

Research Article

Characterisation of Immunostimulation by Trastuzumab in Responders and Non-Responders

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Abstract

It is often difficult to predict the likelihood of immunostimulation by monoclonal antibodies and identify who is at risk based on preclinical results. We explored the feasibility of an *ex vivo* cytokine release assay to detect trastuzumab-induced immunostimulation by correlating *in vivo* and *ex vivo* results. Also the effect of trastuzumab on human umbilical vein endothelial cells (HUVECs) was studied.

Healthy male volunteers were selected from a previous clinical trial where they had been exposed to trastuzumab. Five donors had experienced fever (responders) and six had not (non-responders). Additionally, ten male donors were selected from a healthy, trastuzumab-naïve population. Heparinised whole blood samples were collected and incubated for 24 h with 0.2 mg/mL trastuzumab. HUVECs were grown under a constant laminar flow for 7 days, followed by incubation with 2 mg/mL trastuzumab alone or in combination with TNF- α .

Trastuzumab induced a very mild IL-6 response in the supernatant of the cell cultures, which was more pronounced in the responders. A correlation was detected between the *in vivo* maximum body temperature (T_{max}) and the IL-6 response with Pearson's $r = 0.83$ (95% confidence-interval: 0.45 – 0.95), $p = 0.0017$. Virtually no TNF- α response was observed, although a linear relationship to T_{max} was suggested in responders: $r = 1.00$ (0.93 - 1.00), $p = 0.0004$. No increased conversion of C5 to C5a was observed. The trastuzumab-naïve population did not show any cytokine or complement response. Trastuzumab did not increase E-selectin expression in HUVECs.

Individuals who demonstrate clinical signs of trastuzumab-associated immunostimulation show an elevated *ex vivo* IL-6 response to trastuzumab. Both IL-6 and TNF- α responses are correlated with T_{max} , yet do not explain the full clinical picture.

INTRODUCTION

Some monoclonal antibodies (MAbs) are known to induce an inflammatory response during or shortly after administration. The most common causative MAbs include rituximab (incidence 77%), alemtuzumab (>80%), and trastuzumab (40%) [1]. Frequently, the severity of the inflammatory reactions wanes with each subsequent infusion and the majority of patients experiences a reaction upon the first administration only. Although these events are usually only mildly discomforting to patients and easily manageable with anti-inflammatory agents, severe reactions occur in up to 1% of treated patients, which can even be fatal as a result of uncontrolled activation of the immune system occurs, similar to sepsis and anaphylaxis [1].

It is often difficult to predict the likelihood of such reactions and identify who is at risk. Over recent years, especially after the disastrous TGN1412 case, progress has been made in developing *ex vivo* and *in vitro* models that have increased our knowledge on this topic [2,3]. These models typically utilize primary

human immune cells or (human) cell lines transfected with innate immune receptors that are thought to play a role. Some models incorporate other cell types such as endothelial cells or the complement system [3]. An EMA workgroup [4], recognised cytokine release assays as a valuable tool, yet also identified numerous sources of variability, including donor selection, type of test matrix, and read-out parameters, adding to the complexity of designing and interpreting such assays.

For rituximab and alemtuzumab, which both target lymphocytes, *in vitro* test systems that mimic the clinical response are available [5,6]. Also, the T-cell stimulation by TGN1412 can be predicted by *ex vivo* models [7,8]. However, far less efforts have been devoted to trastuzumab, and some of the applied models fail in detecting a response to trastuzumab [9].

We explored the feasibility of an *ex vivo* cytokine release assay in whole blood to detect trastuzumab-induced inflammatory responses in a group of healthy donors. The donor population was divided into a group of individuals who had demonstrated clinical

signs of an inflammatory reaction induced by *in vivo* trastuzumab administration (responders) and a group of individuals who had not (non-responders). This allowed the correlation of the outcome of an *ex vivo* whole blood cytokine release with the observed clinical response following trastuzumab administration in the same person. Whereas the *ex vivo* cytokine release assay reflects the drug response of circulating immune cells, the *in vivo* inflammatory response is the result of the interaction between circulating immune cells and other immunological organs such as the liver and endothelium. In co-cultures, the combination of peripheral blood mononuclear cells (PBMCs) and endothelial cells has been shown to either be critical for the development of or to enhance the *ex vivo* response towards an exogenous stimulus [3,10]. Therefore, we also tested direct endothelial activation by trastuzumab in an 'artificial vessel' with a quiescent monolayer of endothelial cells grown inside that closely resembles the physiological situation *in vivo*.

