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CD28-Independent Costimulation of T Cells by OX40 Ligand and CD70 on Activated B Cells

Hisaya Akiba,*† Hideo Oshima,*† Kazuyoshi Takeda,*† Machiko Atsuta,*† Hiroyasu Nakano,*† Atsuo Nakajima,§ Chiyoko Nohara,* Hideo Yagita,*† and Ko Okumura2*†

OX40 and its ligand (OX40L) have been implicated in T cell-dependent humoral immune responses. To further characterize the role of OX40/OX40L in T-B cell interaction, we newly generated an anti-mouse OX40L mAb (RM134L) that can inhibit the costimulatory activity of OX40L transfectants for anti-CD3-stimulated T cell proliferation. Flow cytometric analyses using RM134L and an anti-mouse OX40 mAb indicated that OX40 was inducible on splenic T cells by stimulation with immobilized anti-CD3 mAb in a CD28-independent manner, while OX40L was not expressed on resting or activated T cells. OX40L was inducible on splenic B cells by stimulation with anti-IgM Ab plus anti-CD40 mAb, but not by either alone. These activated B cells exhibited a potent costimulatory activity for anti-CD3-stimulated T cell proliferation and IL-2 production. Anti-CD80 and anti-CD86 mAbs partially inhibited the costimulatory activity, and further inhibition was obtained by their combination with RM134L and/or anti-CD70 mAb. We also found the anti-IgM Ab- plus anti-CD40 mAb-stimulated B cells exhibited a potent costimulatory activity for proliferation of and IL-2 production by anti-CD3-stimulated CD28- T cells from CD28-deficient mice, which was substantially inhibited by RM134L and/or anti-CD70 mAb. These results indicated that OX40L and CD70 expressed on surface Ig- and CD40-stimulated B cells can provide CD28-independent costimulatory signals to T cells. The Journal of Immunology, 1999, 162: 7058–7066.

In humoral immune responses, the cognate interaction between Ag-specific T cells and B cells is critical not only for B cell activation but also for T cell activation. Optimal activation of Ag-specific T cells requires engagement of the TCR with Ag/MHC and a costimulatory signal provided by APC (1, 2). It has been shown that CD28 on T cells and its ligands (CD80 and CD86) on APC play a major role in providing a costimulatory signal for T cells (3–5). However, CD28-deficient mice are not fully defective in humoral immune responses (6, 7). It has also been demonstrated that activated B cells exhibited a potent costimulatory activity for proliferation of CD4+ T cells from CD28-deficient mice (8). These results suggested the existence of a CD28-independent costimulatory pathway.

We previously demonstrated that CD27, which is a member of the TNF receptor superfamily and is constitutively expressed on most T cells, can transmit a CD28-independent costimulatory signal upon interaction with its ligand CD70 expressed on a mouse B lymphoma cell line (9). In addition, we and others have shown that OX40 ligand (OX40L),3 which is another member of the TNF superfamily, can provide a costimulatory signal to T cells, resulting in increased proliferation and cytokine production (10–12). OX40 was originally identified as a cell surface Ag on activated CD4+ T cells in rats (13). cDNA cloning of rat, mouse, and human OX40 showed that it belongs to the TNF receptor superfamily (14–16). It has been reported that expression of OX40 is restricted to activated T cells in rats, humans, and mice (13, 15, 17). On the other hand, expression of OX40L has been found on mouse activated B cells (15, 18), human dendritic cells (DC) (19), human vascular endothelial cells (20), and human and rat HTLV-1-transformed T cells (10, 21). Recent studies demonstrated that ligation of OX40L on human DC enhanced their maturation and production of cytokines (19) and that blockade of OX40L during naïve T-DC interaction suppressed the development of IL-4-producing T cells (22), suggesting that OX40-OX40L may play an important role in T-DC interaction. In addition, OX40L expressed on activated B cells has been shown to transmit a signal that enhances the proliferation of and Ig secretion by B cells (18). This suggested that the cross-linking of OX40L on B cells by OX40 on T cells might deliver a B cell differentiation signal, and thus, the OX40-OX40L system might play an important role in T-B interaction. To support this idea, it has been reported that administration of an anti-OX40 Ab inhibited the T cell-dependent humoral immune response in mice, possibly by interrupting the OX40-OX40L interaction (23).

