Clinical Cancer Research

Clinical and Biological Effects of an Agonist Anti-CD40 Antibody: A Cancer Research UK Phase I Study

Peter Johnson[1,2], Ruth Challis[2], Ferdousi Chowdhury[2], Yifang Gao[1], Melanie Harvey[1], Tom Geldart[1], Paul Kerr[1], Claude Chan[1], Anna Smith[2], Neil Steven[3], Ceri Edwards[4], Margaret Ashton-Key[2], Elisabeth Hodges[2], Alison Tutt[1], Christian Ottensmeier[1,2], Martin Glennie[1,2], and Anthony Williams[1,2]

Abstract

Purpose: This phase I study aimed to establish the biologic effects and MTD of the agonistic IgG1 chimeric anti-CD40 antibody ChiLob7/4 in patients (pts) with a range of CD40-expressing solid tumors and diffuse large B-cell lymphoma, resistant to conventional therapy. Potential mechanisms of action for agonistic anti-CD40 include direct cytotoxic effects on tumor cells and conditioning of antigen-presenting cells.

Experimental Design: ChiLob7/4 was given by IV infusion weekly for 4 doses at a range from 0.5 to 240 mg/dose. Validated ELISAs were used to quantify ChiLob7/4 in serum and test for anti-chimeric MAb (HACA) responses. Pharmacodynamic assessments included quantitation of T-cell, natural killer–cell, and B-cell numbers and activation in blood by flow cytometry and a panel of cytokines in plasma by Luminex technology. Planned dose escalation was in cohorts of 3 patients until MTD or biologic effect, defined as reduction of peripheral blood $CD19^+$ B cells to 10% or less of baseline.

Results: Twenty-nine courses of treatment were given to 28 subjects. The MTD was 200 mg × 4, with dose-limiting toxicity of liver transaminase elevations at 240 mg. At 200 mg (range between 2.1 mg/kg and 3.3 mg/kg based on patient body weight), the trough level pretreatment was above 25 μg/mL. Grade 1-2 infusion reactions were seen above the dose of 16 mg, but could be prevented with single-dose corticosteroid premedication. HACA responses were seen after doses between 1.6 mg and 50 mg, but not above this. There were dose-dependent falls in blood B-cell numbers accompanied by reduced expression of CD21, and transient reductions in NK cell numbers with increased CD54 expression from 50 mg upward. MIP-1β and IL12 plasma concentrations rose after doses above 16 mg. Fifteen of 29 treatments were accompanied by disease stabilization for a median 6 months, the longest for 37 months.

Conclusions: ChiLob7/4 can activate B and NK cells at doses that can be administered safely, and should be tested in combination with other antibodies and chemotherapy agents. *Clin Cancer Res; 21(6); 1321–8. ©2015 AACR.*

Introduction

CD40 is a membrane protein belonging to the TNFR superfamily. It is expressed primarily on antigen-presenting cells (APC) such as dendritic cells, B lymphocytes, and monocytes, but has also been found on most B-cell lymphomas and a substantial number of epithelial malignancies. Bidirectional CD40–CD40L interactions are central to the generation of both T-cell–dependent, humoral immune responses and cytotoxic T-cell responses, licensing APC to present antigen to and activate responding $CD8^+$ cytotoxic T-cell precursors (1). The expression pattern of CD40 on a broad range of malignancies and the important immunostimulatory role of CD40 *in vivo* make this an attractive target for agonistic antibody therapy, supported by data from mouse models which showed that effective and longlasting immune responses could be evoked against a variety of tumor types (2). Potential mechanisms of action include recruitment of immune effectors such as complement-dependent cytotoxicity (CDC) and natural killer (NK) cells, direct signaling leading to apoptosis in malignant cells, and licensing of antigen presentation, bypassing the need for specific $CD4^+$ T-cell help to activate $CD8^+$ cytotoxic T-cell precursors (3, 4).

ChiLob7/4 is a chimeric IgG1 anti-human CD40 antibody which has shown growth-inhibitory effects *in vitro* against a variety of CD40 expressing human malignant lymphoma and epithelial cell lines (5). It was effective in assays of both CDC and ADCC, and the parent murine antibody showed agonistic activity in upregulation of costimulatory molecules in a dendritic cell culture system (6). A phase I trial was undertaken to define the effects of administration of ChiLob7/4 for the first time in humans.

[1]Cancer Research UK Centre, University of Southampton, Southampton, United Kingdom. [2]NIHR/CRUK Experimental Cancer Medicine Centre, University of Southampton, Southampton, United Kingdom. [3]NIHR/CRUK Experimental Cancer Medicine Centre, University of Birmingham, Birmingham, United Kingdom. [4]Drug Development Office, Cancer Research UK, London, United Kingdom.

Prior presentation: The American Association for Cancer Research Annual meeting 2013; Chicago, IL.

Corresponding Author: Peter Johnson, University of Southampton, Somers Cancer Research Building, MP 824, Southampton General Hospital, Southampton SO16 6YD, United Kingdom. Phone: 44-2381206186; Fax: 44-2381205152; E-mail: johnsonp@soton.ac.uk

doi: 10.1158/1078-0432.CCR-14-2355

Translational Relevance

CD40 is expressed on antigen-presenting cells (APC) and some malignancies. On APC, ligation results in upregulation of costimulatory molecules, potentially bypassing the need for T-cell help in an antitumor immune response. Anti-CD40 antibodies produce substantial responses and durable anticancer immunity in animal models, an effect mediated by cytotoxic T cells. The IgG1 chimeric antibody ChiLob7/4 was developed as an agonist for CD40, and this first-in-man dose-escalation study describes its pharmacokinetic and pharmacodynamic characteristics. An MTD of 200 mg weekly × 4 was established, yielding concentrations that showed physiologic activity including depletion and activation of peripheral blood B and NK cells, increases in MIP-1β and IL12 levels, and disease stabilization in half of those treated. The treatment was well tolerated, with infusion reactions readily controlled by single-dose corticosteroid premedication. Dose-limiting toxicity was a reversible hepatic transaminitis. This study provides the basis for exploring the activity of ChiLob7/4 in combination studies with chemotherapy, tumor antigen vaccines, and other immunomodulatory antibodies.

