Ex-vivo assays of dendritic cell activation and cytokine profiles as predictors of in vivo effects in an anti-human CD40 monoclonal antibody ChiLob 7/4 Phase I trial.

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Abstract

Immunostimulatory antibodies entering the clinic create challenges not only in terms of pharmacodynamics for monitoring anticipated mechanism but also in pre-determining cytotoxicity. We demonstrate the use of ex-vivo whole blood samples to predict the activation requirements, cytokine signature and adverse events of an anti-human-CD40 chimeric IgG1 antibody, ChiLob7/4. Assessments were initially undertaken on human myeloid (mDC1) and plasmacytoid (pDC) dendritic cells where an absolute need for cross-linking was demonstrated through the up-regulation of activation markers CD83 and CCR7. Subsequent cytokine secretion evaluations of ex-vivo whole blood showed the cross-linked antibody induced increases in MIP1β, IL-8, IL-12, TNFα, and IL-6. This cytokine signature compared favorably to the TLR ligand LPS, where levels of TNFα and IL-6 were significantly higher, suggesting a less intense pro-inflammatory response and possible modified cytokine-release-syndrome when used in human trials. Following first-in-human use of this agent within a dose escalation study, in-vivo evaluations of DC activation and secreted cytokines closely matched the predetermined immunomonitoring endpoints. Patients showed a comparable pattern of MIP1β, IL-8, and IL-12 secretion but no TNFα, and IL-6 was identified. Mild symptoms relating to a cytokine-release-syndrome were seen at an equivalent dosage to that observed for DC activation and cytokine release. In summary, ChiLob7/4 induces a distinctive pattern of DC activation and cytokine secretion in ex-vivo assays that can be predictive of in-vivo responses. Such pre-clinical approaches to monoclonal antibody evaluation may inform both the starting dosages and the anticipated cytokine release events that could occur, providing a valuable adjunct for future first-in-human assessments of immunostimulatory antibodies.
Introduction

Immunotherapy has advanced rapidly over recent years with several monoclonal antibodies entering the clinic for treatment of autoimmunity and cancer. In particular, immunostimulatory antibodies are showing promise in enabling an individual’s own immune repertoire to mount a successful immune response specific against cancer; however, their use comes with a high risk of serious cytotoxicity as was notably encountered with the TeGenero TGN1412, anti-CD28 super-agonist antibody in which a near fatal cytokine storm was experienced by all participants (1). The dilemma that researchers face is the ability to design and pre-determine whether an immunostimulatory antibody is able to activate the cells they are targeting without triggering a cytotoxic storm. We have therefore investigated whether ex-vivo assays are predictive of the ability of an agonistic anti-CD40 monoclonal antibody, ChiLob 7/4, to activate dendritic cells (DC) and monitored for the simultaneous expression of cytokines.

CD40 is a 48kDa, type I membrane protein belonging to the tumor necrosis factor receptor (TNFR) super-family. It is expressed primarily on antigen presenting cells (APCs) such as dendritic cells, B lymphocytes and monocytes but, it has also been found on endothelial and epithelial cells (2,3). The natural ligand for CD40, CD154 (CD40L) is a member of the tumor necrosis factor (TNF) family and exists in both soluble and membrane bound forms where it appears to form a trimeric protein structure (4). CD154 is expressed predominantly on activated CD4+ T lymphocytes. An adaptive immune response is triggered when resting CD4+ T cells are activated following the recognition of MHC-antigenic peptide complex presented by DC in concert with co-stimulatory molecules. Once activated, CD4+ T cells proliferate and further induce immune and inflammatory responses via the secretion of cytokines and the expression of a variety of cell surface molecules that include CD154. It is through the CD154-CD40 interaction that CD4+ T helper cells signal back to the DC leading to their maturation and licensing to activate CD8+ cytotoxic T cells (5-8). Preclinical in-vivo work in syngeneic mouse models of malignancy suggest that one of the most potent therapeutic effects of agonistic anti-CD40 antibodies relates to their ability to effectively license or condition DC, to completely bypass the need for specific CD4+ T cell help to directly activate CD8+ cytotoxic T cell precursors (9,10).

The important functional role of CD154-CD40 interaction in-vivo has made CD40 an attractive target for cancer immunotherapy and several clinical trials have been conducted with different agonistic anti-CD40
antibodies (11-13). More recently the potential of anti-CD40 antibody therapy to engage non T cell dependent immune effector responses has been highlighted through studies on pancreatic cancer (14). Comprehensive human and murine studies support the role of antibody-activated myeloid cells within the tissue stroma as being crucial to the successful outcome of the treatment (15).

As part of a Phase I clinical trial (NCT01561911), we have monitored the effects of ChiLob 7/4 on the activation of human myeloid DC (mDC1) and plasmacytoid DC (pDC) and monitored the associated plasma cytokine profiles (16). We have shown that the effects of ChiLob 7/4 on DC activation and the cytokine release profiles using ex-vivo whole blood stimulation assays are reflective of changes seen in the participants on the ChiLob 7/4 Phase I study. Furthermore, we observed a mild cytokine release syndrome at the first observable biological effect level using in vivo end point assessments that were consistent with our pre-clinical evaluations. The activation of DC as measured by CD83 and CCR7 up-regulation and the release of MIP1β and IL-12 suggest the initiation of the desired Th1 type immune environment without the presence of an uncontrolled cytokine storm.

