

Monoclonal antibody-induced cytokine-release syndrome

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Monoclonal antibodies (mAbs) are widely used in anti-inflammatory and tumor therapy. Although effective, mAbs can cause a variety of adverse effects. An important toxicity seen with a few mAbs is cytokine-release syndrome (CRS). These mAbs include: alemtuzumab, muromonab-CD3, rituximab, tosituzumab, CP-870,893, LO-CD2a/BTI-322 and TGN1412. By contrast, over 30 mAbs used clinically are not associated with CRS. In this review, the clinical aspects of CRS, the mAbs associated with CRS, the cytokines involved and putative mechanisms mediating cytokine release will be discussed. This will be followed by a discussion of the poor predictive value of studies in animals and the prospects for creating *in vitro* screens. Finally, approaches to decreasing the probability of CRS, decreasing the severity or treating CRS, should it occur, will be described.

KEYWORDS: cytokine storm • cytometry • Fc receptors • mice • nonhuman primate

Definition & clinical aspects of cytokine-release syndrome

Recombinant monoclonal antibodies (mAbs) and fusion proteins are widely used in anti-inflammatory and tumor therapy. They are highly efficient in certain diseases, but can cause a variety of adverse side effects [1]. One of the more important adverse effects seen with a number of mAbs is a cascade of systemic cytokine release that has been termed cytokine-release syndrome (CRS) or cytokine storm.

Cytokine-release syndrome describes an exaggerated systemic immune response involving the potential release of more than 150 inflammatory mediators (cytokines, chemokines, oxygen radicals, complement factors and coagulation) [2–4]. Although its origins are not completely understood, CRS is believed to be due to the activation of a variety of cell types, including monocytes/macrophages, T cells and B cells. CRS is characterized by a rise in TNF- α and IFN- γ within 1–2 h followed by increases in IL-6 and IL-10 and, in some cases, IL-2 and IL-8. Patients report a number of adverse events including fatigue, headache, urticaria, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling, rhinitis, nausea, vomiting, flushing, fever, chills, hypotension, tachycardia and asthenia at sites of disease [5]. Regardless of the target antigen or immunoglobulin (Ig) class or subclass of a particular mAb, the profiles of events (and their timing in relation to initiating the first dose infusion) are generally similar.

In addition to inducing CRS, mAbs can induce other types of acute infusion reactions. These range from mild infusion reactions to severe IgE-mediated type I hypersensitivity reactions (anaphylactic reactions) or anaphylactoid reactions not mediated by IgE. True anaphylactic reactions usually do not occur upon first exposure and require prior sensitization. By contrast, anaphylactoid reactions such as CRS appear to be secondary to the release of cytokines following the binding of antibody to cells.

Although the clinical manifestations of infusion reactions, anaphylactic reactions, anaphylactoid reactions and CRS can overlap, they are discriminated by their scope, duration, severity, and response to therapy. This has been codified in the glossary report of the National Cancer Institute Common Toxicity Criteria (NCI-CTC) for Adverse Events v3.0 [301], which defines CRS as an idiosyncratic inflammatory reaction produced by cells of the immune system seen during or immediately after infusion of an agent.

Cytokine-release syndrome is, in most cases, a first-dose phenomenon. For example, with rituximab that targets CD20 (a B-cell antigen), infusion-related symptoms of fever and chills/rigor occur in the majority of patients during the first infusion of rituximab. The incidence of infusion-related events decreased from 70 to 80% during the first infusion to less than 40% with subsequent infusions. In most patients, the symptoms are mild to moderate in severity

and are managed easily. However, some patients may experience severe, life-threatening or even fatal reactions that result from the massive release of cytokines [5].

Monoclonal antibodies associated with CRS

Listed in TABLE 1 are the mAbs most commonly associated with CRS.

Muromonab-CD3

Muromonab-CD3, a murine mAb directed to human CD3, is indicated for the treatment of acute renal, steroid-resistant cardiac, or steroid-resistant hepatic allograft rejection [6] and carries a US FDA designated black box warning for CRS. According to the drug label, most patients receiving muromonab-CD3 develop some form of infusion reaction that ranges from a mild, self-limited, 'flu-like' illness to severe CRS; the milder reactions being more common. The severe reactions can be shock-like and life-threatening, and may include serious cardiovascular and CNS manifestations. CRS is associated with increased serum levels of cytokines (e.g., TNF- α , IL-2, IL-6 and IFN- γ) that peak between 1 and 4 h following administration.

LO-CD2a (BTI-322)

With LO-CD2a, a rat IgG2b antihuman CD2 mAb, tested for the prevention or treatment of kidney allograft rejection, cytokine release (TNF- α and IFN- γ) was observed after the first injection, which was correlated with some side effects, but the effects were never severe or life threatening (fever, chills and vomiting) [7].

Tositumomab

Tositumomab is a murine IgG2a- λ mAb directed against the CD20 antigen. The therapeutic regimen (tositumomab and I¹³¹-tositumomab) is indicated for the treatment of patients with CD20 antigen-expressing relapsed or refractory, low-grade, follicular, or transformed non-Hodgkin's lymphoma (NHL), including patients with rituximab-refractory NHL (Bexxar, GlaxoSmithKline, NC, USA). A constellation of symptoms, including fever, rigors or

chills, sweating, hypotension, dyspnea, bronchospasm and nausea, have been reported during or within 48 h of infusion. Although all patients in the clinical studies received pretreatment with acetaminophen and an antihistamine, the value of premedication in preventing infusion-related toxicity was not evaluated in any of the clinical studies. Infusional toxicities were managed by slowing and/or temporarily interrupting the infusion [5,8].

Rituximab

Rituximab, a chimeric anti-CD20 mAb, is approved to treat CD20-positive NHL, and in combination with methotrexate is also approved for rheumatoid arthritis. Rituximab is thought to deplete B cells through antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and possibly signaling for apoptosis. Severe CRS has occurred in patients receiving rituximab. Characteristic symptoms are severe dyspnea, fever and blood pressure changes occurring within a few hours of the start of treatment. The mechanism through which these adverse events occur has been proposed to be a rapid release of cytokines into the periphery. Byrd *et al.* showed that in chronic lymphocytic leukemia (CLL) patients with rituximab-mediated CRS, release of TNF- α , IL-6 and IFN- γ was maximal 2 h after the start of the infusion [9].

There have been fatal cases of cytokine-release syndrome described with rituximab treatment and approximately 80% of fatal infusion reactions occurred in association with the first infusion [10]. Following approval of the drug in the USA (November 1997) and in Europe (June 1998), there were reports of eight fatalities resulting from CRS in an estimated 12,000–14,000 treated patients [11]. Seifert *et al.* described a fatal case report on a 14-year-old boy with a very early relapse of pre-B-acute lymphoblastic leukemia (ALL) and anaplastic astrocytoma [12]. After a small fraction of the standard dose had been administered, the infusion had to be interrupted owing to an acute attack of pain in the lumbar region. A total of 2 days later, after resumption of the therapy, he developed a fatal course of systemic inflammatory response syndrome (SIRS) and died, possibly due to CRS associated with sepsis [12]. Tournamille *et al.* reported the case of a 46-year-old patient, presenting with familial cardiomyopathy at the time of the second infusion of rituximab, who died a few minutes after having developed CRS [13]. These case reports underlined the need for special attention when using rituximab among patients with cardiac risk factors (reassessment of the risk–benefit ratio, specific monitoring and premedication (see '5-year view')). Lim *et al.* reported a fatal case of CRS that occurred in a 71-year-old woman with CLL who suffered a cardiopulmonary collapse and died 9 h after the administration of rituximab [14]. These authors concluded that in order to avoid serious complications associated with rituximab therapy, it

Table 1. Monoclonal antibodies associated with cytokine-release syndrome (excluding some anti-CD3).

Antibody	Antigen	Class	Ref.
LO-CD2a/BTI-322	CD2	Rat IgG2b	[7]
Muromonab-CD3	CD3	Murine IgG2a	[6]
Tositumomab	CD20	Murine IgG2a	[5,8]
Rituximab	CD20	Murine–human chimeric IgG1	[15,112]
Ibritumomab	CD20	Murine IgG1	[113,114]
TGN1412	CD28	Human IgG4	[24]
CP-870,893	CD40	Human IgG2	[25]
Alemtuzumab	CD52	Humanized IgG1	[115]
Basiliximab	IL-2 R	Murine–human chimeric IgG1	[26,27]
Daclizumab	IL-2 R	Humanized IgG1	[26]

is important to identify risk factors; for example, the presence of circulating tumor cells and lymphocyte counts greater than $50 \times 10^9/l$ [15], and avoid treatment in patients at risk.

In contrast to these severe fatal cases of systemic CRS, Perez-Gala *et al.* reported two localized cases of cutaneous CRS during the first infusion of rituximab without any systemic symptoms in patients with primary cutaneous lymphoma [16]. Both cases shared the clinical feature that the urticarial reaction was limited to lymphoma cutaneous lesions and excision scars of former tumor nodules.

Alemtuzumab

Alemtuzumab is a recombinant DNA-derived humanized IgG1- κ mAb that targets the CD52 antigen expressed on normal and malignant B and T lymphocytes, as well as natural killer (NK) cells, monocytes and macrophages [17]. Alemtuzumab is approved as a single agent for the treatment of CLL. Alemtuzumab administration is accompanied by a characteristic first-dose reaction due to cytokine release involving TNF- α , IFN- γ and IL-6 that consists of fever, rigors, rash and, at times, dyspnea and hypotension [18,19]. It has been described that subcutaneous administration of alemtuzumab to patients with pancreas–kidney transplantation avoids this first-dose reaction while achieving a similar concentration to intravenous administration. It is thought that the avoidance of infusion-associated hypotension is particularly important in pancreas transplantation where venous thrombosis is a major problem [20]. It should be noted that alemtuzumab is not FDA-approved for subcutaneous administration.