Materials and Methods

Patients and study design

Patients with tumors showing expression of CD40 on IHC, for which no curative treatment options existed and who gave written informed consent were enrolled in this National Research Ethics (NRES), UK-approved Phase I clinical trial (NCT01561911). Expression of CD40 was determined by staining with IgG2a anti-CD40 monoclonal antibody Lob 7/6, after proteinase K-based enzyme pretreatment and the horseradish peroxidase ChemMate EnVision Detection Kit, (Dako). Expression was scored as strong, weak or absent, focal or diffuse by a single observer (MA-K). Inclusion criteria included a good performance status (Eastern Cooperative Oncology Group 0-1; ECOG) and adequate hematologic and biochemical indices, including a total blood lymphocyte count of at least 0.5×10^9/L, creatinine clearance of at least 40 mL/minute, hepatic transaminases no more than three times the upper limit of normal (ULN) and bilirubin up to 1.5 times ULN. Treatment was by intravenous infusion of the ChiLob7/4 antibody weekly for four doses, given over 30 minutes for the first three dose levels, and thereafter initially at 10 mg/hour, with planned acceleration in the absence of any infusion reaction after the first 30 minutes. The starting dose level was 0.5 mg per dose, chosen as being the equivalent human dose to one four-hundredth of the highest rat anti-mouse CD40 monoclonal antibody given to mice without toxicity. The dose escalation scheme proceeded to 1.6, 5, 16, 50, 160, and 240 mg per dose, with three patients planned to receive treatment at each dose level, and expansion of the levels at which depletion of peripheral blood B cells confirmed a clear biologic effect. Escalation to the next dose level was only permitted when at least 3 patients had completed 4 weeks of treatment without dose-limiting toxicity. No routine premedication was mandated initially, but a protocol amendment introduced the prophylactic administration of 100 mg hydrocortisone, 1 g acetaminophen, and 10 mg chlorpheniramine before treatment with doses of 16 mg or more.

Evaluation of response

Patients underwent standard tumor assessments by clinical examination and cross-sectional imaging at baseline, and at 8 weeks from the first dose of antibody. Weekly clinical assessments were undertaken during the 4 weeks of treatment, together with routine hematology, biochemistry, measurement of immunoglobulin levels, and urinalysis, also repeated at the final study visit at week 8.

Pharmacokinetic analysis

Serum ChiLob7/4 antibody levels were measured by a validated ELISA technique as described previously (7) on samples taken predose and at 30 minutes, 1, 3, 6, 24, 48, and 72 hours after the start of each infusion. The lower level of quantification (LLOQ) for the assay was 2 ng/mL and the upper level of quantification was 20 ng/mL. Test samples were diluted 1:2,000 for each assay so the effective working range of the assay was 4 to 40 μg/mL. Pharmacokinetic parameters (half-life, AUC and C_{max}) were estimated using best-fit regression analysis (one phase decay), assuming noncompartmental decay using GraphPad Prism 6 software.

Pharmacodynamic assessments

Human anti-chimeric antibody (HACA) serum responses were measured on weekly serum samples obtained before each dose using a validated semiquantitative ELISA, as previously described. (7)

Pharmacodynamics were also assessed by flow cytometry where changes in peripheral blood leukocytes (PBL) were measured in whole blood samples taken preinfusion and at day 4 postinfusion of each cycle in all patients treated with doses up to and including 160 mg ChiLob7/4. At the two higher dose cohorts, a protocol amendment was incorporated, measuring PBL changes at 3 hours after first infusion. Follow-up samples were also taken at day 49 after first infusion. These whole blood samples were stained to assess changes in T-, B-, and NK-cell numbers using the BD Multitest CD3 FITC/CD16 + CD56 PE/CD45 PerCP/CD19 APC Reagent (Becton Dickinson), and DC number and activation as described previously (8). Samples were acquired using FACSCanto flow cytometers (Becton Dickinson).

Cytokine levels in plasma samples were measured using a human custom multiplex-10 bead array assay kit for Luminex purchased from Life technologies to measure the following cytokines: IL2, IL4, IL6, IL8, IL10, IL12p70, IFNγ, TNFα, macrophage inflammatory protein (MIP)-1α and MIP-1β, as per the manufacturer's instructions and as described previously (9) using a Luminex 100 instrument. Appropriate dilutions of the samples in assay diluent were made as required. Each sample was assayed in duplicate and cytokine standards supplied by the manufacturer were used to calculate the concentrations of the samples. Cytokine levels were measured in plasma or serum sample taken before each infusion, and at 3 hours, 6 hours, day 4, day 8, and week 8 postinfusion at each week during treatment.

Peripheral blood mononuclear cell isolation and cryopreservation

Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep centrifugation (Axis-shield) at day 1 (preinfusion)

and day 4 during weeks of treatment and cryopreserved at ≤150°C until use.

