold lower than the NOAEL in cynomolgus monkeys (Duff, 2006). Recently, an *in vitro* cell culture stimulation assay using human cells, resting T cells to original reactivity (RESTORE) assay and a receptor occupancy assay allowed to rationally select a safe dose for TGN1412 (rebranded TAB08), which enabled its return to the clinic (Tkach and Writer, 2015). In the RESTORE assay, TAB08 triggers detectable cytokine release at 0.06 μg/mL or more and Treg-cell activation with as little as 0.015 μg/mL of TAB08, suggesting that TAB08 has a potential therapeutic window (Tabares *et al.*, 2014). Though the dose used in the TGN1412 trial occupied between 45–80% of CD28 in human, the RESTORE assay recommended the dose of 5% receptor occupancy (Tkach and Writer, 2015). Furthermore, corticosteroids eliminated the cytokine release by TAB08, but Treg activation was corticosteroid-resistant (Tabares *et al.*, 2014; Tyrsin *et al.*, 2016). These encouraging non-clinical studies led to the design of a new TAB08 clinical trial in which the first-in-human dose was set at 0.1 μg/kg (1000-fold less than applied in the TGN1412 trial). The results of the clinical study of TAB08 in healthy volunteers shows that the threshold dose, where toxic cytokine release is minimized but Treg-cell activation is effective, is around 5 μg/kg, which is 20-fold below the one applied in the TGN1412 trial of 2006 (Tabares *et al.*, 2014). In the TGN1412 case, appropriate first-in-human dose setting can prevent severe CRS.

In addition to the identification of the potential risk of CRS, appropriate risk mitigation strategies should be investigated. The anti-CD19/CD3 bispecific construct, blinatumomab, induces cytokine release at low concentrations in *in vivo* and *in vitro* studies, and the MABEL approach was used for starting dose selection, but the initial clinical trial was terminated early due to toxicity, severe CRS and neurotoxicity (Yuraszeck *et al.*, 2017). Based on the insight from the initial clinical trial of blinatumomab, the following trials used a prophylactic protocol consisting of premedication of corticosteroids, dose adjustment including intra-patient ascending dosing, and continuous intravenous infusion, and cytoreduction rather than symptomatic treatment (Yuraszeck *et al.*, 2017; Shimabukuro-Vornhagen *et al.*, 2018). In the study described in the last chapter, I investigated the effectiveness of ascending dosing to reduce CRS caused by the anti-GPC3/CD3 construct, ERY22, in cynomolgus monkeys. As for ERY22, cynomolgus monkeys were considered to be a relevant animal species because of the similar binding affinities and functional properties between humans and cynomolgus monkeys in a similar way as ERY974 (Ishiguro *et al.*, 2017). Although a single dose of 1 mg/kg ERY22 caused fetal CRS, daily ascending dosing up to 1 mg/kg dramatically mitigated CRS in cynomolgus monkeys. Serum cytokine levels, transient lymphocyte decrease, and clinical symptoms were improved by ascending dosing, but the higher body temperature and CRP elevation were observed possibly because of the moderate increase of IL-6. Daily ascending dosing of ERY22 avoided severe CRS, and low grade CRS is considered to be manageable by the symptomatic treatment with anti-histamines, antipyretics and fluids (Shimabukuro-Vornhagen *et al.*, 2018). In addition, repetition of the ascending dosing was not required for the following weekly doses of ERY22, which is consistent with the previous report that severe CRS mostly occurred within 24 h of the first infusion, and the incidence declined in accordance with the repetition of dosing (Pangalis *et al.*, 2001; Chung, 2008; Klinger *et al.*, 2012). These results provide useful information that can be extrapolated for humans, and will support future application for CD3 bispecific constructs. Previous studies indicate that rate of

change, fold increases, or net increases in cytokine levels may be better correlated with CRS severity than absolute cytokine levels (Davila *et al.*, 2014; Lee *et al.*, 2014). Ascending doses can decrease rate of change in blood concentration of mAbs, which may hinder T cells releasing extensive cytokines and may mitigate CRS. Li *et al.* (2019) demonstrated that a CD3 bispecific construct induced T cell exhaustion, and these T cells retain their cytotoxic activity despite their inability to release cytokines. Thus, the CRS tolerance acquired with daily ascending doses may be attributed to T cell exhaustion. The mechanism behind the induction and maintenance of the CRS tolerance remains unclear, and further investigations are needed to mitigate CRS in clinical use.

CRS, the potentially fatal or life-threatening immunotoxicitiy caused by mAb pharmaceuticals, stimulates the immune system through multiple mechanisms (Fig. 17). Thus, it is necessary to identify the potential risk of CRS before first-in-human studies. However, preclinical studies using experimental animals may not be enough to predict CRS because of species difference in the immune system. The use of *in vitro* cytokine release assays with human cells are useful to identify the potential risk of CRS, and I proposed the use of the WBCA as a hazard identification tool for CRS in the first study. It is important to set an appropriate assay design in the consideration of variability attributed to individual differences. In the second study, I compared the cytokine-producing cells after stimulation with CD28SA in the two *in vitro* assays (the WBCA and the PBMC assay), and indicated that different immune cells generate a positive response in the two *in vitro* assays, causing differences in the response to CD28SA. Therefore, it is necessary to understand the characteristics of *in vitro* assays and establish an optimal method that suits the mechanism of a therapeutic mAb candidate. To date, it has been possible to detect the CRS hazard, but there still remain difficulties in managing CRS especially in the cancer immune therapy. Careful dose selection for first-in-human clinical trials is essential to prevent unexpected severe CRS, but CRS mitigation is also necessary for CD3 bispecific constructs

in clinical use. In the last study, I investigated the effectiveness of ascending dose regimens for CRS mitigation in cynomolgus monkeys, and the results suggest that ascending doses can mitigate CRS. Further investigations are necessary to reveal the mechanism behind the CRS mitigation and apply effective CRS mitigation strategies.

