



Extracorporeal human whole blood in motion, as a tool to predict first-infusion reactions and mechanism-of-action of immunotherapeutics



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ABSTRACT

First infusion reactions along with severe anaphylactic responses can occur as a result of systemic administration of therapeutic antibodies. The underlying mechanisms by which monoclonal antibodies induce cytokine release syndrome (CRS) can involve direct agonistic effects via the drug target, or a combination of target-engagement along with innate receptor interactions. Despite the wide variety of pathways and cells that can play a role in CRS, many currently used assays are devoid of one or more components that must be present for these responses to occur. One assay that has not been assessed for its capacity to predict CRS is the modified Chandler loop model. Herein we evaluate a plethora of commercially available monoclonal antibodies to evaluate the modified Chandler loop model's potential in CRS prediction. We demonstrate that in a 4-hour loop assay, both the superagonistic antibodies, anti-CD3 (OKT3) and anti-CD28 (ANC28.1), display a clear cytokine response with a mixed adaptive/innate cytokine source. OKT3 induce TNF α and IFN- γ release in 20 out of 23 donors tested, whereas ANC28.1 induce TNF- α , IL-2 and IFN- γ release in all donors tested ($n = 18$ – 22). On the other hand, non-agonistic antibodies associated with no or low infusion reactions in the clinic, namely cetuximab and natalizumab, neither induce cytokine release nor cause false positive responses. A TGN1412-like antibody also display a clear cytokine release with an adaptive cytokine profile (IFN- γ and IL-2) and all donors ($n = 9$) induce a distinct IL-2 response. Additionally, the value of an intact complement system in the assay is highlighted by the possibility to dissect out the mechanism-of-action of alemtuzumab and rituximab. The loop assay can either complement lymph node-like assays or stand-alone to investigate drug/blood interactions during preclinical development, or for individual safety screening prior to first-in-man clinical trial.

1. Introduction

First infusion reactions can be severe and life-threatening reactions, which may develop following therapeutic monoclonal antibodies infusion. These reactions can, however, be managed with administration of steroids or by adjusting the dose and/or the infusion rate prior to drug administration [1,2]. Reactions can develop over time and the target of the antibody is not the sole reason for cytokine release. The sugar composition of the antibody, the Fc domain along with other factors can cause unexpected responses [3,4]. Cytokine release has historically been predicted by a variation of whole blood or peripheral blood mononuclear cell (PBMC) cultures with antibodies in an aqueous phase. However, these types of assays failed to predict the TGN1412 clinical

trial responses, where six healthy volunteers were infused with the anti-CD28 antibody, which led to anaphylactic responses and multi-organ failure [5]. The predictive in vitro assay systems along with the choice of performing toxicity tests in cynomolgus macaques led to the conclusion that the drug was safe to administer. Later, it was found that the toxicity can be enhanced upon Fc receptor cross-linking [4] and that CD28 + CD4 memory T cells were the main responder cells. These cells are CD28 negative in macaques and thus did not respond to stimulation [6,7]. The disastrous clinical trial spurred the development of novel human derived cytokine release assays on blood or blood components [8–10]. Commonly, these assays have been developed to study either anti-CD28 mediated toxicity or alemtuzumab-induced cytokine release. Alternative whole blood systems that have been used to study blood/

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material and blood/islet interactions, specifically studies of the instant-blood-mediated inflammatory reaction (IBMIR) [11], but have until now not been used to study antibody induced cytokine release, are variants of the modified Chandler-loop model [12–15]. Studies of a therapeutic virus and blood interactions along with studies using a Toll-like receptor 9 stimulating oligo have been performed in this system [16,17]. Most standard cytokine release assays (CRAs) are devoid of one or several important blood components, such as the cascade systems (complement and coagulation), a lack of neutrophils and in some cases deficient of endogenous antibodies (mainly PBMC cultures). Herein we hypothesized that the modified Chandler loop model, which comprises these components, can provide an additional tool for CRA and mode-of-action (MOA) studies of biologics where complement-dependent cytotoxicity (CDC) can play a role.

Whereas a static whole blood plate assay use high heparin concentrations to inhibit coagulation, which additionally inhibit the complement system [18], the modified Chandler-loop model applied herein makes use of heparin-conjugate attached to the inner surface of plastic tubings to prevent contact activation of the coagulation system. This prevents clotting and allows for fresh blood with intact complement system to be kept in circulation.

2. Material and methods

2.1. Materials

The characteristics and sources of the antibodies used herein are listed in Table 1. The antibodies were incubated in whole blood at concentrations and time points indicated in the figures and figure legends. The positive control LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich (USA). Heparin (Heparin, LEO), cortisone (Betapred) and hydrocortisone (Solu-Cortef(R)) were purchased from Apoteket AB (Sweden). The C3 inhibitor compstatin (Ac-ICV(1MeW) QDWGAHRCT) [19] was a kind gift from JD Lambris (University of Pennsylvania, School of Medicine, USA) and the C1q binding peptide (CEGPFGRHDLTFCW) [20] was a kind gift from JW Drijfhout (Leiden University, The Netherlands). EGTA and EDTA were purchased from Sigma-Aldrich (USA) and eculizumab was a kind gift from K Nilsson Ekdahl (Uppsala University, Sweden).

2.2. Blood loop

Blood from healthy donors was taken in an open system and immediately mixed with heparin (Leo Pharma AB, Sweden) to a final concentration of 1 IU/ml. All materials in direct contact with whole

blood were surface heparinized in accordance with the manufacturer's protocol (Corline, Sweden). Whole blood (2 ml) was added to PVC-tubings, which, with a surface heparinized metal connector, form a loop. The antibodies were added (diluted 1:100 in the blood), and the loops were set to rotate on a wheel at 37 °C. Blood aliquots were sampled, and EDTA was added to a final concentration of 10 mM to stop reactions at a given time-point. The samples were kept on ice, and plasma was collected by centrifugation at 2000 × g at 4 °C for 20 min. The plasma was stored at –70 °C until the time of analysis. For experiments involving corticosteroids and blocking agents, the blocking agents C1q peptide (100 µM), compstatin (10 µM), eculizumab (100 µg/ml) or anti-CD16 Fab'2 (25 µg/ml) were added approximately 10 min before the stimulatory agents (LPS or alemtuzumab). For blood loops aimed at intracellular flow cytometry, Brefeldin A was added to the blood loops to reach a final concentration of 10 µg/ml.

2.3. Plate assay

Blood from healthy donors was drawn and mixed into standard heparin vacutainer tubes (with a final heparin concentration in blood of 17 IU/ml) (368884, BD Vacutainer). The plate assay was started within 1 h of blood acquisition. 245 µl of blood and 5 µl antibody in PBS was added to round bottom plates (Corning Inc. 3879). The blocking agents; C1q-binding peptide (100 µM), compstatin (10 µM), eculizumab (100 µg/ml) or anti-CD16 Fab'2 (25 µg/ml) were added approximately 10 min before stimulatory agents (alemtuzumab, OKT3 or ANC28.1).

2.4. Blood cell counting

The automated hematology analyzer XP-300 (Sysmex) was used to assess white blood cell (WBC) killing by performing WBC count at different time points indicated in Figs. 4, 5 and in figure legends. The automated analyzer was also used to assess the stability of blood platelet count between pre- and post-experiment.

2.5. Flow cytometry

The following anti-human fluorochrome-labeled antibodies from Biolegend (USA) were used for surface and intracellular staining: anti-CD3 (clone: UVHT1), anti-CD4 (clone: OKT4), anti-CD8 (clone: SK1), anti-CD45RO (clone: UCHL1), anti-CD56 (clone: NCAM), anti-CD19 (clone: HIB19), anti-CD14 (clone: HCD14), anti-CD66b (clone: G10F5), anti-TNF-α (clone: Mab11), anti-IFN-γ (clone: 4S.B3) and anti-IL-2 (clone: MQ1-17H12 and 5344.111). Cell viability was analyzed using Aqua Zombie Fixable Viability Kit (Biolegend). A volume of 100 µl of

Table 1

Table 1 lists all antibodies used in the presented work.

Antibody	Trade name	Target	Isotype	CRS ^a	Source	Ref.
Adalimumab	Humira®	TNF-α	IgG1	No	AbbVie, USA	[43]
Alemtuzumab (ALM)	Lemtrada®	CD52	IgG1κ	Yes	Sanofi, France	[1]
Anakinra	Kineret®	IL-1R	IL-1R antagonist	No	Swedish Orphan Biovitrum, Sweden	[44]
Cetuximab (CET)	Erbix®	EGFR	Chimeric IgG1	Low (1.2% IgE-related)	Merck, Germany	[3,21]
Etanercept	Enbrel®	TNF-α	mIgG1	No	Pfizer, USA	[45]
Golimumab	Simponi®	TNF-α	Fully human IgG1	No	Merck Sharp & Dohme (MSD), USA	[46]
Infliximab	Remsima®	TNF-α	Chimeric IgG1	Low	Orion Pharma, Finland	[47]
Natalizumab	Tysabri®	α4-integrin	IgG4	No	Biogen Idec, USA	[48]
Pembrolizumab	Keytruda®	PD-1	IgG4κ	No	Merck Sharp & Dohme, UK	[49]
Rituximab (RTX)	MabThera®	CD20	Chimeric IgG1	Low ^b	Roche, Switzerland	[50]
Siltuximab	Sylvant®	IL-6	Chimeric IgG1κ	Low	Janssen-Cilag, Belgium/Switzerland	[51]
Tocilizumab	RoActemra®	IL-6R	IgG1	No	Roche, Switzerland	[52]
TGN1412-like Ab	–	CD28	IgG4κ	Yes	Evitria, Switzerland	–
OKT3	–	CD3	mIgG2ak	Yes	Biolegend, USA	[53]
ANC28.1/5D10	–	CD28	mIgG1κ	Yes	Ancell, USA	[36]

^a Cytokine release syndrome.