METHODS

Clinical results

Clinical data were collected in a randomised trial of parallel design, where healthy male volunteers received a single intravenous dose of either placebo or trastuzumab [11]. For the purpose of this analysis, participants who received the registered form of trastuzumab (Herceptin®, hereafter referred to as "trastuzumab") ($n = 46$) were compared with participants who received placebo ($n = 8$). The investigated dose of trastuzumab was 6 mg/kg (6.44 mg/kg based on actual protein content). A subgroup analysis was performed on responders and non-responders, i.e. subjects who experienced fever (body temperature $\geq 38^\circ\text{C}$) or not. Adverse events that were reported within the first 24 h after administration were analyzed, as were vital signs and laboratory assessments (haematology and clinical chemistry) during the same period, and details on the exposure to trastuzumab (maximum plasma concentration and area under the curve [AUC]).

Ex vivo stimulation

For the *ex vivo* stimulation, healthy male volunteers were recruited from the cohort of 46 subjects who were previously exposed to trastuzumab (non-naïve population) and from the community (naïve population). Subjects were selected based on the severity of the inflammatory reaction upon *in vivo* exposure. This resulted in a group size of 11 for the non-naïve population, of whom 5 had experienced fever (responders), and 6 not (non-responders). The naïve population included 10 healthy male volunteers. The *ex vivo* stimulation was executed two years after the clinical trial was completed.

Heparinised whole blood samples (18 mL, BD Vacutainer, Becton Dickinson, Breda, The Netherlands) were collected and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2 , before flash-freezing the supernatant for subsequent analysis. Tested conditions were 0.2 mg/mL trastuzumab (Herceptin®), 2 ng/mL lipopolysaccharide (LPS) gel extracted from *E. coli* serotype O111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA), blank (unstimulated control). The trastuzumab concentration is similar to the maximum plasma concentration

observed after an *in vivo* dose of 6 mg/kg. LPS concentration is based on the EC90 of maximum TNF- α release and serves as a positive control. Conditions were made in RPMI with 25 mM HEPES and L-glutamine (GIBCO products from Life Technologies Europe, Bleiswijk, the Netherlands). Final dilution of whole blood required to achieve the tested concentrations was 1%.

Inflammatory cytokines, IL-6 and TNF α , were measured by multiplex electrochemiluminescence using a human ultra-sensitive cytokine panel (Meso Scale Discovery, Rockville, Maryland, USA). C5a, a late product of complement activation, was measured by ELISA (eBioscience, San Diego, California, USA).

Endothelial stimulation

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords by 0.25% trypsin treatment. HUVECs were cultured in EGM2 medium (Lonza, Basel, Switzerland) containing antibiotics and antimycotics (Life technologies) at 37°C and 5% CO_2 and used for experiments on passage 3-4. To obtain a confluent, quiescent endothelial monolayer that closely resembles the physiological situation, HUVECs were grown under a constant laminar flow of 10 dyne/cm² for 7 days using a closed perfusion Ibidi flow system (ibiTreat 0.4 μ -Slide VI Luer) (Ibidi, Martinsried, Germany).

The endothelial monolayer was incubated with TNF- α (R&D, Abingdon, UK) or trastuzumab in medium or in medium only (blank control) for 5 h. Tested concentrations were 10, 1, 0.1 ng/mL for TNF- α and 2 mg/mL for trastuzumab (comparable to concentration in infusate).