Flow cytometry from cryopreserved PBMC

Cryopreserved PBMC from a subset of patient cohorts and timepoints was stained in a three panels, B-cell subset and panel, NK-cell panel, and APC panel with the following monoclonal antibodies: B-cell subset and panel: V450-conjugated CD20, clone L27; FITC-conjugated IgD, clone IA6-2; phycoerythrin (PE)-conjugated CD27, clone M-T271; allophycocyanin (APC)-conjugated CD38, clone HIT2; PE-Cy7–conjugated CD24, clone ML5; APC-Cy7–conjugated CD19, clone SJ25C1 all from BD Pharmingen, and PerCP/Cy5.5-conjugated CD21, clone Bu32 from Biolegend. NK-cell panel: PE-conjugated CD54, clone LB-2; PerCP-conjugated CD3, clone SP34-2; PE-Cy7–conjugated CD56, clone B159; APC-Cy7–conjugated CD16, clone 3G8 all from BD Pharmingen. APC panel: V450-conjugated CD11c, clone B-ly6; V500-conjugated HLA-DR, clone G46-6; FITC-conjugated CD14, clone M5E2; PE-conjugated CD54, clone LB-2; PerCP-conjugated CD3, clone SP34-2; PerCP-conjugated CD19, clone SJ25C1; PE-Cy7–conjugated CD56, clone B159; APC-Cy7–conjugated CD16, clone 3G8 all from BD Pharmingen, and APC-conjugated CD303, clone 201A from Biolegend. Data acquisition was performed on a FACSCanto II flow cytometer (Becton Dickinson) fitted with three lasers (488-nm 20-mW solid state; 633-nm 17-mW HeNe; and 405-nm 30-mW solid state). The data were analyzed using the FACSDiva software version 6.3.1 (Becton Dickinson) or FlowJo software versionX (Treestar).

Results

Twenty-eight patients received treatment in the study, and dose escalation continued up to 240 mg per dose. One patient who experienced prolonged disease stabilization after receiving treatment at 1.6 mg was re-treated after an interval of 3 years with 240 mg. The clinical characteristics of the patients are shown in Table 1. All patients had progressing tumors, and were treated at an interval of between 1 month and 12 months from their most recent prior therapy (median 3 months). CD40 was expressed on the malignant cells, with strong staining in 23 cases and weak staining in 6. All but four subjects completed 4 weeks of planned treatment. One patient was withdrawn from the study having developed neurologic symptoms from previously undiagnosed brain metastases shortly after the first infusion of 0.5 mg. Both patients treated at 240 mg developed grade 3 liver transaminase rises after the first dose and were withdrawn from treatment. Following this, the dose was reduced to 200 mg for expansion of the cohort, at which one patient out of 6 developed a grade 3 rise in γ-glutamyl transferase after two doses and also stopped treatment.

Adverse events and hematologic parameters

A total of 240 adverse events (AE) were reported (Table 2). Of these, 96 were considered possibly related to ChiLob7/4. No AEs related to the antibody were of grade 4 or 5. Only 5 AEs of grade 3 were considered possibly related to ChiLob7/4: 2 rises in alanine aminotransferase at 240 mg, 1 rise in γ-glutamyltransferase at 200 mg, and 1 episode of hypokalemia at 16 mg in a patient previously treated with cisplatin for mesothelioma, and 1 episode

Table 1. Clinical characteristics of patients

Patient ID	Age at entry	Primary tumor (CD40 staining)	Performance status at entry (ECOG)	Time from diagnosis to treatment (months)	Prior therapy (S: surgery; C: chemotherapy; R: radiotherapy)	Time from most recent therapy (mo)	Antibody dose level (each dose: mg)
1	66	Colorectal cancer (strong diffuse)	1	38	S C R	2	0.5
2	60	Melanoma (weak focal)	1	9	S C	1	0.5
3	55	NSCLC (strong diffuse)	0	20	C R	1	0.5
4	67	Mesothelioma (strong focal)	0	11	C R	2	1.6
5	64	Thymic carcinoma (strong diffuse)	0	24	S C R	2	1.6
6	66	Mesothelioma (weak diffuse)	0	9	C R	4	1.6
7	60	Melanoma (strong focal)	0	11	S C R	3	5
8	72	Melanoma (strong diffuse)	1	85	S C R	4	5
9	52	Cervix (strong diffuse)	0	55	S C R	2	5
10	42	Cervix (strong diffuse)	1	100	C R	3	16
11	60	Mesothelioma (strong diffuse)	0	10	R	10	16
12	64	Mesothelioma (strong diffuse)	1	8	C R	4	16
13	66	Mesothelioma (strong diffuse)	0	15	C R	8	16
14	72	Mesothelioma (weak focal)	1	12	C	6	50
15	73	Mesothelioma (weak diffuse)	1	9	C	1	50
16	59	Laryngeal squamous carcinoma (strong diffuse)	1	34	S C R	3	50
17	68	Mesothelioma (strong diffuse)	0	9	R	2	160
18	66	Mesothelioma (strong focal)	1	16	C R	5	160
19	39	Laryngeal squamous carcinoma (weak diffuse)	1	12	C R	4	160
20	71	Mesothelioma (strong focal)	0	47	R	18	160
21	63	Colorectal cancer (strong focal)	0	51	S C R	9	160
22	70	Mesothelioma (weak diffuse)	1	48	C R	9	240
23	66	Diffuse large B-cell lymphoma (strong diffuse)	0	4	C	1	240
24	90	Diffuse large B-cell lymphoma (strong diffuse)	1	9	C R	6	200
25	69	Esophageal adenocarcinoma (strong diffuse)	1	13	C R	3	200
26	68	Esophageal adenocarcinoma (strong diffuse)	0	19	S C R	3	200
27	72	Mesothelioma (strong diffuse)	1	45	C R	10	200
28	57	Pancreatic adenocarcinoma (strong diffuse)	1	7	C	2	200
29	62	Pancreatic (strong diffuse)	0	26	S C	1	200