In patients with relapsed or refractory CLL, those with massive lymphadenopathy were more prone to signs of CRS [21]. Premedication with acetaminophen and antihistamines has been compulsory in recent studies, significantly reducing the first-dose effects. However, in a study with alemtuzumab in Behcet's disease, five out of 18 patients experienced CRS during alemtuzumab infusion, despite appropriate prophylaxis [22]. The package insert instructs to gradually escalate to the maximum recommended dose of 30 mg at initiation of dosing or if dosing is held over 7 days during treatment, and to premedicate with oral antihistamine and acetaminophen prior to first infusion, and each dose escalation [302].

TGN1412

TGN1412, a novel 'superagonist' anti-CD28 humanized mAb that directly stimulates T cells, was intended to be developed for the treatment of hematological malignancies such as CLL, and autoimmune diseases such as rheumatoid arthritis [23,303]. In the highly publicized first-in-human study, within 90 min after receiving a single intravenous dose of the drug, all six healthy volunteers experienced a systemic inflammatory response characterized by a rapid induction of proinflammatory cytokines, and accompanied by headache, myalgias, nausea, diarrhea, erythema, vasodilatation and hypotension. Within 12–16 h after infusion, they became critically ill, with pulmonary infiltrates and lung injury, renal failure, and disseminated intravascular coagulation. All six patients were

transferred to an intensive care unit at a public hospital, where they received intensive cardiopulmonary support (including dialysis), high-dose methylprednisolone and an anti-IL-2 receptor antagonist antibody. Fortunately, all six volunteers survived [24].

CP-870,893

CP-870,893 [25] is a fully human CD40 agonist IgG2 mAb. CP-870,893 has been tested intravenously in a Phase I study in 29 patients with advanced solid tumors. The most common adverse event was CRS (grade 1–2), occurring in 55% of patients and associated with acute elevations in serum TNF- α and IL-6. The CRS was clinically evident within minutes to hours after infusion, manifested by varying combinations of chills, rigors, fever, rash, nausea, vomiting, muscle aches and back pain. Although the mechanism of CRS after CP-870,893 infusion was not fully understood, it was concluded that it was likely related to the mAb binding its target on immune or vascular cells rather than an antibody-mediated hypersensitivity reaction.

Basiliximab & daclizumab

Two anti-IL-2 receptor (IL-2R) mAbs currently in clinical use, basiliximab and daclizumab [26], have also been associated with CRS. Basiliximab is a chimeric mAb to the IL-2R α receptor of T cells and is indicated for the prophylaxis of acute organ rejection in patients receiving renal transplantation. The prescribing information mentioned that severe acute hypersensitivity reactions, including CRS, have been reported during postmarketing surveillance [27]. Bamgbola *et al.* reported three cases of pulmonary edema in adolescent renal transplant patients who received basiliximab, potentially as a result of cytokine release with increased capillary permeability, volume overload and ischemic-reperfusion injury [28]. Daclizumab is a humanized IgG1 anti-IL-2R mAb directed against the α -subunit (CD25) of the IL-2 receptor that is expressed on the surface of activated T lymphocytes. Like basiliximab, it is used to prevent rejection of organ transplantation in kidney transplants. The prescribing information also mentioned that severe acute hypersensitivity reactions, including CRS, have been reported during post-marketing surveillance. The relationship between these reactions and the development of antibodies is unknown [29].

Monoclonal antibodies not associated with CRS but associated with other infusion reactions

Monoclonal antibodies that are associated with infusion reactions but not typically thought of as causing CRS are listed in TABLE 2. As seen in the table, some of these reactions can be rather severe, and as clinical experience with them grows, some may have to be reclassified. It is beyond the scope of this review to deal with these antibodies in detail.

Monoclonal antibodies not associated with CRS or infusion reactions

Listed in TABLE 3 are antibodies where we were unable to make an association with CRS or infusion reactions (although most, if not all, will have a warning for infusion reactions in their package

inserts). For several of these antibodies, the available data are quite sparse. As clinical experience with them grows, some may have to be reclassified. Again, it is beyond the scope of this review to deal with these antibodies in detail.

Mediators of CRS

Although there are numerous effector molecules involved in CRS, cytokines are thought to be the primary initiators of the cascade and mediators of toxicity. The principle cytokines involved in CRS, the cells of origin and functions are listed in TABLE 4. As seen in the table, cells of both the innate and adaptive immune systems are believed to be involved in CRS.

Molecular mechanisms of CRS

There are four putative molecular mechanisms by which an antibody can activate an effector cell and cause the release of cytokines by:

- Binding of the soluble antibody via its complementarity determining regions (CDRs) to its cognate antigen on the effector cell;

- A pre-existing anti-IgG (usually restricted to a human anti-mouse IgG) cross-linking the cognate antigen;
- An antibody bound to a noneffector cell via its crystallizable fragment (Fc) domain binding and clustering the cognate antigen on the effector cell via its CDRs;
- Antibodies bound to an antigen on a noneffector cell activating Fc receptors (FcRs) on the effector cell.

These mechanisms are illustrated in cartoon form in FIGURE 1. As an aid to understanding, it is useful to think of these as distinct mechanisms. However, *in vivo*, CRS is most likely mediated by one or more of these mechanisms operating simultaneously. The affinity of the antibody for its cognate antigen, the specific epitope bound by the antibody on that antigen, the nature of the antigen, that is, a transmembrane protein with a cytoplasmic tail capable of interacting with signal transduction pathways versus nontransmembrane protein, and interactions with FcRs will all probably influence the 'mix' among these mechanisms. It follows that the different mechanisms are likely to lead to distinct patterns of cytokine release, and thus different clinical manifestations of CRS. As will be discussed later, CRS is not an 'all or nothing' phenomenon and an understanding of the molecular mechanisms for each mAb will likely provide the basis for predicting a clinical response.

Another unresolved question is whether the cytokines mediating CRS are preformed and secreted upon activation or must be synthesized *de novo*. Some cytokines (e.g., IL-1 β [30]) are formed as pro-proteins and are released in their activated form upon proteolytic cleavage. Others must be in a specific conformation to be active (e.g., trimeric TNF- α [31]) and still others (e.g., IFN- γ [32]) must be synthesized *de novo*. The rapidity of the onset of symptoms in CRS in some patients (30 min–1 h) probably does not allow sufficient time for the *de novo* synthesis of cytokines, that is, ligand binding, signal transduction, transcription and translation. This suggests that the earliest symptoms of CRS are caused by preformed mediators. The later symptoms are likely caused by a cascade of cytokines synthesized *de novo*.

CRS mediated by direct binding of an antibody to its cognate antigen

Examples where an antibody binding its cognate antigen may contribute to CRS are muromonab-CD3 (OKT3) [6] and rituximab [33]. Muromonab-CD3 is a murine mAb that binds the T-cell receptor/CD3 (TCR/CD3) complex found on mature

Table 2. Monoclonal antibodies associated with acute reactions (not cytokine-release syndrome).

Monoclonal antibody	Antigen	Class	Finding	Ref.
Keliximab	CD4	Human–monkey chimeric IgG1	Infusion-related rigors, chills and fever Leukocytoclastic vasculitis	[116]
Tremelimumab	CD152 (CTLA-4)	Human IgG2	Immune-mediated diarrhea and pruritus	[117]
Ipilimumab	CTLA-4	Human IgG1	Rashes, diarrhea and hepatitis	[118]
Trastuzumab	c-erbB-2	Humanized IgG1	Infusion reactions	[119]
Panitumumab	EGF receptor	Human IgG2	Infusion reactions Severe dermatologic toxicity Diarrhea	[120]
Cetuximab	EGF receptor	Human/murine chimeric IgG1	Rare severe infusion reactions	[121]
Matuzumab	EGF receptor	Humanized IgG1	Leukopenia, fever and rash	[122]
Nimotuzumab	EGF receptor	Humanized IgG1	Chills, nausea, fever, asthenia, anorexia, somnolence, cephalgia and increase of transaminases, or alkaline phosphatase	[123]
Omalizumab	IgE	Humanized IgG1	Anaphylactoid reaction	[124]
Basiliximab	IL-2 receptor	Human/murine chimeric IgG1	Pulmonary edema	[28]
Tocilizumab	IL-6 receptor	Humanized IgG1	Infusion reactions	[125]
Infliximab	TNF- α	Human/murine chimeric IgG1	Infusion reactions	[126]