After stimulation HUVECs were fixed in 4% paraformaldehyde in HBSS for 10 min at room temperature, washed twice with 1% BSA in HBSS, and blocked with 5% normal goat serum (Dako, Carpinteria, California, USA) in HBSS for 30 min. Cells were permeabilised with TRITON X-100 (Merck, Darmstadt, Germany). Cultures were incubated for at least 60 minutes at 4°C with 10 $\mu\text{g}/\text{mL}$ TRITC-labelled wheat germ agglutinin (WGA) (Sigma-Aldrich, 1:100), Hoechst 33258 (Sigma-Aldrich, 1:5000), and a mouse anti-human E-selectin antibody (R&D, 1:100) diluted in HBSS + 1% BSA. Next, the cell layer was washed three times with HBSS + 1% BSA, counterstained with an Alexa488-coupled anti-mouse-IgG (Thermo Fisher, Waltham, Massachusetts, USA, 1:500), washed again, and imaged using a Leica SP5 confocal microscope.

Confocal 12-bit gray-scale axial image stacks (xyz dimensions, $0.08 \times 0.08 \times 0.13 \mu\text{m}$) that covered $6724 \mu\text{m}^2$ of surface area per image and a height of 5 to $10 \mu\text{m}$ above the EC nuclear plane were recorded using LAS-AF image software (Leica). The image stacks were analysed with the public domain National Institutes of Health IMAGE program (<http://rsb.info.nih.gov/nih-image>). Cross-sections of the cell-layer were generated using this software.

Statistical analysis

Data are summarized as mean (standard deviation) or number (percentage), unless otherwise specified. Testing for linear relationships was done by calculating the Pearson product moment correlation coefficient, r . P-values and 95% -confidence

intervals for the true population value, ρ , were obtained under the assumption of normal distribution. Because of the relatively small sample size, Spearman's rank correlation test was also performed. Fisher transformation was used to estimate the 95%-confidence interval of the corresponding coefficient, r_s . All analyses were performed with R (v2.15.2, R Foundation for Statistical Computing, Vienna, Austria, 2012 [R Development Core Team, 2012]).

RESULTS

Clinical response

Following trastuzumab administration 9 (19.6%) subjects developed fever (responders) (Table 1). Maximum body temperature (T_{max}) was reached at 4.7 (2.6) h after the start of infusion. Seven subjects had a $T_{max} \geq 38.5^\circ\text{C}$, and four $\geq 39.0^\circ\text{C}$.

Influenza-like symptoms (rigors, nausea, myalgia, fatigue) were reported by 5 subjects, of whom 1 without fever.

Leucocytes were observed to increase by 39% (28) at 24 h, predominantly neutrophils (increase 101% [57]), accompanied by a decrease in lymphocytes of 35% (14). These changes were more prominent in subjects with influenza-like symptoms or fever (Figure 1). There were no differences between both subgroups in the maximum plasma concentration (C_{max}) and area under the curve (AUC) for trastuzumab.

Ex vivo response

Incubation of whole blood with 0.2 mg/mL trastuzumab induced a very mild IL-6 response, but virtually no TNF- α response (Table 2). The increase in IL-6 was more pronounced in the responders than the non-responders, whereas this difference

Table 1: Population characteristics.