^b Risk profile in patients with high tumor burden in blood.

whole blood was added to FACS tubes (BD, USA), mixed with 50 μ l master mix of surface staining antibodies (diluted in PBS) and incubated for 15 min at room temperature (RT). Red blood cells were lysed by mixing blood with 2 ml of $1 \times$ FACS lysing solution (BD, USA) for 10 min at RT. The cells were then pelleted and washed in PBS twice by centrifugation. Before the intracellular staining, the cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD, USA) for 20 min. The cells were centrifuged twice with perm/wash buffer (BD, USA) before the intracellular antibody cocktail (diluted in BD perm/wash buffer) was added to the cells and incubated for 30 min at 4 °C. Finally the cells were centrifuged twice, once with perm/wash buffer (BD, USA) and once in PBS (1% BSA, 3 mM EDTA), and analyzed with a Canto II flow cytometer (BD, USA).

2.6. Cytokine, C3a and C5a analysis

Plasma harvested after 0 and 4 h was analyzed for IFN- γ , TNF- α , IL-1 β , IL-2, IL-6 and IL-8 with a Mesoscale V-plex kit (MSD Discovery®, USA) according to the manufacturer's instructions. Complement analysis (C3a and C5a) was performed on plasma collected 15 min after assay start with ELISA kits from BD (USA) or Hycult Biotech (USA) according to the manufacturer's instructions.

2.7. Ethical considerations

Collection of blood from healthy donors was approved by the Regional Ethical Committee in Uppsala.

2.8. Statistics and scoring calculations

Statistical analyses were performed using GraphPad Prism version 7.02 software (GraphPad software). Statistical analyses were performed with paired *t*-test, one-way ANOVA with Dunnett's multiple comparison test or two-way ANOVA with Sidak's multiple comparisons test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0005 (ns = not significant). The threshold for a positive individual cytokine response was set at above the 95th percentile of the calculated ratio PBS/baseline, where baseline values are samples taken at zero time-point and PBS values represent a control group where no cytokine release is expected. Values calculated from ratio antibody/baseline ratio above this threshold were scored as a positive cytokine release response for an individual cytokine (Table 2).

Table 2

Table 2 provides a scoring (positive or negative) of the predicted cytokine release for a certain cytokine and a given antibody.

	Adaptive		Innate	
	IFN γ	IL-2	IL-6	TNF
	^a Threshold fold > 2.80	^a Threshold fold > 1.47	^a Threshold fold > 15.7	^a Threshold fold > 3.93
Cetuximab (150 μ g/ml)	0/16	0/13	0/16	0/16
OKT3 (1 μ g/ml)	23/23	15/19	18/23	21/23
ANC28.1 (1 μ g/ml)	22/22	18/18	15/22	22/22
Natalizumab (1–1.5 μ g/ml)	0/9	1/9	0/9	0/9
TGN1412 (1–1.5 μ g/ml)	8/9	9/9	2/9	5/9
Alemtuzumab (3 μ g/ml)	18/18	9/10 ^b	18/18	18/18
Pembrolizumab (200 μ g/ml)	0/6	0/6	0/6	1/6

^a The threshold for a positive antibody response was set at above the 95th percentile of the calculated ratio of PBS/baseline value. The ratio value of drug X divided by baseline was set to positive if above the threshold. The threshold was calculated based on data from 37 donors of which 8 of them were run more than one time on separated occasions at least one month apart (54 runs in total). Cetuximab; two donors were run two and four times on separate occasions. OKT3; one donor was run twice on separate occasions. ANC28.1; one donor was run four times on separate occasions. TGN1412-like antibody; one donor was run twice separated by more than one year.

^b The alemtuzumab-induced IL-2 was close to or below the limit of detection (measured by MSD Discovery® multiplex) and therefore data have higher uncertainty.

3. Results

3.1. Cytokine release in the loop model

To assess if a modified Chandler loop model can predict an immediate cytokine release the superagonistic anti-CD28 antibody ANC28.1 and the anti-CD3 antibody OKT3 were incubated in the loop assay (all antibodies are listed in Table 1). Cetuximab (anti-EGFR), with low incidence of induction of immediate cytokine secretion [21,22], was used as a negative control. Both ANC28.1 and OKT3 induced a rapid cytokine release of IFN- γ , IL-2, TNF- α and IL-6 after 4 h (Fig. 1a). The IFN- γ , TNF- α and IL-6 profile of ANC28.1 is similar to OKT3, while IL-2 response is stronger for ANC28.1 than for OKT3 (Fig. 1a). Cetuximab on the other hand did not induce a CRS profile, consistent with its low incidence first-infusion reactions in patients [21]. The cellular sources of TNF- α and IFN- γ in response to OKT3 and ANC28.1 were investigated by an intracellular flow cytometry staining of T cells, NK cells, B cells, monocytes and granulocytes. Memory (CD45RO+) CD4+ and CD8+ T cells were identified as the major source of TNF- α and IFN- γ released in response to ANC28.1 (Fig. 1b). OKT3 induced cytokine release mainly from non-memory (CD45RO-) CD8+ cells (Fig. 1b). Monocytes produced low amounts of TNF- α , whereas NK cells, B cells and granulocytes did not produce any TNF- α and IFN- γ (data not shown). Antibodies with low incidence of inducing CRS in patients and/or with low severity grade of infusion reactions, including tocilizumab, etanercept, and infliximab (Table 1), did not induce a cytokine release in the loop (Fig. 1c). To evaluate the potential of the modified Chandler loop system to predict a CRS in humans we set the 95th percentile of the calculated ratio of the control group where no cytokine release was expected and baseline as a threshold (described in Material and methods). An antibody/baseline ratio above this threshold was scored as a positive response for any given cytokine as illustrated in Table 2. After only 4 h of incubation in the loop assay, OKT3 induced cytokine release above threshold with a mixed adaptive/innate cytokine source (TNF- α and IFN- γ release) in 21 out of 23 donors tested whereas ANC28.1 displayed a clear CRS (TNF- α , IL-2 and IFN- γ release) in all donors tested (*n* = 18–22). Non-agonistic antibodies associated with no or low infusion reactions in the clinic neither induced cytokine release nor caused false positive responses (Fig. 1c and Table 2).

3.2. TGN1412-like antibody in the loop

A TGN1412-like antibody, but not the IgG4 isotype-matched control natalizumab, induced a rapid release of IFN- γ , IL-2, IL-6 and TNF- α at antibody concentrations between 1 and 10 μ g/ml after only 4 h of incubation (Fig. 2a). At a concentration of 1 and 1.5 μ g/ml, the TGN1412-

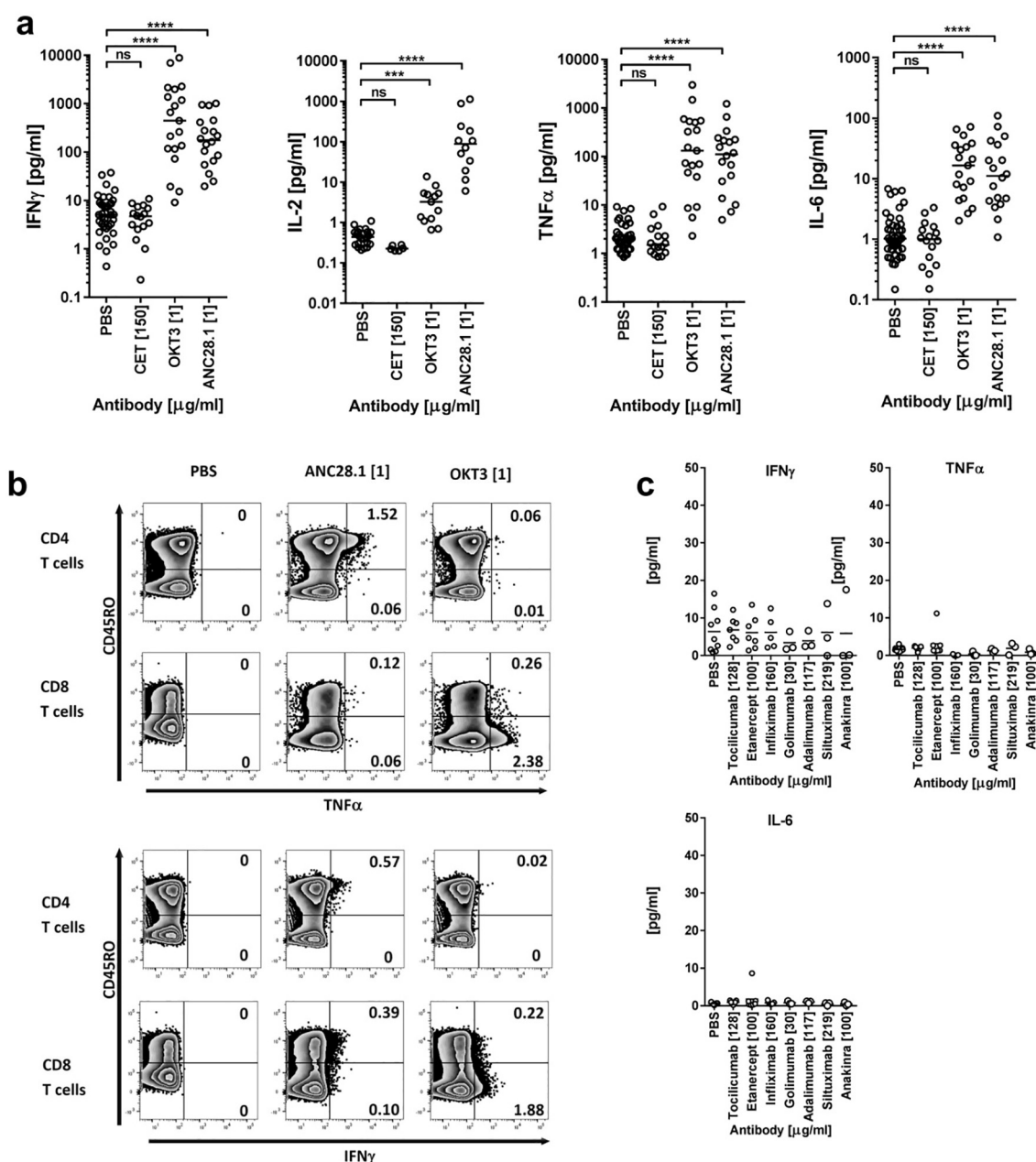


Fig. 1. Cytokine release in response to monoclonal antibodies in a human whole blood loop assay.

