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Affinity and Epitope Profiling of Mouse Anti-CD40 Monoclonal Antibodies

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Abstract

The CD40–CD40L interaction plays a critical role in both humoral and cellular immune responses and interfering antibodies have been suggested as an effective approach for the treatment of lymphomas and autoimmune diseases. In this study we have profiled a panel of mouse antihuman CD40 monoclonal antibodies (MoAbs), regarding their CD40 binding affinity and epitope-specificity relative to the CD40L binding in relation to their cellular activating potential. Despite a rather similar domain-recognition profile, the MoAbs blocked the CD40L binding to a varying degree, with MoAb 5C3 being the poorest inhibitor. There was no correlation between affinity and cellular activation potential. In contrast, a correlation between the ability to block CD40L-binding and activation potential could be seen. We believe that this analysis of several mouse anti-CD40 antibodies can be used to develop strategies for producing new human anti-CD40 antibodies that can more effectively induce or block B-cell proliferation.

Introduction

CD40 is a 48 kDa membrane glycoprotein expressed on B cells, monocytes, dendritic cells, thymic epithelium as well as on certain carcinomas. It belongs to the tumour necrosis factor receptor (TNFR) superfamily, a group of type I transmembrane molecules. The characteristics of these proteins are the presence of multiple cystein-rich repeats, consisting of approximately 40 amino acids in the extracellular domain. CD40 consists of four such cystein-rich repeats that have been suggested to form four predicted protein domains. It has furthermore been shown that every domain is built of two modules with five different forms of modules being identified [1, 2]. The ligand for CD40 (CD40L) belongs to the tumour necrosis factor (TNF) family and is predominantly expressed on activated T cells, although variable expression has been reported on mast cells, B cells, monocytes and basophils. CD40L exists as a homotrimer [3] in the cell membrane and ligation of the CD40 on B cells leads to oligomerization of the receptor. The CD40–CD40L interaction is an important regulatory mechanism in the development of a T-cell dependent B-cell response. It also effects B-cell proliferation, initiation of homotypic adhesion and B-cell differentiation. Furthermore, it can rescue germinal centre B cells from undergoing spontaneous apoptosis *in vitro* [4].

In vitro ligation of CD40 can be studied using soluble CD40L or different anti-CD40 monoclonal antibody (MoAb). However, different antibodies activate B cells to different extent and in the past few years a number of groups have investigated the signalling pathways in B cells activated by different MoAbs directed against CD40. The action of intracellular TNF receptor-associated factors (TRAFs) as well as activation of different protein kinases (phosphatidylinositide-3 (PI3), stress-activated protein kinase (SAPK), c-Jun N-terminal Kinase (JNK) and mitogen activated protein kinase (MAPK)) [5] in the signalling pathway has been elucidated and light has been shed on their effect on different transcription factors such as nuclear factor (NF)-κB, signal transducers and activator of transcription-3 (STAT-3) and STAT-6 [6–8].

From an immunotherapeutic point of view, antibodies that block the activation of CD40 might be of great value in autoimmune diseases like multiple sclerosis, as the CD40–CD40L interaction has proven to be critical for the initiation of this disease of its animal model counterpart experimental autoimmune encephalomyelitis (EAE) [9–11]. However, little is known of the binding mechanism of such nonactivating MoAbs. Therefore, we investigated if there is a correlation between the activating

potential and the binding properties of antibodies directed against CD40 and we studied a panel of different mouse antihuman CD40 antibodies, exhibiting a great variation regarding functional effects on cells.