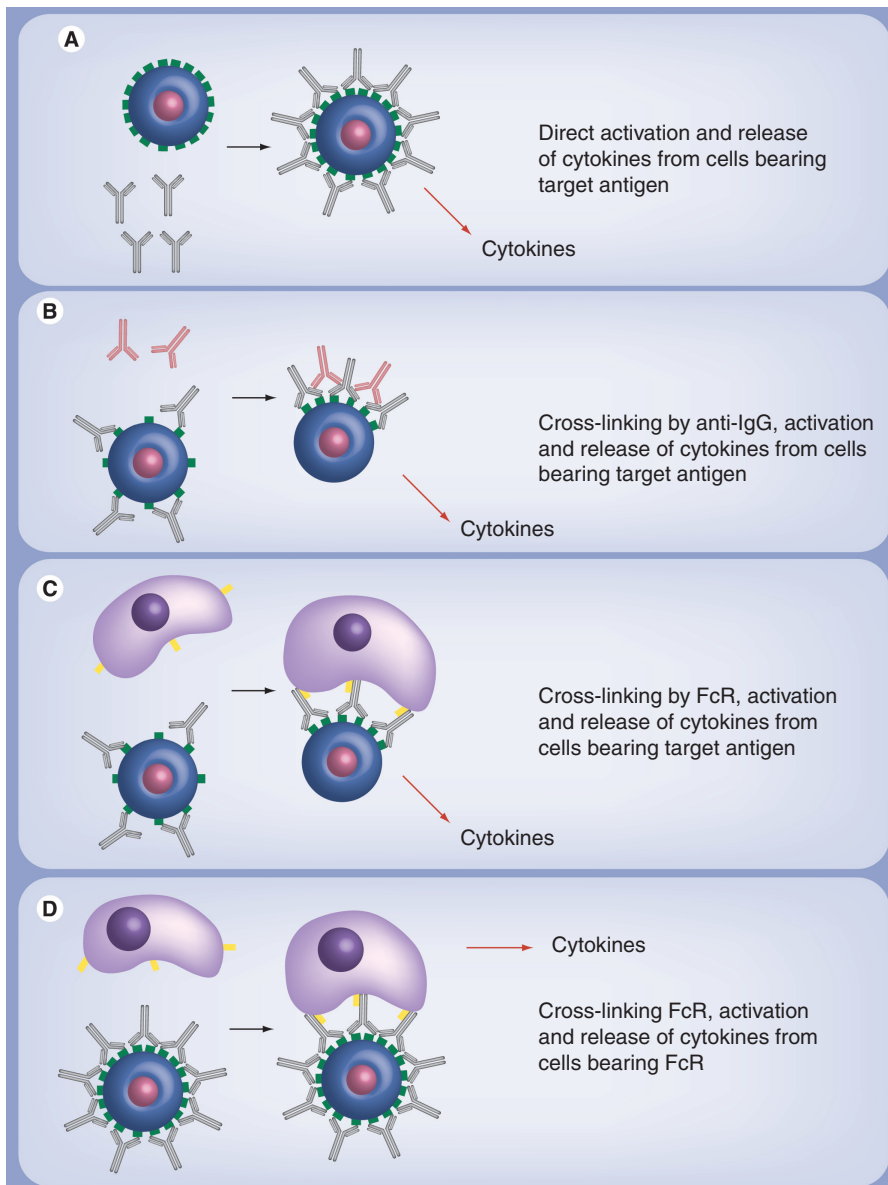


Figure 1. Mechanisms of action of cytokine-release syndrome owing to infusion with therapeutic monoclonal antibodies. Four methods of cytokine release are depicted in the figure. **(A)** The soluble antibody binds directly through its complementarity determining regions to its cognate receptor on the effector cell. The binding causes stimulation of the cells and thus the release of cytokines from the effector cells. **(B)** The soluble antibody binds its receptor and is cross-linked by a pre-existing anti-IgG. This, in turn, leads to cross-linking of the cognate receptor and activation of the effector cell. **(C)** The soluble antibody binds weakly to the effector cell. Moreover, in the vicinity of a noneffector cell, the weakly bound antibodies bind to the noneffector cells through its Fc. This leads to clustering of the antigens on the effector cells and increased stimulation of the cell, leading to cytokine release from the effector cell. **(D)** The soluble antibody binds to its antigen on its effector cells, forming clusters of antibody–antigen sites. In the vicinity of a noneffector cell, the antibodies on the effector cell bind the Fc portion of the noneffector cell. Due to the clustering of the antibodies on the effector cell, there is increased binding of the Fc site on the noneffector cell and thus activation of the cell, leading to cytokine release. Although **A**, **B**, **C** and **D** represent three distinct mechanisms by which CRS occurs, it is not hard to imagine a combination of these processes occurring in CRS. FcR: Fc receptor.

T cells and medullary thymocytes, and acts by blocking the generation and function of cytotoxic T cells [34]. Rituximab is a murine–human chimeric antibody that binds CD20, a transmembrane, a nonglycosylated phosphoprotein, located on pre-B and mature B lymphocytes, and NHL cells, but not on hematopoietic stem cells or normal or malignant plasma cells [35]. Although anti-CD3 and anti-CD20 mAbs result in an effective immunosuppression and modulation of autoimmune disease, the initial treatments with muromonab-CD3 and rituximab are often associated with CRS.

Although limited, there is some evidence that direct binding of CD3 can cause T-cell activation and the release of cytokines. In *in vitro* experiments with various anti-CD3 constructs, Carpenter *et al.* have shown that anti-CD3 antibodies in their soluble form can cause release of IL-2, IL-4, IL-10, TNF- α or IFN- γ [36]. In particular, Xu *et al.* studied a group of five huIgG1- and huIgG4-based OKT3 antibodies, and their corresponding Fc mutants with altered residues at amino acids 234, 235 and 318 [37]. Two of these antibodies huOKT3 γ 1 (A234, A235) and huOKT3 γ 4 (A234, A235) showed at least a 100-fold reduced binding to Fc γ RI and Fc γ RII (see later), and although less potent than the corresponding wild-type antibodies, the mutants retained some ability to induce T-cell activation and cytokine release.

Similarly, evidence that direct binding of CD20 can cause B-cell activation and release of cytokines comes from a number of *in vitro* experiments. Cardarelli *et al.* incubated a number of B-cell cell lines with tositumomab or rituximab [38]. Tositumomab showed direct induction of apoptosis in all B-cell lines tested. Cross-linking cell-bound anti-B1 antibody with a goat antimouse Ig did not further enhance the percentage of cells undergoing apoptosis and an antigen-binding fragment dimer (F[ab']₂) fragment of tositumomab induced apoptosis, while the antigen-binding fragment monomer (Fab) fragment did not, suggesting that dimerization of CD20 may be sufficient for the induction of apoptosis. By contrast, rituximab, which binds to an overlapping epitope on CD20 but with lower affinity than tositumomab, did not efficiently induce apoptosis in the absence of cross-linking. Rituximab

Table 3. Antibodies not associated with cytokine-release syndrome (excluding some anti-CD3).

Monoclonal antibody	Antigen	Class	Ref.
Natalizumab*	$\alpha 4$ integrin	Humanized IgG4	[127]
Arcitumomab*	Carcinoembryonic antigen	Murine Fab	[128]
Efalizumab*	CD11a	Humanized IgG1	[129]
IC14	CD14	Murine chimeric IgG4	[130]
IDEC-131	CD154	Humanized IgG1	[131]
Siplizumab*	CD2	Human IgG1	[132]
Ofatumumab	CD20	Human IgG1	[133]
Epratuzumab	CD22	Humanized IgG1	[132]
Iratumumab	CD30	Human IgG1	[132]
Gemtuzumab*	CD33	Humanized IgG4	[134]
Dacetuzumab (SGN-40)	CD40	Humanized IgG1	[132]
Galiximab	CD80	Human/monkey chimeric IgG1	[132]
Eculizumab*	Complement component C5	Humanized IgG1	[135]
Abciximab*	GP IIb/IIIa	Human/murine chimeric Fab	[136]
Fanolesomab*	Granulocytes	Murine IgM	[137]
BT563	IL-2 R	Murine IgG1	[138]
Daclizumab	IL-2 R	Humanized IgG1	[26]
Apolizumab	Polymorphic epitope on HLA-DR β	Humanized IgG1	[70]
Palivizumab*	RSV fusion protein	Humanized IgG1	[139]
Certolizumab*	TNF	Humanized-pegylated Fab	[127]
Nofetumomab*	Tumor antigen expressed by many cancers	Fab fragment of murine NR-LU-10	[140]
Bevacizumab*	VEGF	Humanized IgG1	[141]
Ranibizumab*	VEGF	Humanized Fab fragment	[142]

*Searching MedLine and the EMEA (May 2009) for 'cytokine release', 'infusion reaction' or anaphylaxis yielded no relevant references. References listed are for general information on the monoclonal antibody. TNF: Tumor necrosis factor.

has also been shown to inhibit the p38 mitogen-activated protein kinase, nuclear factor- κ B, extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (AKT) anti-apoptotic survival pathways, all of which result in the upregulation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and Raf kinase inhibitor protein, and in the downregulation of anti-apoptotic gene products (particularly Bcl-2, Bcl-xL and Mcl-1) [39]. Similarly, in an *in vitro* antibody-dependent cellular cytotoxicity (ADCC) assay, Lefebvre *et al.* showed that macrophage recruitment by rituximab-opsonized tumor cells did not result in enhanced cytokine secretion, suggesting that CRS observed in rituximab-treated patients is not caused by macrophage activation [40].

Szodoray *et al.* cultured isolated peripheral blood B cells from normal humans and patients with rheumatoid arthritis with rituximab, and measured apoptosis within the naive, memory and IgD⁺CD27⁺ B-cell subpopulations [41]. Rituximab induced apoptosis in all B-cell subsets in both arthritics and controls. Although rituximab induced the apoptosis of B cells in both rheumatoid factor (RF)-positive and -negative arthritics, *in vivo* serum levels of IL-1 β , IL-4, IL-10 and IL-13 were increased in RF-positive patients compared with RF-negative patients.