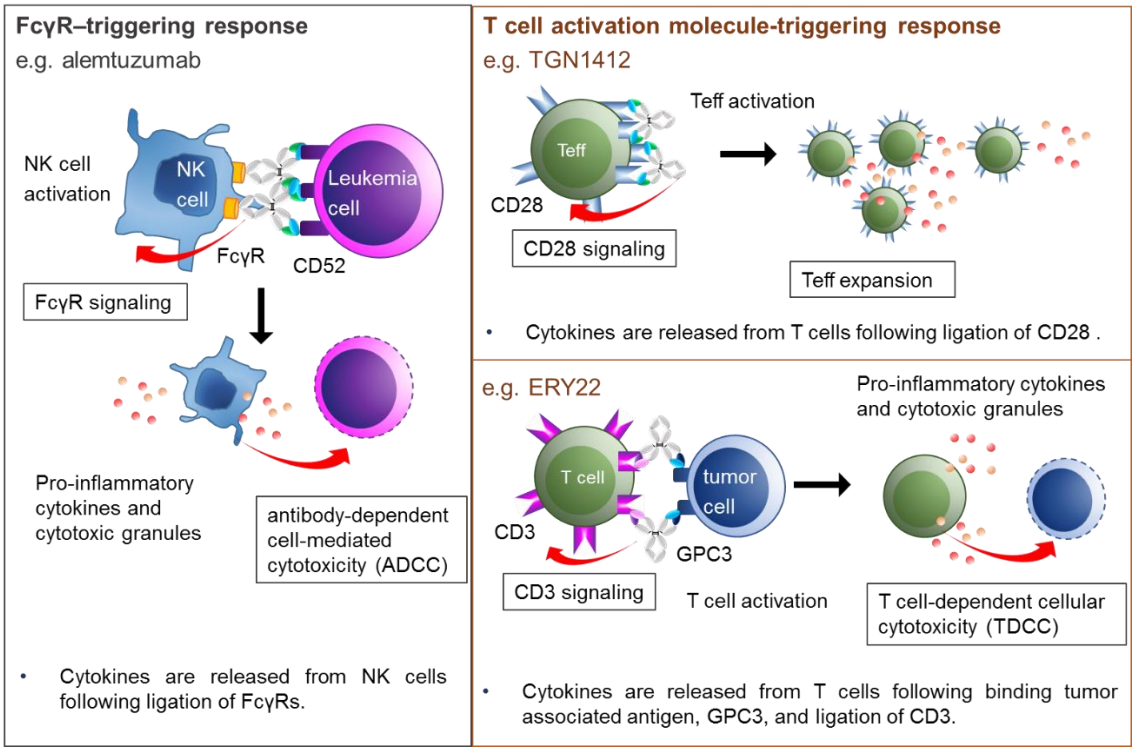


Figure 17. Multiple CRS mechanisms underlying CRS caused by alemtuzumab, TGN1412, and ERY22.

There are multiple mechanisms that drive CRS, a part of which were uncovered in this study. In order to further understand the pathophysiology of CRS, it is necessary to continue biological studies on the effects of mAb pharmaceuticals. In addition, the practical use of *in vitro* cytokine release assays needs to be established for each mAb pharmaceutical. Strategies and experimental approaches to ensure the safety of clinical trial subjects should be science-based.

**Acknowledgements**

I am deeply grateful to Professor Tetsuo Hashimoto and Professors Kentaro Nakano, Ryuhei Harada, Kisaburo Nagamune, and Ikuko Yuyama, University of Tsukuba, for guiding my work and valuable discussions through my doctoral program.

I would also like to express my gratitude to Dr. Masayuki Mishima, Chugai Pharmaceutical Co., Ltd., for giving me constructive comments and warm encouragement; coauthors Drs. Asako Harada, Masanori Sasaki, Toshiko Hara, Chiyomi Kubo, Tomoaki Inoue, Junko Taketo, Sho Akai, Takahiro Ishiguro, Atsushi Narita, and Akihisa Kaneko for permission to use the three research papers for my doctoral thesis; Ms. Mariko Yano for technical assistance; Editage (www.editage.com) for English language editing; Drs. Mitsuyasu Tabo and Masaki Honda for their understanding and supporting on my doctoral program.

Finally, my heartfelt appreciation goes to my family for supporting my life in University of Tsukuba.

## References


Abdulkhaleq, L.A., Assi, M.A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y.H., Hezmee, M.N.M., 2018. The crucial roles of inflammatory mediators in inflammation: A review. Veterinary world **11**, 627-635.