Materials and methods

Reagents. The 5D12 mouse MoAb, human CD40-Fcγ antigen, human CD40L-mouse CD8 (expressed in insect cells containing monomers, dimmers and trimers of CD40L) and a polyclonal anti-CD40 serum were provided by (Tanox Pharma, Amsterdam, The Netherlands). Human monomeric CD40L was purchased from (Pepro-Tech EC Ltd, London, England). Mouse MoAb 5C3 was purchased from (Pharmingen, San Diego, CA, USA), mouse MoAb EA5 was purchased from Biosource International (Camarillo, CA, USA), mouse MoAb S2C6 was kindly provided by Staffan Pauli (Stockholm University, Sweden) and mouse MoAb G28-5 was purified, using protein G, by affinity chromatography from a hybridoma supernatant. The G28–5 hybridoma was a kind gift from Jesper Zeuten (Danish Cancer Society, Copenhagen, Denmark). Human monoclonal anti-AD 2 immunoglobulin G (IgG) (ITC88) was kindly provided by Mats Ohlin (Lund University, Lund, Sweden). Mouse IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Cell lines. The human B-cell line, BJAB [12], a mouse fibroblast L-cell line transfected with CD40L [13] was kindly provided by John Pound (Birmingham, UK) and COS-7 was purchased from American Type Culture Collection (ATCC) (CRL-1651). The cells were maintained in RPMI 1640, supplemented with 2 mM glutamine, 1% v/v 100 × nonessential amino acids and 10% fetal calf serum (FCS) (BRL Life Technology, Täby, Sweden).

Isolation of B cells. Human tonsils were obtained from children undergoing tonsillectomy at Malmö Academic Hospital (Malmö, Sweden). The tonsills were cut into pieces in RPMI 1640 (BRL Life Technology) and enriched on 50% isotonic Percoll (Pharmacia Biotech, Uppsala, Sweden). B cells were positively selected on anti-CD19 coated magnetic beads (Dynal A/S, Oslo, Norway). The beads were subsequently removed from the cells using Detachabeads (Dynal A/S). The enriched B-cells routinely were >98% pure, as determined by FACScan analysis (BD, San Jose, CA, USA).

B-cell proliferation assay. Tonsillar B cells (10^5/well) were cultured in 200 µl R10-medium (RPMI 1640, containing 10% FCS, 2 mM L-glutamine, 1% nonessential aminoacids and 50 µg/ml gentamicin, BRL Life Technologies), together with mouse IgG (20 µg/ml). Anti-CD40 antibodies were added at 1 µg/ml. B-cell proliferation was measured after three days by adding [methyl-^{3}H]thymidine, 0.5 µCi/well (Pharmacia Biotech) and incubating at 37 °C for 16 h.

CD40 domain constructs. The CD40 domain constructs were made as described by Ellmark *et al.* [14]. Briefly, the CD40 leader sequence was amplified together with the AD-2 epitope sequence [15] by overlap extension of polymerase chain reaction (PCR) of oligonucleotides (BRL Life technology) followed by reamplification with flanking primers. AD-2 is a short (13 amino acids) tag that was used to detect surface expression. The resulting fragment, consisting of the leader sequence fused to the AD-2 epitope was digested with NheI and NotI and subcloned into the corresponding sites in pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands). After amplification the fragments were digested with NotI and XbaI and inserted 3′ of the AD-2 epitope sequence in the vector described above. Similar constructs without the AD-2 epitope were also constructed. See Fig. 1B for schematic structures of the resulting molecules.

