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Epitope-dependent synergism and antagonism between CD40 antibodies and soluble CD40 ligand for the regulation of CD23 expression and IgE synthesis in human B cells

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Background: The induction of IgE synthesis in naïve B cells requires two T-cell-derived signals: one delivered through CD40 and the other via interleukin-4 (IL-4). The natural counterstructure to CD40 is the CD40 ligand (CD40L). We have asked about the interplay between CD40L and CD40 mAb that recognize distinct epitopes in delivering signals for regulating IL-4-dependent IgE synthesis and the expression of CD23, the low-affinity IgE receptor, in resting B cells.

Methods: After culture of purified human tonsillar B cells with CD40 agonists and IL-4, surface CD23 was determined by flow cytometric analysis. CD23 levels in cell lysates and supernatants were quantified by ELISA, as were those of secreted IgE.

Results: With regard to both induction of CD23 and IgE production, soluble CD40L trimer (sCD40LT) showed synergistic interaction with two mAb to CD40 which bind to epitopes located outside the ligand binding site (EA5 and 5C3), but not with a mAb (G28-5) which effectively competes for CD40L binding to CD40. Each of the two noncompeting mAb to CD40 was able to cooperate strongly with sCD40LT in promoting high-level induction of CD23 even in the absence of IL-4, an effect mirrored in the promotion of strong homotypic clustering and high-rate DNA synthesis. G28-5, uniquely, induced a down-regulation in IL-4-induced CD23 expression with time, a change that was accompanied by an increase in the amount of soluble CD23 detected. While the two noncompeting mAb consistently synergized with sCD40LT for the promotion of IL-4-dependent IgE synthesis, sCD40LT and G28-5 (which, by itself, was the most potent of the CD40 mAb at inducing IL-4-dependent IgE production) exhibited mutual antagonism in this regard, the level of which could be quite profound.

Conclusions: This study demonstrates that appropriate targeting of CD40 can modulate IgE synthesis either positively or negatively.

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The promotion of IgE synthesis from uncommitted naïve B cells requires a minimum of two signals: the first, from interleukin (IL)-4 (or IL-13), induces the expression of germ-line epsilon transcripts; the second, delivered through B-cell-expressed CD40, results in productive IgE synthesis and secretion [reviewed in ref. 1]. Both the soluble cytokines and the engagement of CD40 by CD40L can be provided by either activated T_H cells or basophils and mast cells (1). Mice engineered to be deficient in IL-4 or either one of the molecules involved in the CD40–CD40L pairing fail to produce IgE, as do patients with the X-linked hyper-IgM syndrome where the CD40L gene is functionally inactive (2–5).

Each of the signals that participate in inducing IgE synthesis also promotes the expression of CD23, the low-affinity receptor for IgE, both as a membrane-bound surface molecule and as proteolytically cleaved soluble fragments (6). The contribution CD23 makes to the regulation of IgE production remains unclear. CD23-deficient mice exhibit elevated IgE responses (7), whereas the addition of the cleaved molecule to cultures *in vitro* can either promote or reduce the amount of IgE produced (8). Others have reported that the binding of membrane-bound CD23 to another of its ligands, CD21, might contribute to enhanced levels of IgE production (9). The confusion as to the precise role of CD23 in regulating IgE synthesis probably relates, in part, to different functions for the various forms of CD23; the promiscuous binding of this C-type lectin to a relatively large number of functional counterstructures; and the diversity and, often, complexity, of the biological systems in which the CD23 function has been analysed [reviewed in ref. 10].