**Materials and Methods**

**Patient Treatment and Monitoring**

Patients with CD40-positive malignancies who were refractory to conventional anti-cancer treatments were enrolled into the Phase I dose-escalation study to evaluate the chimeric anti-CD40 monoclonal antibody (mAb), ChiLob7/4. The study protocol and patient consents were approved by the Cancer Research UK (CRUK) Central Institutional Review Board (CIRB) and the local research and ethics committee (LREC). The primary objective of the study was to establish the safety and tolerability of the ChiLob 7/4 mAb. All patients had tumour assessments by computerised tomography (CT) scan within 4 weeks prior to treatment. The dosing schedule evaluated cohorts of 3 patients at the dose levels of 0.5mg (cohort 1), 1.6mg (cohort 2), 5mg (cohort 3), 16mg (cohort 4), 50 mg (cohort 5) and 160mg (cohort 6) given by weekly intravenous (IV) infusion over a four week period. Twenty one patients were recruited into the study and the demographics of these are shown in Supplementary Table 1. Patients received 4 weekly IV infusions of the chimeric IgG1 ChiLob7/4 mAb at study days 1, 8, 15, 22 with follow up at day 56. Total infusion times for the 6 escalating doses were 30,30,30,71,139 and 273 minutes respectively. Premedication with oral paracetamol and IV chlorpheniramine (10mg) was administered as standard. IV hydrocortisone was administered if signs of a cytokine release syndrome were noted.
Exceptions to this schedule were noted within cohort 4 at the 16mg dose level where adverse reactions potentially attributable to a cytokine release syndrome (CRS) were noted (Supplementary Table 2). The initial patient (subject 10) of this cohort developed a probable Grade 2 non serious CRS reaction on the first dose, requiring IV steroid treatment and a 50% reduction in infusion rate through to completion. Due to the presence of an adverse event within a cohort the trial protocol required that a further patient was recruited to this cohort, before escalating the dose for cohort 5. This individual proceeded through the second infusion with no CRS but had a milder episode during infusions 3 and 4 that were treated similarly to the initial adverse reactions. The second patient (subject 11) in this cohort had a probable Grade 2 non serious CRS reaction on the first dose but following steroids and 50% rate reduction completed the infusion. No further CRS events occurred during the 3 additional infusions. The third patient (subject 12) of this cohort did not experience any probable CRS events during the treatment period. The last patient (subject 13) in this cohort experienced a probable CRS event on the first infusion which was treated with IV steroids and a 50% rate reduction through to completion. No further CRS events occurred during the 3 additional infusions. Following on from this experience the premedication was adjusted to include IV hydrocortisone prior to the first infusion for cohort 5 (50mg) and cohort 6 (160mg). No further CRS events were recorded from these 5 individuals.

Sample collection

Anti-coagulated EDTA (ethylenediaminetetraacetic acid) whole blood samples were collected following informed consent from patients enrolled on the Phase I study, ethically approved by the National Research Ethics Service, UK and conducted in accordance with the Declaration of Helsinki. Samples were taken at pre-infusion and at various time-points post-infusion, and analysed immediately by flow cytometry. Samples for cytokine analysis were centrifuged at 300g for 15 minutes and the plasma removed and stored at -70°C. Whole blood samples from fully anonymous, consented healthy subjects were used for the ex-vivo assays.

Ex-vivo whole blood stimulation assay

Ex-vivo whole blood stimulation assays were set up within 1 hour of blood collection from healthy subjects. One ml of the anti-coagulated whole blood was stimulated with ChiLob7/4 (+/- cross-linking), with either no additional ligand, or with the addition of Toll-like receptor (TLR) ligands and incubated at 37°C, 5%CO2. ChiLob 7/4 was cross-linked using a 1:1 ratio of antibody to goat anti-human IgG Fc-fragment specific antibody (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) and incubated for 30 minutes at room temperature in PBS. The TLR
ligands used as established DC activators were unmethylated cytosine and guanine oligodeoxynucleotide 5'-
gggggACGATCGTggggg-3' (CpG2216) (MWG Biotech) and lipopolysaccharide (LPS) (Sigma-Aldrich Co. Ltd, UK). At the end of 4 hours incubation, 300μl samples were removed for flow cytometry analysis. A further 500μl of each sample was removed and centrifuged at 300g for 15 minutes. The plasma was removed and stored at -70oC.