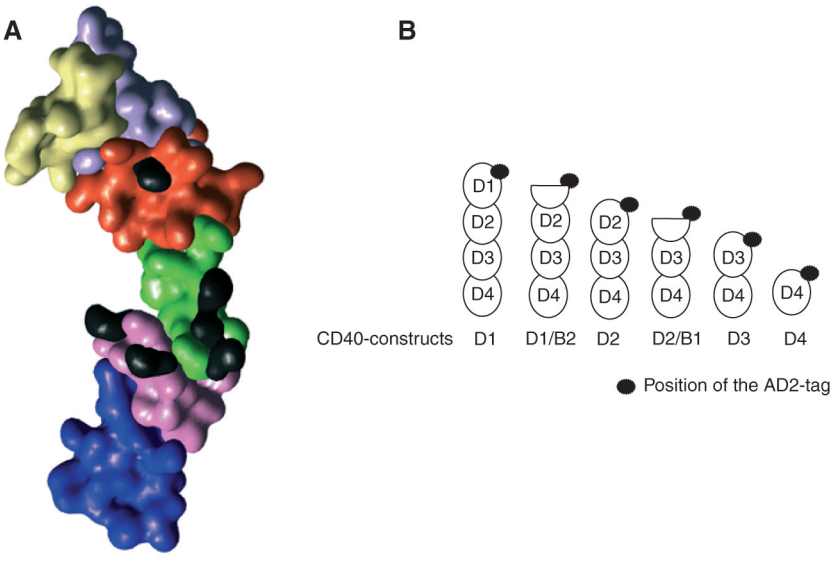

Figure 1 (A) A three dimensional model of the three outermost domains of CD40 as determined by Bajorath *et al.* [1], based on the crystal structure of tumour necrosis factor receptor (TNFR) as a homologue. The amino acids suggested to be critical for the CD40–CD40L interaction are depicted in dark grey [1, 2, 22]. Domain 1: module A1 is depicted in yellow, module B2 in purple, Domain 2: module A1 in red, module B1 in green, Domain 3: module A2 in pink and module B1 in blue.(B) Models of the extracellular part of the different CD40 constructs. Each domain can be subdivided into 1A and 1B module. The constructs are named after the last domain or module they contain. AD-2 is a short (13 amino acids) tag that was used to detect surface expression [15]. All constructs, except D4 have also been expressed without the AD-2 epitope.

Transient transfections and domain mapping using FACScan analysis. The domain mapping was performed essentially as described by Ellmark *et al.* [14]. Briefly, the CD40 constructs were transiently transfected into COS-7 cells, using lipofectamin (BRL Life technology), by mixing 2 μg of DNA with 5 μl of lipofectamin followed by incubation in 200 μl of medium without serum for 45 min in room temperature. The mixture was added to 10^6 COS-7 cells and then incubated for three days in 37 °C.

On day three, the cells were stained with monoclonal anti-CD40 antibodies (1 μg/ml), CD40L-CD8 (25 μl, which corresponds to a five times excess over saturation levels) or anti-AD 2 antibody (ITC88) [15]. Fluorescently labelled second-step conjugates were antimouse IgG-phycoerythrin (PE) (DAKO A/S, Glostrup, Denmark), antimouse CD8-PE (Caltag, Burlingame, CA, USA) and antihuman IgG-fluorescein isothiocyanate (FITC) (DAKO A/S). Cells were analysed on a FACScan instrument (BD, San Jose, CA, USA).

Determination of kinetic parameters and affinity constants using Biacore analysis. The CD40-Fcγ molecule was immobilized to the Biacore sensorship at 200, 650 and 2100 RU, using conventional amine coupling [16]. The mouse MoAbs and the human CD40L (both monomeric CD40L and multimeric CD40L-CD8) were analysed for binding at 3, 6, 12, 25, 50 and 100 nM in Hepes Complete buffer (10 mM Hepes, 3,4 mM ethylenediamine tetraacetic acid (EDTA), 0,15 M NaCl, 0,05% Biacore surfactant P20, pH = 7,4) at a flow rate of 3 μl/min. The association was followed for 11 min and the dissociation for 10 min Regeneration was performed using 100 mM HCL for 3 min The kinetic parameters and the affinity constants were calculated using BIAEVALUATION software version 3.0.

Epitope mapping using Biacore. The CD40-Fcγ molecule was immobilized to the Biacore sensorship at approximately 600 RU, using conventional amine coupling [16]. The CD40 surface was saturated with the first molecule by injecting 45 μl (100 nM in Hepes Complete buffer), followed by 15 μl of the same molecule to confirm that the surface was saturated. Directly after this, 15 μl of the second molecule was injected at 100 nM in Hepes Complete buffer at 3 μl/min. The signal of this second molecule (competing binding signal) was compared with the signal obtained when 15 μl was injected directly on the CD40 surface without any first molecule present (possible binding signal).