Finally, in clinical studies with rituximab, an association of elevated lymphocyte counts with severity of reactions suggests that the toxicity may be directly related to antibody binding to CD20 on B lymphocytes [15]. More recently, Lee *et al.* investigated whether rituximab could induce a specific gene expression signature, using genomic-scale gene expression profiling of B cells from CLL patients receiving their first rituximab infusion [42]. They concluded that rituximab was associated with the activation of interferon response genes that contributed to cytokine release.

Taken together, these data suggest that although simple binding to its cognate antigen may contribute to CRS with anti-CD3 and anti-CD20 antibodies, clustering of the antigen by and activation of FcRs are probably more important mechanisms of CRS (see later).

CRS mediated by FcR-dependent clustering of an antigen

Fc γ R

Receptors for the Fc region of IgG (Fc γ R) provide a link between the humoral and cellular branches of the immune system

[43,44]. Fc γ R play a role in maintaining the balance between simultaneous triggering of activating and inhibitory signals to generate a well-balanced immune response. In addition to their role in signaling in Fc γ R-bearing cells, Fc γ R can mediate the clustering of antibodies bound to their cognate antigens and subsequent activation of the target cell. Much of what is known about the affinity of the various FcRs for the various IgGs is based on binding studies with antibodies in soluble form. One should keep in mind that, *in vivo*, we are often dealing with the interaction of FcR with immune complexes, either on the cell surface or in soluble form. Either way, the avidity of the FcR for the immune complex may play an important role in determining the severity of CRS.

Table 4. Principal cytokines involved in cytokine-release syndrome, cells of origin and function.

Cytokines	Released by	Acting on	Physiologic function	Ref.
TNF- α	Macrophages T-cells	Macrophages, endothelial cells and T cells	Vascular permeability, cell death, cytokine-release cascade	[15,24,25,91,143,144]
IFN- γ	Activated Th1 and NK cells	Neutrophils, macrophages and NK cells	Activation of macrophages, inhibits proliferation of Th2 cells, increased MHC expression	[24,145]
IL-1 β	Macrophages/ monocytes T cells	T cells, B cells and NK cells	Costimulation, proliferation and maturation	[146]
IL-2	T cells	T cells and B cells	Expansion of B cells and activation of cytotoxic T cells	[3]
IL-6	Macrophages/ monocytes T cells	Multiple cell types	Augment immune response	[15,24,25,91,143,147]
IL-8	Macrophages and endothelial cells	Neutrophils	Chemotaxis	[24,147]
IL-10	Activated Th2 cells and macrophages	Macrophages, B cells	Inhibits cytokine production, suppresses cellular immunity	[24,148,149]

NK: Natural killer.

Examples of Fc γ R-mediated cytokine release can be found in the case of the anti-CD3 antibodies. Most anti-CD3 antibodies result in CRS while some do not (TABLE 5). Typically, CRS results in the release of several cytokines, primarily IL-2, IL-6, TNF- α

and IFN- γ . Binding of the anti-CD3 antibody to its antigen is not sufficient to cause T-cell activation; other signals primarily through Fc γ Rs are required, including signals delivered by activated monocytes and other accessory cells [45]. As described

Table 5. Anti-CD3 antibodies and cytokine-release syndrome.

Antibody	Class	Finding	Ref.	Expected interaction with human Fc γ R	Ref.
Rabbit antithymocyte globulin	Rabbit polyclonal	CRS	[143]	I–II	[150]
Muromonab-CD3	Murine IgG2a	CRS	[6]	I–II	[36]
BMA 031 (TCR/CD3)	Murine IgG2b	CRS	[151]	II	[36]
OC/TR (MOv18/CD3 bispecific)	F(ab') ₂	CRS	[152]	None?	NA
T3.G2a	Murine IgG2a	CRS	[153]	I–II	[36]
WT31	Murine IgG1	CRS in some but not all patients. Correlated with Fc γ R1a polymorphism	[47]	III	[56]
Visilizumab	Humanized IgG2 aa 234, 237 Val-Ala mutant	Mild-to-moderate CRS	[154,155]	III?	[156]
T3.A	Murine IgA	Mild CRS	[153]	None	[157]
huOKT3 γ 1 (Ala-Ala)	Humanized IgG1(Ala-Ala)	Mild CRS	[95]	None	[95]
HuM291	Humanized IgG2 M3	Mild-to-moderate headache, nausea, chills and fever	[155]	None	[36]
T10B9 (T10B9.1A31)	Murine IgM	Mild cytokine release	[158]	None	[159]
Monovalent anti-CD3	Monovalent rat IgG2b	No CRS	[96]	I–II	[160]
YTH12.5 (CAMPATH-3)	Rat IgG1 aa 279 mutant	No CRS	[161]	None?	[162]

I, II and III refer to the different Fc receptors.

aa: Amino acid; CRS: Cytokine-release syndrome.

in the previous section, binding of the anti-CD3 antibody on its own cannot fully explain the CRS due to these antibodies. The use of modified anti-CD3 antibodies with limited binding affinity to FcγRs have shown fewer side effects compared with IgG1 and IgG4 counterparts (TABLE 5). Xu D *et al.* have shown that anti-CD3 constructs with IgG1 and IgG4 background are potent in the activation of T-cell proliferation, as well as cytokine release [37]. When mutant constructs were created on the same IgG subclass Fc backgrounds, immunosuppressive effects were retained, despite a 100-fold reduced binding to FcγRI and FcγRII, reduced T-cell activation and decreased CRS. Cole *et al.* introduced mutations into the upper heavy chain constant region-2 (CH2) of IgG2 and expressed the altered Fc as chimeric OKT3 antibodies [46]. Compared with chimeric OKT3 IgG1, IgG2, IgG3 and IgG4, the IgG2 mutants were less mitogenic to T cells, and they did not induce the release of TNF-α, IFN-γ or IL-2. Tax *et al.* have also shown that after treatment with murine IgG1 anti-CD3/T-cell-activating antibody WT31, some patients exhibited CRS characterized by elevated levels of multiple cytokines including TNF-α and IL-6 [47]. The study also showed that the presence of high levels of FcγRIIA was associated with CRS.

FcRn

Another type of Fc receptor is the neonatal Fc receptor (FcRn) [48]. FcRn is believed to play a critical role in the transfer of IgG across the placenta and absorption of intact IgG by the neonatal gut. In the adult, FcRn is expressed by endothelial cells, where it is believed to play a role in rescuing pinocytosed IgG from lysosomal degradation and thus prolonging immunoglobulin circulation time.

An example of a mAb where clustering of the bound cognate antigen by FcRn may contribute to CRS is TGN1412. TGN1412 binds CD28, a dimeric transmembrane glycoprotein present on T cells that acts as a costimulatory molecule [49]. TGN1412 was designed as a 'superagonist' for CD28, activating T cells without the need for additional engagement of the TCR/CD3 complex. A role for FcRn in CRS with TGN1412 is suggested by the work of Stebbings *et al.* [50]. Working *in vitro*, this group showed that TGN1412 in soluble form or aggregated with anti-IgG antibodies failed to elicit the release of cytokines by cultured human peripheral blood mononuclear cells (PBMCs) or human whole blood. By contrast, when TGN1412 was added to a mixed culture of PBMCs and human umbilical cord-derived endothelial cells, a strong cytokine response was measured by a battery of

cytokine-specific ELISAs. As TGN1412 is an IgG4, it would not be expected to bind to FcγR with high affinity (TABLE 6). The fact that endothelial cells could mediate cytokine release strongly suggests a role for FcRn in CRS caused by TGN1412.

CRS mediated by activation of FcR

There are a number of ways activating cells via FcRs can mediate CRS. These can be grouped according to the type of FcR involved: FcεR or FcγR.

FcεR

Fc receptors that binds IgE (FcεRs) may also play a role in CRS. FcεRs are expressed on the surface of mast cells, and ligation of the receptor mediates mast cell degranulation, releasing histamine, serotonin and a number of other inflammatory mediators; for example, arachidonic acid metabolites such as leukotrienes [51]. Hypersensitivity reactions to the xenogenic component of murine, chimeric and humanized antibodies can occur upon the first dose of antibody, and following repeated exposure. For example, rare cases of anaphylaxis have been reported with abciximab (an Fab derived from a chimeric anti-αVβ3) [52], IC14 (chimeric anti-CD14) [53], cetuximab (chimeric anti-EGFR) [54] and basiliximab (chimeric anti-IL-2 R) [55]. In the case of cetuximab, the IgE was against an oligosaccharide in the Fab portion of the heavy chain of the mAb. In the other cases, it is most likely a pre-existing IgE that recognizes the murine portion(s) of the therapeutic mAb that mediates the anaphylactic reaction.

There are two potential mechanisms by which activation of FcR other than FcεR can lead to CRS; activation by cell-bound antibodies and activation by circulating immune complexes. Classically, immune complexes have been shown to cause activation of FcγR and increased expression of cytokines by monocyte/macrophages and NK cells [56]. Based on the presence of activating and inhibitory motifs in their cytoplasmic regions, FcγRs can be classified as activating (FcγRI, FcγRIIA, FcγRIIC, FcγRIIA and FcγRIIB) or inhibitory (FcγRIIB) FcRs. The cellular response depends on the relative expression of activating and inhibitory receptors, which is highly influenced by the cytokine milieu. Innate effector cells such as monocytes/macrophages, dendritic cells, basophils and mast cells express both activating and inhibitory FcγRs. By contrast, NK cells express only activating FcγRs and B cells only inhibitory FcγRs.