**Flow Cytometry Reagents**

The following directly conjugated mAb were used: anti-CD1c-(BDCA-1)-PE, anti-CD303-(BDCA-2)-FITC and anti-IL-12 APC from Miltenyi Biotec (Bergisch Gladbach, Germany), anti-CD14-PE-Cy5 and anti-CD19-PE-Cy5 from Beckman Coulter (Fullerton, CA), anti-CD45-(APC-Cy7), anti-CD83-APC, and anti-MIP-1β-APC from BD Biosciences, (Oxford, UK). FACS Lysing solution and Fix Perm kit from BD Bioscience, and Fixation and Dead Cell Discriminator Kit from Miltenyi Biotec were used according to the manufacturer’s instructions.

**DC flow cytometry analysis**

300μl of anti-coagulated blood samples were labelled with single-fluorochrome conjugated antibodies recognising cell surface markers and dead cell discriminator and incubated on ice for 10 minutes under a 60W bulb. Antibodies conjugated to tandem-dyes were then added and the tubes incubated for a further 10 minutes on ice in the dark. Red blood cell lysis was performed using BD FACS lysing solution, followed by 3 washes in wash buffer (PBS/BSA/azide) with the final pellet resuspended in 300μl wash buffer, 150μl fix buffer and 5μl discriminator stop reagent. Samples were acquired using the BD FACS Canto II, and analysed using the FACS Diva software. The gating strategy used was as reported (17). Briefly, leukocytes were gated using a side-scatter versus CD45 gate. This population was assessed for CD19/CD14 expressing cells and high side scatter, to gate out the unwanted populations of B cells, monocytes and granulocytes respectively. The remaining cells were gated (P1 on Fig 1a), and the BDCA-1 or BDCA-2 positive cells were identified as mDC-1 and pDC respectively. Changes in their level of CD83 and CCR7 expression were monitored to determine activation status.

**Luminex® cytokine detection**

All cytokine analyses were performed on frozen plasma samples. Luminex human custom 10-plex kits (for IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFNγ, TNFα, MIP-1β and MIP-1α) were purchased from Invitrogen Life Sciences. The validation of this 10-plex custom kit has been reported previously (18). All reagents were provided
with the kit and prepared according to the manufacturers’ protocol booklet. The assays were performed in 96-well filter bottom plates and analyzed on the Luminex xMAP 100 system (Luminex Corp., USA), using StarStation software version 2.0 (Applied Cytometry Systems). Each sample was run in duplicate and the mean concentration of each analyte was calculated using a 4 or 5-parameter logistic fit curve generated from the standards.

**Intracellular cytokine analysis**

For intracellular cytokine measurements, stimulation assays were set up as described above. For intracellular DC cytokine analysis, Brefeldin A was added after 1 hour stimulation and the tubes incubated for a further 4 hours. Cells were labeled for cell surface markers as described, followed by red blood cell lysis and washing. The final cell pellet was resuspended in 100μl BD fix/perm buffer and incubated on ice for 20 minutes in the dark. After washing twice with perm wash buffer, the antibodies against the intracellular cytokines were added and the tubes incubated in the dark at room temperature for 20 minutes and then washed once in perm wash followed by one wash with FACS buffer. The samples were analysed on a BD FACS Canto II flow cytometer.

**Statistical Analysis**

Differences between groups were assessed for statistical significance using the student’s t-test. Differences were considered significant when p<0.05. The Pearson’s correlation coefficient (r) was used to determine the relationship between two properties with r >0.5 considered as high positive correlation. Error bars show standard error of the mean.

**Results**

**Effect of ChiLob 7/4 on DC activation and cytokine profiles in *ex-vivo* whole blood**

Unmanipulated anti-coagulated whole blood samples were used for all the *ex-vivo* assays as this was considered to be most physiologically reflective of systemic changes that would occur in peripheral blood. To determine whether ChiLob 7/4 was able to activate circulating mDC1 and pDC, whole blood assays were performed using a titrated concentration of ChiLob 7/4, either soluble or cross-linked with a goat anti-human IgG Fcγ-specific antibody. Using flow cytometry, changes in CD83 surface expression were measured as an early indicator of dendritic cell activation (Figure 1A). Soluble ChiLob 7/4 was unable to upregulate CD83 on pDC and mDC1, however, upon cross-linking there was a significant upregulation on both populations (Figure 1B). The level of upregulation had a positive correlation with increasing doses of ChiLob 7/4 (r>0.6). The level of CD83
upregulation on pDC did not reach the equivalent level seen on mDC1. No change in CD83 expression was found on mDC1 or pDC when ChiLob 7/4 was air-dried on plates prior to addition of whole blood (data not shown).

Plasma from the same whole blood activation assays were removed and analysed for changes in secreted cytokines using the Luminex 100 platform. Cytokine profiles were generated for a panel of 10 cytokines using a custom 10-plex kit (Figure 1C). Little change was measured in samples activated with soluble ChiLob 7/4, but when cross-linked increases were seen in IL-12, MIP1α, MIP1β, IL-8, IL-6 and TNFα showing strong correlation ($r>0.9$) with increasing concentration of cross-linked ChiLob7/4. The pattern of cytokine secretion for ChiLob 7/4 was distinctive with MIP1β expressed most abundantly and earliest, accompanied by the other cytokines at the 50μg concentration. Low levels of IL-4 and IL-10 were measurable at the highest cross-linked concentration of antibody (Figure 1D). No cytokines were detected when ChiLob 7/4 was air-dried on plates, emphasizing the particular importance of cross linking with a secondary antibody to observe the activating nature of this antibody.