For the competition experiments with CD40L, the first molecule was always present in the solution with the second molecule (at 100 nM) in order to assure the saturation of the surface with the first molecule.

Epitope mapping using a B-cell line and FACScan analysis. The MoAbs used in these experiments were antibodies against CD40, either unconjugated (when used as primary antibodies) or biotinylated (when used as secondary antibodies). The concentration of the primary antibody was 5 μg/ml and of the second was 1 μg/ml. When using CD40L, instead of antibody, either 25 or 100 μl (5–20 times saturation level) of insect cell supernatant containing CD40L-mouse CD8 was used. Fluorescently labelled conjugates were Streptavidin-PE (DAKO A/S) or antimouse CD8-PE (Caltag). BJAB cells (3×10^5 cells/sample) were incubated with the first antibody for 30 min at 4 °C and thereafter the second antibody was included for an additional 30 min The cells were washed and then stained by second-step fluorescent reagents. Cells were analysed on a FACScan instrument (Becton Dickinson) and the mean fluorescent intensity (MFI) was determined. Samples with only the secondary antibodies were used as the maximum MFI.

Epitope mapping using CD40L transfected fibroblasts and FACScan analysis. Biotinylated CD40-Fcγ (4 μg/ml) was incubated together with the different MoAb against CD40 (10 μg/ml) in 50 μl for 1 h at 4 °C. CD40L transfected fibroblasts (3×10^5 cells in 100 μl) were added and the cells were incubated additionally for 30 min Thereafter, the cells were washed and then stained with streptavidin-PE (DAKO A/S). The cells were analysed on a FACScan instrument (Becton Dickinson).

Results

Determination of the activation potential of mouse antihuman CD40 MoAbs

The activating potential of a panel of mouse antihuman CD40 MoAbs was determined by measuring the incorporation of [methyl-^{3}H]thymidine in purified tonsillar B cells. Anti-CD40 antibodies were added at 1 μg/ml and a representative result is shown in Fig. 2. To avoid any effect caused by the fragment crystallizable (Fc) part of the antibodies in the proliferation assay [17, 18], purified mouse IgG (20 μg/ml) was added to the B cells in all reactions.

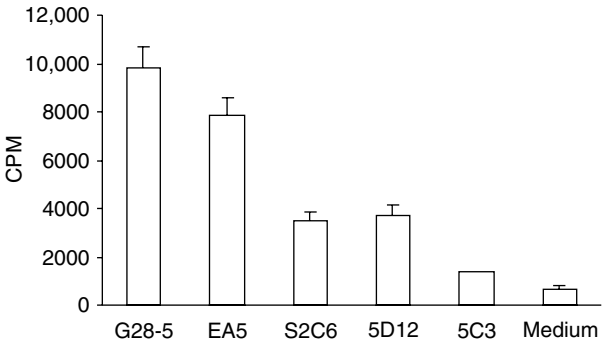

Figure 2 Activation potential of a panel of anti-CD40 antibodies measured as [methyl-^{3}H]thymidine uptake of tonsilla B cells after addition of anti-CD40 monoclonal antibodies (MoAbs). The experiments were performed using 1 μg/ml in three independent experiments.

G28-5 and EA5 are potent activators of B cells, while 5D12 and S2C6 are moderate activators and 5C3 is the least potent MoAb in this panel of anti-CD40 antibodies. These results are in good agreement with data reported by Pound *et al.* [19], who investigated all MoAbs but 5D12. The activation potential of MoAb 5D12 was first reported by Kwekkebom *et al.* in 1993 [20] and was considered to be a nonactivator. In our proliferation assay, 5D12 seems to induce a moderate level of proliferation. However, in the proliferation assay used by Kwekkeboom *et al.* the B-cells where stimulated with CD40 antibodies in the presence of anti-IgM coupled to Sepharose beads or anti-IgM together with rInterleukin-2 (rIL-2).