Freshly acquired whole blood was incubated with the antibodies cetuximab (CET), OKT3 or ANC28.1 in a circulating loop assay (as described in [Material and methods](#)). The final concentrations of the antibodies in blood are displayed in brackets in the figure [μ g/ml]. After 4 h blood samples were collected and further activation was inhibited by mixing the blood with EDTA. Plasma was collected and analyzed for cytokines with MSD Discovery[®] multiplex (a). CET; $n = 6$ (IL-2) and $n = 16$ (IFN- γ , TNF- α and IL-6), of which two donors were run more than one time on separate occasions. OKT3; $n = 13$ (IL-2) and $n = 19$ (IFN- γ , TNF- α and IL-6), of which one donor was run twice on separate occasions. PBS; $n = 38$ of which three donors were run more than one time on separate occasions. An assay interference with detecting drug bound TNF- α is plausible, since it has been reported for other methods [23]. For intracellular staining of cytokines Brefeldin A was added 1 h after the antibodies and after a total of 4 h blood was stained for surface markers and intracellular cytokines (as described in [Material and methods](#)). The blood cells were analyzed by flow cytometry and the data shown is for one representative donor out of three (b). Other antibodies listed in [Table 1](#) were analyzed as described above (c, $n = 3$ –7; PBS $n = 9$). The data were analyzed with a paired t -test on log-transformed values. *** $p < 0.001$ and **** $p < 0.0005$ (ns = not significant).

like antibody induced a clear cytokine release with an adaptive cytokine profile (IFN- γ and IL-2) and all donors ($n = 9$) scoring the positive IL-2 response and majority of donors (8/9) giving IFN- γ response above threshold. On the other hand, natalizumab at 1–1.5 μ g/ml, had no IL-2 (1/9) or IFN γ (0/9) responses above the threshold ([Table 2](#)). Blood cells including; T cells, NK cells, B cells, monocytes and granulocytes were stained for intracellular IFN- γ , TNF- α and IL-2 and analyzed by flow cytometry to identify the cellular source of the cytokines. The memory (CD45RO+) CD4+ and CD8+ T cells were the sources of the IFN- γ and TNF- α in response to the TGN1412-like antibody ([Fig. 2b](#)). IL-2 was

produced by memory CD4+ T cells in response to TGN1412-like antibody but was not detectable in the memory CD8+ T cells ([Fig. 2b](#)). Non-memory (CD45RO-) CD4+ and CD8+ T cells did not produce cytokines in response to the TGN1412-like antibody ([Fig. 2b](#)) and no cytokine production was detected from B, NK cells, monocytes or granulocytes (data not shown).

3.3. LPS-induced cytokine release and complement activation in the loop

To evaluate if the circulating whole blood loop assay can predict the

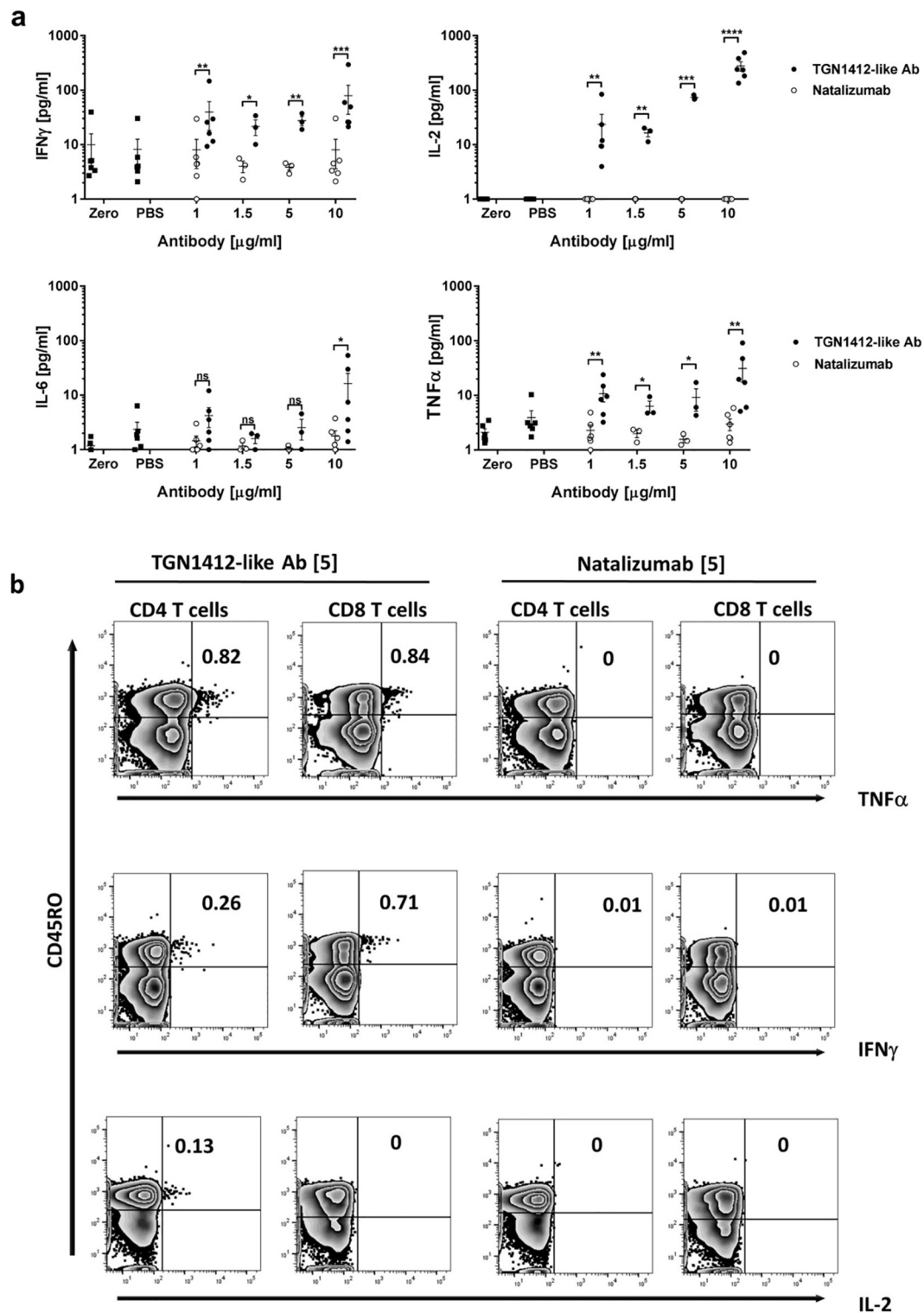


Fig. 2. Cytokine release in response to a TGN1412-like Ab in a human whole blood loop assay.

Fresh whole blood from healthy donors was incubated with TGN1412-like antibody, natalizumab or PBS for 4 h. Plasma cytokines were measured by the MSD Discovery® multiplex platform (a) and intracellular flow cytometry was performed on blood cells (b). Plasma levels of IFN- γ , TNF- α , IL-2 and IL-6 after a 4 h stimulation are shown (a, $n = 3-6$), of which one donor was run twice on separate occasions. Flow cytometric intracellular cytokine staining for memory/non-memory CD4 + and CD8 + T cells in response to TGN1412-like antibody and natalizumab at 5 μ g/ml (b, one representative donor out of three). The data were analyzed with a paired t -test on log-transformed values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0005$ (ns = not significant).

effects of immune modulating agents, their ability to inhibit LPS-induced immune activation was assessed in the loop assay. LPS alone induced a rapid cytokine release in the loop assay after 4 h (Fig. 3a). The immune modulating agents, etanercept (TNF α inhibitor), cortisone

(betamethasone) and hydrocortisone were pre-incubated before addition of LPS to the loop to assess their ability to attenuate LPS-mediated cytokine release. All three agents blocked LPS-induced TNF- α and IL-8 release (Fig. 3b). Additionally, cortisone and hydrocortisone inhibited