Although both the flow cytometry and Luminex assays were able to measure responses when the antibody was cross-linked, the flow-based assay was able to detect mDC1 activation with the soluble form of the antibody before any cytokine response was detected. This suggests that the antibody has the potential to activate its target cells without the onset of excessive cytokine release. The pDC were not activated unless the antibody was cross-linked, suggesting a higher activation threshold, as more antibody or aggregated CD40-CD154 interaction was needed to trigger a response. Both assays provide an effective approach for screening the response to ChiLob 7/4 antibody, flow cytometry to monitor target cell activation and Luminex to monitor cytokine release syndrome.

**Comparison of ChiLob 7/4 DC activation with TLR ligands**

We wanted to determine how ChiLob 7/4 compared to other DC activating ligands. Therefore, the activation capacity of ChiLob 7/4 was compared to the level of activation triggered by known DC activating Toll-like receptor (TLR) ligands, specifically oligodeoxynucleotide (ODN) containing cytosine–phosphate–guanine (CpG) and lipopolysaccharide (LPS) (Fig 2A). Up-regulation of CD83 was measured as an early indicator of DC activation, and up-regulation of CCR7 as an indicator of DC maturation and migration. As anticipated the level of activation varied between individuals, however, the changes induced by each ligand were consistent within each cohort and correlated with that expected by the ligand. CpG2216 which activates via TLR-9 was found to be a weak DC activator in terms of CD83 and CCR7 up-regulation, whilst LPS which activates via TLR-4 was a potent activator of mDC1 but also demonstrated reasonable pDC activation. In comparison, soluble ChiLob 7/4
only minimally activated mDC1 and pDC, and although non-significant was at the equivalent level of the weaker TLR ligand CpG2216. When cross-linked, ChiLob7/4 was at least as effective as the more potent TLR ligand LPS in activating the mDC1 and pDC, with CD83 up-regulation reaching the equivalent level induced by LPS (r>0.9), although CD83 was still consistently lower with the cross-linked ChiLob 7/4 in comparison to LPS (Fig 2B). Our previous studies have shown CCR7 upregulation to occur 24 hours post LPS stimulation (16), and the results of this study would suggest the kinetics of mDC1 CCR7 upregulation with x-linked ChiLob 7/4 occur more rapidly, detectable at 4 hours. It is clear from these results that in an ex-vivo setting Chilob 7/4 is most effective when cross-linked, and its ability to activate is similar to LPS with both DC subsets becoming activated, mDC1 more so than pDC, but having the ability to trigger chemotactic markers on DC more dynamically than LPS.

To determine whether the ChiLob7/4 activity could synergistically or otherwise enhance TLR ligand responses, whole blood assays were performed combining soluble or cross-linked ChiLob 7/4 with LPS or CpG. Although cross-linked ChiLob 7/4 and LPS were each highly effective on their own at activating DC, in combination there was no significant additive effect on DC activation. In combination with cross-linked ChiLob 7/4, CpG2216 which triggered low CD83/CCR7 activation on its own, did not enhance the levels induced by cross-linked ChiLob 7/4 alone. Similarly, there was no additional up-regulation with the addition of cross-linked ChiLob 7/4 to LPS. These results suggest that the addition of ChiLob7/4 (even in the highly activating cross-linked form) would not exacerbate any pre-existing activation of DC.

Comparison of cytokine profiles induced by ChiLob 7/4 and TLR ligands

The plasma removed from the whole blood assays was used to generate 10-plex cytokine profiles (Figure 3). We wanted to assess whether during the process of DC activation the cytokine profile generated by ChiLob 7/4 was similar to that generated by LPS or CpG. The amount of each cytokine produced was variable between individuals however the same group of cytokines were induced/secreted by the same ligand. Soluble ChiLob 7/4 produced a similar cytokine expression profile as the weaker TLR ligand CpG2216, with only MIP1α, MIP1β, IL-8 and TNFα being minimally increased above the unstimulated levels, with MIP1β already having a relatively high concentration in the unstimulated control sample. Cross-linked ChiLob 7/4 generated a profile similar to the more potent TLR ligand, LPS, producing high levels of MIP1α (>5000pg/ml), MIP1β (>10,000pg/ml), TNFα (>5000pg/ml), IL-6 (>5000pg/ml), IL8 (>1000pg/ml) and IL-12 (>250pg/ml); notably the level of IL-6...
expression was ~8 fold less than that induced by LPS (50,000pg/ml), suggesting an overall less pro-inflammatory response.

Combinations of soluble and cross-linked ChiLob 7/4 with either CpG or LPS showed a similar pattern as the changes seen in the CD83 and CCR7 expression. That is, CpG or LPS in combination with cross-linked ChiLob 7/4, resulted in cytokines being produced at the same level as cross-linked ChiLob 7/4 alone and LPS alone, respectively with no indication of a synergistic effect (data not shown).