Determination of kinetic parameters and affinity constants

The kinetic binding parameters as well as the affinity constants for the binding between CD40 and the panel of mouse anti-CD40 MoAbs were determined by Biacore analysis and compared with the binding parameters for the interaction between CD40 and CD40L (Table 1). Three different concentrations of immobilized CD40-Fc were tested in order to avoid mass transport limitations, and diffusion effects and optimal signals were obtained for 650 RU of immobilized CD40-Fcγ. The affinities of the antibodies ranged from 5×10^9 1/M for 5D12 down to 8×10^6 1/M for S2C6, a difference close to 600 times. The largest contribution to this difference in affinity constants lies in the association rate constants, which spanned over three orders of magnitude, while the dissociation rate constants differed only three times.

No obvious correlation between activation potential and binding affinity could be observed. For example the high affinity of 5D12, being a moderate activator, is mainly caused by an extremely high association rate (2×10^6 $1/(M \times s)$), while the dissociation rate was of the same magnitude as for the other MoAbs. Furthermore, MoAb S2C6, which is also a moderate activator showed the lowest binding affinity, while G28-5, being a potent activator of B cells has a rather low affinity. Another potent activator is MoAb EA5, which had a moderate to high affinity and the least potent activator (5C3) had an affinity constant in the same range as EA5.

CD40L, which is a potent activator had a lower affinity for CD40 than all the anti-CD40 MoAb tested in this study, which was because of both lower association rate and faster dissociation rate. However, the recombinant CD40L used was monomeric and this is not the naturally occurring format of the molecule. Rather, it is believed that the CD40L forms trimers in the cell membrane [3, 21]. For this reason we tested another preparation of CD40L, which contained monomers, dimmers and trimers as analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). This preparation had the same dissociation rate constant as the monomeric preparation, but the association rate was much slower than for the monomeric CD40L preparation, giving an overall lower affinity for the interaction.

Domain mapping using FACScan analysis

To elucidate a possible correlation between activation potential and binding specificity, we mapped the domain binding sites of the CD40 MoAb. Figure 1A shows a three dimensional model of the CD40 molecule as determined by Bajorath *et al.* [1], based on the crystal structure of TNFR as a homologue. The amino acids suggested to be critical for the CD40–CD40L interaction are depicted in dark grey [1, 2, 22]. To elucidate the binding sites for the mouse MoAbs on the CD40 molecule, we expressed domains of CD40 molecule on the surface of COS cells, as outlined in Fig. 1B, and determined the interaction of the different mouse MoAbs and CD40L with these constructs. All CD40 variants were cloned with and without an N-terminal recognition tag (AD-2), that was used to verify surface expression of the molecule. As is shown in Table 2, all of the truncated CD40 variants were detected on the cell surface with an antibody (ITC88) binding to the recognition tag (AD-2). Furthermore, the functionality of the constructs was analysed with polyclonal anti-CD40 sera which bound to the D1, D1/B2, D2 and D2/B1 variants.

Table 1 Kinetic parameters and affinity constants for the interaction between immobilized CD40 molecule (650 RU), anti-CD40 mouse monoclonal antibody (MoAb) and CD40L, respectively

	k_{ass} (1/(M × s))	k_{diss} (1/s)	K_A (1/M)	Activation potential
5D12	2×10^6	4.2×10^{-4}	4.7×10^9	++
EA5	2×10^5	5.8×10^{-4}	3.5×10^8	+++
5C3	1×10^5	6.0×10^{-4}	1.8×10^8	+
G28-5	3×10^4	7.6×10^{-4}	4.0×10^7	+++
CD40L monomeric	2.5×10^4	1.2×10^{-3}	2×10^7	ND
CD40L multimeric	$<10^4$	1.5×10^{-3}	$<10^7$	+++
S2C6	2×10^3	2.4×10^{-4}	8.2×10^6	++

Activation potential is based on Fig. 2. +++, potent activator; ++, moderate activator; + poor activator; ND, not determined.