An example of where a cell-bound antibody activating FcγR may contribute to CRS is alemtuzumab (CAMPATH-1H).

Alemtuzumab is a humanized IgG1 mAb, which binds the glycolipid-anchored CD52 antigen on T, B and NK cells but not on stem cells [19]. It was designed to eliminate host T cells and keep hematopoietic stem cells to prevent graft rejection and graft-versus-host disease during organ transplant (primarily renal transplant). Alemtuzumab has been used for therapy of leukemia and lymphomas, and in the treatment of autoimmune diseases, including rheumatoid arthritis [57,58].

Table 6. Human Fc receptors most relevant to cytokine-release syndrome.

Receptor type	Relative binding affinity for human immunoglobulin	Relative binding affinity for murine immunoglobulin	Ref.
FcγRI	IgG3>IgG1>IgG4>IgG2	IgG2a≈IgG3>IgG1≈IgG2b	[163]
FcγRII	IgG3>IgG1>IgG2≈IgG4	IgG2a≈IgG2b≈IgG1	[163]
FcγRIII	IgG1≈IgG3>IgG2≈IgG4	IgG3>IgG2a>IgG2b>IgG1	[163]
FcRn	IgG1≈IgG2≈IgG3≈IgG4	IgG2b>IgG2a>IgG1	[164,165]

Wing *et al.* showed that cytokine release due to anti-CD52 binding was both antibody-specific as well as isotype-specific [18,59]. Whole-blood cultures were incubated with rat IgG2b (CAMPATH-1G), rat IgM (CAMPATH-1M), humanized IgG1 (alemtuzumab) and humanized IgG4 mAb antibodies to CD52. TNF- α , IFN- γ and IL-6 were elevated with CAMPATH-1G and alemtuzumab but not with the other antibodies. The importance of the Fc region of the antibody was further emphasized, as an F(ab')₂ fragment of alemtuzumab failed to stimulate cytokine release. Wing *et al.* further showed that by blocking the Fc γ RIII (CD16) using an anti-CD16 F(ab')₂ fragment resulted in 85% inhibition of cytokine release. In the case of alemtuzumab, it was shown that binding of antibody to CD52 combined with the ligation of Fc γ RIII on CD56⁺ NK cells resulted in cytokine release. This was a case where the antibody did not bind to the Fc γ R on the same cell containing CD52 moiety, but rather the Fc γ R on the NK cells.

A final mechanism of CRS is via activation of FcR by circulating immune complexes. In this case, preformed or natural antibodies can bind the therapeutic antibody and the resultant immune complexes activate FcR-bearing cells. An example of this is the report of anaphylaxis in patients receiving cetuximab [54]. Although we have found no specific examples of CRS caused by this mechanism, all mAbs expressed in rodent cell systems, for example, CHO cells, have the potential to contribute to CRS via immune complex formation. This is because recombinant proteins expressed by rodent cells may have a pattern of terminal glycosylation that is 'foreign' to humans [60] and some patients may have antibodies that recognize this 'rodent' pattern of glycosylation. Sometimes referred to as Hanganutziu–Deicher (HD) or heterophile antibodies [61], although rare in normal humans, these antibodies can be present in a number of chronic diseases including rheumatoid arthritis, liver disease and cancer [62]. Interestingly, HD antibodies can lead to false-positive findings in cytokine assays, and thus may complicate the diagnosis of CRS [63].

In summary, the role of Fc γ Rs in CRS is complex, and likely involves a combination of interactions between the Fc γ Rs and the antibody–ligand pair. This complexity is illustrated by the extensive clinical data on anti-CD3 antibodies. As shown in TABLE 5, not all anti-CD3 antibodies are associated with CRS, and there is no simple relationship with antibody isotype and the ability of an anti-CD3 antibody to cause CRS.

Other consequences of CRS

Activation of the complement cascade

Activation of complement is believed to contribute to the adverse effects of anti-CD3 [64]. Similarly, Van der Kolk *et al.* reported a study in five relapsed low-grade NHL patients, where infusion of rituximab induced rapid complement activation, preceding the release of TNF- α , IL-6 and IL-8, and concluded that complement played a pivotal role in the pathogenesis of side effects of rituximab treatment [65]. Hills *et al.* investigated the possibility that in severe infusion reactions rituximab could lead to cross-linking and a rapid drop in circulating lymphocyte count [66]. The findings of their *in vitro* study supported the hypothesis that B-cell aggregation may occur *in vivo* during rituximab infusion

in patients with high lymphocyte counts, and may be associated with complement fixation and localized cytokine production. They also suggested that these cell aggregates in the circulation lead to the occlusion of pulmonary blood vessels, resulting in the severe infusion related clinical adverse events.

Activation of coagulation

As a downstream effect of cytokine release, the coagulation system can become activated. In the TGN1412 trial, evidence of disseminated intravascular coagulation (DIC) was found [24], and CRS with rituximab has been associated with a fivefold to tenfold increase of fibrin D-dimers and a prolongation of prothrombin time [15]. This may be a consequence of cytokines increasing expression of tissue factor on the surface of monocytes and endothelial cells [67].

Nonclinical aspects of CRS

As described in the International Committee on Harmonization (ICH) guidance on nonclinical testing of therapeutic proteins (ICH S6), testing in a species in which the agent has been shown to be pharmacologically active is expected for all human therapeutics [304]. Owing to limited cross-species binding in rodents, in general this will mean that a mAb will be tested in nonhuman primates. In special circumstances, the antihuman mAb will be active in rodents, and nonclinical studies may be conducted in mice or rats. More novel systems, for example, transgenic mice or homologous mAbs, may also play a role in the nonclinical evaluation of mAbs. As shown in TABLE 7 and illustrated in detail by selected examples, the predictive value of nonclinical studies for human CRS is limited. In some cases, the animals showed clinical signs of CRS. In others, although clinical signs of CRS were not observed, analysis of serum showed that cytokines were released and, in others still, no evidence of release of cytokines was found. Thus, overall the predictive value of *in vivo* studies in rodents and nonhuman primates for CRS is very poor. A number of potential explanations for the poor predictive value of nonclinical studies are listed in TABLE 8.

Predictive value of nonhuman primates

Cynomolgus and Rhesus macaques are the most commonly used test systems for toxicity testing of mAbs. Some, but by no means all mAbs directed toward human targets, bind their macaque homologues. In addition, there has been some work conducted in chimpanzees. However, as shown in TABLE 7, binding the epitope and even expression of desired pharmacologic activity does not guarantee the expression of CRS in nonhuman primates. Some examples are described in detail later (New world monkeys, e.g., squirrel monkeys [*Saimiri sciureus*] and marmosets [*Calithrix jacchus*] have been suggested by some workers to have potential for toxicologic evaluations [68,69]. However, we have not found any examples of their use with mAbs).

Macaques

Shi *et al.* studied the humanized mAb Hu1D10 (remitogen) that recognizes a polymorphic determinant of HLA-DR expressed on normal B cells in rhesus macaques [70]. They found that not all monkeys expressed the target antigen. In monkeys preselected to

Table 7. Predictive value of preclinical studies for cytokine-release syndrome in humans.

Antigen	Antibody	Species	Results	Ref.	Human mAb	Results	Ref.
CD2	Anti-murine CD2 (12–15)	Mice	Prolonged allograft survival No CRS	[166]	LO-CD2a/BTI-322	Depletion of T cells and NK cells CRS	[7]
CD3	Anti-murine CD3 (145–2C11) HuM291	Mice Chimpanzee	CRS Cytokine release No CRS	[107] [72]	Muromonab-CD3 HuM291	CRS Mild-to-moderate headache, nausea, chills and fever	[6] [155]
CD3/CD19	MT103 MEDI-538 bscCD19xCD3	Chimpanzee	Increased expression of CD69 and MHC class II Increased serum levels of IFN- γ , IL-6 and IL-2 No CRS	[167]	MT103 MEDI-538 bscCD19xCD3	Lymphopenia, pyrexia, neutropenia, thrombocytopenia, transient increase of liver enzymes, confusion, disorientation and tremor	[168]
CD4	Keliximab	Chimpanzee HuCD4Tg mouse	No CRS	[76,169]	Keliximab	Infusion-related rigors, chills and fever Leukocytoclastic vasculitis	[116]
CD11a	Anti-rhesus H2C12	Rhesus	No CRS	[170]	Efalizumab	Headache, chills and, to a lesser extent, nausea, myalgia, pain and fever are the common adverse events associated with efalizumab	[171]
CD20	Anti-muCD20 Rituximab Rituximab Rituximab Rituximab Rituximab Ibritumomab	Normal and FcR-deficient mice HuCD20Tg/ NOD mice HuCD20Tg/ MRL/lpr Cynomolgus Rhesus Baboon Cynomolgus	Decreased B cells No CRS Decreased B cells No CRS Decreased B cells No CRS Decreased B cells No CRS Toxic epidermal necrolysis No CRS Decreased B cells No CRS Decreased B cells No CRS	[172] [173] [174] [175] [176] [177] [306]	Rituximab Ibritumomab	CRS Pain, fever, chills	[112] [306]

CRS: Cytokine-release syndrome; NK: Natural killer.

Table 7. Predictive value of preclinical studies for cytokine-release syndrome in humans (cont.)