Given its importance in mediating the symptoms associated with allergic asthma and immediate-type hypersensitivity, it is crucial to understand the control mechanisms leading to IgE production. In this paper, we describe the ability of different mAb to CD40 either to synergize or antagonize soluble CD40L for driving IL-4-promoted IgE synthesis in purified, resting human B cells; these opposing outcomes are reflected in a differential regulation of CD23 expression and appear to be dependent on the CD40 epitope recognized. One of the CD40 mAb, G28-5, has been shown to compete effectively for the CD40L binding site on CD40. Two other mAb, EA5 and 5C3, while both binding outside the CD40L site, each recognize different epitopes of CD40 (11). Thus, EA5, but not 5C3, competes with the S2C6 mAb for CD40 binding; moreover, 5C3, but not EA5, is able to enhance the binding of CD40L to its cognate receptor (11, 12). While previous reports have examined the interplay

between different CD40 mAb in regulating various aspects of B-cell function (13–15), this is the first study to address the possibility of distinct CD40 mAb modulating CD40L-driven IgE synthesis.

Material and methods

Reagents

Mouse monoclonal CD40 mAb, EA5, 5C3, and G28-5 were all of the IgG1 isotype and were purified from mouse ascites fluid by ion exchange chromatography on DE52 (Whatman Ltd, Maidstone, UK). The G28-5 clone was obtained from the American Type Culture Repository (Rockville, MD, USA). 5C3 was a kind gift of Prof. H. Kikutani (Osaka University, Japan), and EA5 was generously provided by Prof. T. LeBien (University of Minnesota, MN, USA). Mouse monoclonal CD23 mAb, BU38 (IgG1), was from D. Hardie (Immunology Department, University of Birmingham, Birmingham, UK) and was biotinylated by a standard method. Biotin and FITC-conjugated streptavidin were from Sigma. Soluble human CD40LT was produced in COS cells, as described by Fanslow et al. (16), and used at the dilutions indicated; supernatants from COS cells transfected with vector alone were used as controls. Human recombinant IL-4 was from R&D Systems (Oxford, UK) and was added to cultures at 20 ng/ml.

Cells

B cells were isolated from tonsils taken at routine tonsillectomy by negative selection protocols, exactly as described previously; resting B cells were separated as the high buoyant density fraction from a 60% Percoll (Pharmacia Biotech) gradient and were always more than 98% CD19⁺ve (17). For all assays, B cells were cultured at 37°C in a humidified incubator in 5% CO₂/95% air. Culture medium (CM) was RPMI-1640 containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), 2 mM glutamine (Gibco), and 10% (v/v) FCS.

DNA synthesis

DNA synthesis was determined by thymidine incorporation. After culture at 10⁵ cells per 200 µl in flat-bottom, 96-well microplates with additions for the times specified in the Results section, cells were pulsed for 16–18 h with [³H]thymidine (Amersham International, Amersham, UK,

10 $\mu\text{Ci}/\text{ml}$ in culture medium, 50 μl per well) and harvested on a Skatron cell harvester (Helis Bio Ltd, Newmarket, UK). Assays were performed in triplicate and means given, with replicates usually being within 10% and always within 15% of each other.

CD23 production

Surface CD23 expression was measured by indirect immunofluorescence, as described elsewhere (18), with BU38-biotin in the first layer and FITC-streptavidin in the second: 50 000 stained cells were analysed on a FACS IV (Becton Dickinson, Cowley, UK), and the mean fluorescent intensity (MFI) was determined. CD23 levels in cell lysates and supernatants were determined by a sandwich ELISA assay on duplicate samples, exactly as described previously (18).

IgE synthesis

For assays of IgE synthesis, resting B cells isolated as described above were further depleted of any remaining adherent cells by incubating on tissue-culture plastic for 2 h at 37°C and decanting the nonadherent cells. These cells were then cultured for 12 days at 5×10^4 per well in 50 μl of culture medium contained in round-bottom microwells before collecting supernatants. The culture medium was as described above except that Iscove's modified Dulbecco medium supplemented with human transferrin at 40 $\mu\text{g}/\text{ml}$ (Sigma) was used in place of RPMI-1640. IL-4 at 20 ng/ml was always included. These conditions had been determined previously as being optimal for the promotion of IgE synthesis from purified resting tonsillar B cells. IgE concentration in the harvested supernatants was determined by quantitative ELISA (The Binding Site, Birmingham, UK). Results are given as means of quadruplicate determinations that were always within 15% of each other.