Table 2 Epitope mapping of monoclonal antibody (MoAb) using different CD40 domains

	CD40L	5D12	G28-5	S2C6	5C3	EA5	Polyclonal anti-CD40	ITC88
D1	+	+	+	+	+	+	+	+
D1/B2	+	+	+	(+)	–	–	+	+
D2	–	–	–	–	–	–	+	+
D2/B1	–	–	–	–	–	–	+	+
D3	–	–	–	–	–	–	–	+
D4	–	–	–	–	–	–	–	+

The ability of the anti-CD40 scFv antibodies to bind to truncated CD40 constructs transiently expressed on COS-7 cells was measured using FACScan. –, no binding, <10% of the population that displayed the constructs was positive compared with the negative control; (+), weak binding, 30% of the population that displayed the constructs was positive compared with the negative control; +, strong binding, >80% of the population that displayed the constructs was positive compared with the negative control.

All the mouse anti-CD40 MoAbs and the CD40L depend on residues in domain D1 or D1/B2 for binding. CD40L and MoAb 5D12, S2C6 and G28-5 are all moderate to potent activators and all binds to the D1/B2 variant, indicating that the binding site of these molecules depends on residues in the D1/B1 module. Moreover, MoAb EA5 (a potent activator) and 5C3 (the least potent activator) both show identical domain binding pattern, as these antibodies do not tolerate removal of even the distal module. Consequently, the epitopes of both these antibodies are probably located near the distal end of CD40. The fact that both the polyclonal sera and one previously described antibody (14) binds to the D2 and the D2/B1 constructs, indicates that these constructs are correctly folded and that all the mouse anti-CD40 antibodies indeed depend on residues located in domain 1. All the mouse anti-CD40 MoAbs and CD40L showed identical binding pattern to the CD40 constructs with or without the AD-2 epitope.

Epitope mapping using Biacore analysis

To further investigate how these antibodies and CD40L competed for epitopes on the CD40 molecule we took advantage of Biacore analysis (Table 3). The result shows that MoAb G28-5 is the antibody that influences the binding of CD40L (dimmer/trimer) to CD40 the most, while MoAb 5C3 is the antibody with the least effect. These findings are comparable with the results achieved by Pound *et al.* [19] and correlates to their activation potential of B cells. However, when comparing the results from all the MoAbs there was no statistically significant correlation with the activation potential.

In the competition experiment with two antibodies, only the MoAb 5C3 allowed a secondary antibody to bind the CD40 molecule, which agrees with the assumption that the 5C3 epitope is spatially different from the others.

Epitope mapping using a B-cell line and FACScan analysis

In the Biacore assays immobilized CD40-Fcγ was used and therefore we performed similar epitope mapping experiments using a B-cell line that expresses CD40. The relative position of the epitopes of the different MoAbs and the CD40L were mapped by measuring the interactions using FACScan analysis. The result is shown in Table 4 and most of the data agrees well with the results achieved using Biacore analysis. In summary, MoAb 5D12 and 5C3 seem to bind further away from the CD40L binding site whereas the MoAb G28-5 more significantly affect the binding of CD40L. The ability of the antibodies to block the subsequent binding of CD40L correlates

Table 3 Competition analysis determined by Biacore analysis

	Human CD40L–mouseCD8†	5D12†	G28-5†	S2C6†	5C3†	EA5†
Human CD40L–mouse CD8*	0	100	78	74	100	100
5D12*	67	0	0	0	0	0
G28-5*	52	6	0	0	0–10	0
S2C6*	53	8	2	0	0	2
5C3*	71	43	36	39	0	20
EA5*	68	21	7	5	0	0

*First molecule.
†Second molecule.
The first molecule was allowed to bind the CD40 surface and thereafter the second molecule was added. The amount of bound second molecule (RU) was determined and compared with the amount bound first molecule (RU). Mean values of three independent experiments are shown. All numbers are given in percentage of bound second molecule.