Table 2. Frequency of adverse events

Event	Total events reported	Total possibly related to ChiLob7/4	Grade 3 or worse related to drug	Maximum grade of related AE
Any	240	96	5	3
Seen in more than 10% of patients:				
Fatigue	17	13	0	2
Chest pain	11	3	0	2
Headache	11	4	0	2
Dyspnea	10	1	0	1
Diarrhea	8	7	0	1
Infection	12	1	0	1
Rigors	6	4	0	2
Raised ALT	5	6	2	3
Fever	5	3	0	1
Cough	7	1	0	2
Pruritus	5	2	0	1
Anorexia	3	1	0	2
Nausea	3	1	0	1
Vomiting	3	0	0	-
Raised AST	3	3	0	2
Infusion reaction	8	8	0	2

of raised C-reactive protein at 240 mg. There were few clinically significant AEs related to the antibody other than infusion reactions. Mild headache was reported by 11 patients, and considered possibly related to the drug in four cases. Chest pain was reported in 11 patients with chest wall involvement by tumor, and considered possibly related to the treatment in three cases. There were no significant changes in hematologic indices such as hemoglobin, platelet count or total white blood cell count. Immunoglobulin levels showed no significant changes during the period of study.

HACA responses were detected in 7 patients during the study: 1 of 3 patients at 1.6 mg (33%), 1 of 3 patients at 5 mg (33%), 3 of 4 patients at 16 mg (75%), and 2 of 3 patients at 50 mg (66%; data not shown). The positive responses were first detected in six cases before treatment at week 4, but in one case (at 5 mg), a reaction was detected at week 3. All positive responses remained detectable at week 8. None of the patients treated with 160 to 240 mg developed a positive HACA response, and in the 50-mg cohort, there was no apparent effect upon the pharmacokinetics of the antibody, with the HACA-negative patient showing similar levels to the two with positive results.

Infusion reactions and cytokine release

The antibody treatment was well tolerated, but infusion reactions (grade 1 and 2 respectively) occurred in the first two patients treated at the 16 mg dose level. The reactions consisted of flushing, low-grade pyrexia, and mild rigors, requiring interruption of the infusion and treatment with intravenous corticosteroids, after which the symptoms settled rapidly and the infusions could be completed without further symptoms. Following these findings, premedication was given routinely as described above. This prevented further infusion reactions at all doses up to the 200 mg dose level, at which three of the six subjects experienced grade 1-2 reactions, as did one of those treated at 240 mg. In all cases the infusion was successfully completed following a further dose of intravenous corticosteroids. These reactions were investigated by cytokine measurements in plasma or serum samples taken at baseline (preinfusion) 3, 6, 24 hours and day 4 and 8 after the start of antibody infusion. No rises in IL2, IFNγ, TNFα, or IL6 were detected (data not shown), but a consistent finding was of transiently raised levels of MIP-1β, maximal at 3 to 6 hours, and increases in IL12 most evident at the highest doses (Fig. 4A and B). The changes in these cytokines were seen in the patients treated at 16 mg without premedication who experienced infusion reactions, and in subsequent patients who received corticosteroid prophylaxis and who remained asymptomatic.

Table 3. Summary pharmacokinetic data from first infusion

Dose groups	Half life (h)	AUC (hmg/mL)	C_{max} (μg/mL)
50 mg	2.40 ± 2.00	0.40 ± 0.33	17.35 ± 2.75
160 mg	12.96 ± 9.89[a(2/5)]	1.96 ± 0.44	47.01 ± 12.08
200 mg	42.16 ± 35.65[a(5/6)]	2.83 ± 0.62	63.69 ± 12.36
240 mg	25.76[a(1/2)]	3.27 ± 1.49	65.83 ± 20.26

[a]Patients with half-life greater than 80 hours gave ambiguous regression analyses, and are therefore not included in the calculated half-life; numbers in parentheses describe the number of patients with >80-h half-life out of the number of patients within the dose group.

Pharmacokinetics

Serum ChiLob7/4 was detected and quantifiable only at the 50 mg dose level and above (Fig. 1). Serum ChiLob7/4 concentration peaked within the first 3 hours after the infusion. Half lives for the decay of Chilob7/4 were estimated where possible and are presented in Table 3 (half-life estimation after the first infusion was not possible for a proportion of patients since the plateau phases were not reached in these patients before 72 hours). There was a linear relationship between dose level and the calculated AUC. The only patient to develop grade 3 toxicity in the 200-mg cohort showed unusually slow clearance of the antibody after the first dose, with a half-life of over 80 hours. Repeat measurements during week 4 prolonged elimination, suggesting depletion of target antigen (data not shown). Patients treated at 160 mg had trough antibody levels of 10 to 25 μg/mL before administration of the fourth dose, and at 200 mg, the range was 27 to 52 μg/mL. No antibody could be detected at the 8 week visit in any cohort.