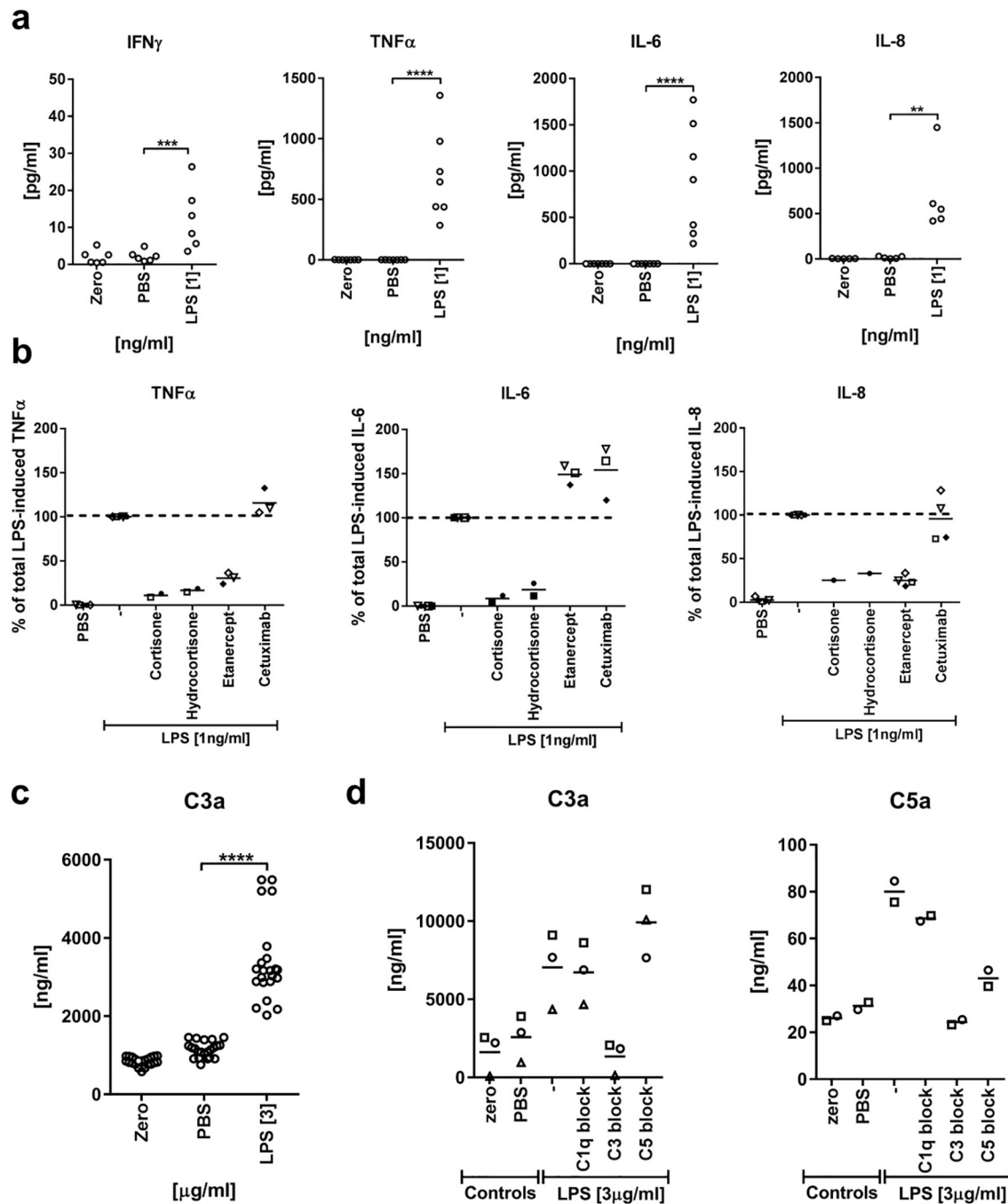


Fig. 3. LPS-induced cytokine release and complement activation in a human whole blood loop assay.

Freshly acquired whole blood was incubated with LPS (1 μ g/ml) in a circulating blood loop assay (as described in [Material and methods](#)). After 15 min and 4 h blood samples were collected and cascade systems were inhibited by mixing the blood with EDTA. Plasma was harvested and analyzed for cytokines with MSD Discovery[®] multiplex (a, n = 5–7). Blood was pre-incubated for 15 min with cortisone (4 μ g/ml) or hydrocortisone (50 μ g/ml), etanercept (100 μ g/ml) or cetuximab (150 μ g/ml) before addition of LPS (1 ng/ml) and the plasma was analyzed for cytokine release after 4 h as described above (b, n = 1–6). Plasma samples acquired after 15 min were analyzed for C3a by ELISA (c, n = 21). C1q block (C1q binding peptide), C3 block (compstatin) and C5 block (eculizumab) were incubated for 10 min before addition of LPS and the plasma (15 min samples) were analyzed for C3a and C5a by ELISA (d, n = 2–3). The data were analyzed with a paired *t*-test on log-transformed values (a and c) **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0005 (ns = not significant).

IL-6 release in the loop model ([Fig. 3b](#)). Cetuximab was used as an unspecific control, and it did not inhibit the LPS-induced cytokine release. LPS is known to activate the complement system [24] which was detected in the loop model by analyzing C3a and C5a release ([Fig. 3c](#) and [d](#)). Furthermore, LPS-induced complement activation was inhibited with a C3-specific peptide (compstatin) and a C5-specific antibody (eculizumab) ([Fig. 3d](#)).

3.4. Circulating blood versus a static blood plate assay

In a Chandler-loop model, the circulation of blood is a key to prevent coagulation, whereas undiluted blood in static plate assays requires a high dose of heparin to prevent coagulation. Blood assays used prior to the disastrous TGN1412 trial were commonly performed with diluted blood and have in our hands displayed a poor prediction of cytokine release (data not shown). To determine if undiluted blood in a static plate assay version, or as a loop assay, differs with respect to

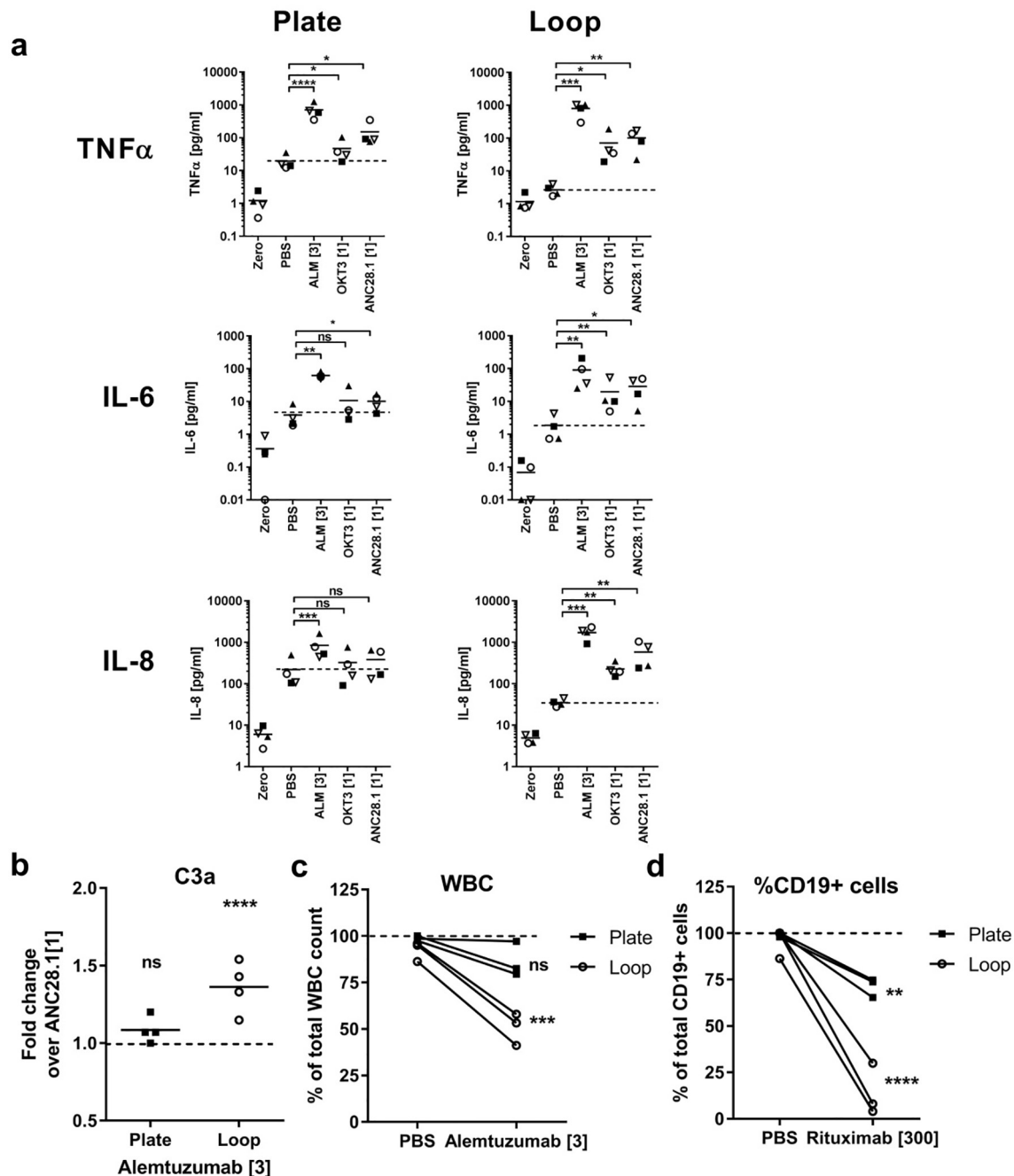


Fig. 4. Cytokine release and complement activation in human whole blood on a static plate versus a blood in circulation.