Table 4 Competition analysis determined by FACScan

	Human CD40L–mouseCD8†	5D12†	G28-5†	S2C6†	5C3†	EA5†
Human CD40L–mouseCD8*	0	70	30	10	30	20
5D12*	93	0	8	7	2	5
G28-5*	5	10	0	0	0	10
S2C6*	86	7	11	0	0	18
5C3*	180	11	45	3	0	15
EA5*	70	0	4	0	0	0
CD40L monomer*	0	72	100	13	85	28

*First molecule.
†Second molecule.
The first molecule was incubated with B-cells expressing the CD40 molecule, thereafter the second molecule was included and the mean fluorescent intensity (MFI) was determined. Samples with only the second molecule were used as the maximum MFI. Mean values of three independent experiments are shown. All numbers are given as percentage of bound second molecule.

significantly with the activation potential ($r = 0.903$, $P\ 0.05 = 0.878$ for $n = 5$). In this assay we also included a monomeric CD40L in the competition assay. Although the monomeric fraction is not the natural ligand it does provide information on the specific epitope recognition. The result obtained with the monomeric CD40L correlates with the result obtained with CD40L (dimmer/trimer) except for MoAb G28-5. Using the monomeric CD40L, CD40 binding by G28-5 is not affected at all whereas the trimeric version of the CD40L inhibits quite well. The MoAb 5C3 inhibition is also affected less by monomeric CD40L that agrees with the finding that 5C3 might recognize an epitope outside of the ligand-binding site. Another interesting observation is that 5C3 enhances binding of CD40L to the CD40 molecule, when bound prior to the CD40L, something that was also observed by Pound *et al.* [19]. This might indicate that MoAb 5C3 induces a conformational change in the CD40 molecule that exposes the site for CD40L binding or that 5C3 is able to cross-link CD40 in a manner that stabilizes the CD40–CD40L complex.

Epitope mapping using CD40L transfected fibroblasts and FACScan analysis

In a further approach we used a competition assay based on CD40L transfected fibroblasts. The system is reversed, as compared with the previously described methods, in that CD40L rather than CD40 is expressed on a cell surface. Biotinylated CD40 was preincubated with each of the anti-CD40 MoAbs and thereafter binding of CD40 to the CD40L transfected fibroblasts was analysed, using PE-streptavidin for detection. The results are summarized in Table 5, showing the highest inhibitory effect by MoAb S2C6 and the lowest by 5C3. When comparing all of the MoAb the ability to block the CD40–CD40L interaction there is no significant correlation with the ability to promote proliferation. However, the poorest activator (5C3) showed the lowest ability to inhibit the CD40/CD40L interaction, which is in agreement with the results from the epitope mapping analysis above.

Discussion

There is an increasing interest in monoclonal anti-CD40 antibodies for therapy, both for blocking the CD40–CD40L interaction [10] as well as for inducing signals via CD40 [23]. This has led us to conduct a detailed analysis of the properties of a set of widely used mouse antihuman-CD40 antibodies.

Our data clearly indicates that there is no correlation between high affinity of the MoAb and activation potential of B cells. Furthermore, dissecting the affinity into the individual kinetic binding constants did not give any clear relation between binding parameters and cellular activation. The affinity of the CD40–CD40L interaction is low, as indicated in earlier reports [24], but as CD40L forms trimers in the membrane [3, 21] it is likely that its high activation potential depends on its capacity to cross-link CD40.

The domain mapping experiments showed that the D1 domain is critical for CD40L binding. This is rather surprising as the ligand-binding pocket for CD40L is suggested to be formed by the D2 and D3 domain of CD40 [1]. The important residues for binding have been

Table 5 Inhibitory ability of mouse monoclonal antibodies (MoAbs) on the binding of CD40 to CD40L using transfected fibroblasts

MoAb	Inhibitory ability (%)	Activation potential
G28-5	81	+++
S2C6	84	++
EA5	72	+++
5D12	77	++
5C3	10	+

Mean values of three independent experiments are shown. Activation potential is based on Fig. 2. +++, potent activator; ++, moderate activator; +, poor activator.