Results

Interplay between CD40 mAb and sCD40LT in promoting CD23 production and release

In the first set of experiments, the capacity of the ligand-competing G28-5 mAb and the noncompeting EA5 mAb to promote IL-4-dependent and -independent CD23 production and release in resting B cells was compared to that of sCD40LT. While the two mAb and sCD40LT were each

incapable of inducing significant CD23 production in the absence of IL-4 (data not detailed), they all synergized with IL-4 for enhancing CD23 synthesis and release (Fig. 1). Differences were observed, however, in that the amount of IL-4-dependent cell-associated CD23 provoked by G28-5 was much lower than that encouraged with EA5 and sCD40LT; the amount of soluble CD23 generated was, however, similar with all three CD40 agonists. These data therefore indicate that there was a higher rate of CD23 cleavage from IL-4-stimulated cells in the presence of G28-5 than with either of the other two reagents. Optimal IL-4-dependent CD23 production was obtained with 1 $\mu\text{g}/\text{ml}$ of each of the mAb and with a 1:25 dilution of sCD40LT, respectively. In separate experiments (not detailed), the other noncompeting CD40 mAb, 5C3, was shown to act similarly to EA5 and sCD40LT in promoting high-level augmentation of CD23 production and release in IL-4-stimulated B cells with G28-5 again being exceptional in provoking a much higher ratio of soluble:cell-associated CD23.

We have previously shown that, at 1 $\mu\text{g}/\text{ml}$, each of the CD40 mAb binding outside the CD40L binding site (EA5 and 5C3) synergizes with sCD40LT for promoting DNA synthesis in resting tonsillar B cells (15). We asked here about the dose response of this effect and compared the ability of all three mAb to induce DNA synthesis whether alone, with IL-4, or with sCD40LT. The results presented in

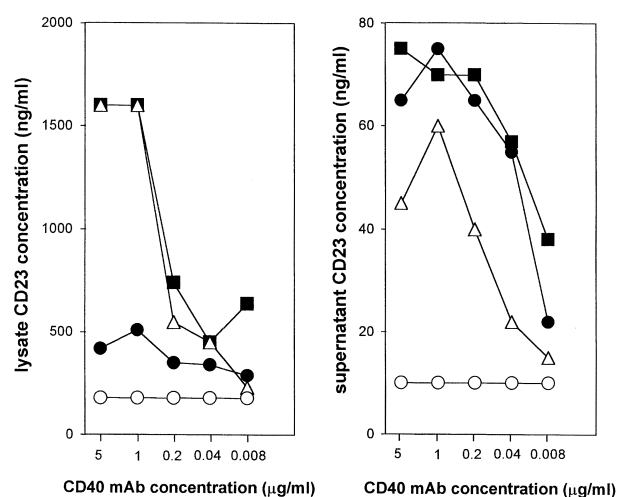


Figure 1. Ability of CD40 mAb and sCD40LT to enhance IL-4-dependent CD23 production. Resting B cells ($10^6/\text{ml}$) were cultured for 3 days with IL-4 (20 ng/ml) and either culture medium alone (○), G28-5 (●), EA5 (■), or sCD40LT (△), for which initial concentration represented 1:5 dilution of transfected COS cell-conditioned medium. Culture supernatants were then harvested (right-hand panel) and cells lysed (left-hand panel) for determination of CD23 levels by ELISA.