Whole blood was acquired either through a heparin vacutainer tube (plate assay) or open system (loop assay) (as described in [Material and methods](#)) and incubated with PBS, Alemtuzumab (ALM), OKT3 or ANC28.1 on a static plate (a, on the left) or in a circulating blood loop assay (a, on the right). The final concentration of the antibodies in blood is displayed in brackets in the figure [$\mu\text{g/ml}$]. After 15 min and 4 h blood samples were collected and cascade systems were inhibited by mixing the blood with EDTA. Plasma samples acquired after 4 h were analyzed for cytokines with MSD Discovery[®] multiplex (a, $n = 4$). Plasma samples acquired after 15 min were analyzed for C3a by ELISA (b, $n = 4$) and fold increase in C3a levels of Alemtuzumab in comparison to ANC28.1 is presented. WBC count after 4 h was calculated using Sysmex XP-300 and plotted as % of zero time point (c, $n = 3$). B cell counts were counted by flow cytometry (CD19+) (d, $n = 3$). The dotted line is drawn from the mean of the PBS group in each graph. The data were analyzed with a paired t -test on log-transformed values (a) or two-way ANOVA with Sidak's multiple comparison test (b and c) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0005$ (ns = not significant).

cytokine release, an experiment was performed where blood was sampled from the same donors for both assays, and the experiments were run in parallel. All agonistic antibodies tested (alemtuzumab, OKT3 and ANC28.1) induced similar levels of cytokines in both assays (Fig. 4a). However, there was a marked TNF- α , IL-6 and IL-8 release in the PBS group using the plate assay (marked with a dotted line in Fig. 4a). With this high background of cytokine release, the plate assay displayed a poor sensitivity for scoring cytokine release compared to the loop assay, and only alemtuzumab could score a positive response in the plate

assay, while OKT3 and ANC28.1 had responses comparable to the negative control (PBS) for most of the cytokines.

The plate assay requires high heparin concentration to prevent coagulation and this is known to inhibit the complement system [18]. In the circulating loop model with a low free heparin content, complement activation induced by LPS (Fig. 3c and d) or antibodies (Figs. 4b and 5d) can be assessed. Alemtuzumab [25] induce complement activation when applied in the loop, but not in the counterpart plate assay (Fig. 4b). ANC28.1 (mouse IgG1) was used as a negative

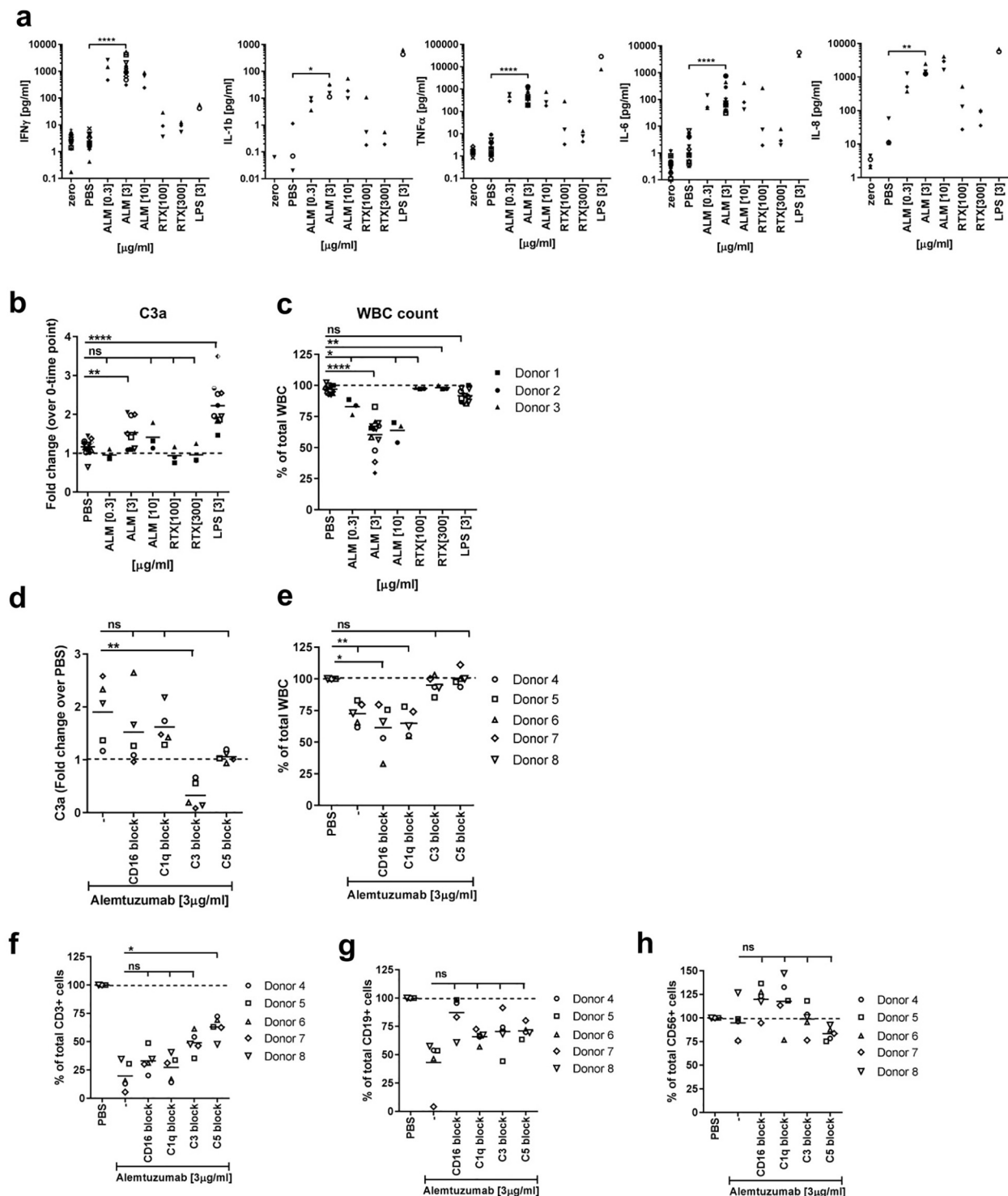


Fig. 5. Cytokine release and mechanism-of-action of alemtuzumab in human whole blood.

Freshly acquired whole blood was incubated with alemtuzumab (ALM), rituximab (RTX) or LPS in a circulating blood loop assay (as described in [Material and methods](#)). The final concentration of the antibodies in blood is displayed in brackets in the figure [μ g/ml]. After 15 min and 4 h blood samples were collected and cascade systems were inhibited by mixing the blood with EDTA. Plasma samples acquired after 4 h were analyzed for cytokines with MSD Discovery[®] multiplex (a, $n = 2-14$). Plasma samples acquired after 15 min were analyzed for C3a by ELISA (b, $n = 3-13$). WBC count after 4 h was calculated using Sysmex XP-300 and was plotted as the % of the zero sample (c, $n = 3-13$). CD16 block (anti-CD16 Fab'2), C1q block (C1q binding peptide), C3 block (compstatin) and C5 block (eculizumab) were incubated for 10 min before addition of alemtuzumab (d-h, $n = 5$). The data were analyzed with a paired t -test of log-transformed values (a-c) or one way ANOVA with Dunnett's multiple comparison test (d-f) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0005$ (ns = not significant).

comparator when looking at complement activation as it has low affinity to human C1q [26,27] and does not induce detectable complement activation in our system (data not shown). Furthermore, both alemtuzumab and rituximab, which deplete CD52 + cells and B cells

respectively, displayed more prominent depletion of their target cells in the loop assay compared to the plate assay (Fig. 4c and d). A 4-hour loop assay did not affect cell viability for individual white blood cell populations (T cells, B cells, NK cells, monocytes and granulocytes as

shown in Supplementary Fig. 1) if not a cell depleting antibody or LPS stimuli was added to the system. Alemtuzumab displayed a variation in cell killing capacity between donors, as visualized with the increased standard deviation. Blood pH at zero time point was 7.4 and it remained stable to up to 6 h in the loop assay. Blood values for bicarbonate (HCO_3), partial pressure of carbon dioxide (PCO_2) and base excess (BE) were similar between zero and a 6 h time-point.

3.5. Alemtuzumab-induced cytokine release and mechanism-of-action in the loop

Alemtuzumab is associated with immediate cytokine release in patients [1] and consistently induced $\text{IFN-}\gamma$, $\text{IL-1}\beta$, $\text{TNF-}\alpha$, IL-6 and IL-8 release in the loop assay (Fig. 5a). Two different MOAs of alemtuzumab have been suggested, more specifically antibody-dependent cellular cytotoxicity (ADCC) [28,29] and CDC [25]. In the loop assay alemtuzumab activated the complement system by inducing C3a in a concentration-dependent manner (Fig. 5b) in line with published data (25). Furthermore, the alemtuzumab-induced C3a levels correlated with the reduction of WBC count (Fig. 5c). Depletion of the T and B cell populations was also observed by flow cytometry analysis (Fig. 5f/g and Supplementary Fig. 1). Rituximab, an IgG1 antibody, did not induce detectable complement activation, a finding also consistent with in vitro published data (25) and no drop in WBC was observed (Fig. 5b and c). A toll-like receptor stimulus such as LPS, activated complement without profoundly affecting the WBC count (Fig. 5b and c), however increased killing of monocytes was visible through flow cytometry analysis (Supplementary Fig. 1). To further evaluate the MOA of alemtuzumab, both ADCC and CDC were modulated by specifically blocking CD16 (ADCC) or the complement components C1q, C3 and C5 (CDC). By blocking C3 and C5, but not CD16 and C1q, alemtuzumab-induced increased C3a levels and the drop in WBC count was abolished, suggesting killing via CDC (Fig. 5e and f). As CD52 is expressed on several cell-types in blood, the mechanism of killing was also investigated by flow cytometry where CD3^+ cells were to some degree rescued via complement inhibitors suggesting a CDC mediated alemtuzumab induced T cell depletion (Fig. 5f). On the contrary, CD19^+ cells displayed a trend towards a depletion mechanism via ADCC, as B cell were rescued by blocking CD16 although this did not reach statistical significance (Fig. 5g). NK cells were not affected by alemtuzumab in the loop assay (Fig. 5h) which can be explained by their low expression of CD52 [30]. Although alemtuzumab at $3\text{ }\mu\text{g/ml}$ did not clearly affect the numbers of monocytes (CD14^+) and granulocytes (CD66b^+), monocytes showed decreased viability when analyzed with viability dye (Aqua Zombie) by flow cytometry (Supplementary Fig. 1).