To further characterize the role of OX40/OX40L in T-B cell interaction, we established a functional blocking mAb against mouse OX40L, which will be useful for interrupting the OX40-OX40L interaction in vivo. Our present study formally defined the expression of OX40 and OX40L on activated T and B cells. We found that the expression of OX40 on activated T cells is CD28 independent, and the CD28-independent costimulatory activity of surface Ig- and CD40-activated B cells is coordinately mediated by OX40L and CD70. The physiological relevance of these findings is discussed.

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Materials and Methods

Animals and cell lines

Six- to seven-week-old male DBA/2 and C57BL/6 mice and 6- to 8-week-old female SD rats were purchased from Charles River Japan (Atsugi, Japan). Six- to seven-week-old C57BL/6 CD28-deficient (−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Murine mastocytoma P815, murine T lymphoma L5178Y, murine B lymphomas A20.2J and 2PK-3, and murine myeloma P3U1 (F3 x 63Ag8U.1) were purchased from American Type Culture Collection (Manassas, VA). The hybridomas producing mAbs against MHC class II (MS5.114), heat stable Ag (J11d), B220 (RA3-3A1), CD8 (53-6.7), CD4 (RM4-4), Thy1.2 (J1), and Thy1.2 (J1) were also obtained from American Type Culture Collection. The hybridoma OX86 producing anti-mouse OX40 mAb (24) was obtained from European Collection of Cell Cultures (Wiltshire, U.K.). The hybridoma producing mAb against CD4 (RL172) was provided by Dr. T. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). A murine B lymphoma cell line BCL1-B20 (25) was provided by Dr. K. Takatsuki (Institute of Medical Science, University of Tokyo, Tokyo, Japan). A murine T lymphoma cell line MBL2 (26) was provided by Dr. T. Nishimura (Tokai University, Kanagawa, Japan). These cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM t-glutamic acid, 1 mM sodium pyruvate, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

Preparation and culture of splenic T cells and B cells

Six- to seven-week-old male DBA/2 and C57BL/6 mice and 6-wk-old BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). Mice were housed in a pathogen-free facility at the National Institute of Medical Science, Tokyo, Japan. A murine T lymphoma cell line MBL2 (26) was provided by Dr. T. Nishimura (Tokai University, Kanagawa, Japan). These cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM t-glutamic acid, 1 mM sodium pyruvate, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

Antibodies

PE-conjugated goat anti-human and anti-rat IgG Abs were purchased from Caltag (South San Francisco, CA). Purified anti-CD16/32 (2.4G2), CD3 (145-2C1), CD19 (3.2B, 3.2C), CD8 (3.155), B220 (RA3-6B2), CD11b (M1/70) mAbs, PE-conjugated goat anti-human and anti-rat IgG Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). A fusion protein consisting of the extracellular portion of murine OX40 and the Fc portion of human IgG1 (OX40-Fc) was prepared as described previously (10).

Preparation of mouse OX40L transfectants

A cDNA fragment encoding the entire open reading frame of mouse OX40L was cloned by limiting dilution. RM134L was purified from ascites by standard procedures with caprylic acid, and purity was verified by SDS-PAGE analysis.

Preparation and culture of splenic T cells and B cells

Six- to seven-week-old male DBA/2 and C57BL/6 mice and 6-wk-old BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). Mice were housed in a pathogen-free facility at the National Institute of Medical Science, Tokyo, Japan. A murine T lymphoma cell line MBL2 (26) was provided by Dr. T. Nishimura (Tokai University, Kanagawa, Japan). These cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM t-glutamic acid, 1 mM sodium pyruvate, 0.1 mg/ml penicillin and streptomycin, and 50 μM 2-ME.