Fig. 2 demonstrate that of the three mAb studied, G28-5 was superior to 5C3 and EA5 at promoting IL-4-dependent DNA synthesis in resting B cells, but, again, only EA5 and 5C3 synergized with sCD40LT to this end and could do so even at relatively low concentrations of the mAb. Synergy with

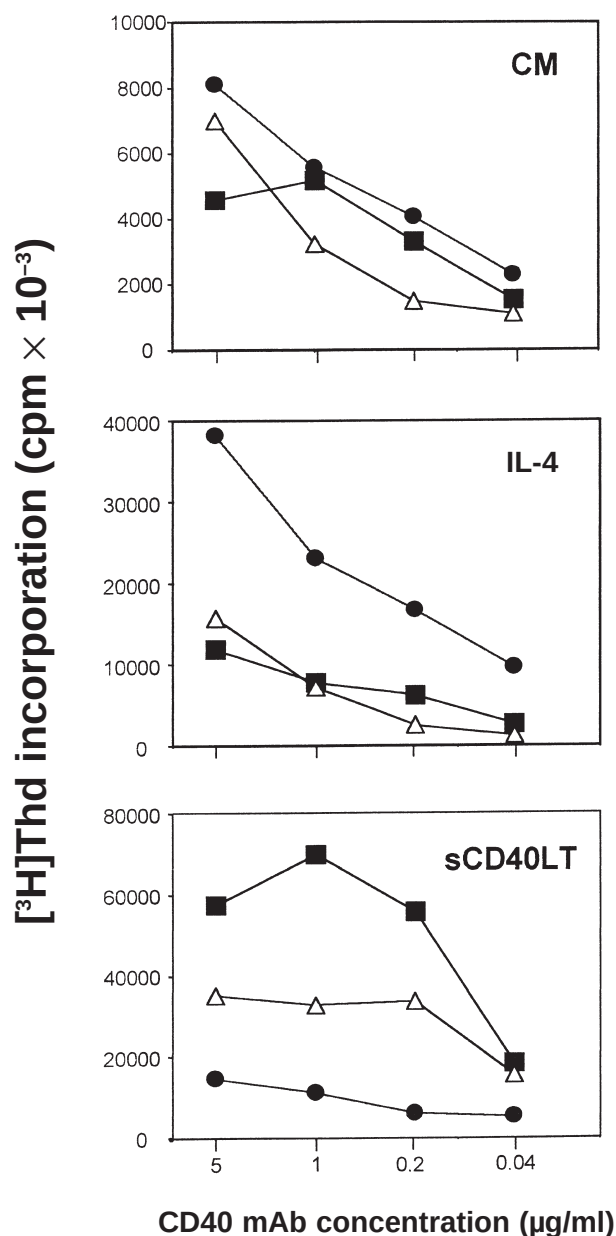


Figure 2. Ability of CD40 mAb to promote DNA synthesis alone, with IL-4, or together with sCD40LT. Resting B cells were cultured for 3 days in microwells for measurement of DNA synthesis, as described in Material and methods. G28-5 (●), EA5 (■), and 5C3 (△) were added at concentrations shown, together with culture medium alone (CM, upper panel), IL-4 (20 ng/ml, middle panel), or sCD40LT (1:25 dilution of transfected COS cell conditioned medium, lower panel). Levels of incorporation of radioactivity without mAb were as follows. CM, 568 cpm; IL-4, 1215 cpm; sCD40LT, 6798 cpm. Results are means of triplicate determinations that were always within 10% of each other. Thd: thymidine.

sCD40LT at 1:25 was optimal with 1 µg/ml for both EA5 and 5C3, and on the basis of this and the above study (Fig. 1), these concentrations were chosen for further investigation of the interactive effects of the mAb with CD40L. It should be noted that, in contrast to G28-5, which induces rapid homotypic clustering among resting B cells (17), neither of the two noncompeting mAb encouraged strong cluster formation, but they each substantially enhanced the moderate level of clustering promoted in response to sCD40LT ([11] and data not detailed).

As seen from Table 1, the actual amounts of CD23 produced in response to the various agonists could be quite variable among individual samples. Nevertheless, a consistent pattern emerged in the interplay of the different mAb with sCD40LT for promoting this phenotype. Thus, EA5 and 5C3 each synergized with sCD40LT for promoting CD23 production, whereas G28-5 failed to demonstrate any such effect. The amount of CD23 induced by each of the noncompeting mAb in combination with sCD40LT was particularly marked for cell-associated (lysate) material and approached or equalled that seen in B cells cultured with IL-4.

