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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

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ABSTRACT

The administration of several monoclonal antibodies (mAbs) to humans has been associated with acute adverse events characterized by clinically significant release of cytokines in the blood. The limited predictive value of toxicology species in this field has triggered intensive research to establish human *in vitro* assays using peripheral blood mononuclear cells or blood to predict cytokine release in humans. A thorough characterization of these assays is required to understand their predictive value for hazard identification and risk assessment in an optimal manner, and to highlight potential limitations of individual assay formats.

We have characterized a whole human blood cytokine release assay with only minimal dilution by the test antibodies (95% v/v blood) in aqueous presentation format, an assay which has so far received less attention in the scientific world with respect to the evaluation of its suitability to predict cytokine release in humans. This format was compared with a human PBMC assay with immobilized mAbs presentation already well-characterized by others. Cytokine secretion into plasma or cell culture supernatants after 24 h incubation with the test mAbs (anti-CD28 superagonist TGN1412-like material (TGN1412L), another anti-CD28 superagonistic mAb (ANC28.1), a T-cell depleting mAb (Orthoclone™), and a TGN1412 isotype-matched control (Tysabri™) not associated with clinically-relevant cytokine release) was detected by a multiplex assay based on electrochemiluminescent excitation.

We provide proof that this whole blood assay is a suitable new method for hazard identification of safety-relevant cytokine release in the clinic based on its ability to detect the typical cytokine signatures found in humans for the tested mAbs and on a markedly lower assay background and cytokine release with the isotype-matched control mAb Tysabri™ – a clear advantage over the PBMC assay. Importantly, quantitative and qualitative differences in the relative cytokine responses to the individual mAbs, in the concentration-response relationships and the prominent cytokine signatures for individual mAbs in the two formats reflect diverging mechanisms of cytokine release and different levels of dependency on high density coating even for two anti-CD28 super-agonistic antibodies. These results clearly show that one generic approach to assessment of cytokine release using *in vitro* assays is not sufficient, but rather the choice of the method, i.e. applying the whole blood assay or the PBMC assay needs to be well considered depending on the target characteristics and the mechanistic features of the therapeutic mAbs being evaluated.

1. Introduction

Monoclonal antibodies and other recombinant proteins that target receptors expressed on immune cells represent promising therapeutic agents to modulate immune responses with high specificity in an increasing number of disease indications [1–4]. Consequently, there is a growing need for the development of methods for hazard identification and potentially quantitative risk assessment for on-target immunostimulatory effects in humans

Abbreviations: CD, cluster of differentiation; Cs, calibrator samples; CV, coefficient of variation; Fc, fragment crystallizable; IFN, interferon; IL, interleukin; LLOQ, lower limit of quantification; mAb, monoclonal antibody; MSD, Meso Scale Discovery; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PEI, polyethylenimine; QCs, quality control samples; RT, room temperature; SEM, standard error of the mean; TGN1412L, TeGenero1412-like material; TNF, tumor necrosis factor; ULOQ, upper limit of quantification.

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that can result in clinical toxicity [5]. The failure to predict the cytokine release syndrome induced by the anti-CD28 superagonist TGN1412 in humans has dramatically demonstrated the limitations of pre-clinical animal models [6] and has triggered a broad spectrum of activities to develop and qualify *in vitro* tests with high predictive value using human systems [7]. The test formats include assays in whole or diluted human blood and methods using isolated PBMCs with mAbs presented in aqueous or immobilized form. In order to mimic the *in vivo* situation for a specific mAb as closely as possible, it should be considered that whole blood in contrast to PBMCs contains certain cell populations such as erythrocytes and granulocytes as well as factors such as complement which may act as contributors to or modulators of cytokine responses. Autologous plasma containing soluble factors can be added back to the PBMC culture medium at a certain percentage, but this does not reflect their original concentrations in undiluted plasma. However, using whole blood may not always eliminate these limitations outlined for PBMC based assays, especially when interactions with endothelial cells are of importance or if the majority of target cells are located in tissues. Although PBMCs were shown to produce distinct responses upon air-dried presentation of mAbs known to induce cytokine release *in vivo* [8], this approach may tend to overestimate risks and produce false positive results for some antibodies such as Tysabri™ and Avastin™ that can infrequently cause infusion reactions in humans which generally lack a causative link to cytokine release syndrome. For example, Findlay et al. [8] revealed marked cytokine release with Tysabri™ using a PBMC-based assay, although Tysabri™ is associated with a relatively low incidence of infusion reactions not related to adverse cytokine release but frequently related to the development of anti-drug antibodies and hypersensitivity reactions [9,10]. In addition, the method of mAb presentation may strongly influence the results. In this context, it is questionable, whether cytokine release stimulated by high density coating, e.g. by immobilization onto the plate via air-drying, provides relevant data for risk assessment of a new mAb, when a physiological correlate to this presentation form cannot be identified. The retrospective testing of TGN1412 in different assay formats was mainly focused on reproducing the patterns and levels of cytokines seen *in vivo* as closely as possible. However, the finding that TGN1412 must be presented in an immobilized form for hazard identification [11] raises the question as to what the *in vivo* correlate could be. In an assay using PBMCs or diluted blood (20% v/v) with TGN1412 added to the aqueous phase, very small responses almost similar to the isotype control were found [11,12]. There is the possibility that responses were not detected upon aqueous presentation due to the high dilution of human blood.