4. Discussion

There is a need for human-based in vitro assay to perform a risk-based evaluation of infusion reactions of monoclonal antibodies, which should imaginably be composed of all in vivo components such as the target cells, $\text{Fc}\gamma\text{Rs}$, circulating antibodies, intact complement, neutrophils, and may also include endothelial cells, all which can contribute to adverse events. As a blood/endothelial cell system is difficult to achieve in an autologous setting, immune cell readouts becomes a challenge and thus assays devoid of endothelial cells are commonly used [31]. Current available assays such as PBMC, high density cultures [4,32] and whole blood plate assays all lack at least one of the above mentioned components. We therefore evaluated a modified Chandler loop model for its potential to predict first infusion reactions as well as MOA of immunotherapeutics. Traditionally extracorporeal blood systems have been used to study blood/material interactions along with studies of the IBMIR reaction (11), studies where coagulation and complement cascades can have a great role in the outcome. It is however clear that these system can be of importance when interpreting blood/drug interactions (Unpublished data from our group and

reference [16,17,33,34]). Here, the system takes the advantage of the circulatory movement of the blood and incorporates a heparin conjugate attached to the plastic/metallic surfaces that comes in contact with the blood preventing stagnation as well as contact induced clotting while retaining the integrity of the complement and coagulation cascades.

In the loop assay, the superagonistic antibodies OKT3 and ANC28.1 induced proinflammatory cytokine release and a TGN1412-like Ab induced IL-2 release by memory CD4^+ T cells after 4 h, in line with what has been seen in patients, and the cause of the adverse events of healthy individuals in the clinical trial with TGN1412 [6]. The TGN1412-induced IL-2 release has been found to be potentiated by $\text{Fc}\gamma\text{RIIb}$ [4], which can to some extent be mimicked by coating the antibody to the surface [35]. Although, the mechanism of crosslinking TGN1412 in the loop system is unknown, IL-2 was released in the loop system more rapidly than in what was described in vitro plate assays with both diluted [35] and minimally diluted whole blood [36]. IL-2 release was clearly induced in all donors tested (measured by mesoscale analysis from plasma), albeit only a small fraction of memory CD4^+ T cells appeared to be the producers of these cytokines as visualized by the intracellular staining. A plausible explanation could be that these few cells are potent in their IL-2 production.

Until now, PBMC-based assays have been more sensitive in cytokine release prediction in response to anti-CD28 stimuli, whereas the whole blood assays have shown great sensitivity, when it comes to alemtuzumab-induced cytokine release [35–37]. When comparing a static whole blood plate assay with the blood loop assay the later was more sensitive to score a positive response in each donor, as the background cytokine induction was greatly reduced. In the loop assay, the cytokine release is prominent and in addition, complement activation in response to a certain drug can be evaluated.

As both CDC and ADCC are active in the loop assay, we assessed the MOA of IgG1 antibodies such as alemtuzumab and rituximab. The MOA of alemtuzumab in the loop assay was through CDC, consistent with published data (25). Surprisingly, our data indicate that alemtuzumab-induced cell death required C3 and C5, but not C1q, suggesting a killing mechanism via the alternative or the lectin pathway rather than the classical pathway. N-glycans on mouse IgG1 antibodies have been shown to initiate alternative pathway activation in mice [38]. Another possible explanation is complement activation via the lectin pathway [39], something that requires further evaluation. By specifically looking at T cells, B cells and NK cells in flow cytometry, there appears to be separate mechanisms by which alemtuzumab induce killing of the target cells, at least for individual donors. T cells were depleted by CDC whereas B-cells appeared to be depleted via ADCC, as T or B cells were, at least partly, rescued from alemtuzumab-induced cell death by complement blockade or CD16 blockade respectively. The potential difference in killing mechanism of T cells and B cells may be explained by different expression levels of CD52 on B and T cells [30,40], as the efficiency of CDC is proportional to the level of target expression. ADCC however, is less dependent on the target expression and has been reported to be complemented by CDC [41]. Thus, the lower expression of CD52 on B cells makes them less susceptible to CDC and thereby ADCC might be the main cell killing mechanism. Rituximab also efficiently kills B cells in the loop assay although this was only visible through flow cytometry and not in the WBC count. This is due to that B cells constitutes a minor fraction in blood out of the complete leucocyte count, therefore a drop in B cell count might not be apparent when counting the complete WBC count. Similarly, the absence of detectable complement activation with rituximab in the loop system, despite its apparent role for inducing B cell depletion, is also most likely due to the B cell population constituting a minor part of the WBC of a healthy individual. This is in line with the reported observation that rituximab-mediated infusion reactions are commonly prevalent in B cell lymphoma patients where the increasing numbers of tumor cells, and thereby also the target expression and possibly target density, correlate to increased

cytokine release and adverse effects [42]. The reason for the full rescue of WBC counts with alemtuzumab plus complement inhibitors when analyzed by the hematology analyzer, and the lack of complete rescue of individual cells when using flow cytometry as read-out is something we found challenging to completely understand. One explanation is that the hematology analyzer counts WBCs based on cell size and therefore overestimate the number of cells that are rescued by the C3/C5 block, an alternative explanation is that CD3 expression is downregulated in response to alemtuzumab which may affect the flow cytometry data to some degree. CD52 is expressed on monocytes and alemtuzumab affects also viability of monocytes, however a drop in monocyte cell number is not as distinct as for T and B cells, which can be explained by their higher expression of complement inhibitors compared to lymphocytes [30]. In the clinic, immune modulating agents like cortisone and hydrocortisone are used to reduce infusion reactions of immunotherapeutics [2]. Both corticosteroids and etanercept inhibited LPS-induced cytokine release in the loop assay, validating the assays potential use in predicting immune modulating effects of immunotherapeutics. Similar to any other artificial system, the whole blood loop system is not devoid of limitations, such as absence of the endothelial cells or limited oxygen exchange and nutrients supply to the blood in the loops. The minimal gas-exchange, which can limit prolonged incubation times suggest that the assay is best suited to score immediate infusion reactions. It is of importance to note that longer incubation times may be required to monitor adverse events that develop over a longer time period. Notably, despite minimal gas exchange, we could not find that a 4-hour assay harmed individual immune cell populations. Nevertheless, the loop model proved to be successful in predicting CRS at the 4 h time point, in concordance with the clinical observation where treatment with monoclonal antibodies may cause cytokine release in the first hours after infusion. We suggest that a modified Chandler loop model can be used to assess immune modulating effects that take place in circulation and that lymphoid tissue-like culture can complement this to provide a complete picture of the effects an antibody has in circulation versus a tissue-based effect [33].

In conclusion, we have shown that a circulating whole blood loop assay is a powerful human-based extra-corporal assay to predict CRS. It was superior to a standard plate assay in presenting distinctively lowered background of cytokine release, which translates to increased sensitivity of the assay. Presence of intact complement and coagulation system adds an extra dimension to the loop model, not available in other currently used *in vitro* methods. The advantage of having all blood components and intact cascade systems in one assay makes the loop model a unique and important tool for evaluating the risk of infusion reactions and studying MOA of immunotherapeutics.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2017.10.021>.

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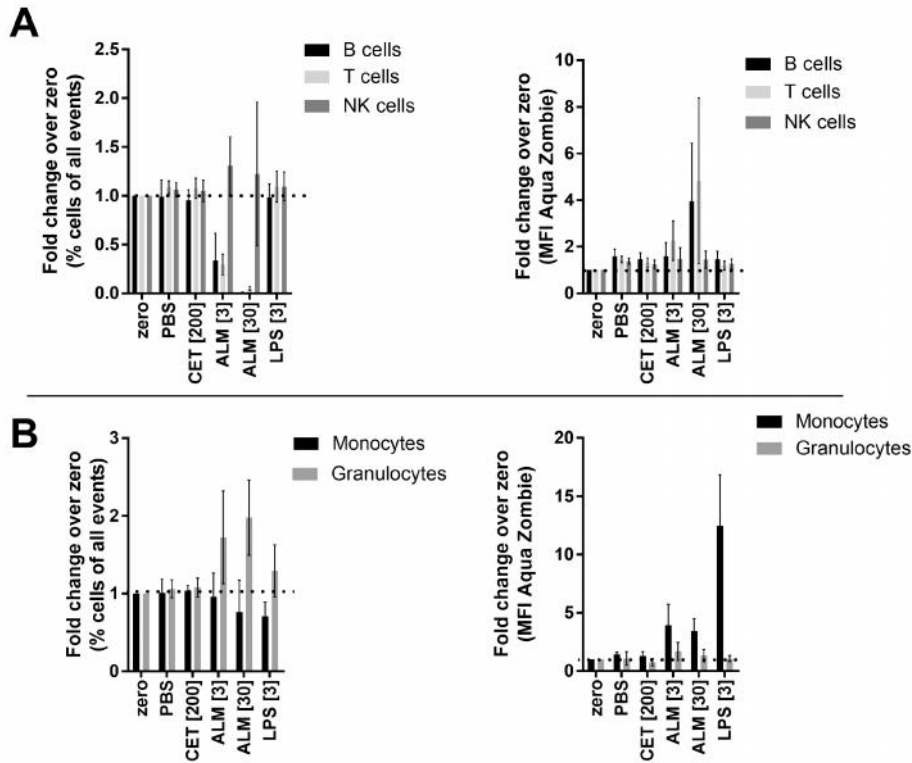
Declaration of interest

EF is the founder/co-owner of Immuneed AB, SM is the founder/owner, CSO and a board member of Immuneed AB. FL, GT and JLJ are employees of Immuneed AB.