**In vivo dose effect of ChiLob 7/4**

Peripheral blood samples from patients treated with ChiLob 7/4 were analysed for CD83 and CCR7 expression on mDC1 and pDC, and cytokine profiles were generated from plasma samples. Baseline samples were taken at pre-infusion (day 1) and at day 4 post-infusion for each weekly cycle of treatment over 4 weeks to analyse the changes in DC activation. Changes above the baseline level of expression of DC activation markers were measurable at a minimal dose of 16mg (~3.2 ug/ml) (Fig 4A). Changes in CD83 and CCR7 were evident in mDC1 whereas pDC only showed changes in CCR7 (Figure 4B). The 16mg dose (cohort 4) was the first point in the study where a cytokine release syndrome was recorded. Three of the four patients in this cohort experienced a first dose infusion reaction. Two of these patients progressed through further infusions with no additional adverse reactions whilst the third patient (subject 10) experienced milder reactions following the third and fourth infusions at 16 mg.

Cytokine responses were evaluated at earlier timepoints than the flow analysis, sampling at 3, 6 and 24 hours post completion of the ChiLob 7/4 infusions. The cytokine responses of the comparable cohorts at 1.6mg (cohort 2), 16 mg (cohort 4) and 160 mg (cohort 6) also show a significant positive response at the 16mg dose where the DC activation and adverse reactions first occur (Figure 4C). The cytokine signature of the *in-vivo* responses displayed similarities as the previous *ex-vivo* observations with significant increases in MIP1β and IL-12 and smaller increases above baseline for MIP1α and IL-8. The kinetics of the responses were also shared across the cohort with an early rise in MIP1β at 3 hours, reaching a maximum at 6 hours and the IL-12 and IL-8 responses appearing at 6 hours. These responses were replicated at the 160mg dose and although the amount of cytokine measured varied between individuals, the group of measurable cytokines produced demonstrated a clear pattern with MIP1β showing the most rapid and largest spike at 3 hours and dropping at 24 hours, and IL-12 continuing to increase even at the 24 hour time-point. The cytokine profiles were remarkably consistent between patients at
the 16mg and 160mg doses despite the introduction of premedication steroids for the latter group. The pro-
inflammatory cytokines associated with a cytokine storm, notably TNFα and IL-6, although measurable in the ex-
vivo assays with cross-linked ChiLob7/4, were undetectable in patient samples at both 16mg and 160 mg doses.
This may either relate to a true absence of these cytokines in the cytokine release syndrome of this antibody or be
secondary to the introduction of steroids and increased infusion time for those experiencing a first dose reaction at
16mg and the subsequent introduction of pre-medication steroids for the cohort at 160mg. However the
 persistence of a reproducible cytokine signature across these 2 cohorts may also suggest that the cytokines being
 released are not in-line with a classical cytokine release syndrome, typically T cell focused with variable FcγR
 positive innate cell involvement, and are secondary to the targeting of a different cellular subset with a milder
 adverse reaction outcome.

**In vivo effect of ChiLob 7/4 on circulating DC activation and intracellular cytokine profile**

Although there was no consistent pattern in the kinetics of DC activation by phenotyping, the individual results
from the 4 patients on the highest dose (160mg) showed comparable fluctuating changes above baseline in both
CD83 and CCR7 expression for mDC1, and only CCR7 for pDC (Fig 5A). This pattern across a heterogeneous
patient group is consistent with our ex-vivo results where pDC changes in CCR7 expression were more
pronounced than those observed for CD83, which only showed minimal levels of up-regulation.

Our results from the multiplex cytokine analysis identified MIP1β and IL-12 as the main cytokines being
produced in both the ex-vivo activation assays and the patient samples. Both of these cytokines can be produced
by activated DC and we therefore, performed intracellular flow cytometry analysis on samples from healthy
subjects to determine whether the activated DCs could produce these cytokines. Following stimulation with LPS
both MIP1β and IL-12 could be identified from a significant number of mDC (Figure 5B) and a smaller
population of pDC (Figure 5C). Activation with cross linked ChiLob 7/4 induced a smaller response for MIP1β
and IL-12 production that was only present in the mDC population and not observed in the pDC population. This
supports the association of DC activation, principally of mDC, with the production of MIP1β and IL-12 that may
contribute to the cytokine release profile of ChiLob 7/4. This distinctive cytokine profile may contribute to the
proposed immunomodulatory effects of CD40 targeting as well as modulating the severity of a cytokine release
syndrome.