Aerts, J.G., Hegmans, J.P., 2013. Tumor-specific cytotoxic T cells are crucial for efficacy of immunomodulatory antibodies in patients with lung cancer. Cancer Res **73**, 2381-2388.

Amgen Inc. (2015): Vectibix® (panitumumab) [package insert]. Thousand Oaks, CA.

Andisheh-Tadbir, A., Ashraf, M.J., Gudarzi, A., Zare, R., 2019. Evaluation of Glypican-3 expression in benign and malignant salivary gland tumors. Journal of oral biology and craniofacial research **9**, 63-66.

Attarwala, H., 2010. TGN1412: From Discovery to Disaster. Journal of young pharmacists : JYP **2**, 332-336.

Bachmann, M.F., Hunziker, L., Zinkernagel, R.M., Storni, T., Kopf, M., 2004. Maintenance of memory CTL responses by T helper cells and CD40-CD40 ligand: antibodies provide the key. Eur J Immunol **34**, 317-326.

Bailey, L., Moreno, L., Manigold, T., Krasniqi, S., Kropshofer, H., Hinton, H., Singer, T., Suter, L., Hansel, T.T., Mitchell, J.A., 2013. A simple whole blood bioassay detects cytokine responses to anti-CD28SA and anti-CD52 antibodies. Journal of pharmacological and toxicological methods **68**, 231-239.

Ball, C., Fox, B., Hufton, S., Sharp, G., Poole, S., Stebbings, R., Eastwood, D., Findlay, L., Parren, P.W., Thorpe, R., Bristow, A., Thorpe, S.J., 2012. Antibody C region influences TGN1412-like functional activity in vitro. J Immunol **189**, 5831-5840.

Bargou, R., Leo, E., Zugmaier, G., Klinger, M., Goebeler, M., Knop, S., Noppeney, R., Viardot, A., Hess, G., Schuler, M., Einsele, H., Brandl, C., Wolf, A., Kirchinger, P., Klappers, P., Schmidt, M., Riethmuller, G., Reinhardt, C., Baeuerle, P.A., Kufer, P., 2008. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science **321**, 974-977.

Barrett, D.M., Teachey, D.T., Grupp, S.A., 2014. Toxicity management for patients receiving novel T-cell engaging therapies. Current opinion in pediatrics **26**, 43-49.

Barrington, R., Zhang, M., Fischer, M., Carroll, M.C., 2001. The role of complement in inflammation and adaptive immunity. Immunol Rev **180**, 5-15.

Beers, S.A., Glennie, M.J., White, A.L., 2016. Influence of immunoglobulin isotype on therapeutic antibody function. Blood **127**, 1097-1101.

Bertoletti, A., Ferrari, C., Fiaccadori, F., Penna, A., Margolskee, R., Schlicht, H.J., Fowler, P., Guilhot, S., Chisari, F.V., 1991. HLA class I-restricted human cytotoxic T cells recognize

endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc Natl Acad Sci U S A **88**, 10445-10449.

Beyersdorf, N., Gaupp, S., Balbach, K., Schmidt, J., Toyka, K.V., Lin, C.H., Hanke, T., Hunig, T., Kerkau, T., Gold, R., 2005a. Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. J Exp Med **202**, 445-455.

Beyersdorf, N., Hanke, T., Kerkau, T., Hunig, T., 2005b. Superagonistic anti-CD28 antibodies: potent activators of regulatory T cells for the therapy of autoimmune diseases. Annals of the rheumatic diseases **64 Suppl 4**, iv91-95.

Boehm, T., 2011. Design principles of adaptive immune systems. Nat Rev Immunol **11**, 307-317.

Boyman, O., Purton, J.F., Surh, C.D., Sprent, J., 2007. Cytokines and T-cell homeostasis. Curr Opin Immunol **19**, 320-326.

Brekke, O.H., Sandlie, I., 2003. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov **2**, 52-62.

Brennan, F.R., Morton, L.D., Spindeldreher, S., Kiessling, A., Allenspach, R., Hey, A., Muller, P.Y., Frings, W., Sims, J., 2010. Safety and immunotoxicity assessment of immunomodulatory monoclonal antibodies. MAbs **2**, 233-255.

Bugelski, P.J., Achuthanandam, R., Capocasale, R.J., Treacy, G., Bouman-Thio, E., 2009. Monoclonal antibody-induced cytokine-release syndrome. Expert Rev Clin Immunol **5**, 499-521.

Buysmann, S., Hack, C.E., van Diepen, F.N., Surachno, J., ten Berge, I.J., 1997. Administration of OKT3 as a two-hour infusion attenuates first-dose side effects. Transplantation **64**, 1620-1623.

Cambier, J.C., Gauld, S.B., Merrell, K.T., Vilen, B.J., 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat Rev Immunol **7**, 633-643.

Cassidy, J.T., Nordby, G.L., Dodge, H.J., 1974. Biologic variation of human serum immunoglobulin concentrations: sex-age specific effects. Journal of chronic diseases **27**, 507-516.

Chung, C.H., 2008. Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. Oncologist **13**, 725-732.

Cole, D.S., Morgan, B.P., 2003. Beyond lysis: how complement influences cell fate. Clinical science **104**, 455-466.