References

- [1] A. Osterborg, C. Karlsson, J. Lundin, E. Kimby, H. Mellstedt, Strategies in the management of alemtuzumab-related side effects, *Semin. Oncol.* 33 (2 Suppl. 5) (2006) S29–35.
- [2] D.W. Lee, R. Gardner, D.L. Porter, C.U. Louis, N. Ahmed, M. Jensen, S.A. Grupp, C.L. Mackall, Current concepts in the diagnosis and management of cytokine release syndrome, *Blood* 124 (2) (2014) 188–195.
- [3] C.H. Chung, B. Mirakhor, E. Chan, Q.T. Le, J. Berlin, M. Morse, B.A. Murphy, S.M. Satinover, J. Hosen, D. Mauro, R.J. Slebos, Q. Zhou, D. Gold, T. Hatley, D.J. Hicklin, T.A. Platts-Mills, Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose, *N. Engl. J. Med.* 358 (11) (2008) 1109–1117.
- [4] K. Hussain, C.E. Hargreaves, A. Roghanian, R.J. Oldham, H.T. Chan, C.I. Mockridge, F. Chowdhury, B. Frendeus, K.S. Harper, J.C. Strefford, M.S. Cragg, M.J. Glennie, A.P. Williams, R.R. French, Upregulation of Fc γ RIIb on monocytes is necessary to promote the superagonist activity of TGN1412, *Blood* 125 (1) (2015) 102–110.
- [5] G. Suntharalingam, M.R. Perry, S. Ward, S.J. Brett, A. Castello-Cortes, M.D. Brunner, N. Panoskaltis, Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412, *N. Engl. J. Med.* 355 (10) (2006) 1018–1028.
- [6] D. Eastwood, C. Bird, P. Dilger, J. Hockley, L. Findlay, S. Poole, S.J. Thorpe, M. Wadhwa, R. Thorpe, R. Stebbings, Severity of the TGN1412 trial disaster cytokine storm correlated with IL-2 release, *Br. J. Clin. Pharmacol.* 76 (2) (2013) 299–315.
- [7] D. Eastwood, L. Findlay, S. Poole, C. Bird, M. Wadhwa, M. Moore, C. Burns, R. Thorpe, R. Stebbings, Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4+ effector memory T-cells, *Br. J. Pharmacol.* 161 (3) (2010) 512–526.
- [8] L. Findlay, D. Eastwood, R. Stebbings, G. Sharp, Y. Mistry, C. Ball, J. Hood, R. Thorpe, S. Poole, Improved *in vitro* methods to predict the *in vivo* toxicity in man of therapeutic monoclonal antibodies including TGN1412, *J. Immunol. Methods* 352 (1–2) (2010) 1–12.
- [9] R. Stebbings, D. Eastwood, S. Poole, R. Thorpe, After TGN1412: recent developments in cytokine release assays, *J. Immunotoxicol.* 10 (1) (2013) 75–82.
- [10] L. Findlay, G. Sharp, B. Fox, C. Ball, C.J. Robinson, C. Bird, R. Stebbings, D. Eastwood, M. Wadhwa, S. Poole, R. Thorpe, S.J. Thorpe, Endothelial cells co-stimulate peripheral blood mononuclear cell responses to monoclonal antibody TGN1412 in culture, *Cytokine* 55 (1) (2011) 141–151.
- [11] H. Johansson, M. Goto, D. Dufrane, A. Siegbahn, G. Elgue, P. Gianello, O. Korsgren, B. Nilsson, Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation, *Am. J. Transplant. Off. J. Am. Soc. Transplant. Am. Soc. Transplant Surg.* 6 (2) (2006) 305–312.
- [12] W. Bennet, B. Sundberg, C.G. Groth, M.D. Brendel, D. Brandhorst, H. Brandhorst, R.G. Bretzel, G. Elgue, R. Larsson, B. Nilsson, O. Korsgren, Incompatibility between human blood and isolated islets of Langerhans: a finding with implications for clinical intraportal islet transplantation? *Diabetes* 48 (10) (1999) 1907–1914.
- [13] J. Gong, R. Larsson, K.N. Ekdahl, T.E. Mollnes, U. Nilsson, B. Nilsson, Tubing loops as a model for cardiopulmonary bypass circuits: both the biomaterial and the blood-gas phase interfaces induce complement activation in an *in vitro* model, *J. Clin. Immunol.* 16 (4) (1996) 222–229.
- [14] R. Larsson, G. Elgue, A. Larsson, K.N. Ekdahl, U.R. Nilsson, B. Nilsson, Inhibition of complement activation by soluble recombinant CR1 under conditions resembling those in a cardiopulmonary circuit: reduced up-regulation of CD11b and complete abrogation of binding of PMNs to the biomaterial surface, *Immunopharmacology* 38 (1–2) (1997) 119–127.
- [15] J. Andersson, J. Sanchez, K.N. Ekdahl, G. Elgue, B. Nilsson, R. Larsson, Optimal heparin surface concentration and antithrombin binding capacity as evaluated with human non-anticoagulated blood *in vitro*, *J. Biomed. Mater. Res. A* 67 (2) (2003) 458–466.
- [16] A. Danielsson, G. Elgue, B.M. Nilsson, B. Nilsson, J.D. Lambris, T.H. Totterman, S. Kochanek, F. Kreppel, M. Essand, An *ex vivo* loop system models the toxicity and efficacy of PEGylated and unmodified adenovirus serotype 5 in whole human blood, *Gene Ther.* 17 (6) (2010) 752–762.
- [17] S.M. Mangsbo, J. Sanchez, K. Anger, J.D. Lambris, K.N. Ekdahl, A.S. Loskog, B. Nilsson, T.H. Totterman, Complement activation by CpG in a human whole blood loop system: mechanisms and immunomodulatory effects, *J. Immunol. (Baltimore, Md: 1950)* 183 (10) (2009) 6724–6732.
- [18] G.L. Logue, Effect of heparin on complement activation and lysis of paroxysmal nocturnal hemoglobinuria (PNH) red cells, *Blood* 50 (2) (1977) 239–247.
- [19] M. Katragadda, P. Magotti, G. Sfyroera, J.D. Lambris, Hydrophobic effect and hydrogen bonds account for the improved activity of a complement inhibitor, compstatin, *J. Med. Chem.* 49 (15) (2006) 4616–4622.

