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Ligation of CD28 In Vivo Induces CD40 Ligand Expression and Promotes B Cell Survival

Deling Yin,* Liying Zhang,* Ruoxiang Wang,* Laszlo Radvanyi,2§ Christian Haudenschild, † Qiding Fang,* Marilyn R. Kehry,‡ and Yufang Shi3*

Functional activation of T cells requires ligation of Ag receptors with specific peptides presented by MHC molecules on APCs concurrent with appropriate contacts of cell surface accessory molecules. Among these accessory molecules, interactions between CD28/CTLA-4 with B7 family members (CD80 and CD86) and CD40 with CD40 ligand (CD40L) play a decisive role in regulating the progression of balanced immune responses. However, most information regarding the role of accessory molecules in immune responses has been derived in the context of signals from the TCRs. Little understanding has been achieved regarding the consequence of ligation of costimulation molecules in absence of signals from the TCR. By employing an in vivo murine system, we show, herein, that ligation of CD28 alone with anti-CD28 Abs leads to a dramatic enlargement of the peripheral lymphoid organs characterized primarily by the expansion of B cells. B cells from anti-CD28-treated mice are resistant to spontaneous and anti-IgM-induced apoptosis. These cells are also unsusceptible to FasL-mediated apoptosis. Interestingly, this in vivo effect of CD28 on B cells is largely mediated by inducing the expression of CD40L, since coadministration of a blocking Ab against CD40L inhibited CD40L-mediated B cell survival and expansion. Therefore, CD28-mediated expression of CD40L may play an important role in the regulation of lymphocyte homeostasis.

(23–26). As a consequence, T cell-dependent B cell growth and differentiation were consistently augmented, when anti-CD3-stimulated T cells were simultaneously activated with anti-CD28 (27). This T cell-mediated B cell growth was found to be dependent on CD28 costimulation-induced increases of CD40L on T cells. However, it is not known whether the increase in CD40L expression on T cells is due to CD28 ligation alone, or also requires TCR ligation. We have studied the effect of ligation of CD28 in vivo by administration of anti-CD28 Abs. We have previously shown that this treatment could inhibit activation-induced apoptosis in T cells (28). We report herein that administration of anti-CD28 induces the expression of CD40L on T cells and splenomegaly characterized by the expansion of B cells. These B cells are resistant to spontaneous and activation-induced apoptosis. The expansion of B cells could be inhibited by coinjection of a blocking Ab to CD40L. Therefore, CD28 ligation in absence of exogenous Ags induces the expression of CD40L, which in turn regulates B cell homeostasis.

Materials and Methods

Mice and Abs

Four- to 6-wk-old male BALB/c mice were obtained from the National Cancer Institute (Frederick, MD) and were maintained in the vivarium of the Holland Laboratory of the American Red Cross, a facility accredited by the American Association for the Accreditation of Laboratory Animal Care, Inc. (AAALAC). CD28 knockout mice were kindly provided by Dr. Tak Mak (Ontario Cancer Institute, Toronto, Canada). Mice were allowed to acclimatize to the new environment for at least one week after shipping. Animals were age matched in each experiment.

Anti-CD28 was purchased from PharMingen (La Jolla, CA), or was produced by a B cell hybridoma, 37.51, kindly provided by Dr. James P. Allison (University of California at Berkeley, CA). Hamster anti-murine CD40L, B cell hybridoma, MR1, was provided by Dr. Randolph Noelle (Dartmouth Medical School, Hanover, NH). mAbs were purified by chromatography with protein G-coupled sephadex. In some cases, hybridoma culture supernatent was precipitated with saturated (NH₄)₂SO₄ (pH 7.2), redissolved in PBS, and dialyzed extensively against PBS. The anti-CD28 Ab prepared in our laboratory was assessed for the presence of endotoxin by AlerCHEK (Portland, ME) and found to have negligible amount (<2.0 EU/ml). Normal hamster Ig obtained from Sigma (St. Louis, MO) was used as a control. PE-anti-CD40L and FITC-anti-CD3 were purchased from PharMingen. FITC-labeled goat anti-murine IgM was a gift of Dr. David Scott (The Holland Laboratory of the American Red Cross, Rockville, MD).