Parameter	Placebo (n = 8)	all (n = 46)	Trastuzumab	
			non-responders (n = 37)	responders (n = 9)
Age (year)	25.3 (3.6)	24.1 (5.8)	23.2 (5.0)	27.8 (7.8)
Height (cm)	182 (7)	182 (6)	182 (6)	182 (6)
Weight (kg)	76.3 (12.6)	77.1 (10.2)	77.3 (10.2)	76.5 (10.6)
BMI (kg/m ²)	23.1 (3.3)	23.2 (2.7)	23.2 (2.8)	23.2 (2.8)
t = 0 h				
Leucocytes (10 ⁹ /L)	6.0 (1.8)	5.4 (1.2)	5.4 (1.1)	5.6 (1.6)
Neutrophils	3.5 (1.3)	2.8 (0.8)	2.7 (0.6)	3.1 (1.3)
Lymphocytes	1.8 (0.5)	1.9 (0.6)	1.9 (0.6)	1.8 (0.3)
Monocytes	0.50 (0.17)	0.46 (0.14)	0.46 (0.14)	0.44 (0.13)
Eosinophils	0.17 (0.13)	0.24 (0.25)	0.26 (0.27)	0.17 (0.10)
Basophils	0.03 (0.03)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
t = 24 h				
Leucocytes (10 ⁹ /L)	6.0 (1.1)	7.3 (1.3)	7.1 (1.4)	8.2 (0.7)
Neutrophils	3.4 (0.8)	5.4 (1.4)	5.1 (1.4)	6.5 (0.8)
Lymphocytes	1.9 (0.4)	1.2 (0.4)	1.2 (0.4)	1.1 (0.2)
Monocytes	0.53 (0.12)	0.43 (0.16)	0.44 (0.16)	0.39 (0.17)
Eosinophils	0.16 (0.05)	0.27 (0.30)	0.29 (0.32)	0.17 (0.12)
Basophils	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)
Ratio 0/24 h				
Leucocytes	0.99 (0.11)	1.39 (0.28)	1.36 (0.27)	1.54 (0.31)
Neutrophils	1.04 (0.17)	2.01 (0.57)	1.94 (0.55)	2.28 (0.60)
Lymphocytes	0.92 (0.09)	0.65 (0.14)	0.66 (0.15)	0.59 (0.06)
Monocytes	1.01 (0.16)	0.97 (0.29)	0.98 (0.28)	0.92 (0.36)
Eosinophils	1.04 (0.17)	1.17 (0.32)	1.21 (0.32)	1.00 (0.27)
Basophils	0.92 (0.57)	0.94 (0.45)	0.91 (0.45)	1.03 (0.48)
T_{max} (°C)	36.9 (0.2)	37.6 (0.7)	37.3 (0.4)	38.8 (0.4)
C_{max} (µg mL ⁻¹)	-	185 (34)	185 (33)	184 (39)
AUC_{inf} (day µg mL ⁻¹)	-	1609 (224)	1635 (229)	1503 (174)

Data are presented as mean (standard deviation).

Leucocytes at 24 h post administration and corresponding ratio were not available for 3 placebo subjects.

AUC_{inf} , area under the (trastuzumab plasma concentration-time) curve extrapolated until infinity; C_{max} , maximum trastuzumab plasma concentration; T_{max} , maximum body temperature.

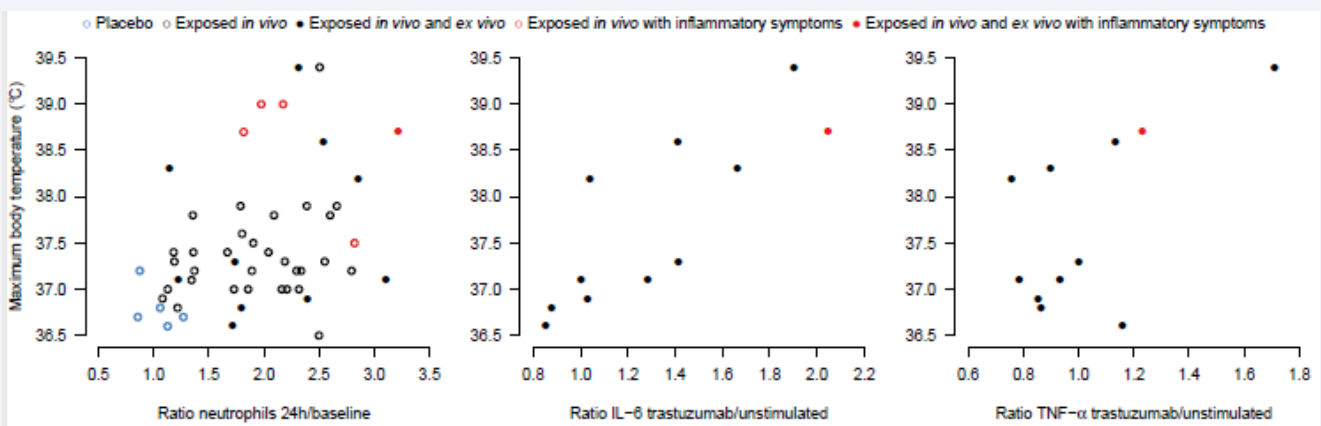


Figure 1 *In vivo* and *ex vivo* response. Ratio of neutrophil concentration at 24 h post administration over pre-dose value for total population exposed *in vivo* and ratios of trastuzumab *ex vivo* stimulated IL-6 and TNF- α levels over background (unstimulated level) for a subset of responders (maximum body temperature $\geq 38^{\circ}\text{C}$) and non-responders, who were previously exposed *in vivo*.