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Flow cytometric analysis

Cells (5 x 10^6) were first preincubated with anti-CD16/32 mAb to avoid nonspecific binding of Abs to FcγR and then incubated with a saturating amount of OX40-Ig, FITC- or PE-labeled mAb, or biotinylated mAb. After washing with PBS twice, the cells were incubated with PE-conjugated goat anti-human IgG Ab for OX40-Ig or PE-labeled streptavidin for biotinylated mAb. After washing with PBS twice, the stained cells (live-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACScan (Becton Dickinson, San Jose, CA), and data were processed using the CellQuest program (Becton Dickinson).

FIGURE 1. Characterization of RM134L mAb. A, Reactivity of RM134L to mouse OX40L transfectants and cell lines. NRK-52E- and L5178Y-derived transfectants (OX40LNRK, OX40LL5178Y, mock/NRK, and mock/L5178Y), a T lymphoma cell line (MBL2), and B lymphoma cell lines (BCL1-B20, A20.2J, and 2PK-3) were stained with biotinylated RM134L followed by PE-labeled streptavidin. The shaded histograms indicate background staining with RM134L and PE-labeled anti-human IgG without OX40-Ig. B, RM134L inhibits OX40-Ig binding to OX40L transfectants. OX40L/L5178Y cells were pretreated with or without RM134L and then stained with OX40-Ig followed by PE-labeled goat anti-human IgG Ab. The control histogram represents background staining with RM134L and PE-labeled anti-human IgG without OX40-Ig.
Results

Establishment of anti-mouse OX40L mAb

To isolate murine OX40L cDNA, we first tested the reactivities of various mouse cell lines with OX40-Ig. A B lymphoma cell line, BCL1-B20, exhibited high reactivity with OX40-Ig (data not shown). Then, OX40L cDNA was cloned into a mammalian expression vector from RNA of BCL1-B20 cells by RT-PCR and transfected into NRK-52E, L5178Y, and P815 cells. Stable expression of OX40L on the cDNA transfectants (OX40L/NRK, OX40L/L5178Y, and OX40L/P815), but not on mock-transfected cells (mock/NRK, mock/L5178Y, and mock/P815), was verified by binding of OX40-Ig (data not shown). We then immunized an SD rat with OX40L/NRK cells and fused the splenocytes with P3U1 myeloma cells. The hybridomas producing anti-OX40L mAb were screened by specific reactivity to OX40L/L5178Y cells. As represented in Fig. 1A, one mAb, designated RM134L, was obtained, which bound to OX40L/L5178Y and OX40L/NRK cells but not to mock/L5178Y or mock/NRK cells. A number of mouse cell lines were also tested for OX40L expression by staining with RM134L and Northern blot analysis. BCL1-B20 and T lymphoma cell line MBL2, which expressed OX40L mRNA as estimated by Northern blot analysis (not shown), were reactive with RM134L (Fig. 1A), but the other T (WR19L, YAC-1, and BW5147) or B (A20.2J, 2PK-3, CH1, and WEHI231) lymphoma cell lines, which did not express OX40L mRNA, were not reactive with RM134L (Fig. 1A and data not shown). Macrophage cell lines (J774.1 and P388D1) also did not express OX40L mRNA or react with RM134L (not shown). These results indicated a strict coincidence of OX40L mRNA expression and RM134L reactivity. Moreover, preincubation with RM134L blocked OX40-Ig binding to OX40L/L5178Y cells (Fig. 1B), further substantiating that RM134L is specific for mouse OX40L.