identified through site-directed mutagenesis [1, 22, 25]. One potential explanation might be that the D1 domain of CD40 in homology with the closely related TNFR and Fas [26] contains a preligand-binding assembly domain (PLAD). In the case of TNFR and Fas, preassociation via the PLAD seems to be necessary for binding of the corresponding ligand even though the PLAD is physically distinct from the ligand-binding domains [27, 28]. In our experiments, by excluding the D1 domain we might lose the PLAD-mediated self-oligomerization of the CD40 molecule and thereby lose the CD40L binding ability, in analogy with these reports. When we removed the outermost module of CD40 (the D1/B2 construct) we retained CD40L binding, which is unexpected because Siegel *et al.* [28] have shown that almost all FasL binding was abolished when the outermost module from Fas was removed. However, they had grafted a HA-tag 5′ of their truncated constructs for detection and we have observed that certain antibodies do not bind CD40 if tagged with the AD-2 epitope, although they do bind when the tag is removed [14].

There was no correlation between the ability to activate B cells and the location of the epitope on CD40, as determined by domain mapping. However, the domain mapping experiments only reveal which domain (or module) the MoAb binds to whereas the epitope mapping indicates the spatial relationship.

The results from the FACScan analysis showed that ability to block the CD40L from binding to CD40 correlates well with the ability to promote activation of germinal centre B-cells. This is in agreement with the results presented by Barr and Heath [29] who investigated a panel of MoAb binding to mouse CD40 and came to the conclusion that the functional activity correlates with their position of binding, relative to CD40L.

When analysing how the antibodies and CD40L compete for binding to the CD40 molecule, the results differed somewhat, depending on the method used. In addition, they only correlate with the activation potential in the analysis where the ability to block CD40L to CD40 expressed on cells was measured. The absolute differences in inhibitory effect between the two methods (Biacore and FACScan) can be explained by their different nature. In the FACScan analysis all molecules are present under the same conditions and there is also an excess of the first molecule during all the incubation steps. In the Biacore, however, the second counterpart is injected into a continuous flow and during this phase the relative differences between the association and dissociation constant of the first and the second molecule will effect the detected level of blocking. This fact can also explain why the percentage of binding in Biacore is changing dependent on which of the two interacting molecules is immobilized and which is in the continuous flow. It can, however, not explain why the results from the epitope mapping using FACScan differ depending on which molecule is applied first. It is likely that the molecule that first binds to CD40 can effect the ability of the second molecule to bind in other ways than directly masking its epitope. Sterical hindrance, owing to the large size of the MoAb compared with the CD40 molecule is likely to affect the binding pattern. It is also possible that conformational changes induced by the first molecule can affect the ability of the second molecule to bind. Furthermore, as most of the molecules used in this study are bivalent or trivalent, it is possible that the nature of the CD40 complexes formed by the first molecule can either stabilize or destabilize the binding of the second molecule. This shows that comparative binding studies require careful analysis and preferentially several different methods should be used. Each molecule used in such a study should be applied in both orders to obtain a complete profile of the antibody.

The MoAbs used in most reports are all of mouse origin. To avoid human antimouse antibodies (HAMA) effects *in vivo* and to increase the circulation half-life of MoAbs to be used in therapy, antibodies of human origin would be of great advantage [30]. With established technologies like, e.g. phage display of human antibodies, these goals can today easily be obtained [31]. Consequently, findings presented in this study could prove valuable when designing selection strategies to develop human MoAbs to be used in therapy. Our data reveal the nature of several of the most widely used anti-CD40 antibodies and indicate that there is a correlation between cellular activation and ability to block CD40/CD40L interactions. Using these findings, it may be possible to devise schemes for selection of human anti-CD40 antibodies with certain properties, from phage display libraries, using a specific domain of CD40 as antigen and/or using one of the mouse antibodies described in this study to either block unwanted epitopes or specifically elute phages that binds to a certain epitope.

Acknowledgments

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