Davila, M.L., Riviere, I., Wang, X., Bartido, S., Park, J., Curran, K., Chung, S.S., Stefanski, J., Borquez-Ojeda, O., Olszewska, M., Qu, J., Wasielewska, T., He, Q., Fink, M., Shinglot, H., Youssif, M., Satter, M., Wang, Y., Hosey, J., Quintanilla, H., Halton, E., Bernal, Y., Bouhassira, D.C., Arcila, M.E., Gonen, M., Roboz, G.J., Maslak, P., Douer, D., Frattini, M.G., Giralt, S., Sadelain, M., Brentjens, R., 2014. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Science translational medicine **6**, 224ra225.

Dettmar, K., Seitz-Merwald, I., Lindemann, C., Schroeder, P., Seimetz, D., Atz, J., 2012. Transient lymphocyte decrease due to adhesion and migration following catumaxomab (anti-EpCAM x anti-CD3) treatment in vivo. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico **14**, 376-381.

Dilek, N., Poirier, N., Hulin, P., Coulon, F., Mary, C., Ville, S., Vie, H., Clemenceau, B., Blancho, G., Vanhove, B., 2013. Targeting CD28, CTLA-4 and PD-L1 costimulation differentially controls immune synapses and function of human regulatory and conventional T-cells. PLoS One **8**, e83139.

Drouin, S.M., Kildsgaard, J., Haviland, J., Zabner, J., Jia, H.P., McCray, P.B., Jr., Tack, B.F., Wetsel, R.A., 2001. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. J Immunol **166**, 2025-2032.

Duff, G.W.C., 2006. EXPERT SCIENTIFIC GROUP ON PHASE ONE CLINICAL TRIALS.

Eastwood, D., Bird, C., Dilger, P., Hockley, J., Findlay, L., Poole, S., Thorpe, S.J., Wadhwa, M., Thorpe, R., Stebbings, R., 2013. Severity of the TGN1412 trial disaster cytokine storm correlated with IL-2 release. Br J Clin Pharmacol **76**, 299-315.

Eastwood, D., Findlay, L., Poole, S., Bird, C., Wadhwa, M., Moore, M., Burns, C., Thorpe, R., Stebbings, R., 2010. Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells. Br J Pharmacol **161**, 512-526.

EMA, 2007. GUIDELINE ON STRATEGIES TO IDENTIFY AND MITIGATE RISKS FOR FIRST-IN-HUMAN CLINICAL TRIALS WITH INVESTIGATIONAL MEDICINAL PRODUCTS. EMEA/CHMP/SWP/28367/07

EMA, 2017. Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products. EMEA/CHMP/SWP/28367/07 Rev. 1

Finco, D., Grimaldi, C., Fort, M., Walker, M., Kiessling, A., Wolf, B., Salcedo, T., Faggioni, R., Schneider, A., Ibraghimov, A., Scesney, S., Serna, D., Prell, R., Stebbings, R., Narayanan, P.K., 2014. Cytokine release assays: current practices and future directions. Cytokine **66**, 143-155.

Findlay, L., Eastwood, D., Stebbings, R., Sharp, G., Mistry, Y., Ball, C., Hood, J., Thorpe, R., Poole, S., 2010. Improved in vitro methods to predict the in vivo toxicity in man of therapeutic monoclonal antibodies including TGN1412. J Immunol Methods **352**, 1-12.

Foreback, J.L., Remick, D.G., Crockett-Torabi, E., Ward, P.A., 1997. Cytokine responses of human blood monocytes stimulated with Igs. Inflammation **21**, 501-517.

Franks, Z., Carlisle, M., Rondina, M.T., 2015. Current challenges in understanding immune cell functions during septic syndromes. BMC immunology **16**, 11.

Friberg, G., Reese, D., 2017. Blinatumomab (Blincyto): lessons learned from the bispecific t-cell engager (BiTE) in acute lymphocytic leukemia (ALL). Annals of oncology : official journal of the European Society for Medical Oncology **28**, 2009-2012.

Gerlach, H., 2016. Agents to reduce cytokine storm. F1000Res **5**, 2909.

Giavridis, T., Van der Stegen, S.J.C., Eyquem, J., Hamieh, M., Piersigilli, A., Sadelain, M., 2018. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. Nat Med **24**, 731-738.

Gulati, K., Ray, A. Immunotoxicity. Handbook of Toxicology of Chemical Walfare Agents, Section III - Target Organ Toxicity, Chapter 40

Grignani, G., Maiolo, A., 2000. Cytokines and hemostasis. Haematologica **85**, 967-972.

Grimaldi, C., Finco, D., Fort, M.M., Gliddon, D., Harper, K., Helms, W.S., Mitchell, J.A., O'Lone, R., Parish, S.T., Piche, M.S., Reed, D.M., Reichmann, G., Ryan, P.C., Stebbings, R., Walker, M., 2016. Cytokine release: A workshop proceedings on the state-of-the-science, current challenges and future directions. Cytokine **85**, 101-108.

Guo, F., Iclozan, C., Suh, W.K., Anasetti, C., Yu, X.Z., 2008. CD28 controls differentiation of regulatory T cells from naive CD4 T cells. J Immunol **181**, 2285-2291.