- [20] A. Roos, A.J. Nauta, D. Broers, M.C. Faber-Krol, L.A. Trouw, J.W. Drijfhout, M.R. Daha, Specific inhibition of the classical complement pathway by C1q-binding peptides, *J. Immunol. (Baltimore, Md: 1950)* 167 (12) (2001) 7052–7059.
- [21] D. Cunningham, Y. Humblet, S. Siena, D. Khayat, H. Bleiberg, A. Santoro, D. Bets, M. Mueser, A. Harstick, C. Verslype, I. Chau, E. Van Cutsem, Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer, *N. Engl. J. Med.* 351 (4) (2004) 337–345.
- [22] B.H. O'Neil, R. Allen, D.R. Spiegel, T.E. Stinchcombe, D.T. Moore, J.D. Berlin, R.M. Goldberg, High incidence of cetuximab-related infusion reactions in Tennessee and North Carolina and the association with atopic history, *J. Clin. Oncol.* 25 (24) (2007) 3644–3648.
- [23] H.W. Verspaget, A.M. van der Zon, Q. Gao, R.A. van Hogezaand, Infliximab for Crohn's disease, *Aliment. Pharmacol. Ther.* 15 (12) (2001) 2041–2042.
- [24] D.P. Fine, Activation of the classic and alternate complement pathways by endotoxin, *J. Immunol. (Baltimore, Md: 1950)* 112 (2) (1974) 763–769.
- [25] N.A. Baig, R.P. Taylor, M.A. Lindorfer, A.K. Church, B.R. Laplant, E.S. Pavey, G.S. Nowakowski, C.S. Zent, Complement dependent cytotoxicity in chronic lymphocytic leukemia: ofatumumab enhances alemtuzumab complement dependent cytotoxicity and reveals cells resistant to activated complement, *Leuk. Lymphoma* 53 (11) (2012) 2218–2227.
- [26] J. Seino, P. Eveleigh, S. Warnaar, L.J. van Haarlem, L.A. van Es, M.R. Daha, Activation of human complement by mouse and mouse/human chimeric monoclonal antibodies, *Clin. Exp. Immunol.* 94 (2) (1993) 291–296.
- [27] P. Koolwijk, J.H. Boot, R. Griep, B.J. Bast, Binding of the human complement subcomponent C1q to hybrid mouse monoclonal antibodies, *Mol. Immunol.* 28 (6) (1991) 567–576.
- [28] Y. Hu, M.J. Turner, J. Shields, M.S. Gale, E. Hutto, B.L. Roberts, W.M. Siders, J.M. Kaplan, Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model, *Immunology* 128 (2) (2009) 260–270.
- [29] Z. Zhang, M. Zhang, C.K. Goldman, J.V. Ravetch, T.A. Waldmann, Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD52 monoclonal antibody, Campath-1H, *Cancer Res.* 63 (19) (2003) 6453–6457.
- [30] S.P. Rao, J. Sancho, J. Campos-Rivera, P.M. Boutin, P.B. Severy, T. Weeden, S. Shankara, B.L. Roberts, J.M. Kaplan, Human peripheral blood mononuclear cells exhibit heterogeneous CD52 expression levels and show differential sensitivity to alemtuzumab mediated cytotoxicity, *PLoS One* 7 (6) (2012) e39416.
- [31] D.M. Reed, K.E. Paschalaki, R.D. Starke, N.A. Mohamed, G. Sharp, B. Fox, D. Eastwood, A. Bristow, C. Ball, S. Vessillier, T.T. Hansel, S.J. Thorpe, A.M. Randi, R. Stebbings, J.A. Mitchell, An autologous endothelial cell/peripheral blood mononuclear cell assay that detects cytokine storm responses to biologics, *FASEB J.* 29 (6) (2015) 2595–2602.
- [32] P.S. Romer, S. Berr, E. Avota, S.Y. Na, M. Battaglia, I. ten Berge, H. Einsele, T. Hunig, Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412, *Blood* 118 (26) (2011) 6772–6782.
- [33] S. Vogel, E. Grabski, D. Buschjager, F. Klawonn, M. Doring, J. Wang, E. Fletcher, I. Bechmann, T. Witte, M. Durisin, B. Schraven, S.M. Mangsbo, K. Schonfeld, N. Czeloth, U. Kalinke, Antibody induced CD4 down-modulation of T cells is site-specifically mediated by CD64(+) cells, *Sci Rep* 5 (2015) 18308.
- [34] D. Yu, J. Leja-Jarblad, A. Loskog, P. Hellman, V. Giandomenico, K. Oberg, M. Essand, Preclinical evaluation of AdVince, an oncolytic adenovirus adapted for treatment of liver metastases from neuroendocrine cancer, *Neuroendocrinology* (2016).
- [35] R. Stebbings, L. Findlay, C. Edwards, D. Eastwood, C. Bird, D. North, Y. Mistry, P. Dilger, E. Liefvooghe, I. Cludts, B. Fox, G. Tarrant, J. Robinson, T. Meager, C. Dolman, S.J. Thorpe, A. Bristow, M. Wadhwa, R. Thorpe, S. Poole, "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics, *J. Immunol. (Baltimore, Md: 1950)* 179 (5) (2007) 3325–3331.
- [36] B. Wolf, H. Morgan, J. Krieg, Z. Gani, A. Milicov, M. Warncke, F. Brennan, S. Jones, J. Sims, A. Kiessling, 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 (3) (2012) 828–837.
- [37] B. Wolf, H. Morgan, F. Brennan, J. Krieg, Z. Gani, S. Jones, A. Kiessling, 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 (1) (2013) 473–475 (discussion 476).
- [38] N.K. Banda, A.K. Wood, K. Takahashi, B. Levitt, P.M. Rudd, L. Royle, J.L. Abrahams, G.L. Stahl, V.M. Holers, W.P. Arend, Initiation of the alternative pathway of murine complement by immune complexes is dependent on N-glycans in IgG antibodies, *Arthritis Rheum.* 58 (10) (2008) 3081–3089.
- [39] R. Malhotra, M.R. Wormald, P.M. Rudd, P.B. Fischer, R.A. Dwek, R.B. Sim, Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein, *Nat. Med.* 1 (3) (1995) 237–243.
- [40] L. Ginaldi, M. De Martinis, E. Matutes, N. Farahat, R. Morilla, M.J. Dyer, D. Catovsky, Levels of expression of CD52 in normal and leukemic B and T cells: correlation with in vivo therapeutic responses to Campath-1H, *Leuk. Res.* 22 (2) (1998) 185–191.
- [41] T. van Meerten, R.S. van Rijn, S. Hol, A. Hagenbeek, S.B. Ebeling, Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity, *Clin. Cancer Res.* 12 (13) (2006) 4027–4035.
- [42] L.E. van der Kolk, A.J. Grillo-Lopez, J.W. Baars, C.E. Hack, M.H. van Oers, Complement activation plays a key role in the side-effects of rituximab treatment, *Br. J. Haematol.* 115 (4) (2001) 807–811.
- [43] Y. Bouhnik, F. Carbonnel, D. Laharie, C. Stefanescu, X. Hebuterne, V. Abitbol, M. Nachury, H. Brixi, A. Bourreille, L. Picon, A. Bourrier, M. Allez, L. Peyrin-Biroulet, J. Moreau, G. Savoye, M. Fumery, S. Nancey, X. Roblin, R. Altwegg, G. Bouguen, G. Bommelaer, S. Danese, E. Louis, M. Zappa, J.Y. Mary, Efficacy of adalimumab in patients with Crohn's disease and symptomatic small bowel stricture: a multicentre, prospective, observational cohort (CREOLE) study, *Gut* (2017).
- [44] M.H. Schiff, G. DiVittorio, J. Tesser, R. Fleischmann, J. Schechtman, S. Hartman, T. Liu, A.M. Solinger, The safety of anakinra in high-risk patients with active rheumatoid arthritis: six-month observations of patients with comorbid conditions, *Arthritis Rheum.* 50 (6) (2004) 1752–1760.
- [45] K. Peppel, D. Crawford, B. Beutler, A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity, *J. Exp. Med.* 174 (6) (1991) 1483–1489.
- [46] P. Rutgeerts, B.G. Feagan, C.W. Marano, L. Padgett, R. Strauss, J. Johanns, O.J. Adedokun, C. Guzzo, H. Zhang, J.F. Colombel, W. Reinisch, P.R. Gibson, W.J. Sandborn, Randomised clinical trial: a placebo-controlled study of intravenous golimumab induction therapy for ulcerative colitis, *Aliment. Pharmacol. Ther.* 42 (5) (2015) 504–514.
- [47] F.F. Yalniz, M. Hefazi, K. McCollough, M.R. Litzow, W.J. Hogan, R. Wolf, H. Alkhateeb, A. Kansagra, M. Damraj, M.M. Patnaik, Safety and efficacy of infliximab therapy in the setting of steroid-refractory acute graft versus host disease, *Biol. Blood Marrow Transplant.* (2017).
- [48] C.H. Polman, P.W. O'Connor, E. Havrdova, M. Hutchinson, L. Kappos, D.H. Miller, J.T. Phillips, F.D. Lublin, G. Giovannoni, A. Wajgt, M. Toal, F. Lynn, M.A. Panzara, A.W. Sandrock, A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis, *N. Engl. J. Med.* 354 (9) (2006) 899–910.
- [49] E.W. Alley, J. Lopez, A. Santoro, A. Morosky, S. Saraf, B. Piperdi, E. van Brummelen, Clinical safety and activity of pembrolizumab in patients with malignant pleural mesothelioma (KEYNOTE-028): preliminary results from a non-randomised, open-label, phase 1b trial, *Lancet Oncol.* 18 (5) (2017) 623–630.
- [50] J.R. Brown, B. Tesar, L. Yu, L. Werner, N. Takebe, E. Mikler, H.M. Reynolds, C. Thompson, D.C. Fisher, D. Neuberg, A.S. Freedman, Obatoclax in combination with fludarabine and rituximab is well-tolerated and shows promising clinical activity in relapsed chronic lymphocytic leukemia, *Leuk. Lymphoma* 56 (12) (2015) 3336–3342.
- [51] M. Vernon, D. Robinson Jr., D. Trundell, J. Ishak, M.H. Jen, J. Brazier, Deriving health utility values from a randomized, double-blind, placebo-controlled trial of siltuximab in subjects with multicentric Castleman's disease, *Curr. Med. Res. Opin.* 32 (7) (2016) 1193–1200.
- [52] S. Yamada, A. Tsuchimoto, Y. Kaizu, M. Taniguchi, K. Masutani, H. Tsukamoto, H. Ooboshi, K. Tsuruya, T. Kitazono, Tocilizumab-induced remission of nephrotic syndrome accompanied by secondary amyloidosis and glomerulonephritis in a patient with rheumatoid arthritis, *CEN Case Rep.* 3 (2) (2014) 237–243.
- [53] S. Sgro, Side-effects of a monoclonal antibody, muromonab CD3/orthoclone OKT3: bibliographic review, *Toxicology* 105 (1) (1995) 23–29.



Supplementary Figure 1. Blood cell viability in a human whole blood loop assay

Freshly acquired whole blood was incubated with the antibodies cetuximab (CET), alemtuzumab (ALM), LPS and PBS in a circulating loop assay (as described in Materials and Methods). The final concentrations of the antibodies in blood are displayed in brackets in the figure [$\mu\text{g/ml}$]. After 4 hours blood samples were collected and further activation was inhibited by mixing the blood with EDTA. Blood was stained for surface markers and viability dye Aqua Zombie (as described in Materials and Methods). The blood cells (B cells, T cells, NK cells, monocytes and granulocytes) were analyzed by flow cytometry ($n=4$) to estimate percent of cell populations out of all events (left) and MFI of Aqua Zombie staining (right), where A shows the results for lymphocytes and B for granulocytes and monocytes. Fold change of each group over mean value of zero samples is presented along with SD.