Pharmacodynamics

Infusion of ChiLob7/4 was associated with dose-related and transient decreases in peripheral lymphocytes, including NK

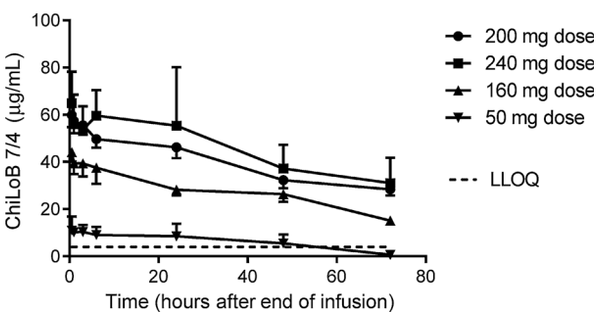

Figure 1.
Serum concentration of ChiLob7/4 after a single, intravenous infusion at 50 mg ($n = 3$ patients), 160 mg ($n = 5$ patients), 200 mg ($n = 6$ patients), and 240 mg ($n = 2$ patients). Bars, SEM for patients at each dose level. Patients treated at doses below 50 mg had serum levels of ChiLob7/4 that were not measurable since below LLOQ.

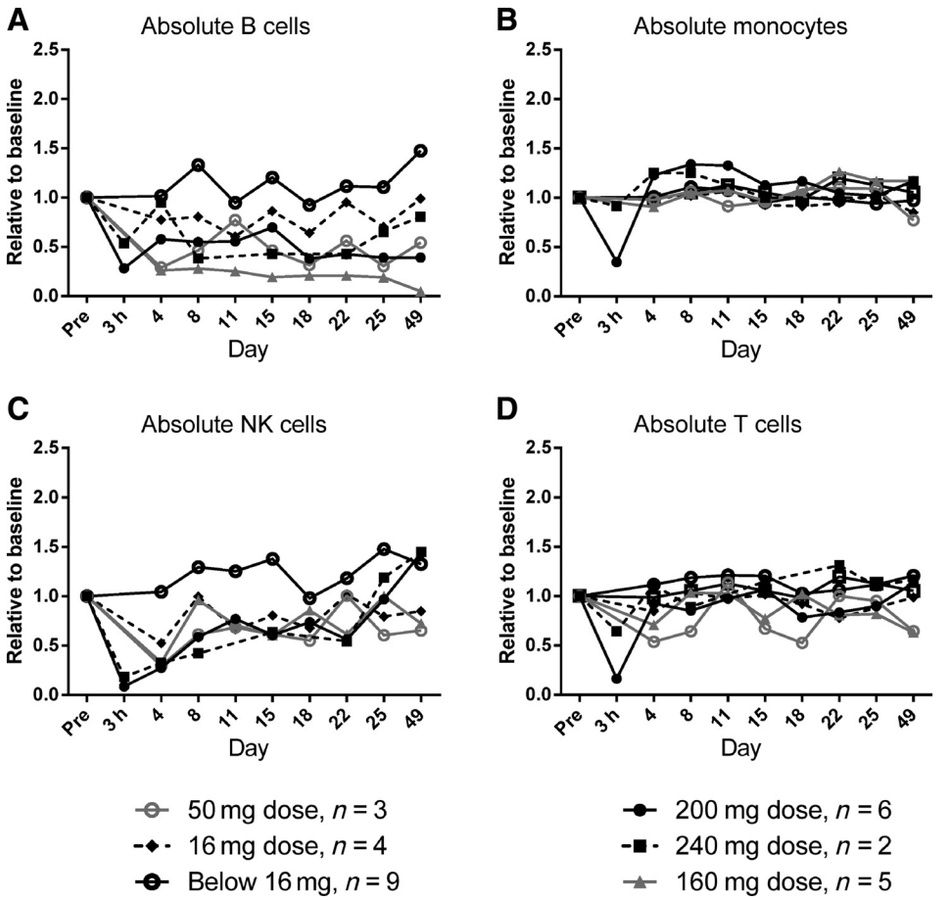

Figure 2.
Change relative to baseline in PBMC populations. A–D, data in the three lowest dose cohorts of 0.5 mg, 1.6 mg, and 5 mg (clinically nonresponding) have been grouped together ($n = 9$; solid black line with open circle), and all higher dose groups are grouped for all patients within a given dose level cohort and reported as mean values. Black dashed lines, diamond symbol: 16 mg ($n = 4$); gray solid line, open symbol: 50 mg ($n = 3$); gray solid line, triangle symbol: 160 mg ($n = 5$); black solid line, solid circle symbol: 200 mg ($n = 6$); black dashed line, square symbol: 240 mg ($n = 2$).

cells and monocytes, which were most evident at 3 hours after infusion (Fig. 2). Absolute numbers of $CD19^+$ B cells in the peripheral blood were reduced, with partial depletion seen in one patient treated at 16 mg and further depletion at the higher doses (Fig. 3). In all cases where patients enrolled onto trial with normal absolute B-cell numbers (between $0.1 - 0.5 \times 10^9$ cells/L), and received four drug infusions, B cells remained depleted (absolute number $< 0.1 \times 10^9$ cells/L) at 8 weeks after first infusion. No consistent alterations were observed in the phenotype of circulating dendritic cell populations (data not shown).

Elevated plasma concentrations of the cytokine MIP-1β were observed at 3 to 6 hour after infusion in most of the higher dose groups (Fig. 4A; range at 3–6 hours in 16-mg dose cohort 216–6,776 pg/mL MIP-1β; baseline levels range, 48–200 pg/mL). Similar rises were also seen in the higher dose cohorts in which premedication prevented infusion reactions; 50 mg (296–1,456 pg/mL); 160 mg (488–660 pg/mL); 200 mg (98–1,136 pg/mL), and 240 mg (342–416 pg/mL) cohorts. Figure 4B shows the plasma levels of IL12, which rose in the 16-mg cohort during the first 24 hours, between 1.32-fold and 2.8-fold from baseline (range, 260–1,068 pg/mL), with similar findings at the 50 mg dose, with levels 1.52- to 2.38-fold above baseline (424–660 pg/mL). The rises in IL12 were not sustained in the lower-dose cohorts, but at higher doses, they continued to rise, peaking during the second week for 160 mg doses at 1.74- to 3.14-fold (226–480 pg/mL), and at 200 mg at 1.84- to 12.4-fold (99–415 pg/mL).