In vivo Ab administration

Mice were injected i.p. with purified anti-CD28, normal hamster Ig, or anti-CD40L (MR1) at indicated doses and dosage regimes. Splenocytes were isolated for the assessment of apoptosis and histological studies at indicated times. Splenocytes were also analyzed for the expression of cell surface proteins by flow cytometry or Western blotting.

In vitro activation of splenocytes

Freshly isolated spleens were made into single cell suspensions by pressing them between frosted ends of two microscope slides. Splenocytes (3 × 10⁶/well) were activated with anti-IgM at 30 µg/ml (29) in 96-well tissue culture plates in 100 µl RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine, 50 µM mercaptoethanol, 50 µg/ml gentamicin, and 10% heat-inactivated FCS (Sigma). After 24 h of incubation at 37°C in the presence of 5% CO₂, cells were harvested for the assessment of genomic DNA integrity.

Fas ligand-mediated apoptosis

The sensitivity to Fas ligation-induced apoptosis in splenocytes was determined by coculturing with cells expressing sense Fas ligand (FasL) or antisense FasL (kindly provided by Dr. T. A. Ferguson, Washington University School of Medicine, St. Louis, MO) (30). Cells were harvested at 12 h, and apoptosis was determined by flow cytometric DNA content analysis as described below.

Flow cytometry

Single splenocyte suspensions were prepared for different treatments and washed with PBS supplemented with 1% FCS and 0.02% sodium azide (staining buffer). Cells (1 × 10⁶) were stained with FITC-anti-CD3, FITC-anti-IgM, or PE-anti-CD40L (PharMingen) in staining buffer at 4°C for 30 min, washed twice with PBS, and fixed with 1% formalin in PBS. Cells were analyzed for fluorescence intensity on a FACScan flow cytometer with a single argon laser and log-logarithmic intensity scales using the CellQuest program (Becton Dickinson, San Jose, CA).

Flow cytometric analysis was also employed to assay cellular DNA content, where apoptotic cells were shown as a hypodiploid peak. After treatments, splenocytes were fixed with 70% ethanol for 30 min at 4°C, followed by two washes with PBS. The fixed splenocytes were then incubated in PBS containing propidium iodide (Sigma) at 50 µg/ml and RNase (Boehringer Mannheim, Indianapolis, IN) at 0.1 mg/ml at room temperature for 30 min. DNA content was determined by flow cytometry on FACScan (Becton Dickinson). The FL2 intensity was plotted as histograms on a linear scale.

Western blotting

Equal numbers of cells were lysed in RIPA lysis buffer, which was composed of 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 µM orthovanadate (Fisher Scientific, Fairlawn, NJ), 50 mM ZnCl₂, 2 mM EDTA, 2 mM PMSF, 0.1% SDS, and 0.1% deoxycholate. Samples were incubated at 4°C for 10 min and then centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were transferred, mixed, and boiled in SDS sample buffer. The lysates were separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1× TBS (10 mM Tris, pH 8.0, and 140 mM NaCl) for 1 h. The membrane was then incubated with the blocking solution containing anti-CD40L (1 µg/ml) for 4 h at room temperature. After washing three times with TBS for 5 min, the blot was then incubated with a HRP-conjugated protein A in the blocking solution. The blot was again washed three times with TBS before being exposed to ECL (Amersham, Arlington Heights, IL).

Histology sections

Spleen samples were fixed in 10% formalin in PBS and embedded in paraffin. Three-micron sections were stained with hematoxylin and eosin and viewed under light microscope, and representative areas were microphotographed.