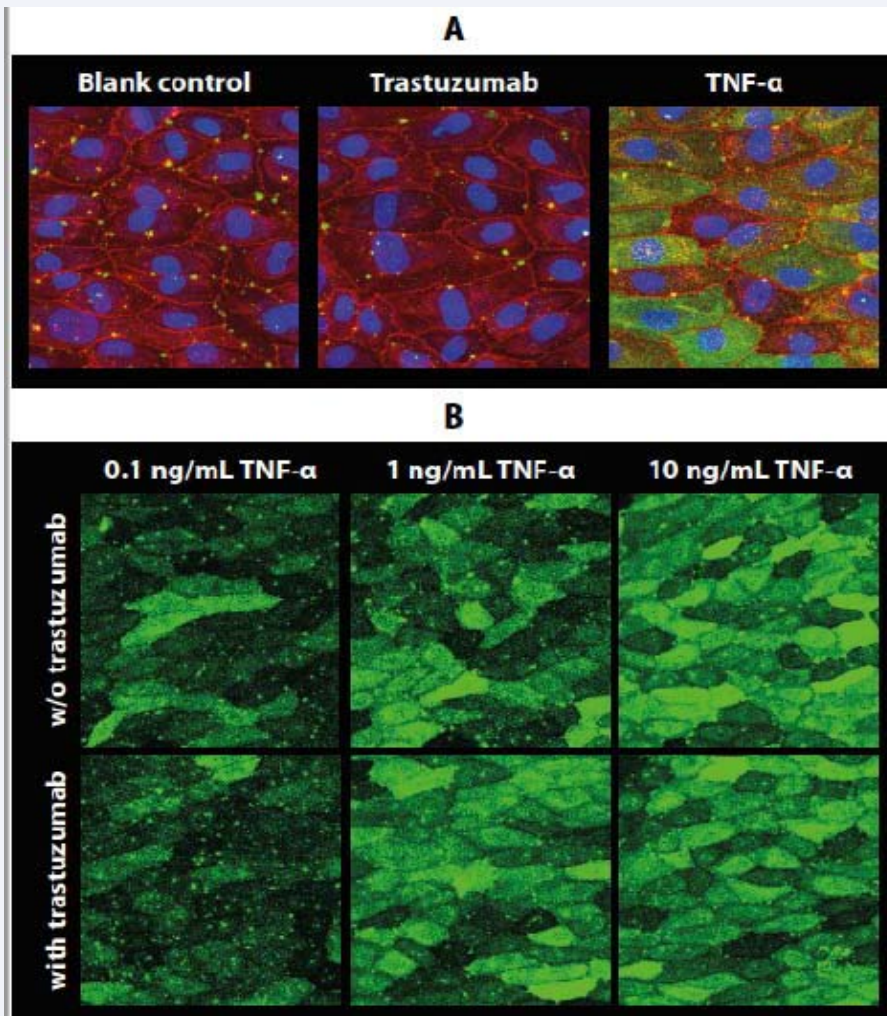


Figure 2 Endothelial stimulation. (A) After five hours incubation with medium alone, trastuzumab, or TNF- α human umbilical vein endothelial cells (HUVECs) were stained with Hoechst 33258 (nucleus, blue), TRITC-labelled wheat germ agglutinin (WGA) binding the carbohydrate N-acetyl galactosamine on the cell surface (red), and a mouse anti-human E-selectin antibody (green). (B) Results after HUVECs were incubated with different concentrations of TNF- α for five hours in the presence or absence of trastuzumab; only the green Alexa488 is displayed for clarity.

Table 2: *Ex vivo* stimulation.