**Discussion**
Agonistic anti-CD40 antibody has an important role in triggering the activation of adaptive immune cells that can be directed towards an antitumour response. Although other agonistic anti-CD40 antibodies entering the clinic have been shown to activate DC with the use of model systems (19-21), we have used *ex-vivo* assays to uniquely show biological activity of ChiLob 7/4 on unmanipulated peripheral DC and performed a direct comparison with a first-in-human study. *Ex-vivo*, we have shown that ChiLob 7/4 is most potent when cross-linked and is able to activate DC to the level induced by the potent TLR ligand, LPS. The activation and cytokine profiles for cross-linked ChiLob7/4 in our *ex-vivo* studies are most reflective of the *in-vivo* setting, suggesting that some form of antibody cross-linking may be occurring in these patients as the antibody is provided to the participants in a soluble non-cross-linked form. This is perhaps unsurprising as CD40 and CD154 each form a trimeric protein structure for successful cell signaling (4) which we can simulate *ex-vivo* when cross-linking via the antibody’s Fc receptor. This highlights that although *ex-vivo* assays may be able to predict clinical effect, knowledge of the antibody: target interaction is critical to ensure the assay design is reflective of *in-vivo* mechanisms to correctly assess the potential biological activity. In addition, several murine studies have highlighted the importance of understanding the role of FcγR cross-linking for monoclonal antibody function. The agonistic activity of anti-CD40 antibody has already been shown to be dependent on the interaction with FcγRIIB, an inhibitory FcR, (22, 23), and appears specific to IgG1 antibodies, which may be providing a structural scaffold to enable stable CD40 signaling. Other studies have also shown cytokine release syndrome elicited by CAMPATH-1 to be isotype dependent, with human IgG1 and rat IgG2b antibodies eliciting the strongest response which could be inhibited with an anti-CD16 antibody (FcRγIII), to block cross-linking with the CD16 FcR (24). There is great interest to understand fully the role of cross-linking and FcR usage, and how this relates to the different IgG isotypes that maybe targeting the same antigen. For anti-CD40 targeting there are different anti-CD40 isotype antibodies already in the clinic and they may trigger different responses. These functional differences between similar therapeutic antibodies may require novel immunomonitoring assays to establish the profiles and mechanism that might be operating *in vivo*, together with the risk for different types of cytokine release syndrome. Furthermore, the scope for Fc engineering to enhance efficacy of existing antibodies provides a promising avenue for improved therapy with reduced cytotoxicity.

Immunostimulatory antibodies that are designed to enhance a person’s own immune response against cancer provide a promising alternative when conventional treatments are exhausted (25). However, predicting toxicity of
these agents is not always successful. TGN1412, an anti-CD28 super-agonist antibody is perhaps the most well-known first-in-human clinical trial of an immunostimulatory antibody where pre-clinical assessments failed to predict an almost fatal cytokine storm (1). Although researchers had assessed the risks, the assay designs had not accounted for all the different antibody interactions and species variability, in this case the differences in CD28 expression on CD4+ effector memory T-cells (26), which led to a massive cytokine-mediated inflammatory response. In addition, the dose had been determined using the ‘no observed adverse effect level’ (NOAEL) method which calculates dose based on risk, and where no adverse effect is observed in pre-clinical studies this dose can be over-estimated. Since the TGN1412 trial, the ‘minimally anticipated biological effect level’ (MABEL) has been recommended as a more appropriate basis for determining dose and allows dose-escalation studies to be based on minimal activation (27). In the aftermath of the trial, cytotoxicity was investigated by different researchers using different assay set-ups and found that the cytokine storm could actually have been predicted using human PBMC with adapted assays (28-29). The reports that followed highlighted the need to advance with caution when translating immunostimulatory antibodies to the clinic and to better assess cytotoxicity of such high-risk agents to not only predict mechanism of action, but to also better predict cytotoxicity before use in clinical studies: in particular ex-vivo assays were identified as a potential pre-requisite (30). If appropriate ex-vivo assays had been performed and the MABEL method for determining dose applied, the outcome of the TGN1412 trial may have been very different. Nevertheless, there is still a lack of studies that have demonstrated directly comparable ex-vivo results with corresponding clinical outcomes. When demonstrating the effect of ChiLob 7/4 on targeting and activating DC via CD40, the use of unmanipulated peripheral DC from human ex-vivo whole blood samples presents various obstacles, including low numbers in peripheral blood combined with limited DC specific markers and inherent biological variability. Nevertheless, in this study, the ex-vivo experiments were workable and confirmed that ChiLob 7/4 could activate both mDC1 and pDC to the same extent as LPS, but with a reduced inflammatory response as demonstrated by a lower CD83 upregulation and lower production of the inflammatory cytokines IL-6 and TNFα. CD83 expression is often used as a DC early activation marker; however, our results have shown lower CD83 expression on pDC following ex-vivo ChiLob7/4 stimulation, and negligible levels in the patient samples. This suggests CD83 is perhaps not an appropriate marker for pDC activation. Changes in CCR7 expression were measurable and possibly represent a more preferable marker, whose role in DC maturation is better understood due to its
involvement in chemotaxis and as an indicator of early migration (31). Upregulation of CCR7 in the \textit{ex-vivo} assays would suggest ChiLob7/4 is effective in stimulating DC maturation towards a migratory phenotype, the expression of which was reflected in patient samples. Differences in the upregulation of activation markers are perhaps expected, as the two subsets are known to differ in CD40 expression (32). The proportion of mDC expressing CD40 is greater than pDC as well as the individual expression level of CD40, which is higher on the mDC population. This may contribute to the reduced cytokine responses from the pDC in our study. In addition, different ligands can activate DC to produce a different DC outcome (33). This is demonstrated in our study with the pDC population, where LPS activates the upregulation of CD83 and CCR7 whereas cross-linked ChiLob7/4 only upregulates CCR7; LPS induces MIP1\(\beta\) and IL-12 production in pDC but cross-linked ChiLob 7/4 does not. Cytokine production was also only detectable when ChiLob 7/4 was cross-linked. Two of the cytokines that are often associated with a cytokine storm (TNF\(\alpha\) and IL-6) were not detectable in samples from ChiLob 7/4-treated patients. The onset of a cytokine storm is usually fast and immediate and causative cytokines are measurable within the first 24 hours (25). We used patient samples that were taken at early time-points (3-24 hours post-infusion) to monitor for the presence of any immediate cytokine storm. Generally there was more cytokine present at these earlier time-points and these had disappeared at the later day 4 time-point. The pattern of cytokine expression in the first 3 hours post-infusion was directly comparable to that seen in the \textit{ex-vivo} 4 hour stimulation assays with the exception of IL-6 and TNF\(\alpha\). It is possible that the time-points for the clinical samples may have been after the peak expression of IL-6 and TNF\(\alpha\) which may have been \textit{during} the infusion period, with cytokine being produced and dissipated by the time of the post-infusion blood sampling, in line with other studies showing acutely transient plasma cytokine responses post stimulation (34). The constraints of the clinical study where the drug infusion is undertaken over 6 hours at the higher dose, compounded by the reduction in infusion rate for those who experienced a cytokine release syndrome may have contributed to a failure to detect some cytokines. The lack of TNF\(\alpha\) and IL-6 in patients may also be explained by the use of corticosteroids that may have dampened the production of cytokines, although MIP1\(\beta\), MIP1\(\alpha\), IL-8 and IL-12 were still produced in cohort 4 who were therapeutically treated with steroids and cohort 6 who were prophylactically premedicated with steroids. The fact that MIP1\(\beta\) and IL-12 are still produced is particularly encouraging, as both cytokines are known to drive Th1 responses and suggests a distinct controlled cytokine response in these patients. The effect of corticosteroids on specific cytokine response is an area that clearly requires further investigation. In our study, the
16mg (3.2µg/ml) approximates to the 10µg concentration we used in the ex vivo evaluations where we first observed a cytokine response following cross linking of ChiLob 7/4. The emergence of the first symptoms relating to a cytokine release syndrome would be compatible with this approximate equivalence between ex vivo and in vivo evaluations and is supportive of our original starting dose, which was 80 fold less (0.5mg) than this first adverse event related dose. At the 160mg dose the cytokine responses for MIP1β are lower than that seen at 16 mg, despite the ex vivo experiments showing a significant increase over this 10 fold rise, and this may reflect the introduction of prophylactic corticosteroids to this cohort.