Further flow-cytometric analysis was extended in a subset of patients using cryopreserved PBMC. Three panels of antibodies were used to identify modulation or activation of peripheral blood cell types which could contribute to the cytokine profile observed. From these analyses, no change in the proportions of naïve, memory, or antibody isotype class-switched B-cell subtypes was observed (data not shown), but a significant decrease in CD21 (complement receptor type II or CR2) was observed at all the doses tested (50, 160, 200, and 240 mg; Fig. 4C). The CD21 mean fluorescence intensity (MFI) relative to baseline was significantly decreased at day 4 and day 8 in the highest four dose groups, although the levels had returned to baseline by day 49.

We also evaluated NK cell activation using the cell surface marker CD54. We found an increase in CD54 expression in a subset of NK cells, (Fig. 4D), which was coincident with the transient falls in absolute numbers of $CD3^-CD16^+CD56^+$ NK cells. The number of cells in the CD54 bright subset increased at the higher antibody doses, reaching statistical significance in the expanded 200-mg dose cohort ($n = 6$).

Antitumor activity

In fifteen treatment courses, patients had stable disease at the final study visit at week 8, while in 14 cases, the tumors showed

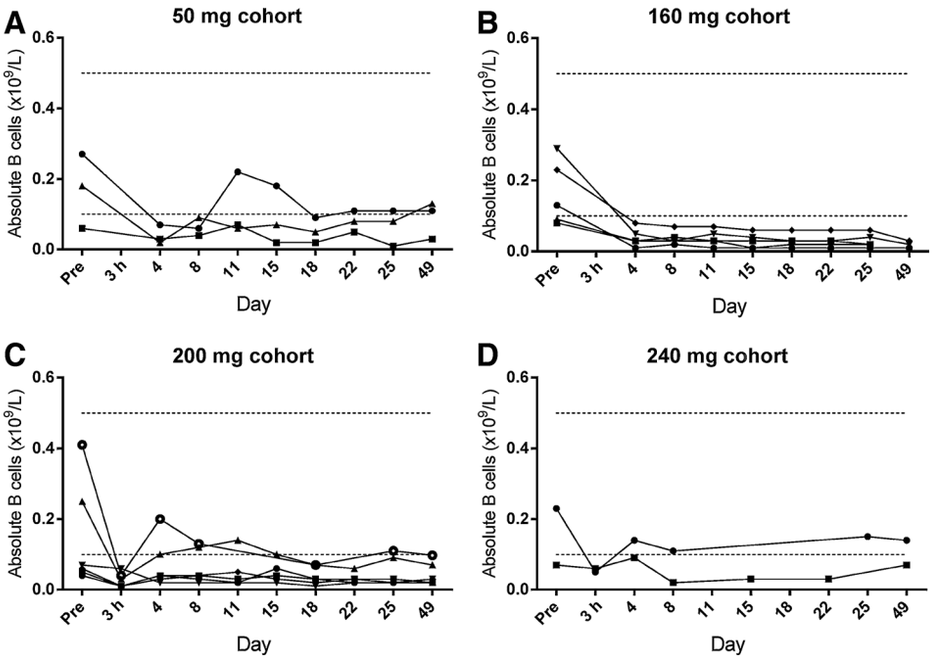

Figure 3.
Absolute B-cell numbers ($\times 10^9$ cells/L blood) in individual patient traces in the four highest dose cohorts. Dotted lines show the upper and lower range of normal absolute B-cell numbers.

evidence of continued progression during the study. Patients with stable disease had a median time to progression of 6 months (range, 5 to over 37), and the overall median survival for all patients was 11 months. There was no correlation between CD40 staining intensity and disease stabilization: 3 of 6 patients with weak CD40 expression showed stable disease. Similarly, there was no correlation between interval from last therapy and tumor stabilization.

Discussion

The aim of this study was to investigate the safety and tolerability of this anti-CD40 antibody, and to explore its biologic effects for the first time in man. We have shown that ChiLob7/4 can be given safely at a dose of up to 200 mg for four weekly doses, and that it is generally well tolerated, provided premedication is given with a single dose of corticosteroid. The infusion reactions seen when this premedication was not used occurred at a much lower dose (16 mg), but were not associated with a specific cytokine release syndrome. This is in contrast with the effects seen after treatment with a different agonistic anti-CD40 antibody, CP-870,893, which led to high levels of TNFα and IL6 production, with dose-limiting toxicity at 0.3 mg/kg (10). In our study, no induction of IL6 or TNFα plasma levels was observed, neither in the presence nor absence of corticosteroid premedication. As was seen with CP-870,893, dose-limiting toxicity was from raised transaminase levels. The two antibodies are of different isotype, with ChiLob 7/4 being an IgG1 whereas CP-870,893 is an IgG2 molecule. Recent evidence suggests that binding to inhibitory Fc receptors may be an important determinant of efficacy, at least in mouse models, implying that the isotype may have a strong influence upon the activity of agonistic antibodies (11). It is possible that the difference in cytokine production with ChiLob7/4 is due to lower agonistic potency than CP-870,893, and this is the subject of an ongoing comparative *in vitro* analysis.