Ratio	Naïve (n = 10)	all (n = 11)	Non-naïve	
			non-responders (n = 6)	responders (n = 5)
IL-6	1.06 (1.40)	1.26 (1.36)	1.06 (1.23)	1.57 (1.31)
TNF- α	1.11 (1.30)	1.00 (1.27)	0.92 (1.15)	1.10 (1.37)
C5a	1.03 (1.13)	1.00 (1.06)	1.02 (1.08)	0.97 (1.03)

Geometric mean (standard deviation) of ratios of stimulated IL-6, TNF- α , and C5a levels over unstimulated (background) levels for the trastuzumab-naïve and non-naïve population.

was much smaller for TNF- α . The trastuzumab-naïve population did not show an IL-6 or a TNF- α response upon stimulation. No increased conversion of C5 to C5a was observed after trastuzumab treatment, compared to the unstimulated control in any (sub) population (Table 2).

A strong positive and statistically significant correlation was detected between T_{max} and the IL-6 response (Figure 1, correlation coefficient, $r = 0.83$ [0.45 – 0.95], $p = 0.0017$). Spearman's rank correlation test gave $r_s = 0.91$ (0.59 – 0.98), $p = 0.0001$. A similar linear relationship was suggested for TNF- α only in the responders (Figure 1, $r = 1.00$ [0.93 – 1.00], $p = 0.0004$, $r_s = 1$), although it should be noted that the ratios of stimulated over unstimulated (background) levels ranged from ~ 0.8 to ~ 1.2 for the majority of subjects, which does indicate no or minimal TNF- α release upon stimulation. A correlation was also observed between the *ex vivo* IL-6 and TNF- α responses: $r = 0.90$ (0.33 – 0.99), $p = 0.0146$; $r_s = 0.85$ (0.41 – 0.97), $p = 0.0341$. The changes in circulating leucocytes following *in vivo* trastuzumab exposure did not correlate with any of the *ex vivo* responses.

Endothelial stimulation

Trastuzumab did not increase E-selectin expression in HUVECs, whereas TNF- α dose-dependently increased E-selectin expression (Figure 2). The combined stimulation (trastuzumab + TNF- α) did not reveal an additive or potentiating effect from trastuzumab.

DISCUSSION

Following exposure to trastuzumab, approximately 20% of the healthy volunteers developed fever with or without accompanying influenza-like symptoms, which is half of that commonly reported in patients [1]. This may be the result of chance as another trial in healthy volunteers also found a lower incidence [12], whereas a trial in healthy Japanese males reported an incidence of $\sim 40\%$ [13]. Batch differences could also have caused the disparity. Nonetheless, virtually all exposed participants showed an increase in neutrophils, paralleled by a decrease in lymphocytes, indicating that true non-responsiveness in terms of trastuzumab-induced immunostimulation is probably rare.

Although the occurrence of fever was associated with greater changes in neutrophil and lymphocyte counts, the opposite was not true. This suggests that more than one pathway is involved in the inflammatory reaction, which contributes to phenotypic

variation. Therefore, the subgroups (with or without fever, termed responders and non-responders) were studied for differences that could explain the observed variability in response. In both groups comparable exposure to trastuzumab, specifically maximum plasma concentration (C_{max}), was seen. Differences in any of the baseline laboratory and other parameters were not detected. Unfortunately, no additional material from the clinical trial was available to further characterise the subpopulations or further delineate their response with biomarkers that might provide mechanistic insight, *e.g.* (baseline) cytokine profiles.

Exposing a number of subjects from each subgroup to trastuzumab *ex vivo* revealed a difference in the responsiveness. The release of IL-6 was greater in responders than in the non-responders, which was also true for TNF- α , albeit the difference in TNF- α release was less pronounced. The IL-6 response upon *ex vivo* stimulation correlated with the maximum body temperature (T_{max}) seen clinically after trastuzumab administration, as did the TNF- α results, but only in the responders. This suggests both IL-6 and TNF- α release are involved in the production of fever. IL-6 correlated with T_{max} in the entire population exposed to trastuzumab *in vivo*, whereas TNF- α showed a linear relationship with T_{max} only within the responders. A relationship between changes in leucocytes and *ex vivo* IL-6 or TNF- α results could not be detected. Thus, the release of these two cytokines from circulating immune cells explains only certain aspects of the clinical picture.