T cell costimulatory activity of mouse OX40L and its blockade by RM134L

It has been shown that OX40L provided a costimulatory signal for T cell proliferation in mice, humans, and rats (10–12). To examine whether RM134L can block the costimulatory activity of mouse OX40L, splenic T cells were cocultured with OX40L/P815 or mock/P815 cells in the presence of anti-CD3 mAb and RM134L, and the proliferative responses were assessed. As shown in Fig. 2A, while OX40L/P815 cells expressed a high level of mouse OX40L, mock/P815 cells also expressed OX40L at a low level, which was verified by Northern blot analysis (not shown). As shown in Fig. 2B, OX40L/P815 cells costimulated the proliferation of anti-CD3-stimulated T cells far more efficiently than mock/P815 cells. The peak proliferative response on day 2 was blocked by RM134L, but not by control IgG, in a dose-dependent manner (Fig. 2C). In addition, the proliferative response costimulated by mock/P815 cells was abrogated by RM134L (data not shown), indicating a substantial contribution of endogenously expressed OX40L to the costimulatory activity of mock/P815 cells. These results indicated that mouse OX40L exhibits a potent costimulatory activity for anti-CD3-stimulated T cell proliferation, which can be blocked by RM134L.

Expression of OX40L and OX40 on activated T cells

We next examined the expression of OX40L and OX40 on splenic T cells by flow cytometric analysis using RM134L and an anti-mouse OX40 mAb (MRC OX86) (24). An anti-CD25 mAb was also included as an activation marker. Purified splenic T cells were

T cell proliferation and IL-2 assay

Purified splenic T cells (1 × 10^5/well) were cocultured with irradiated (100 Gy) transfectants (2 × 10^5/well) or irradiated (20 Gy) preactivated B cells (5 × 10^5/well) in the presence of the absence of anti-CD3 mAb (2C11, 1 μg/ml) in 96-well round-bottomed culture plates at 37°C for 48 h. For estimating proliferative responses, the cultures were pulsed with 0.5 μCi/well of [3H]thymidine (DuPont-New England Nuclear, Boston, MA) for the last 9 h and then harvested using a Micro 96 Harvester (Skatron, Lier, Norway). Incorporated radioactivity was measured in a micro beta counter (Micro β Plus, Wallac, Turku, Finland). To determine IL-2 production, cell-free supernatants were collected at 48 h and subjected to ELISA using OptEIA Mouse IL-2 Set (PharMingen) according to the manufacturer’s instruction.
stimulated for 24–120 h with immobilized anti-CD3 mAb without (A) or with (B) soluble anti-CD28 mAb and were harvested at the indicated periods. Cells were stained with PE-labeled anti-CD25 mAb, biotinylated RM134L, anti-OX40 mAb, or control IgG followed by PE-labeled streptavidin. C. C57BL/6 splenocytes (3 × 10⁶/ml) were stimulated with immobilized anti-CD3 mAb for 48 h and double-stained with FITC-labeled anti-CD4 or CD8 mAb and biotinylated anti-OX40 mAb or control IgG followed by PE-labeled streptavidin. D. Splenic T cells (3 × 10⁶/ml) from CD28-deficient (-/-) mice were stimulated with immobilized anti-CD3 mAb for 48 h and stained with biotinylated RM134L, anti-OX40 mAb, or control IgG followed by PE-labeled streptavidin. In A, B, and D, the shaded histograms indicate staining with the indicated mAb and the blank histograms indicate background staining with control IgG.

**FIGURE 3.** Expression of OX40 and OX40L on activated T cells. Splenic T cells (3 × 10⁶/ml) from C57BL/6 mice were stimulated with immobilized anti-CD3 mAb without (A) or with (B) soluble anti-CD28 mAb and were harvested at the indicated periods. Cells were stained with PE-labeled anti-CD25 mAb, biotinylated RM134L, anti-OX40 mAb, or control IgG followed by PE-labeled streptavidin. C. C57BL/6 splenocytes (3 × 10⁶/ml) were stimulated with immobilized anti-CD3 mAb for 48 h and double-stained with FITC-labeled anti-CD4 or CD8 mAb and biotinylated anti-OX40 mAb or control IgG followed by PE-labeled streptavidin. D. Splenic T cells (3 × 10⁶/ml) from CD28-deficient (-/-) mice were stimulated with immobilized anti-CD3 mAb for 48 h and stained with biotinylated RM134L, anti-OX40 mAb, or control IgG followed by PE-labeled streptavidin. In A, B, and D, the shaded histograms indicate staining with the indicated mAb and the blank histograms indicate background staining with control IgG.