Flow cytometry results proved to be a sensitive method for detecting DC activation, with changes in CD83 and CCR7 being measured in mDC1 without the need for cross-linking, whereas cytokines were only detected when cross-linked. In addition, in-vivo changes in DC activation markers were seen at a lower ChiLob 7/4 dose before reaching a dose able to produce detectable cytokines. Specifically, patient samples taken at corresponding day 1 and day 4 post-infusion time-points for DC flow cytometry and cytokine analysis showed measurable changes in DC activation markers at a 10-fold lower dose (16mg) before any measurable cytokine response at a 160mg dose. Together these results suggest lower stimulation can trigger up-regulation of DC activation markers, but a stronger stimulation is required to trigger cytokine production.

Together, our study demonstrates that ChiLob7/4 is biologically active and can trigger human peripheral DC activation and maturation with the release of Th1 cytokines but without the presence of a classic cytokine storm. We have also shown that in combination with other DC ligands such as LPS and CpG, there is no augmented response. Interestingly, CP-870-893, another anti-CD40 agonistic antibody has been shown to have augmented B-cell activation in combination with CpG TLR9 stimulation (35). This IgG2 antibody has also been reported to produce TNFα and IL-6 in clinical studies (11), suggesting that this antibody may be working in a different way than ChiLob 7/4. Finally and most importantly, we demonstrate that ex-vivo assays provide a useful pre-clinical tool when correctly applied to help determine the effectiveness of an antibody on their target cell (i.e. ChiLob 7/4 on DC activation) in addition to monitoring for the presence of a cytokine response and any potential cytokine storm. The ability of ChiLob7/4 to trigger ‘safe’ DC activation and cytokine release provides a rationale for subsequent assessment in combination with other clinical agents that may trigger specific effector responses to tumour cells.
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References


Figure Legends

Figure 1: Effect of titrated ChiLob 7/4 (+/- cross-linking) on DC activation and cytokine release.