Despite its almost perfect correlation, the role for TNF- α remains more controversial since the majority of individuals had ratios between 0.8 and 1.2, which is usually not considered a response to a stimulus. However, the 24 h duration of the incubation may have been suboptimal for detecting the peak values of this cytokine. We have data demonstrating that the measured TNF- α in the supernatant decreases with prolonged incubation, even after 4 h (data on file). Therefore, the presented TNF- α results could be a relative response rather than an absolute figure.

Because the *ex vivo* study was conducted *after* the *in vivo* study, carry-over effects cannot be excluded, but these are unlikely to have influenced the results. Based on available PK data, trastuzumab is completely cleared from the body two years after administration and thus could not have interfered with the *ex vivo* stimulation. The finding that most inflammatory reactions occur during the first infusion points toward the innate immune system in mediating the response. Furthermore, the *ex vivo* IL-6 response correlated well with the T_{max} observed two years earlier, which also suggests that an individual's responsiveness to trastuzumab does not change over time.

Complement activation was not observed by us upon *ex vivo* stimulation in any of the populations or subgroups. Additionally, trastuzumab did not activate endothelial cells, nor did trastuzumab potentiate TNF- α mediated endothelial activation, which is in agreement with a report by Findlay and colleagues [14]. However, these findings do not preclude the possibility of augmentation of the initially limited inflammatory (IL-6 & TNF- α) response when trastuzumab engages a particular target present on for example endothelium, or the activation of endothelial cells by the cytokines released from circulating immune cells.

Minor responses to trastuzumab were previously elicited in an *ex vivo* model using leucocytes and autologous platelet-poor plasma on top of a HUVEC layer [8], but were not observed in a model that used PBMCs in combination with autologous endothelial cells [9]. Similarly, trastuzumab immobilised onto a plastic surface induced cytokine release from PBMCs, whereas no reaction occurred when PBMCs were exposed to soluble trastuzumab [14].

The method of culturing the endothelial cells in previous studies may also have influenced their results. Cultured endothelial cells are phenotypically different compared to *in vivo* endothelial cells. One of the key factors for this difference is the absence of flow in traditional endothelial cell culture. Since shear stress leads to a more quiescent, less inflammatory and less proliferative state [15-17], it thereby better mimics the healthy *in vivo* situation. Culturing under flow, or perhaps even under flow in 3D cultured vessels, which are currently under development, may therefore improve these studies.

Remarkably, trastuzumab is sometimes used as a negative control in (*ex vivo*) immunostimulation experiments, which may be related to the small number of donors included in the published experiments. Our data suggest that trastuzumab is capable of eliciting an immune response in susceptible humans and therefore should not be used as a negative control in immunostimulation testing.

Main strength of our study is the correlation of known clinical responses with results from *ex vivo* whole blood stimulations within the same individual. Based on *in vivo* data we were able to identify 'responders' and 'non-responders' and indeed observed differences in the reactivity of their circulating immune cells to *ex vivo* trastuzumab exposure. This in contrast to the naïve population ($n = 10$), where no response to trastuzumab could be found, which should not be surprising with a pre-selection incidence of ~20% for fever. Unfortunately, the difference between responders and non-responders in *ex vivo* IL-6 and TNF- α release was too small for this type of test to serve as a general screening assay.

For two MAbs, alemtuzumab and rituximab, the pathogenesis of the inflammatory response has been elucidated. In both cases different mechanism were involved in the immunostimulation; cytokine release (IL-6, TNF- α , and IFN- γ) following alemtuzumab was found to result from an interaction with natural killer (NK) cells via Fc γ -receptors [5,18], whereas rituximab induced complement activation and stimulated MIP-1 β secretion from NK cells [6,19]. Lack of knowledge on the exact mechanism of trastuzumab associated immunostimulation does not facilitate an easy interpretation of our results or the development of a discriminating (*ex vivo*) assay. Nonetheless, by simultaneously studying *in vivo* and *ex vivo* responses the involved pathways may be unravelled.

In conclusion, individuals who demonstrate clinical signs of trastuzumab associated immunostimulation show an elevated *ex vivo* IL-6 response to trastuzumab. Both IL-6 and TNF- α response are correlated with the observed *in vivo* maximum body temperature; yet do not explain the full clinical picture. It remains difficult to predict a clinical response in an individual

or to separate responders from non-responders based on *ex vivo* results.

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