Hale, G., Rebello, P., Brettman, L.R., Fegan, C., Kennedy, B., Kimby, E., Leach, M., Lundin, J., Mellstedt, H., Moreton, P., Rawstron, A.C., Waldmann, H., Osterborg, A., Hillmen, P., 2004. Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. Blood **104**, 948-955.

Hanke, T., 2006. Lessons from TGN1412. Lancet **368**, 1569-1570; author reply 1570.

Hansel, T.T., Kropshofer, H., Singer, T., Mitchell, J.A., George, A.J., 2010. The safety and side effects of monoclonal antibodies. Nat Rev Drug Discov **9**, 325-338.

Herter, S., Herting, F., Mundigl, O., Waldhauer, I., Weinzierl, T., Fauti, T., Muth, G., Ziegler-Landesberger, D., Van Puijenbroek, E., Lang, S., Duong, M.N., Reslan, L., Gerdes, C.A., Friess, T., Baer, U., Burtscher, H., Weidner, M., Dumontet, C., Umana, P., Niederfellner, G., Bacac, M., Klein, C., 2013. Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models. Molecular cancer therapeutics **12**, 2031-2042.

Hoffman, L.M., Gore, L., 2014. Blinatumomab, a Bi-Specific Anti-CD19/CD3 BiTE((R)) Antibody for the Treatment of Acute Lymphoblastic Leukemia: Perspectives and Current Pediatric Applications. Frontiers in oncology **4**, 63.

Hoffmann, P., Hofmeister, R., Brischwein, K., Brandl, C., Crommer, S., Bargou, R., Itin, C., Prang, N., Baeuerle, P.A., 2005. Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. International journal of cancer **115**, 98-104.

Holland, M., Hewins, P., Goodall, M., Adu, D., Jefferis, R., Savage, C.O., 2004. Anti-neutrophil cytoplasm antibody IgG subclasses in Wegener's granulomatosis: a possible pathogenic role for the IgG4 subclass. Clin Exp Immunol **138**, 183-192.

Horton, H.M., Chu, S.Y., Ortiz, E.C., Pong, E., Cemerski, S., Leung, I.W., Jacob, N., Zalevsky, J., Desjarlais, J.R., Stohl, W., Szymkowski, D.E., 2011. Antibody-mediated coengagement of FcgammaRIIb and B cell receptor complex suppresses humoral immunity in systemic lupus erythematosus. J Immunol **186**, 4223-4233.

Hussain, A., Pankhurst, T., Goodall, M., Colman, R., Jefferis, R., Savage, C.O., Williams, J.M., 2009. Chimeric IgG4 PR3-ANCA induces selective inflammatory responses from neutrophils through engagement of Fcgamma receptors. Immunology **128**, 236-244.

ICH, 2011. ICH HARMONISED TRIPARTITE GUIDELINE: PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS S6(R1)

Ishiguro, T., Sano, Y., Komatsu, S.I., Kamata-Sakurai, M., Kaneko, A., Kinoshita, Y., Shiraiwa, H., Azuma, Y., Tsunenari, T., Kayukawa, Y., Sonobe, Y., Ono, N., Sakata, K., Fujii, T., Miyazaki, Y., Noguchi, M., Endo, M., Harada, A., Frings, W., Fujii, E., Nanba, E., Narita, A., Sakamoto, A., Wakabayashi, T., Konishi, H., Segawa, H., Igawa, T., Tsushima, T., Mutoh, H., Nishito, Y., Takahashi, M., Stewart, L., ElGabry, E., Kawabe, Y., Ishigai, M., Chiba, S., Aoki, M., Hattori, K., Nezu, J., 2017. An anti-glypican 3/CD3 bispecific T cell-redirecting antibody for treatment of solid tumors. Sci Transl Med **9**, eaal4291.

Jilani, I., Keating, M., Giles, F.J., O'Brien, S., Kantarjian, H.M., Albitar, M., 2004. Alemtuzumab: validation of a sensitive and simple enzyme-linked immunosorbent assay. Leukemia research **28**, 1255-1262.

Yam-Puc, J.C., Zhang,L., Zhang, Y., Toellner, K.M., 2018. Role of B-cell receptors for B-cell development and antigen-induced differentiation. F1000Research.

Kasahara, K., Strieter, R.M., Chensue, S.W., Standiford, T.J., Kunkel, S.L., 1991. Mononuclear cell adherence induces neutrophil chemotactic factor/interleukin-8 gene expression. J Leukoc Biol **50**, 287-295.

Klinger, M., Brandl, C., Zugmaier, G., Hijazi, Y., Bargou, R.C., Topp, M.S., Gokbuget, N., Neumann, S., Goebeler, M., Viardot, A., Stelljes, M., Bruggemann, M., Hoelzer, D., Degenhard, E., Nagorsen, D., Baeuerle, P.A., Wolf, A., Kufer, P., 2012. Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab. Blood **119**, 6226-6233.

Kontermann, R.E., Brinkmann, U., 2015. Bispecific antibodies. Drug Discov Today **20**, 838-847.

Kulkarni, H.S., Kasi, P.M., 2012. Rituximab and cytokine release syndrome. Case reports in oncology **5**, 134-141.

Lee, D.W., Gardner, R., Porter, D.L., Louis, C.U., Ahmed, N., Jensen, M., Grupp, S.A., Mackall, C.L., 2014. Current concepts in the diagnosis and management of cytokine release syndrome. Blood **124**, 188-195.