Table 1. Interplay between CD40 mAb and sCD40LT for production of CD23^a

CD40 mAb added	Lysate CD23 (ng/ml)		Soluble CD23 (ng/ml)	
	CM	sCD40LT	CM	sCD40LT
Tonsil 1				
None	20	10	20	25
G28-5	25	45	50	85
EA5	35	280	78	230
5C3	15	205	22	153
Tonsil 2				
None	<5	<5	<5	<5
G28-5	<5	<5	<5	<5
EA5	<5	170	<5	28
5C3	<5	175	<5	7
Tonsil 3				
None	7	50	8	40
G28-5	7	38	10	102
EA5	15	>800	20	125
5C3	10	>800	8	82
IL-4	285	>800	30	350

^aResting tonsillar B cells were cultured at 10⁶/ml for 3 days before harvesting supernatant CM and lysing cells for determination of CD23 concentration by ELISA. CD40 mAb were added at 1 µg/ml and sCD40LT as 1:25 dilution of COS supernatant. Each data point represents mean of duplicate samples that were always within 10% of each other. CM: culture medium.

Interplay between CD40 mAb and sCD40LT in inducing surface CD23 expression

By themselves, all of the CD40 mAb and sCD40LT were each able to induce a small increase in the low level of CD23 expressed on resting tonsillar B cells after 48-h culture (Table 2). Similarly, all of the mAb and sCD40LT were each capable of enhancing the already high level of surface CD23 expression induced by IL-4 at 48 h. The G28-5 mAb appeared to be the least active in its ability to enhance IL-4-dependent surface CD23 expression at 48 h. A further study of the kinetics, however, revealed that at 24 h, G28-5 was among the more efficient of the CD40 mAb at enhancing IL-4-promoted CD23 expression, while, by 72 h, its presence appeared to result in a reduction in the amount of CD23 expressed on culture with IL-4. These results, taken together with those presented in the previous section, are consistent with G28-5 promoting a more rapid turnover of CD23 from the cell surface than is obtained with sCD40LT, EA5, or 5C3.

Table 2. Interplay between CD40 mAb and sCD40LT for promoting induction of surface CD23^a

CD40 mAb added	Surface CD23 expression (MFI)			
	CM		IL-4 ^b	
	CM	sCD40LT	CM	sCD40LT
Day 2 (n=4)				
None	17 (1.4)	18 (2.5)	55 (14)	169 (15)
G28-5	20 (2.7)	25 (2.1)	83 (13)	90 (17)
EA5	26 (7.4)	61 (20)	129 (40)	149 (32)
5C3	22 (6.4)	60 (17)	97 (22)	147 (34)
Day 1 (n=2)				
None	10, 19	9, 17	43, 53	145, 154
G28-5	14, 23	13, 23	129, 138	128, 137
EA5	7, 16	15, 24	121, 131	145, 154
5C3	12, 21	15, 24	91, 100	153, 162
Day 3 (n=1)				
None	22	27	88	189
G28-5	23	26	61	65
EA5	25	58	105	207
5C3	28	109	147	202

^aResting B cells were cultured with CD40 mAb and sCD40LT, as in Table 1, and surface expression of CD23 was measured by FCM. Initial experiments were performed after 2 days of culture and results shown as means (SD) for tonsils from four donors. Day 1 results show individual data from tonsils from two donors. Results are mean fluorescence intensities (MFI) after subtraction of isotype control binding.

^bIL-4 was added at 20 ng/ml. CM: culture medium.

When added in combination, both EA5 and 5C3 were each capable of synergizing with sCD40LT for inducing CD23 at the resting B-cell surface. Indeed, by 48 h, the level of surface CD23 induced by this regimen equalled or exceeded that promoted by IL-4 alone. G28-5 failed to augment sCD40LT-promoted CD23 expression significantly. Neither EA5 nor 5C3 was able to enhance further the very high level of CD23 expression induced by sCD40LT and IL-4 together; again, the addition of G28-5 led to a reduction in the amount of CD23 detected at the surface of cells cultured with sCD40LT and IL-4 together (Table 2).