ELISA

Total and hamster Ig-specific mouse Ab were detected by ELISA. To determine total amounts of mouse Ig, serum samples were diluted in a binding buffer (0.05 M Tris, pH 9.0) and incubated in 96-well ELISA plates. After overnight incubation at 4°C, the plates were washed with PBS plus 0.05% Tween 20, and the nonspecific binding sites were blocked with 0.5% gelatin in PBS. The amount of mouse Ig was detected by HRP conjugated to rabbit anti-mouse κ chain (PharMingen). To assess the amount of Ab in mouse serum specific to hamster Ig, ELISA plates were first coated with hamster anti-mouse CD28 mAb (37.51) at 10 µg/ml in the binding buffer. After blocking of the nonspecific binding sites, the plates were further incubated with mouse serum or mouse anti-hamster Ab (Sigma) as standard. Similarly, the amount of mouse Ig binding hamster Ig was detected by HRP conjugated to rabbit anti-mouse κ chain (Sigma) as standard. HRP substrate ABTS (Sigma), the amount of mouse Ig was determined by absorbance reading.

Cell proliferation assay

Freshly isolated splenocytes (5 × 10⁵/well) were incubated in 96-well plates with 200 µl RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM glutamine, 50 mM 2-ME, and 10 mM gentamicin (Life Technologies). [³H]Thymidine was added to the culture at 1 µCi/well. Cells were harvested onto glass-fiber filter paper at 6 h after culture. Cell proliferation in terms of [³H]thymidine uptake was measured by liquid scintillation counting.
Table I. Effects of in vivo administration of anti-CD28 and anti-CD40L on splenic T and B cells

<table>
<thead>
<tr>
<th>Spleen Weight (mg)</th>
<th>Total Cell Number (×10^6)</th>
<th>CD3+ Cell (×10^6)</th>
<th>IgM+ Cells (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87.5 ± 2.2</td>
<td>215.3 ± 3.2</td>
<td>50.2 ± 1.9</td>
</tr>
<tr>
<td>Anti-CD28</td>
<td>272.3 ± 3.6</td>
<td>484.9 ± 4.4</td>
<td>46.7 ± 2.8</td>
</tr>
<tr>
<td>Anti-CD28 + Anti-CD40L</td>
<td>141.3 ± 2.4</td>
<td>301.7 ± 4.2</td>
<td>44.5 ± 3.8</td>
</tr>
</tbody>
</table>

* Groups of four to six mice were injected with 200 μg anti-CD28 with or without 200 μg anti-CD40L (MR1) at 48-h intervals. Mice were euthanized at 24 h after the third injection. Splenocytes were counted and stained with FITC-anti-CD3 or FITC-anti-IgM and analyzed by flow cytometry. Percentage of each population was converted to absolute cell number based on total splenocyte counts.

Results

In vivo administration of anti-CD28 induces splenomegaly in mice

Previous studies have demonstrated that ligation of CD28 could inhibit activation-induced apoptosis in T cells (10, 28, 31, 32). Thus, it is conceivable that CD28 mediates cell survival signals. Indeed, others and we have shown that CD28 induces the expression of Bcl-X, a protein that increases the resistance of cells to apoptosis (10, 31). To assess whether sustained cell survival induced by treatment with anti-CD28 would alter cellular homeostasis in the peripheral lymphoid organs, we injected mice i.p. with three doses of anti-CD28 at 48-h intervals. Mice did not show any clinical abnormality over the course of the treatment. However, this treatment resulted in a marked increase in the size of the spleen and lymph nodes. The size of the spleen increased 3- to 5-fold (28), and the number of splenocytes per spleen also dramatically increased (Table I). When splenocytes of anti-CD28-treated mice were stained with anti-CD3 for T cells and anti-IgM for B cells, flow cytometric analysis revealed that there was a dramatic increase in the percentage of B cells, with a decrease in the percentage of T cells. When the total cell number in each population was calculated, the number of splenic T cells in anti-CD28 treated mice did not change significantly, while the number of splenic B cells increased 4.4-fold. Thus, the enlargement of the spleen is mainly caused by an increase in the number of B cells, suggesting that the ligation of CD28 alone in vivo modulates B cell survival.