The above experiments suggested that the CD28-mediated co-stimulation was facilitating, but not required, for the OX40 expression on anti-CD3-stimulated T cells. This idea was further verified using CD28⁻ T cells from CD28-deficient mice. As shown in Fig. 3D, a comparable level of OX40 expression was found on CD28⁻ T cells to that on wild-type T cells. Collectively, these results indicated that mouse OX40 is expressed on TCR/CD3-stimulated CD4⁺ and CD8⁺ T cells in a CD28-independent manner and that OX40L is not expressed on normal mouse T cells.

**Expression of OX40L and OX40 on activated B cells**

We next examined the expression of OX40L and OX40 on splenic B cells. Purified splenic B cells were stimulated with anti-IgM Ab, anti-CD40 mAb, or both for 24–96 h and stained with RM134L or anti-OX40 mAb. An anti-CD69 mAb was included as an activation marker. As shown in Fig. 4C, OX40L expression was detected on B cells after stimulation with the combination of anti-IgM Ab and anti-CD40 mAb, which appeared at 24 h and reached a peak at 72 h. In contrast, stimulation with either anti-IgM Ab or anti-CD40 mAb alone did not induce OX40L expression (Fig. 4, A and B).
Unexpectedly, marginal expression of OX40 was reproducibly found on anti-IgM-stimulated B cells, which was apparent at 48–72 h (Fig. 4A). The combination with anti-CD40 mAb markedly enhanced OX40 expression (Fig. 4B). These results indicated that OX40L is specifically expressed on surface Ig- and CD40-stimulated B cell and that either stimulation alone was not effective. Moreover, a novel expression of OX40 on B cells was revealed.

Involvement of CD80, CD86, CD70, and OX40L in T cell costimulation by activated B cells

The above observations of a potent T cell costimulatory activity of OX40L and the expression of OX40L on anti-IgM- plus anti-CD40-stimulated B cells suggested that OX40L might play a substantial role in T cell costimulation by activated B cells. It has been shown that surface Ig- and/or CD40-stimulated B cells expressed the CD28 ligands CD80 and CD86 (32–35). We also previously demonstrated that anti-IgM- plus anti-CD40-stimulated B cells expressed CD70, which is another member of the TNF superfamily and provides a costimulatory signal via CD27 constitutively expressed on most T cells (9). Consistent with these observations, the anti-IgM plus anti-CD40-stimulated B cells expressed CD80, CD86, CD70, and OX40L at high levels (Fig. 5A). We then examined the relative contributions of CD80/86, CD70, and OX40L to T cell costimulation by anti-IgM- plus anti-CD40-stimulated B cells. Purified splenic T cells were cocultured with preactivated B cells in the presence of anti-CD3 mAb and combinations of anti-CD80/86, anti-CD70, and/or anti-OX40L mAb for 48 h, and then the proliferative response and IL-2 production were assessed. As shown in Fig. 5, B and C, the anti-IgM- plus anti-CD40-stimulated B cells exhibited a potent costimulatory activity for anti-CD3-stimulated T cell proliferation and IL-2 production. Anti-CD80/86 mAbs only partially inhibited T cell proliferation, and the combination of these two mAbs exhibited a significant inhibitory effect (p < 0.01) comparable to that of anti-CD80/86 mAbs (Fig. 5B). On the other hand, IL-2 production was strongly inhibited by anti-CD80/86 mAbs, and further inhibition was observed by their combination with anti-OX40L and anti-CD70 mAbs (Fig. 5C). Anti-OX40L or anti-CD70 alone significantly inhibited IL-2 production (p < 0.01), and further inhibition was observed by the mixture of these two mAbs (Fig. 5C). These results indicated the substantial contributions of OX40L and CD70 to the costimulatory activity of anti-IgM- plus anti-CD40-stimulated B cells, which can mostly account for the CD80/86-independent part.