Antigen	Antibody	Species	Results	Ref.	Human mAb	Results	Ref.
CD28	Hamster anti-muCD28 (37.51)	NOD mice	Decreased insulinitis No CRS	[178]	TGN1412	Severe CRS	[24]
	anti-muCD28 (JJ316)	Rats	Increased serum levels of cytokines No CRS	[75]			
	TGN1412	Cynomolgus	Moderate elevations of IL-2, IL-5 and IL-6 in individual animals No CRS	[307]			
CD52	TGN1412	Rhesus	No substantial change in IL-5, IL-6, IL-10 and IFN- γ No CRS	[307]			
	Alemtuzumab	Cynomolgus	Lymphopenia, neutropenia, slight-to-moderate hypotension, mild-to-moderate fever No CRS	[302]	Alemtuzumab	Fever, rigor, nausea, vomiting rash and hypotension	[302]

CRS: Cytokine-release syndrome; NK: Natural killer.

express the antigen, administration of Hu1D10 resulted in the rapid and profound depletion of circulating B cells for 7–10 days following the last dose. Bolus injections of Hu1D10 resulted in type 1 hypersensitivity reactions in the majority of animals and one death due to anaphylaxis. Slow infusion of Hu1D10 was associated with mild hypersensitivity reactions after the first dose but not subsequent doses.

Sabaeus monkeys

Nimotuzumab (h-R3), a humanized anti-epidermal growth factor receptor mAb, was evaluated in sabaeus monkeys (*Cercopithecus aethiops*) [71]. This species was chosen because nimotuzumab was found to have a similar binding constant for human and sabaeus monkey EGF receptors in a radioligand competition assay. Monkeys (n = 18) were distributed into three groups, with three animals of each sex in each group. Group I received saline, group II received 2.85 mg/kg of h-R3 and group III received 28.57 mg/kg of the h-R3, which represents 1 and 10-times the human dose, respectively. Monkeys were dosed intravenously, weekly for 26 weeks. During the study there were no deaths, or changes in electroneurophysiological or clinical pathology parameters. Skin biopsies and a detailed clinical inspection of the animals did not detect the presence of cutaneous rash or any other sign of cutaneous toxicity.

Chimpanzees

As our closest evolutionary relative, chimpanzees will usually bind and express the pharmacologic activity of mAbs directed toward human targets. However, despite their close homology, chimpanzees appear to be resistant to CRS. Hsu *et al.* studied a humanized anti-CD3 IgG2 (HuM291 γ 2 M3) with mutations in the upper CH2 region of the Fc domain in chimpanzees [72]. *In vitro*, HuM291 bound to and effectively downmodulated CD3 on chimpanzee PBMCs and stimulated substantially less cytokine secretion and proliferation compared with OKT3. *In vivo*, multiple doses of HuM291 (0.1, 1.0 or 10 mg/dose) were not associated with adverse events, signs of toxicity or CRS, despite cytokine release. As shown in Table 5, in humans this antibody is associated with headache, nausea, chills and fever. Why the response of chimpanzees should have poor predictive value for in humans CRS is not understood. One likely contributing factor is the lack of expression of signaling lectins (siglecs) by humans. Siglecs are a family of sialic acid-recognizing lectins and the CD33-related siglecs (siglec3 and siglec5-11) are thought to downregulate innate immune-cell activation. Most siglecs are expressed by the immune cells of most mammals, including most higher primates (chimpanzees, bonobos, gorillas and orangutans). A notable exception is the lack of expression of CD33-related siglecs by humans [73]. Expression of CD33-related siglecs may thus attenuate cytokine release in nonhuman species and the lack of expression of these siglecs may make humans inordinately sensitive to cell activation and CRS.

However, owing to their status as an endangered species and because there are almost no animals available for scientific study, it is impractical to use chimpanzees except as a last resort.

Table 8. Potential explanations for the poor predictive value of nonclinical studies for cytokine-release syndrome in humans.

Attribute	Specific example	Ref.
FcγR expression	Differential expression of FcγRIII by PBMCs across nonhuman primate species	[179]
FcγR function	Differential binding of human and mouse IgG by human but not murine FcRn	[165]
T-cell subsets	CD4–CD8 double positive common in monkeys, rare in humans	[180]
T-cell receptor utilization	BC1-BJ1 common in a new world monkey (<i>Callithrix jacchus</i>) compared with BC2-BJ2 in humans	[181]
MHC class I and II	Limited homology among humans and monkeys	[182]
Th1 and Th2 balance	Th1 clones high in rhesus, Th2 high in sooty mangabey PBMCs	[183]
Regulatory molecules	Differential expression of siglecs in humans, nonhuman primates and rodents	[73, 184]
Coreceptors and binding proteins	Differential expression of CD16 on human and primate neutrophils	[185]
Signal transduction	Differences in nuclear translocation of glucocorticoid receptor in humans and squirrel monkeys	[186]
Downstream effects	Differential effects of an anti-human CD4 mAb on CD4 ⁺ cells in chimpanzees and cynomolgus macaques	[187]

mAb: Monoclonal antibody; PBMC: Peripheral blood mononuclear cell.

Predictive value of rodents

For rodents to have even the potential to predict CRS in humans, the antibody would have to bind its target. In general, however, owing to their exquisite selectivity for a single epitope, mAbs directed toward human targets fail to bind the homologous target in rodents. Thus, for most mAbs there is no scientific basis for normal rodents to have any predictive value, and as described in the ICH S6 guidance on preclinical safety testing of biopharmaceuticals, toxicity studies in normal rodents are not conducted with human selective mAbs.

Homologous (surrogate) systems

One strategy to circumvent human selectivity is to study a mAb directed toward the rodent homologue of the human target. An example of this is the work of Ferran *et al.*, who studied cytokine release induced by a hamster antimurine CD3 (145–2C11) in mice [74]. In BALB/c mice, 145–2C11 induced high serum concentrations of TNF- α , IFN- γ , IL-2, IL-3 and IL-6 within 1–8 h of intravenous dosing with 10–100 μ g/mouse. This was associated with hypothermia, hypomotility, diarrhea, piloerection and death. By contrast, although 145–2C11 induced significant release of TNF- α and IL-2 in NZW, CBA/J and C3H/HeJ mice, IFN- γ was detected only in BALB/c mice. Interestingly, BALB/c mice showed the greatest physical reaction (hypothermia and hypomotility), suggesting that in mice IFN- γ is a prominent

mediator of CRS. Moreover, these data also suggest that not all strains of mice will have equivalent predictive value for human CRS.

By contrast, Müller *et al.* studied a superagonistic anti-muCD28 (JJ316) in rats and found a dramatic redistribution of T lymphocytes from the periphery to the secondary lymphoid organs [75]. In addition, surface expression of lymphocyte function-associated antigen (LFA)-1 was enhanced, endothelial differentiation sphingolipid G protein-coupled receptor 1 and L-selectin levels were downregulated, and the cells lost their responsiveness to sphingosine 1-phosphate-directed migration. These alterations were accompanied by a transient upregulation of CD25, CD69, CD134 and proinflammatory mediators. They also observed a second phase of activation of CD4⁺CD25⁺FoxP3⁺ regulatory T cells. Despite these changes, no signs of CRS were observed.

Transgenic systems

Another strategy to circumvent human selectivity is to study the human selective mAbs in mice that express the human target protein. An example of this is the work by Bugelski *et al.* who studied anti-

human CD4 mAbs (keliximab [IgG1] and clenoliximab [IgG4]) in murine CD4 knockout/human CD4 transgenic mice [76,77]. In these studies, as expected, the mAbs modulated expression of CD4, the number of CD4⁺ cells and were immunosuppressive. Furthermore, although cytokines were not measured, single doses of these antibodies were well-tolerated and no evidence of CRS was observed.

Human-reconstituted mice

A third strategy to circumvent human selectivity is to study the human-selective mAbs in mice whose immune system has been reconstituted with human cells. The development of humanized mice for preclinical testing has been done since 1988 [78]. Each of these models have severe restrictions on its use such a limited immune response, depleted T and B cells, presence of strong innate immune response and a leaky model. Recently, in order to achieve permanent a human immune reconstitution in mice, Hiramatsu *et al.* injected irradiated NOD/SCID/ $\gamma\gamma$ null mice with CD34⁺ human cord blood cells [79]. This resulted in repopulation of lymphoid tissues including T, B and NK cells. This model may have value in prediction of therapeutic antibodies in its potential for CRS. They possess most of the building blocks required to see a humanized *in vivo* response when treated with an antibody and may prove beneficial to predict CRS in humans. However, although reconstituted mice have cells that express the

target for the mAb and may release cytokines in response to an antihuman mAb, there is still no guarantee of their predictive value for CRS. This is in part because the cytokines that are important in human CRS may not be active in mice or may only express only some of the activity expressed in humans. For example, human IFN- γ is not active in mice [80], and human TNF- α interacts only with the murine p55 receptor and does not mediate signaling by the murine p75 TNF-receptor [81]. Thus, mice can be expected to underestimate the potential of a mAb to cause CRS, and data from experiments with human reconstituted mice should be interpreted with caution since there may well be modifications to the immune system as well as to nonimmune systems.

Potential for *in vitro* screening

As of May 2009, to the best of our knowledge there are no fully validated *in vitro* test systems that can predict CRS. There is thus an unmet need for *in vitro* systems using human cells to be developed to detect the potential of novel mAbs to cause CRS. The strategy of developing such an assay can be divided into three parts: test system, presentation system and detection system.