Leung, S., Liu, X., Fang, L., Chen, X., Guo, T., Zhang, J., 2010. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cellular & molecular immunology **7**, 182-189.

Li, F., Ravetch, J.V., 2012. Apoptotic and antitumor activity of death receptor antibodies require inhibitory Fcgamma receptor engagement. Proc Natl Acad Sci U S A **109**, 10966-10971.

Li, J., Piskol, R., Ybarra, R., Chen, Y.J., Li, J., Slaga, D., Hristopoulos, M., Clark, R., Modrusan, Z., Totpal, K., Junttila, M.R., Junttila, T.T., 2019. CD3 bispecific antibody-induced cytokine release is dispensable for cytotoxic T cell activity. Science translational medicine **11**.

Libby, P., 2002. Inflammation in atherosclerosis. Nature **420**, 868-874.

Litman, G.W., Cannon, J.P., Dishaw, L.J., 2005. Reconstructing immune phylogeny: new perspectives. Nat Rev Immunol **5**, 866-879.

Liu, Q., Zhou, Y.H., Yang, Z.Q., 2016. The cytokine storm of severe influenza and development of immunomodulatory therapy. Cellular & molecular immunology **13**, 3-10.

Luheshi, G., Rothwell, N., 1996. Cytokines and fever. International archives of allergy and immunology **109**, 301-307.

Maggi, E., Vultaggio, A., Matucci, A., 2011. Acute infusion reactions induced by monoclonal antibody therapy. Expert Rev Clin Immunol **7**, 55-63.

Marsh, C.B., Gadek, J.E., Kindt, G.C., Moore, S.A., Wewers, M.D., 1995. Monocyte Fc gamma receptor cross-linking induces IL-8 production. J Immunol **155**, 3161-3167.

Maude, S.L., Barrett, D., Teachey, D.T., Grupp, S.A., 2014. Managing cytokine release syndrome associated with novel T cell-engaging therapies. Cancer journal **20**, 119-122.

Medzhitov, R., 2013. Pattern recognition theory and the launch of modern innate immunity. J Immunol **191**, 4473-4474.

Mimoto, F., Katada, H., Kadono, S., Igawa, T., Kuramochi, T., Muraoka, M., Wada, Y., Haraya, T., Miyazaki, T., Hattori, K., 2013. Engineered antibody Fc variant with selectively enhanced FcγRIIb binding over both FcγRIIaR131 and FcγRIIaH131. Protein Eng Des Sel **26**, 589-598.

Molema, G., Tervaert, J.W., Kroesen, B.J., Helfrich, W., Meijer, D.K., de Leij, L.F., 2000. CD3 directed bispecific antibodies induce increased lymphocyte-endothelial cell interactions in vitro. British journal of cancer **82**, 472-479.

Moreau, T., Coles, A., Wing, M., Isaacs, J., Hale, G., Waldmann, H., Compston, A., 1996. Transient increase in symptoms associated with cytokine release in patients with multiple sclerosis. Brain **119 ( Pt 1)**, 225-237.

Nagele, V., Kratzer, A., Zugmaier, G., Holland, C., Hijazi, Y., Topp, M.S., Gokbuget, N., Baeuerle, P.A., Kufer, P., Wolf, A., Klinger, M., 2017. Changes in clinical laboratory parameters and pharmacodynamic markers in response to blinatumomab treatment of patients with relapsed/refractory ALL. Experimental hematology & oncology **6**, 14.

Nguyen, D.H., Hurtado-Ziola, N., Gagneux, P., Varki, A., 2006. Loss of Siglec expression on T lymphocytes during human evolution. Proc Natl Acad Sci U S A **103**, 7765-7770.

O'Shea, J.J., Murray, P.J., 2008. Cytokine signaling modules in inflammatory responses. Immunity **28**, 477-487.

Pangalis, G.A., Dimopoulou, M.N., Angelopoulou, M.K., Tsekouras, C., Vassilakopoulos, T.P., Vaiopoulos, G., Siakantaris, M.P., 2001. Campath-1H (anti-CD52) monoclonal antibody therapy in lymphoproliferative disorders. Med Oncol **18**, 99-107.

Pilia, G., Hughes-Benzie, R.M., MacKenzie, A., Baybayan, P., Chen, E.Y., Huber, R., Neri, G., Cao, A., Forabosco, A., Schlessinger, D., 1996. Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nature genetics **12**, 241-247.

Piquette, C.A., Robinson-Hill, R., Webster, R.O., 1994. Human monocyte chemotaxis to complement-derived chemotaxins is enhanced by Gc-globulin. J Leukoc Biol **55**, 349-354.

Radtke, F., MacDonald, H.R., Tacchini-Cottier, F., 2013. Regulation of innate and adaptive immunity by Notch. Nat Rev Immunol **13**, 427-437.

Romer, P.S., Berr, S., Avota, E., Na, S.Y., Battaglia, M., ten Berge, I., Einsele, H., Hunig, T., 2011. Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. Blood **118**, 6772-6782.

Saber, H., Del Valle, P., Ricks, T.K., Leighton, J.K., 2017. An FDA oncology analysis of CD3 bispecific constructs and first-in-human dose selection. Regulatory toxicology and pharmacology : RTP **90**, 144-152.