The aim of our study was to evaluate a cytokine release assay in almost undiluted whole blood in combination with aqueous mAb presentation for hazard prediction and potential risk assessment of *in vivo* cytokine release by mAbs with respect to cytokine pattern, magnitude, frequency and concentration dependency of responses. To demonstrate the value of this assay as part of a new *in vitro* cytokine release testing strategy, we conducted a comparative study with a PBMC assay previously shown to predict TGN1412-induced cytokine release upon air-dried coating following the assay protocol published by Findlay et al. [12] as close as the published method description allowed. However, the quantitative determination of secreted cytokines – a commercial multiplexed plate based assay coupled to electrochemiluminiscence detection – differed from the described method, since the authors used ELISA methods with in-house generated recombinant cytokine standards and raised anti-cytokine antibodies not accessible to us. We evaluated internally produced TGN1412-like material (sequence-identical material; TGN1412L), another super-agonistic mouse anti-human CD28 mAb, and Orthoclone™ – a murine anti-human CD3 mAb whose mechanism of cytokine release resembles that of TGN1412 in that the target-bearing cells are expected to be the main source of cytokine release [13]. Tysabri™ was used as an isotype-matched control for TGN1412L in all assays.

2. Materials and methods

2.1. Generation of vector constructs for TGN1412 heavy and light chain

Human heavy and light chain constant regions and TGN1412 heavy and light chain variable regions were synthesized by Geneart (accession numbers of human IgG4: P01861; human kappa light chain: P01834). Human heavy and light chain constant regions were cloned directly after the anti-CD28 TGN1412 variable region to generate the vector constructs [pRS5a-TGN1412-human IgG4 heavy chain] and [pRS5a-TGN1412-human kappa light chain], respectively.

2.2. Transient expression of anti-CD28-TGN1412-hIgG4/kappa

HEK293 Freestyle cells (Invitrogen, Carlsbad, CA, USA) were cultivated in Freestyle 293 medium (Invitrogen) in Roller bottles at 6.5–7.5 rpm, 5% CO_2 and 37 °C to a density of 3.17×10^6 viable cells/ml. The cells were transiently transfected with a vector DNA:PEI (Polyscience, Warrington, PA, USA) mixture, using a 1:3 ratio. For this, [pRS5a-TGN1412-human IgG4 heavy chain], [pRS5a-TGN1412-human kappa light chain] and PEI were diluted in M11V3 medium (Novartis, Basel, Switzerland), sterile-filtered and combined. After an incubation for 10 min at RT, the DNA:PEI mixture was added to the cells.

Six hours after transfection, Freestyle 293 medium was added to the culture. The cells were then further cultivated at 6.5–7.5 rpm, 5% CO_2 and 37 °C. Eleven days after transfection, cells were harvested and the supernatant was sterile filtered through a stericup filter (0.22 μm; Stericup Express™ Plus, Millipore, Billerica, MA, USA). The sterile supernatant was stored at 4 °C.

2.3. Purification of anti-CD28-TGN1412-hIgG4/kappa

Purification was performed on an ÄKTA 100 explorer air chromatography system (GE Healthcare, Glattbrugg, Switzerland) at 4 °C in a cooling cabinet, using a freshly sanitized (0.25 M NaOH) HiTrap ProtA MabSelectSuRe column (GE Healthcare). The column was equilibrated with Dulbecco's PBS, and then the sterile-filtered supernatant was loaded at 4.0 ml/min. After washing with DPBS, the anti-CD28 mAb was eluted with 50 mM Citrate, 70 mM NaCl pH3.2. The eluate was collected in fractions, pooled, adjusted to pH6 with 1 M Tris HCl pH10 and sterile filtered (0.22 μm; Millipore Steriflip). For the second purification step, pools from the first purification were loaded into a freshly sanitized (0.5 M NaOH) and equilibrated (50 mM Citrate, 140 mM NaCl pH6) Sephadex column (Hi Load 16/60 Superdex 200 grade) (GE Healthcare), and the run was performed with the same buffer at 1 ml/min. The total amount was divided in two different pools, one in 50 mM Citrate/140 mM NaCl pH6 and the second pool was neutralized at pH7 with 1 M Tris HCl pH10. These two different formulations were tested in the cytokine release assays.

The optical density at 280 nm of the pooled eluate was measured in a spectrophotometer ND-1000 (NanoDrop), and the protein concentration was calculated based on the sequence data. The final material was tested for purity by SDS-polyacrylamide gel electrophoresis which shows purity above 90%. Limulus amebocyte lysate test revealed an endotoxin level below 0.25 EU/mg. The mAb sequence was verified by mass spectromety. Aggregation