Test system

A number of *in vitro* test systems have been used in the study of cytokine release. These include various cell lines, PBMCs, enriched cell populations and whole-blood assays (TABLE 9). As described in TABLE 9, each of these systems has advantages and disadvantages. To our knowledge, only PBMCs and whole blood have been validated as having potential predictive value for CRS, and only for TGN1412 [50] and anti-CD3 [59]. In our opinion, test systems that include incubation with costimulatory factors, for

example, lipopolysaccharide (LPS) [82], phorbol myristate acetate (PMA) or phytohemagglutinin (PHA) [83], should be avoided in screening for the potential to cause CRS. We believe that inclusion of such stimuli 'on top of' the mAb being tested may lead to an overprediction of the potential to cause CRS and thus cause unwarranted concern.

Presentation system

Once the test system has been identified, many different methods of stimulation are available (TABLE 10). As has been shown for TGN1412, *in vitro* testing mAbs in soluble form may underestimate their potential to cause CRS [50]. Thus, some form of immobilization of the mAb is likely to be required for that mAb to elicit cytokine release *in vitro*. Depending on which of the mechanisms by which one hypothesizes the therapeutic antibody to elicit CRS, the presentation system can be chosen. As with the various *in vitro* test systems, each antibody presentation method has its pros and cons (TABLE 10). By their very nature, all of the presentation systems, with the possible exception of cell-based systems, for example, endothelial cells, are nonphysiologic. As a primary screen, to test the potential of an antibody to cause CRS with rigor, immobilized mAbs are probably advantageous. In a second tier of screening, follow-up assays with the mAb in soluble form and with the mAb bound by Fc γ R (e.g., NK cells [84]) and FcRn (e.g., endothelial cells [50] or monocyte/macrophages [50]) will likely be required to provide a more physiologic stimulus. Moreover, in the second tier, owing to the recognized role of genetic polymorphisms in the function of FcR [45,47,84], the test system should be characterized in terms of FcR genotype, and the use of multiple donors or cell lines may be warranted.

Table 9. Cell culture systems used to study cytokine release.

Culture method	Advantages	Disadvantages	Ref.*
Continuous cell lines (e.g., HUT78)	Unlimited availability Easily standardized	Single cell type May not express all relevant antigens or receptors May not reflect physiologic response	[188]
Clonal cells	Unlimited availability Easily standardized	Single cell type May not express all relevant antigens or receptors May not reflect physiologic response	[189]
PBMC	Readily available from multiple donors Multiple cell types in culture	Contribution of PBMC to CRS undefined Significant donor to donor variability All cell types may not express all relevant antigens or receptors Isolation procedures may activate cells	[190]
Enriched cell populations from peripheral blood (e.g., CD4 T cells)	Readily available from multiple donors Multiple cell types in culture	Contribution to CRS undefined Significant donor-to-donor variability All cell types may not express all relevant antigens or receptors Isolation procedures may activate cells	[18]
Whole blood	Readily available from multiple donors Multiple cell types in culture	Contribution of PBMC to CRS undefined Significant donor-to-donor variability All cell types may not express relevant antigens or receptors	[191]
Tissue (e.g., bronchoalveolar lavage cells)	Rich in many types of mature cells	Limited availability from few donors Significant donor to donor variability Isolation procedures may activate cells	[192]

*Reference for application to cytokine release.

CRS: Cytokine-release syndrome; PBMC: Peripheral blood mononuclear cell.

Detection systems

A number of measurement systems have been used in the study of cytokine release. These are listed in TABLE 11. As described in the table, each of these systems has advantages and disadvantages. In our opinion, to avoid false-negative results, a highly sensitive detection system is probably advantageous as a primary screen to test the potential of an antibody to cause CRS with rigor. To our knowledge, only ELISAs have been validated as having predictive value for CRS and only for TGN1412 [50]. Although as yet unvalidated, flow cytometric analysis of intracellular cytokines may provide a useful alternative to ELISAs. In these systems, cells are stimulated for various periods of time and secretion of cytokines is blocked by brefeldin A and/or monensin [85]. By combining intracellular staining with cell-surface staining for lineage markers, a rather complete picture of cytokine production on a cell-by-cell basis can be accomplished. This is exemplified by the work of Wosinska-Becler *et al.* who stained peripheral blood cells for CD3, CD8, CD14, and the intracellular cytokines IL-4, IL-5, IL-10, IL-12, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN- γ [86]. However, as the cytometric assays are usually semiquantitative and do not measure secreted cytokines, follow-up assays with measurement of secreted cytokines will likely be required to provide a more physiologic read-out of the potential of a mAb to cause CRS.

Validation

Once a test system is identified, a critical final step is to validate the assay. Fortunately, as shown by this review, there are a reasonable number of mAbs that have been tested in humans with a known

probability of causing CRS. These antibodies fall into three classes: those with a high probability of causing CRS, those with a probability of causing non-CRS infusion reactions and those with a low probability of causing any infusion reactions. When validating an assay, this classification will play a crucial role in interpreting the data. Moreover, when validating a screening assay for CRS, one should run as many of the marketed antibodies as possible. This may require the cooperation of the companies that market the antibodies.

Expert commentary

While one should not minimize the impact of causing CRS on patients, neither should one lose perspective. There are some important points in managing the risk of CRS first in human studies and in clinical practice.

CRS is not unique to therapeutic antibodies

Cytokine-release syndrome is not restricted to mAbs and is observed in natural infections, and is associated with other drug therapy. CRS is thought to be a significant part of the pathology associated with pandemic influenza [87,88]. This phenomenon involves the elevation of both pro-inflammatory (TNF- α , IL-1 and IL-6) and anti-inflammatory (IL-10 and IL-1Ra) cytokines. The Jarisch–Herxheimer Reaction is a cytokine-mediated syndrome occurring soon after the first adequate dose of an antimicrobial drug to treat infectious diseases [89]. Seen in Lyme disease, syphilis and relapsing fever, release of material from dying bacteria is believed to trigger a cascade of immune cell activation and cytokine release. CRS is also associated with small molecule drugs, for example, cytarabine and

Table 10. Antibody presentation methods.

Methods of presenting mAbs to effector cells	Advantages	Disadvantages	Ref.*
Soluble mAb	Easy No antibody modification	Random orientation Nonphysiologic presentation Nonspecific protein binding	[193]
Soluble mAb + antihuman Ig Ab	Easy No antibody modification Only antigen binding sites exposed	Random orientation Nonphysiologic presentation Nonspecific protein binding	[194]
Soluble mAb + FcR-bearing cells	Easy No antibody modification Only antigen binding sites exposed Effects of other cells type (e.g., monocytes and endothelial cells captured)	Random orientation Nonphysiologic presentation Nonspecific protein binding	[47,59]
Immobilized mAb adsorbed to polystyrene plate	Easy No antibody modification High-level antibody loading	Random orientation Nonphysiologic presentation Nonspecific protein binding	[193]
Immobilized mAb covalently coupled to activated surface (bead or plate)	Strong/stable coupling	Random orientation Antigen binding site modification	[195]
Immobilized mAb bound to an anti-Ig, Protein A- or G-coated bead	Easy Proper antibody orientation for optimal binding No antibody modification	Fc-mediated effects not fully understood	[196]

*Reference for application to cytokine release.
Ab: Antibody; mAb: Monoclonal antibody.

Table 11. Cytokine detection methods.

Assay method	Advantages	Disadvantages	Ref.*
ELISA from culture supernatants	Well-established technology Quantitative	Limited sensitivity Assays cytokines one at a time Not informative about which cells are producing cytokines	[197]
Multiplexed ELISA from culture supernatants	Established technology Assays multiple cytokines simultaneously Quantitative	Limited sensitivity Not informative about which cells are producing cytokines	[198]
Protein microarray from cultured cells	Emerging technology Assays multiple cytokines simultaneously	Limited sensitivity Not informative about which cells are producing cytokines	[199]
RT-PCR from cultured cells	Established technology Can be made quantitative	Limited sensitivity Assays cytokines one at a time Not informative about which cells are producing cytokines Expression ≠ translation	[197]
cDNA microarray from cultured cells	Emerging technology Assays multiple cytokines simultaneously	Limited sensitivity Not informative about which cells are producing cytokines Expression ≠ translation	[199]
ELISpot of cultured cells	High sensitivity Measures secretion	Technically demanding Semiquantitative Informative about how many cells are producing cytokines, but not cell lineage	[200]
Cytometric analysis of cultured cells	Well-established technology Ultra-high sensitivity for some cytokines Informative about which cells are producing cytokines and about cell lineage	Technically demanding Semiquantitative Translation ≠ secretion	[201]
Polychromatic cytometric analysis of cultured cells	Emerging technology Informative about how many cells are producing cytokines and about cell lineages Allows additional end points (e.g., viability)	Very technically demanding Semiquantitative Translation ≠ secretion	[202]

*Reference for application to cytokine release.
ELISpot: Enzyme-linked immunosorbent spot.

oxaliplatin [90,91], antisense oligonucleotides [92] and polyoxalated castor oil (Cremophor®), used as an excipient with some poorly soluble chemotherapeutics [93].

Severe CRS is not unique to TGN1412

TGN1412 induced severe, life-threatening CRS in all six normal human volunteers that received the first dose level in a planned dose-escalation study [24]. A safe dose level was not identified. Although the TGN1412 case has received extensive treatment in the medical and lay press, as discussed extensively earlier, other mAbs, including some approved for clinical use, can also cause severe CRS. Examples include the rare fatal outcomes with anti-CD20 and anti-CD3 [6,14,94]. Thus, although TGN1412 is the most notorious case of CRS mediated by a mAb, is it not unique.