Interplay between CD40 mAb and sCD40LT for promoting IgE synthesis

As noted by others in previous studies, there could be wide variation in the amount of IgE synthesis induced in B cells from different individuals (Table 3). Basal levels of IgE produced by purified B cells in response to IL-4 alone were never greater than 0.5 ng/ml. Of the different CD40 mAb used, G28-5 was – of itself – the most efficient at enhancing IL-4-dependent IgE synthesis, with levels usually reaching or even exceeding those achieved with sCD40LT. Invariably, however, when G28-5 and sCD40LT were used in combination, there was a resultant mutual antagonism of the IgE synthesis promoted by either reagent alone. In stark

Table 3. Interplay between CD40 mAb and sCD40LT for regulation of IgE synthesis

sCD40LT	IgE (ng/ml)				
	CM	G28-5	EA5	5C3	
Tonsil 1	–	0.26	20.3	8.3	3.0
	+	15.1	1.4	194	180
Tonsil 2	–	0.12	1.7	0.21	0.18
	+	1.5	0.12	28.8	19.8
Tonsil 3	–	0.12	0.15	0.5	0.12
	+	1.2	0.12	31.0	8.0
Tonsil 4	–	0.12	50.4	ND	0.15
	+	0.12	23.7	ND	16

Resting B cells were cultured for 12 days with CD40 mAbs or CM in presence or absence of sCD40LT. Reagent concentrations were as given in Tables 1 and 2. Results are culture supernatant IgE concentrations determined by ELISA for cells from tonsils from four donors. Each data set is mean of quadruplicate analyses that were always within 15% of each other. ND: not determined; CM: culture medium.

contrast, sCD40LT always synergized with either EA5 or 5C3 for promoting IL-4-dependent IgE synthesis in resting tonsillar B cells (Table 3). No detectable IgE synthesis was observed in the absence of IL-4 with any of the CD40 mAb, either alone or in combination with sCD40LT (data not detailed).

Discussion

The CD40-CD40L interaction is central to many facets of T-dependent B-cell responses, including the IL-4-dependent promotion of IgE synthesis (19). The understanding of the processes which lead to IgE synthesis and the ability to modify this response are clearly of major importance given the contribution of this isotype to the development of allergic disease. Here we have shown that, depending upon the epitopes of CD40 engaged, there can be differential outcomes not only with regard to the synthesis of IgE but also the expression and release of CD23, the low-affinity receptor for IgE.

The two mAb which bind to CD40 outside the CD40L-binding site (5C3 and EA5) were able to synergize with sCD40LT for inducing CD23 expression at the cell surface, for provoking CD23 release, and for promoting the synthesis of IgE: these mAb also cooperate with sCD40LT for inducing high-rate DNA synthesis. Despite evidence that these noncompeting mAb bind to different epitopes of CD40 (11, 12), they behaved similarly in the functional studies reported here.

Recent data demonstrate that, at least for the mouse, there exists a strong link between the cycling of B cells and their ability to switch to IgE synthesis, a notion consistent with the observations made here (20). The literature on the role of homotypic adhesions in regulating IgE synthesis is contradictory, with one report indicating a clear enhancement due to clustering while another suggests that clustering suppresses IL-4-dependent CD40-induced IgE production (21, 22). Our data are more compatible with the former where the G28-5 mAb which induced the strongest clustering of the CD40 agonists used also tended, by itself, to promote the highest level of IL-4-dependent IgE synthesis. Moreover, the synergistic interaction between the two noncompeting mAb and sCD40LT for inducing IgE synthesis was reflected in enhanced cluster formation.