Saber, H., Gudi, R., Manning, M., Wearne, E., Leighton, J.K., 2016. An FDA oncology analysis of immune activating products and first-in-human dose selection. Regulatory toxicology and pharmacology : RTP **81**, 448-456.

Sandilands, G.P., Wilson, M., Huser, C., Jolly, L., Sands, W.A., McSharry, C., 2010. Were monocytes responsible for initiating the cytokine storm in the TGN1412 clinical trial tragedy? Clin Exp Immunol **162**, 516-527.

Schrezenmeier, E., Jayne, D., Dorner, T., 2018. Targeting B Cells and Plasma Cells in Glomerular Diseases: Translational Perspectives. Journal of the American Society of Nephrology : JASN **29**, 741-758.

Schutte, R.J., Parisi-Amon, A., Reichert, W.M., 2009. Cytokine profiling using monocytes/macrophages cultured on common biomaterials with a range of surface chemistries. Journal of biomedical materials research. Part A **88**, 128-139.

Seimetz, D., Lindhofer, H., Bokemeyer, C., 2010. Development and approval of the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3) as a targeted cancer immunotherapy. Cancer treatment reviews **36**, 458-467.

Shimabukuro-Vornhagen, A., Godel, P., Subklewe, M., Stemmler, H.J., Schlosser, H.A., Schlaak, M., Kochanek, M., Boll, B., von Bergwelt-Baildon, M.S., 2018. Cytokine release syndrome. Journal for immunotherapy of cancer **6**, 56.

Shiraiwa, H., Narita, A., Kamata-Sakurai, M., Ishiguro, T., Sano, Y., Hironiwa, N., Tsushima, T., Segawa, H., Tsunenari, T., Ikeda, Y., Kayukawa, Y., Noguchi, M., Wakabayashi, T., Sakamoto, A., Konishi, H., Kuramochi, T., Endo, M., Hattori, K., Nezu, J., Igawa, T., 2019. Engineering a bispecific antibody with a common light chain: Identification and optimization of an anti-CD3 epsilon and anti-GPC3 bispecific antibody, ERY974. Methods **154**, 10-20.

Srivastava, R.K., Dar, H.Y., Mishra, P.K., 2018. Immunoporosis: Immunology of Osteoporosis-Role of T Cells. Frontiers in immunology **9**, 657.

Stebbings, R., Eastwood, D., Poole, S., Thorpe, R., 2013. After TGN1412: recent developments in cytokine release assays. J Immunotoxicol **10**, 75-82.

Stebbings, R., Findlay, L., Edwards, C., Eastwood, D., Bird, C., North, D., Mistry, Y., Dilger, P., Liefooghe, E., Cludts, I., Fox, B., Tarrant, G., Robinson, J., Meager, T., Dolman, C., Thorpe, S.J., Bristow, A., Wadhwa, M., Thorpe, R., Poole, S., 2007. "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics. J Immunol **179**, 3325-3331.

Stephenson, J.J., Gregory, C., Burris, H., Larson, T., Verma, U., Cohn, A., Crawford, J., Cohen, R.B., Martin, J., Lum, P., Yang, X., Amado, R.G., 2009. An open-label clinical trial evaluating safety and pharmacokinetics of two dosing schedules of panitumumab in patients with solid tumors. Clinical colorectal cancer **8**, 29-37.

Strome, S.E., Sausville, E.A., Mann, D., 2007. A mechanistic perspective of monoclonal antibodies in cancer therapy beyond target-related effects. Oncologist **12**, 1084-1095.

Suntharalingam, G., Perry, M.R., Ward, S., Brett, S.J., Castello-Cortes, A., Brunner, M.D., Panoskaltsis, N., 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N Engl J Med **355**, 1018-1028.

Tabares, P., Berr, S., Romer, P.S., Chuvpilo, S., Matskevich, A.A., Tyrsin, D., Fedotov, Y., Einsele, H., Tony, H.P., Hunig, T., 2014. Human regulatory T cells are selectively activated by low-dose application of the CD28 superagonist TGN1412/TAB08. Eur J Immunol **44**, 1225-1236.

Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., Lotze, M.T., 2012. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev **249**, 158-175.

Thorpe, S.J., Stebbings, R., Findlay, L., Eastwood, D., Poole, S., Thorpe, R., 2013. How predictive are in vitro assays for cytokine release syndrome in vivo? A comparison of methods reveals worrying differences in sensitivity and frequency of response. Cytokine **64**, 471-472.

Tisoncik, J.R., Korth, M.J., Simmons, C.P., Farrar, J., Martin, T.R., Katze, M.G., 2012. Into the eye of the cytokine storm. Microbiology and molecular biology reviews : MMBR **76**, 16-32.

Tkach, K., Writer, S., 2015. TGN1412: THE NEXT GENERATION. Biocentury Innovations, November 5

Topp, M.S., Kufer, P., Gokbuget, N., Goebeler, M., Klinger, M., Neumann, S., Horst, H.A., Raff, T., Viardot, A., Schmid, M., Stelljes, M., Schaich, M., Degenhard, E., Kohne-Volland, R., Bruggemann, M., Ottmann, O., Pfeifer, H., Burmeister, T., Nagorsen, D., Schmidt, M., Lutterbuese, R., Reinhardt, C., Baeuerle, P.A., Kneba, M., Einsele, H., Riethmuller, G., Hoelzer, D., Zugmaier, G., Bargou, R.C., 2011. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. J Clin Oncol **29**, 2493-2498.