The probability of causing CRS depends on the mAb & the underlying disease

Not all mAbs have the same potential to cause CRS. While some antibodies are commonly associated with first-dose CRS, with others CRS is more rare (see TABLE 5 for a comparison of different anti-CD3 mAbs). Similarly, although comparative data from human trials are limited, some immunoglobulin subclasses and antibodies with mutations in their Fc domains may have a decreased potential to

cause CRS. Moreover, the probability of causing CRS appears to be related to comorbidity factors, for example, high lymphocyte counts in patients receiving rituximab appear to be related to CRS [15].

Safeguards exist to prevent catastrophic events

In the post-TGN1412 era, extra caution is warranted to avoid a similar occurrence. However, in our opinion, the following accepted clinical pharmacology practices (as codified in the recent European Medicines Authority Committee for Medicinal Products for Human Use [EMA CHMP] guidelines [305]) provide adequate safeguards. These include:

- Avoiding over-reliance on *in vivo* nonclinical testing. Both rodents and nonhuman primates may underexpress CRS
- Thorough risk assessment integrating all available *in vitro* and *in vivo* data
- Selecting starting doses based on all available data and avoiding full pharmacologically active dose levels
- Slowing infusion rates to allow adequate monitoring
- Spacing treatment of individual subjects in first-in-human studies by 8–24 h to allow enough time for expression of CRS

Five-year view

Decreasing the potential for new monoclonal antibodies to cause CRS

There are a number of approaches to decreasing the likelihood of catastrophic CRS that can be implemented in the near future. These include: engineering mAbs to decrease FcγR interactions [95] or the potential to cross-link target antigens [96], better application of the principles of physiologic-based pharmacokinetics [97], and improved stratification in volunteer or patient cohorts based on relevant genetic polymorphisms (e.g., FcγR [47]).

Prospects for advancing patient management

The severity of some CRS symptoms can be reduced by careful patient management [98]. Infusion rate reduction, fractionated dosing, steroid premedication and hospitalization for inpatient administration may be necessary [5]. Administration schedules are antibody specific and are detailed in the manufacturers' prescribing information. According to the OKT3 label, the serum levels of cytokines and the manifestations of CRS may be reduced by pretreatment with high-dose steroids given 1–4 h prior to administration of the first dose, and by closely following recommendations for dosage and treatment duration. Acetaminophen and antihistamines given concomitantly with OKT3 may also help to reduce some early reactions [99]. For tositumomab, infusional toxicities are managed by slowing and/or temporarily interrupting the infusion [5]. Similarly, for rituximab, the infusional toxicity has led to strict guidelines of use; that is, infusion times from 4 to 6 h.

According to the rituximab label, premedication consisting of acetaminophen and an antihistamine should be considered before each infusion to attenuate infusion reactions. Premedication with acetaminophen and antihistamines has also been advised for alemtuzumab, in order to significantly reduce the first-dose effects. The alemtuzumab prescribing information instructs to gradually escalate to the maximum recommended dose of 30 mg at initiation of dosing, and to premedicate with oral antihistamine and acetaminophen prior to first infusion and each dose escalation [302]. If any of the more serious presentations of the CRS occur, intensive treatment including oxygen, intravenous fluids, corticosteroids, pressor amines, antihistamines and intubation may be required [100]. An example for acute treatment of severe CRS cases is TGN1412, where all six patients received intensive cardiopulmonary support (including dialysis). In addition, all patients were treated empirically with high-dose methylprednisolone, and an anti-IL-2 receptor antagonist antibody (daclizumab) owing to the expected drug-specific effects of TGN1412 on T cells [24].

The timing and impact of premedication prior to administration of mAb has not been studied in randomized, controlled clinical trials (RCTs). Some labels give very specific instructions. For example, the muronumab-CD3 label is very specific and has a boxed warning that states that pretreatment with methylprednisolone is recommended to minimize symptoms of CRS [100]. The label strongly recommended that methylprednisolone sodium succinate 8.0 mg/kg be administered intravenously 1–4 h prior to the initial dose. In addition, the rituximab label has specific instructions for CLL patients whose lymphocyte counts are more than

$25 \times 10^9/l$, where it is recommended that prednisone should be administered shortly before infusion to decrease the rate and severity of acute infusion reactions, and/or CRS [101]. Other labels give more generic recommendations around the nature (antipyretic, antihistaminic, steroids) and the start timings of premedication, that is, shortly (30 min) before starting the infusion (rituximab, alemtuzumab). The tositumomab label states that all patients in the clinical studies received pretreatment with acetaminophen and an antihistamine. However, the value of premedication in preventing infusion-related toxicity was not evaluated *per se* [102].

Antagonists of TNF-α

Since TNF-α may play a role in the CRS and infusion toxicity associated with mAb therapy, antagonists of TNF-α have been used as potential therapeutic agents that may decrease infusion toxicity. Lin *et al.* reported a Phase II study of the TNF-α inhibitor etanercept and rituximab in 36 patients with relapsed CLL to determine whether inhibition of TNF-α by etanercept increased the clinical activity of rituximab and reduced infusion toxicity [103]. Infusion reactions were mild and were not associated with severe adverse events. In another small-scale study, 14 kidney allograft recipients undergoing prophylactic OKT3 therapy were pretreated with a murine IgG1 anti-TNFα mAb (CB006). None of the CB006-pretreated patients showed any of the common, severe OKT3-associated symptoms, which were observed in 10% of the historical controls [104]. Eason *et al.* reported a Phase I study where 12 renal allograft recipients were randomized to receive OKT3 alone or in combination with a recombinant human-soluble TNF receptor (TNFR:Fc) [105]. Patients treated with TNFR:Fc had significantly fewer symptoms by day 2 of OKT3, and had a lower overall incidence of chills and arthralgias. Similarly, Novak *et al.* found that the administration of TNFR:Fc to 12 renal allograft recipients modestly decreased the severity of CRS symptoms associated with OKT3 [106]. These small-scale studies are promising; however, thus far, no large Phase III randomized, clinical studies have been conducted to show the possible benefits of treatment with anti-TNF-α mAbs for CRS.

Pentoxifylline

The hemorrheologic agent pentoxifylline (PTF) inhibits the synthesis of TNF-α *in vitro* in response to a variety of stimuli, including OKT3. Previous data suggest that PTF administration resulted in a significant reduction in serum TNF levels among OKT3-treated patients [107]. By contrast, a randomized, double-blind trial of PTF during OKT3 induction in recipients of cadaveric renal allografts showed that, despite the achievement of apparently adequate plasma levels of PTF and its active metabolites, no difference was observed in the incidence or severity of clinical manifestations of CRS [108]. Similarly, Vasquez and Pollak evaluated 32 OKT3-treated renal transplant recipients to assess the impact of PTF on OKT3 adverse reactions. They found in their study that oral administration of PTF did not appear to affect the incidence or severity of OKT3-related adverse reactions [109]. In addition, Vincenti *et al.* reported in a controlled, multicenter trial, that pretreatment with oral PTF was ineffective

in attenuating OKT3-related CRS in renal allograft recipients [110]. Therefore, the role of pentoxifylline in treatment of CRS remains is uncertain.

Antagonists of IFN- γ & IL-6

Data in rodents provide evidence that endogenous IFN- γ was important in the early phase of the anti-CD3 syndrome, while endogenous IL-6 seemed to be of lesser importance for the outcome of the CRS. Anti-IFN- γ antibodies were found to protect mice against pathological changes induced by injection of anti-CD3 antibody: incidence of diarrhea, severity of hypothermia and mortality rates were dramatically reduced, and IFN- γ blood levels were significantly reduced at 1.5 h post anti-CD3 challenge. Anti-IL-6 antibodies provided

only marginal protection against hypothermia and mortality. Levels of biologically active IL-6 in serum were not influenced by anti-IL-6 antibody treatment during the first few hours after anti-CD3 challenge, but were significantly increased at later times [111].

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Key issues

- There are a number of issues that are key to avoiding catastrophic cytokine-release syndrome (CRS), decreasing the potential for CRS and managing patients should CRS occur:
 - Avoiding catastrophic CRS
 - Recognition of the poor predictive value of CRS in nonclinical studies in rodent and nonhuman primates
 - Recognition that some monoclonal antibodies (mAbs) have a greater potential to cause CRS than others
 - Recognition that all mAbs have the potential to cause CRS and selecting starting doses for first in-human trials at a fraction of the expected pharmacologically active dose, rather than an arbitrary fraction of the NOAEL in animals
 - Avoiding over-reliance on the ability of antibody engineering; that is, changing immunoglobulin subclass or mutating the Fc region, to eliminate CRS
 - Although of potential, validation of this approach has yet to be achieved and will probably be target antigen specific
 - Development of standardized protocols for first in-human studies
- Decreasing the potential of CRS:
 - Avoiding short infusions or administering mAbs subcutaneously (e.g., subcutaneous administration has been shown to decrease first-dose reactions to alemtuzumab)
 - Further research into one or more *in vitro* assays using human cells to define the potential of a mAb to cause CRS
 - Cross-laboratory validation of one or more potentially predictive *in vitro* assays using known positive and negative antibodies (requires cooperation among pharmaceutical companies and/or sponsorship by regulatory authorities)
- Managing CRS should it occur:
 - Development of improved 'generic' prophylaxis
 - Development of 'specific' antidotal treatments to block or reduce the severity of CRS

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