Considerable uncertainty exists regarding the precise contribution and importance of CD23 to the regulation of IgE synthesis. Some reports indicate the involvement of CD23, possibly through its binding to CD21, in promoting

homotypic adhesion and this, as discussed above, could of itself lead to a modulation of IgE production (22). Its ability to serve as a low-affinity receptor for IgE or, when shed, as an IgE-binding factor has also been implicated in providing a level of regulation for the development of IgE responses (8–10). The relative roles of the membrane and soluble forms and the mechanism of their action remain a question of debate. It seems, while not *a priori* necessary for IgE synthesis (as judged by gene knockout models), that – when present – CD23 can serve to modulate the IgE response (7, 8, 23). Also, signals which influence CD23 expression correspondingly influence IgE production (6). Moreover, inhibition of CD23 cleavage results in a diminution of IgE synthesis, while it has been speculated that the ability of the Der p 1 protease of house-dust mite to cleave CD23 can lead to enhanced IgE production by ablating an otherwise important inhibitory feedback mechanism (24).

In the present study, the noncompeting mAb which synergized with sCD40LT for promoting IgE synthesis also enhanced the level of CD23 produced both as a surface molecule and in its shed form. Interestingly, high levels of CD23 could be induced under such a regimen even in the absence of IL-4. It is also of note that stimulation with the competing mAb, G28-5 – while initially being among the most efficient of the CD40 agonists at enhancing IL-4-dependent CD23 expression on resting B cells – uniquely resulted, with time, in a diminution of expression which was accompanied by the appearance of high levels of soluble CD23 in the cultures. This reduction in membrane CD23, as argued above, could contribute to the relative efficacy of G28-5 in promoting IL-4-dependent IgE synthesis. The apparent high rate of CD23 cleavage in response to G28-5 might also contribute to its increased potency in promoting homotypic adhesion. Thus, Moulder et al. have described a non-CD21-dependent mechanism of CD23-mediated clustering which requires that membrane-bound CD23 is cleaved before the residual cell-associated CD23 moiety is able to engage in homotypic B-cell aggregation (25).

One surprising feature of the study was the mutual antagonism that was observed between G28-5 and sCD40LT in the induction of IgE synthesis, the extent of which could quite dramatically reduce the amount of ensuing IgE to near basal levels. The interpretation of this finding is unclear but it might result from confounding actions of the two CD40 agonists at different stages of the response: differences in the stability of the two reagents over the 12-day culture period might contribute to such a complex interplay. Although demonstrating competitive binding for CD40, G28-5 and sCD40LT do differ in that the former is dimeric and the

latter trimeric. Moreover, as illustrated by the different relative rates of CD23 turnover they induce from the surface of IL-4-cultured cells, they do differ functionally. In the context of the findings presented here, it is of interest to note the study of Splawski et al., who, using the CD40 mAb 626.1, obtained inhibition of the production of all Ig isotypes – including that of IgE – when they stimulated B cells with CD3-activated T cells (which would be expressing CD40L), whereas, in the absence of T cells, the mAb, together with IL-4, promoted high-rate switching to IgE (26).

While the present study provides further insight into the complexity of the factors that can regulate IgE synthesis, it does not address the issue of the physiological relevance of CD40 epitopes that lie outside the CD40L-binding site. Many of the cooperative interactions described in this paper could result from enhanced cross-linking of CD40 when more than one epitope is engaged. Although only preliminary evidence has been presented, it is possible that more

than one ligand for CD40 exists, and that a so far uncharacterized CD40L may engage a site on CD40 distinct from the known ligand-binding site (27). If so, this would have profound implications for the regulation of IgE synthesis.

In conclusion, this study – by focusing on the interplay among signals delivered through different epitopes of CD40 – has highlighted for resting B cells, linkage between the promotion of homotypic adhesions, entry into cell cycle, the expression and shedding of CD23, and the eventual production of IgE. The insights gained could be valuable in attempts to target CD40 as a potential therapeutic modality in IgE-dependent allergic disease, and the ability to modulate IgE production with purely soluble reagents is clearly of interest in this regard.

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