The lack of toxicity allowed escalation of the dose of ChiLob7/4 to a much higher level than was achievable with CP-870,893, resulting in more durable B-cell depletion and prolonged elevation of antibody levels well above 10 ng/mL, the level at which maximal biologic effects were seen *in vitro* (12). At the lower doses, such depletion was transient and partial, in keeping with the findings with CP-870,893. Studies with a different anti-CD40, the humanized IgG1 dacetuzumab, have demonstrated that this too can evoke a cytokine release syndrome, with ocular toxicity and headaches the most prominent symptoms, although higher doses of up to 8 mg/kg could be given following intra-patient escalation and corticosteroid premedication in some cases (13).

We observed several effects in this study that suggest the recruitment of an active immune response. Transient falls in NK cell numbers in the blood at antibody doses as low as 16 mg were associated with rises in MIP-1β, and at higher doses with upregulation of CD54, suggestive of effector cells trafficking out of the circulation following activation. The development of HACA responses occurred at similarly low antibody doses and was seen at an unusually high frequency for a chimeric antibody. This suggests that the agonistic effects of ChiLoB7/4 may be leading to enhanced antigen presentation, in keeping with the changes in production of IL12, although these were only detectable at higher antibody doses. The disappearance of HACA responses in the highest dose cohort reflects the durable B-cell depletion seen at this level, removing the population capable of mounting a humoral response.

Transient activation of B cells was also demonstrated in studies with CP-870,893 either when used as a single agent (10) or in combination with chemotherapy (14). In the present study, we demonstrated B-cell activation as evidenced by the shedding of CD21, also known as complement receptor II or

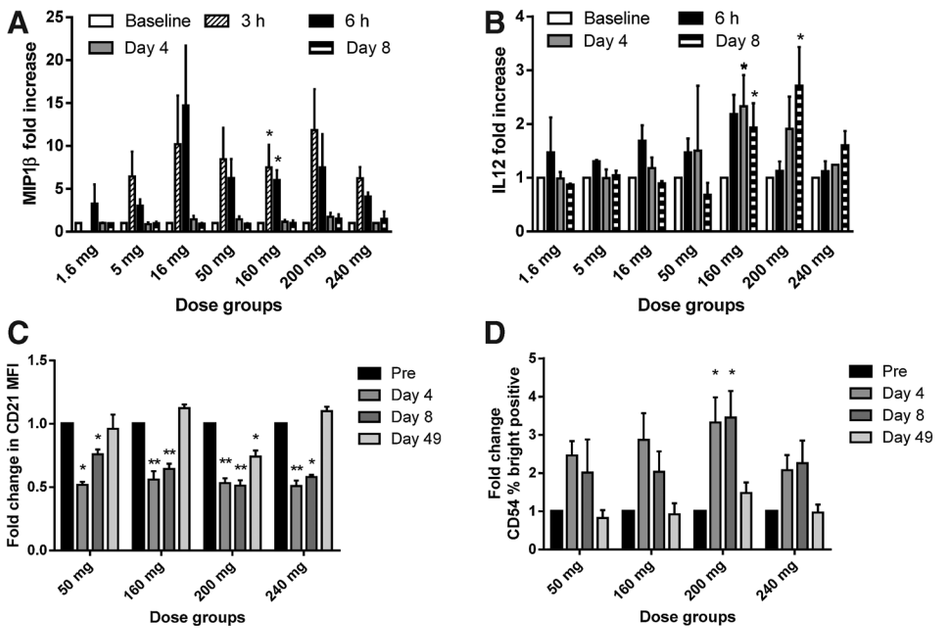

Figure 4.
A and B, change relative to baseline in cytokine concentrations measured in plasma or serum after first infusion of ChiLoB 7/4. A, MIP1; B, IL12. C and D, flow-cytometric data from a subset of time points (baseline, preinfusion "pre," day 4, day 8, and day 49) of the four highest dose groups treated (50–240 mg) measured from cryopreserved PBMC. C, changes in CD21 mean fluorescence intensity (MFI) in the $CD19^{+}CD20^{+}$ B cells relative to baseline. D, changes in CD54% bright positive population in $CD16^{+}$ NK cells relative to baseline. Data in the bar graphs are grouped for all patients in a given dose level cohort and reported as mean values ± SEM. *, $P < 0.05$; **, $P < 0.01$ paired two-tailed *t* tests.

CR2. This is expressed on mature B cells, T cells, and a number of other cell types (15). Ligation of CD21 results in signals critical for normal B-cell responses (16, 17). Activation of B cells by stimulation of the BCR with anti-IgM and anti-CD40 has been shown to induce CD21 shedding (18), which then contributes to the plasma CD21 pool. Soluble CD21 has also been shown to act as a functional ligand for CD23-expressing monocytes, downregulating CD14, enhancing expression of HLA-DR and CD40, and eliciting production of IL6 and TNFα from stimulated monocytes (19).

The use of corticosteroid premedication is a debatable strategy for an immunostimulatory antibody. It might be expected that this could attenuate the effect, but we found no evidence of this upon the NK, T-, or B-cell subsets. HACA responses were seen in the routinely premedicated 50-mg cohort as well as at 16 mg, and progressive rises in IL12, B-cell, and NK cell activation markers were most evident at higher doses, suggesting that these effects at least were not suppressed. Studies of vaccination against viral antigens have previously shown that chronic concomitant administration of corticosteroids did not affect seroconversion rates (20), and the exposure in this study was of brief duration, and much less likely to affect cellular immunity. The use of corticosteroids has allowed progression to a much higher dose than would otherwise have been tolerated, and this may be important for further studies. We have demonstrated that this antibody can be given safely at a dose of up to 200 mg, with evidence of immunostimulatory effects at lower doses, and marked peripheral blood B-cell depletion at higher ones.