Costimulation of CD28T cells by OX40L and CD70 on activated B cells

The above experiment implied that the OX40L and CD70 expressed on activated B cells could costimulate T cells independently of the CD80/86 and CD28 interaction. To further address this idea, we examined the contributions of OX40L and CD70 to costimulation of CD28T cells from CD28-deficient mice by preactivated B cells. As shown in Fig. 6A, the anti-IgM- plus anti-CD40-stimulated B cells from CD28-deficient mice expressed CD80, CD86, CD70, and OX40L at levels comparable to those in B cells from wild-type C57BL/6 mice (Fig. 5A). As shown in Fig. 6, B and C, when costimulated by these preactivated B cells, the CD28T cells exhibited 50–60% reduced proliferation and 60–70% reduced IL-2 production compared with wild-type T cells, which were almost comparable to those of wild-type T cells in the presence of anti-CD80/86 mAbs (Fig. 5, B and C). As expected, the addition of anti-CD80/86 mAbs showed no inhibitory effect. In contrast, both T cell proliferation (Fig. 6B) and IL-2 production (Fig. 6C) were significantly inhibited by either anti-OX40L or anti-CD70 mAb alone. Notably, T cell proliferation and IL-2 production were mostly inhibited by the mixture of these two mAbs (75.8 and 70.6% inhibition, respectively). These results indicated that the CD28-independent T cell costimulatory activity of surface Ig- and CD40-stimulated B cells is mostly mediated by OX40L and
CD70, although a minor contribution of some other molecule was also suggested.

Discussion

In this study we generated an mAb specific for murine OX40L and determined the expression of OX40L and OX40 on murine T and B lymphocytes. We also examined the T cell costimulatory function of OX40L expressed on activated B cells. Some novel findings were obtained, as follows.

OX40 has been originally identified as an activation marker preferentially expressed on CD4⁴ T cells in the rat system (13). In contrast, a recent study demonstrated that OX40 was expressed on both CD4⁺ and CD8⁺ T cells after Con A stimulation of mouse splenocytes, suggesting a differential expression of OX40 on activated rat and mouse T cells (24). We confirmed this in the present study and observed that both CD4⁺ and CD8⁺ T cells expressed OX40 after anti-CD3 stimulation in the mouse system (Fig. 3C). Although the expression of OX40 on human T cells has not been well characterized, our preliminary results showed that OX40 is expressed on both CD4⁺ and CD8⁺ T cells after anti-CD3 stimulation of human peripheral blood lymphocytes (unpublished observations). Therefore, the rather restricted expression of OX40 on CD4⁺ T cells appears to be a unique feature of the rat system. We also examined the stimulatory requirement for OX40 expression on T cells, which has not been determined in previous studies. We found that OX40 can be expressed on anti-CD3-stimulated T cells without costimulation with anti-CD28 mAb (Fig. 3A) and also on CD28⁺ T cells from CD28-deficient mice (Fig. 3D). This independence of OX40 expression from CD28 costimulation underlies the CD28-independent costimulatory activity of OX40L as represented in Fig. 6.