A. Flow cytometry gating strategy for enumeration of mDC1 and pDC subsets. B. Graph shows upregulation of CD83 on mDC1 and pDC using healthy whole blood assays following stimulation with increasing dose of soluble or cross-linked ChiLob 7/4 (p-values show statistical difference between soluble and cross-linked ChiLob 7/4). Supernatants from these assays were screened for cytokine expression using Luminex technology. C. Minimal cytokine levels were detectable following stimulation with soluble ChiLob 7/4. With cross-linked antibody, high concentrations of MIP1β, MIP1α, IL-8, IL-6, IL-12 and TNFα were induced. D. Low concentrations of IL-4 and IL-10 were measurable at the highest dose of cross-linked antibody. (Cytokine data representative of at least 2 donors).

Figure 2. Comparison of ChiLob 7/4 cytokine profiles with TLR ligands and in combination.

Results shown are a mean of 3 independent ex-vivo whole blood experiments, stimulated with +/- cross-linked ChiLob 7/4, LPS or CpG alone or in combination. A. Representative flow cytometry plots showing shift in CD83 and CCR7 expression on mDC1 and pDC. B. Graph shows CD83 upregulation on mDC1 and pDC. Cross-linked ChiLob 7/4 significantly up-regulated CD83 on mDC1 and pDC compared to unstimulated, with mDC1 but not pDC showing good correlation to LPS CD83 upregulation (n=3). C. Graph shows CCR7 upregulation on mDC1 and pDC. There was a significant upregulation of CCR7 above unstimulated with cross-linked ChiLob 7/4 on mDC1 and pDC with good correlation to CCR7 upregulation with LPS (n=3). (r = Pearson’s correlation coefficient)

Figure 3. Comparison of cytokine profiles induced by ChiLob 7/4 and TLR ligands in ex-vivo whole blood stimulation assays. LPS was the most potent ligand for stimulating release of cytokines. Soluble ChiLob 7/4 (CL7/4) and CpG were not effective at producing cytokines above unstimulated levels. The cytokine expression profile for cross-linked ChiLob 7/4 was similar to that produced by LPS, producing significantly high concentrations (**p<0.05) of MIP1α, MIP1β, TNFα, IL-6, IL-8 and IL-12 compared to unstimulated; although, the level of MIP1α, MIP1β, IL-6, IL-8 and IL-12 produced with cross-linked ChiLob 7/4 was significantly lower than that produced by LPS**. Low but significant levels of IL-4 and IL-10 above unstimulated were also produced***. The results show the mean response of 3 donors.
**Figure 4. In vivo dose effect on DC activation and cytokine production.** A,B. Results are shown from one representative patient from 3 different ChiLob 7/4 dose cohorts (160, 16 and 1.6mg total dose) with samples taken at day 1 pre-infusion and day 4 post-infusion over a 4 weekly cycle. A. The level of CD83 and CCR7 expression on mDC1 is shown. Changes in these activation markers above baseline are seen with a 16mg dose and above. B. The CD83 and CCR7 expression on pDC is shown, with only CCR7 but not CD83 levels fluctuating above baseline with the 16mg dose and above. C. The mean cytokine profiles are shown for all patients on each dose cohort (160, 16 and 1.6 mg total dose) over a 24 hour period post-infusion. No changes in cytokine levels above baseline (0) are measured until the 16mg cohort. Increases above baseline (0) in MIP1α, MIP1β, IL-12 and IL-8 occur in the first 24 hours in the 16mg and 160mg cohort. The level of MIP1α, MIP1β and IL-8 peak at 3-6 hours with IL-12 continuing to increase up to 24 hours later at 160mg.

**Figure 5. DC activation from 5 patients on 160mg dose cohort and intracellular cytokine expression.** A. Fluctuating increases in both CD83 and CCR7 in mDC1, and in CCR7 in pDC with negligible levels of CD83 was most evident throughout the course of treatment for patients on the 160mg dose. However, there is no obvious pattern of activation between individuals. The ability of mDC1 (B) and pDC (C) to produce the cytokines, MIP1β and IL-12, was investigated using intra-cellular flow cytometry of ex-vivo stimulated whole blood. Results show pDC only produced these cytokines with LPS stimulation, with mDC1 being able to produce IL-12 and MIP1β with cross-linked ChiLob 7/4, although not at the same level following LPS stimulation.
Figure A: Flow cytometry plots showing the percentage of mDC1 and pDC cells in different conditions. The conditions include unstimulated (unstim), LPS, CD40, and x-linked CD40. The plots show the expression levels of CD83 APC-A and CCR7 PE-Cy7-A.

Figure B: Bar graphs showing the percentage of CD83+ cells in mDC1 and pDC under different conditions. The conditions include unstim, anti-CD40, x-linked CD40, and LPS. The graphs show the correlation coefficients (r) and p-values for each condition.

Figure C: Similar to Figure B, but for different conditions and markers. The correlation coefficients (r) and p-values are also indicated.
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Ex-vivo assays of dendritic cell activation and cytokine profiles as predictors of in vivo effects in an anti-human CD40 monoclonal antibody ChiLob 7/4 Phase I trial

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