It is encouraging that half the patients with previous progressive disease experienced stabilization following treatment with this antibody, and further studies are warranted to define potential antitumor activity either as a single agent or in combination with other immunostimulatory antibodies or chemotherapy. Further studies are required to examine the possible mechanisms of action: as an IgG1 molecule, this antibody is capable of directing ADCC and complement fixation, which may have a direct effect upon CD40-positive tumors, although stabilization of disease was also noted in three cases with much weaker CD40 staining.

Disclosure of Potential Conflicts of Interest

N. Steven is a consultant/advisory board member for GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: P. Johnson, M. Harvey, T. Geldart, C. Chan, M. Glennie

Development of methodology: P. Johnson, R. Challis, F. Chowdhury, M. Harvey, T. Geldart, C. Chan, E. Hodges, C. Ottensmeier, M. Glennie

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Johnson, R. Challis, F. Chowdhury, Y. Gao, T. Geldart, P. Kerr, A. Smith, N. Steven, M. Ashton-Key, E. Hodges, A. Tutt, C. Ottensmeier, A. Williams

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Johnson, R. Challis, F. Chowdhury, Y. Gao, A. Williams

Writing, review, and/or revision of the manuscript: P. Johnson, R. Challis, F. Chowdhury, Y. Gao, M. Harvey, T. Geldart, N. Steven, C. Edwards, M. Ashton-Key, E. Hodges, C. Ottensmeier, M. Glennie, A. Williams

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Johnson, R. Challis, F. Chowdhury, C. Edwards

Study supervision: P. Johnson, N. Steven, C. Edwards, C. Ottensmeier

Other (made the chimeric anti-CD40 antibody used in this trial during 2002 and proposed the initial concept for this phase I trial): M. Harvey

Other (patient recruitment and management): C. Ottensmeier

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 9, 2014; revised January 6, 2015; accepted January 6, 2015; published OnlineFirst January 14, 2015.

References

1. Grewal A, Flavell R. CD40 and CD154 in cell-mediated immunity. Ann Rev Immunol 1998;987:111–35.
2. French RR, Chan C, Tutt A, Glennie MJ. CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nat Med 1999;5:548–33.
3. Diehl L, Th. den Boer A, Schoenberger SP, van der Voort EIH, Schumacher TNM, Melief CJM, et al. CD40 activation *in vivo* overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumour-vaccine efficacy. Nat Med 1999;5:774–9.
4. Schoenberger SP, Toes RM, van der Voort EIH, Offringa R, Melief CJM. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interaction. Nature 1998;393:480–3.
5. Geldart TR, Harvey M, Carr N, Glennie M, Johnson P. Cancer immunotherapy with a chimeric anti-CD40 monoclonal antibody: evidence of preclinical efficacy. Proc ASCO 2004;14:2577.
6. Chowdhury F, Johnson PW, Glennie MJ, Williams AP. 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. Cancer Immunol Res 2014;2:229–40.
7. Chowdhury F, Tutt AL, Chan C, Glennie M, Johnson PW. Development, validation and application of ELISAs for pharmacokinetic and HACA assessment of a chimeric anti-CD40 monoclonal antibody in human serum. J Immunol Methods 2010;363:1–8.
8. Chowdhury F, Johnson P, Williams AP. Enumeration and phenotypic assessment of human plasmacytoid and myeloid dendritic cells in whole blood. Cytometry A 2010;77:328–37.
9. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. J Immunol Methods 2009;340: 55–64.
10. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. J Clin Oncol 2007;25:876–83.
11. White AL, Chan HT, Roghanian A, French RR, Mockridge CI, Tutt AL, et al. Interaction with FcgammaRIIB is critical for the agonistic activity of anti-CD40 monoclonal antibody. J Immunol 2011;187:1754–63.
12. Chowdhury F, Tutt AL, Chan C, Glennie M, Johnson PW. Development, validation and application of ELISAs for pharmacokinetic and HACA assessment of a chimeric anti-CD40 monoclonal antibody in human serum. J Immunol Methods 2010;363:1–8.
13. Advani R, Forero-Torres A, Furman RR, Rosenblatt JD, Younes A, Ren H, et al. Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma. J Clin Oncol 2009;27:4371–7.
14. Vonderheide RH, Burg JM, Mick R, Trosko JA, Li D, Shaik MN, et al. Phase I study of the CD40 agonist antibody CP-870,893 combined with carboplatin and paclitaxel in patients with advanced solid tumors. Oncoimmunology 2013;2:e23033.
15. Carroll MC. CD21/CD35 in B cell activation. Sem Immunol 1998;10:279–86.
16. Cherukuri A, Cheng PC, Pierce SK. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. J Immunol 2001;167:163–72.
17. Fearon DT, Carroll MC. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu Rev Immunol 2000;18:393–422.
18. Masilamani M, Kassahn D, Mikkat S, Glocker MO, Illges H. B cell activation leads to shedding of complement receptor type II (CR2/CD21). Eur J Immunol 2003;33:2391–7.
19. Fremeaux-Bacchi V, Fischer E, Lecoanet-Henchoz S, Mani JC, Bonnefoy JY, Kazatchkine MD. Soluble CD21 (sCD21) forms biologically active complexes with CD23: sCD21 is present in normal plasma as a complex with trimeric CD23 and inhibits soluble CD23-induced IgE synthesis by B cells. Int Immunol 1998;10:1459–66.
20. Kubiet MA, Gonzalez-Rothi RJ, Cottey R, Bender BS. Serum antibody response to influenza vaccine in pulmonary patients receiving corticosteroids. Chest 1996;110:367–70.