A previous report (11) showed that OX40L was expressed on a murine T lymphoma cell line and anti-TCR-stimulated murine splenic T cells as estimated by OX40-Ig binding, whereas staining of the latter was marginal. We found a high expression of OX40L in a Moloney virus-induced T lymphoma cell line MBL2 (Fig. 1A), but not in the other T cell lines tested or in anti-CD3- or anti-CD3/CD28-stimulated splenic T cells as estimated by staining with anti-OX40L mAb. Consistently, we could detect OX40L mRNA in MBL2, but not in anti-CD3- or anti-CD3/CD28-stimulated splenic T cells, by Northern blot analysis (data not shown). Therefore, OX40L appears not to be expressed on splenic T cells under these conditions, although it remains to be determined whether OX40L can be expressed on T cells under a particular condition. In this respect, it is noteworthy that OX40L is frequently expressed on HTLV-1-transformed human and rat T cell lines, possibly due to trans-activation by Tax protein encoded by the pX region of HTLV-1 (10, 21, 36). HTLV-1 is not only the etiological agent of adult T cell leukemia but also causes HTLV-1-associated diseases, such as myelopathy, uveitis, arthropathy, and Sjögren’s syndrome (37). It has been shown that the HTLV-1 pX transgenic mice developed inflammatory arthropathy, neurofibromatosis, and exocrinopathy (38–41). The anti-mouse OX40L mAb we established in

FIGURE 5. Contributions of CD80/86, CD70, and OX40L to costimulation of T cells by anti-IgM plus anti-CD40-stimulated B cells. A, Cell surface expression of costimulatory molecules on preactivated B cells. Preactivated B cells were prepared by stimulating splenic B cells from C57BL/6 mice with anti-IgM Ab plus anti-CD40 mAb for 72 h. Cells were stained with biotinylated mAb against the indicated molecules (shaded histograms) or control IgG (blank histograms) followed by PE-labeled streptavidin. B, Inhibitory effects of anti-CD80/86, anti-OX40L, and/or anti-CD70 mAb on T cell proliferation costimulated by activated B cells. Splenic T-
cells from C57BL/6 mice (1 × 10⁶/well) were cocultured with the irradiated preactivated B cells (5 × 10⁵/well) in the presence of anti-CD3 mAb and 2 µg/ml each of the indicated mAbs (total concentration of mAbs was adjusted to 8 µg/ml with rat IgG) for 48 h. The proliferative response was assessed by pulsing the cultures with 0.5 µCi/well [³H]thymidine for the last 9 h. C, Inhibitory effects on IL-2 production. Cell-free supernatants were collected from the cultures in B at 48 h, and IL-2 content was measured by ELISA. The data in B and C are expressed as the mean ± SD of triplicate wells. Similar results were obtained from three independent experiments. *, p < 0.05; **, p < 0.01.
the present study will be useful to address the pathological function of OX40L in these mouse models.

It has been reported that OX40L, as estimated by OX40-Ig binding, was expressed on mouse splenic B cells upon stimulation with anti-IgD dextran or CD40 ligand (CD40L)-expressing L cells, and the highest expression levels were obtained when stimulated by both (18). In contrast, we could not detect significant levels of OX40L in the presence of anti-CD3 Ab- or anti-CD40 mAb-stimulated B cells (Fig. 4, A and B). This discrepancy may be due to higher cross-linking of surface Ig by anti-IgD dextran than by anti-IgM Ab or a signaling difference between surface IgD and IgM and higher cross-linking of CD40 by CD40L-expressing L cells than anti-CD40 mAb. In our present results, however, a high expression of OX40L was obtained by the stimulation with anti-IgM Ab plus anti-CD40 mAb, consistent with the previous observation that the highest OX40L expression was obtained after stimulation by anti-IgD dextran and CD40L. Therefore, it is likely that OX40L is preferentially expressed on surface Ig-stimulated, Ag-specific B cells upon interaction with CD40L-expressing Th cells, especially when Ig cross-linking by Ags and CD40 cross-linking by CD40L are limiting. Stüber et al. also demonstrated that cross-linking of OX40L on activated B cells by OX40-Ig resulted in an enhanced proliferation and an increase in Ig secretion in vitro (18), and that administration of anti-OX40 Ab profoundly inhibited the anti-hapten IgG response in vivo and the development of parieto-lateral lymphoid sheath-associated B cell foci probably by interrupting the interaction of OX40 on activated T cells with OX40L on activated B cells (23), suggesting a critical role for the OX40-OX40L interaction in differentiation of activated B cells into Ig-producing plasma cells. In this respect, our novel finding of concomitant expression of OX40 and OX40L on activated B cells is intriguing, since it suggests that the OX40/OX40L-mediated proliferation and differentiation could be driven by B–cell interactions in the B cell foci. Further studies are now under way to characterize the function of OX40 expressed on activated B cells.