Tsiantoulas, D., Diehl, C.J., Witztum, J.L., Binder, C.J., 2014. B cells and humoral immunity in atherosclerosis. Circulation research **114**, 1743-1756.

Tyrsin, D., Chuvpilo, S., Matskevich, A., Nemenov, D., Römer, P.S., Tabares, P., Hünig, T., 2016. From TGN1412 to TAB08: the return of CD28 superagonist therapy to clinical development for the treatment of rheumatoid arthritis. Clinical and experimental rheumatology **34**, S45-S48.

Van Gerven, J., Bonelli, M., 2018. Commentary on the EMA Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products. Br J Clin Pharmacol **84**, 1401-1409.

Van Linthout, S., Miteva, K., Tschope, C., 2014. Crosstalk between fibroblasts and inflammatory cells. Cardiovasc Res **102**, 258-269.

Van Spriel, A.B., van Ojik, H.H., van De Winkel, J.G., 2000. Immunotherapeutic perspective for bispecific antibodies. Immunology today **21**, 391-397.

Vessillier, S., Eastwood, D., Fox, B., Sathish, J., Sethu, S., Dougall, T., Thorpe, S.J., Thorpe, R., Stebbings, R., 2015. Cytokine release assays for the prediction of therapeutic mAb safety in first-in man trials - Whole blood cytokine release assays are poorly predictive for TGN1412 cytokine storm. J Immunol Methods **424**, 43-52.

Vogelpoel, L.T., Baeten, D.L., de Jong, E.C., den Dunnen, J., 2015. Control of cytokine production by human fc gamma receptors: implications for pathogen defense and autoimmunity. Frontiers in immunology **6**, 79.

Waibler, Z., Sender, L.Y., Merten, C., Hartig, R., Kliche, S., Gunzer, M., Reichardt, P., Kalinke, U., Schraven, B., 2008. Signaling signatures and functional properties of anti-human CD28 superagonistic antibodies. PLoS ONE **3**, e1708.

Wang, H., Ma, S., 2008. The cytokine storm and factors determining the sequence and severity of organ dysfunction in multiple organ dysfunction syndrome. Am J Emerg Med **26**, 711-715.

Ward, P.A., Cochrane, C.G., Mueller-Eberhard, H.J., 1965. The Role of Serum Complement in Chemotaxis of Leukocytes in Vitro. J Exp Med **122**, 327-346.

Warncke, M., Calzascia, T., Coulot, M., Balke, N., Touil, R., Kolbinger, F., Heusser, C., 2012. Different adaptations of IgG effector function in human and nonhuman primates and implications for therapeutic antibody treatment. J Immunol **188**, 4405-4411.

Wilson, M., Seymour, R., Henderson, B., 1998. Bacterial perturbation of cytokine networks. Infect Immun **66**, 2401-2409.

Wing, M., 2008. Monoclonal antibody first dose cytokine release syndromes-mechanisms and prediction. J Immunotoxicol **5**, 11-15.

Wing, M.G., Moreau, T., Greenwood, J., Smith, R.M., Hale, G., Isaacs, J., Waldmann, H., Lachmann, P.J., Compston, A., 1996. Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. J Clin Invest **98**, 2819-2826.

Wing, M.G., Waldmann, H., Isaacs, J., Compston, D.A., Hale, G., 1995. Ex-vivo whole blood cultures for predicting cytokine-release syndrome: dependence on target antigen and antibody isotype. Ther Immunol **2**, 183-190.

Wolf, B., Morgan, H., Krieg, J., Gani, Z., Milicov, A., Warncke, M., Brennan, F., Jones, S., Sims, J., Kiessling, A., 2012. A whole blood in vitro cytokine release assay with aqueous monoclonal antibody presentation for the prediction of therapeutic protein induced cytokine release syndrome in humans. Cytokine **60**, 828-837.

Wolf, B., Morgan, H., Brennan, F., Krieg, J., Gani, Z., Jones, S., Kiessling, A., 2013. Response to the letter to the editor by Susan Thorpe et al.: how predictive are in vitro assays for cytokine release syndrome in vivo? A comparison of methods reveals worrying differences in sensitivity and frequency of response. Cytokine **64**, 473-475; discussion 476.

Wu, J., Fu, J., Zhang, M., Liu, D., 2015. Blinatumomab: a bispecific T cell engager (BiTE) antibody against CD19/CD3 for refractory acute lymphoid leukemia. Journal of hematology & oncology **8**, 104.

Yuraszeck, T., Kasichayanula, S., Benjamin, J.E., 2017. Translation and Clinical Development of Bispecific T-cell Engaging Antibodies for Cancer Treatment. Clin Pharmacol Ther **101**, 634-645.

Zhu, Z.W., Friess, H., Wang, L., Abou-Shady, M., Zimmermann, A., Lander, A.D., Korc, M., Kleeff, J., Buchler, M.W., 2001. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut **48**, 558-564.