It has been shown that ligation of OX40 by anti-OX40 mAbs or OX40L provided a costimulatory signal for T cell proliferation (10–12). We also observed a potent costimulatory activity of mouse OX40L for proliferation of anti-CD3-stimulated naive T cells (Fig. 2). We then examined the costimulatory function of OX40L expressed on activated B cells. The anti-IgM plus anti-CD40-stimulated B cells expressed multiple costimulatory molecules, including CD80, CD86, CD70, and OX40L (Fig. 5A). Anti-CD80/86 mAbs only partially inhibited the costimulatory activity of activated B cells, and the CD80/86-independent part was mostly inhibited by the combination of anti-OX40L and anti-CD70 mAbs (Fig. 5B), indicating substantial contributions of OX40L and CD70. The residual part may be mediated by other costimulatory molecules, such as CD44H (42) and CD54 (43), which have also been implicated in the costimulatory activity of CD40L-activated B cells. Although it is possible that the anti-OX40L and anti-CD70 mAbs might act in an inhibitory manner by negatively signaling T cells, this is unlikely, since neither mAb inhibited anti-CD3- and anti-CD28-stimulated T cell proliferation (data not shown). The additional experiment with CD28 T cells from CD28-deficient mice (Fig. 6) clearly indicated that OX40L and CD70 expressed on activated B cells can costimulate T cells in a CD28-independent
manner. It has been reported that CD4+ T cells from CD28-deficient mice can express CD40L upon anti-CD3 stimulation (44), which is a prerequisite for the expression of OX40L and CD70 on B cells. We also showed that OX40 can be expressed on CD28− T cells upon anti-CD3 stimulation (Fig. 3). Taken together, these results suggest that OX40 and CD70 may mostly account for the CD28-independent pathway of T cell costimulation during Ag-specific T-B cognate interactions. It has been shown that CD28-deficient mice can be primed with soluble Ags in vivo so as to generate Ag-specific T cell proliferative responses in vitro (6, 7). CD40/CD40L have been implicated as the predominant mediator of the CD28-independent pathway of T cell costimulation (45–47). Although some reports suggested transmission of a costimulatory signal via CD40L into T cells (48, 49), it is generally accepted that CD40L acts indirectly by up-regulating the expression of costimulatory molecules on APC. Our present results suggest that OX40L and CD70 can at least partly account for the CD40/CD40L-dependent pathway of T cell costimulation. We are now addressing this possibility by administrating anti-OX40L and/or anti-CD70 mAb into CD28-deficient or CD40-deficient mice immunized with various Ags.

Our present study revealed substantial contributions of OX40L and CD70 to CD28-independent T cell costimulation. CD28− T cells are not unique to CD28-deficient mice. CD28 is expressed on most CD4+ T cells, but on only half of CD8+ T cells (50, 51). It has been suggested that CD8− CD28+ T cells might represent suppressor T cells in humans (52, 53). It has also been reported that CD4− CD28− T cells accumulated in PBL from SLE patients, which might be relevant to autoantibody production in these patients (51). At present, costimulatory molecules mediating activation of these CD28− T cells have not been well characterized. Our present results suggest that OX40L and CD70 may be responsible for the costimulation of these CD28− T cells.

In the present study we demonstrated the T cell costimulatory function of OX40L expressed on activated B cells. It has been reported that OX40L was also expressed on human DC and endothelial cells. We observed that murine DC and endothelial cell lines express OX40L, which costimulated CD28+ T cells. We also showed that OX40 can be expressed on CD28− T cells into high IL-4-producing effectors. Blood 92:3338.

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References