We also examined the spleens histologically. Paraffin sections of the spleens from anti-CD28-treated mice stained with hematoxylin and eosin showed dramatic changes in the splenic structure as compared with that of normal hamster immunoglobulin-treated mice (Fig. 1). The lymphoid nodules (white pulp), where B cells reside, were extensively expanded. As a consequence, the red pulp was pushed to limited areas (Fig. 1B). A large number of cells in the white pulps had a blast-like morphology. Occasionally, mitotic figures could be observed. The cell size increase was also apparent when analyzed by flow cytometry based on forward and side scatter plotting (data not shown). This change is specific for anti-CD28. Normal hamster Ig and anti-CD40L did not show such changes. Interestingly, anti-CD28-altered splenic structure could be blocked by coadministration of anti-CD40L (discussed below).

We have also shown that the changes in lymphoid organs induced by anti-CD28 were not due to the contamination with bacterial endotoxin in our Ab preparations, since acid treatment (1 M HCl, which destroys proteins but not endotoxins) diminished the effect of anti-CD28 in inducing splenomegaly (28). Furthermore, when our Ab preparations were examined for the presence of endotoxin (by AlerCHECK, Portland, ME), it was found that the amount of endotoxin was essentially negligible (<2EU/ml), being only about 10% of that in FCS. When CD28 “knockout” (CD28−/−) mice were treated with anti-CD28, no enlargement of the spleen was observed, while CD28+/+ littermates showed dramatic enlargement of their spleens (Fig. 2), indicating that the effect of anti-CD28 was exerted through CD28 ligation. Furthermore, injection of control Ab, anti-CD4 (GK1.5) prepared with the same method as anti-CD28, or normal hamster Ig, did not induce any alteration in the size of lymphoid organs (data not shown). Thus the above changes in the spleen induced by anti-CD28 were specific for CD28.

FIGURE 1. Histological analysis of the alterations of the spleen induced by in vivo administration of anti-CD28 and the inhibitory effect of blocking CD40L. Six-wk-old mice were injected i.p. with 200 μg anti-CD28 with or without 200 μg anti-CD40L (MR1) at 48-h intervals. Mice were euthanized at 24 h after the third injection. Spleens were fixed with 10% formalin, and 3-micron sections were stained with hematoxylin and eosin. A, Normal hamster Ig. B, Anti-CD28. C, Anti-CD28 and anti-CD40L.
Since the anti-CD28 Ab used in our studies is originated from hamster, it is possible that its foreign nature plus the specificity to CD28 may activate T cells and B cells. To test whether B cells are activated by this Ab, we examined the total amount of serum Ig- and hamster Ig-specific Ab. Though anti-CD28 induced more than a 4-fold increase in splenic B cells, the serum Ig level was not significantly elevated, as detected by ELISA with rabbit anti-mouse κ L chain-specific Abs (~1.5 times that of the untreated mice). To test whether this increase represents Abs specific to hamster Ig, we also tested the amount of serum Ab specific to the anti-CD28 Ab. Commercial mouse anti-hamster Ig (HG-31) was used as standard. Anti-CD28 did not induce the production of anti-hamster Ig Ab (below 200 ng/ml for both control and anti-CD28 treated). Therefore, anti-CD28 treatment did not activate B cell Ag receptor to produce measurable amounts of Ab. The dramatic increase in B cell number in anti-CD28-treated mice is not due to the antigenic activation.

**Anti-CD28 in vivo induces the expression of CD40L**

It has been shown that CD28 ligation enhances TCR-induced expression of CD40L on the T cell surface in vitro (23). In addition, constitutive expression of CD80 on L cells could enhance the expression of CD40L on CD4+ T cells; however, the exact role of CD28 in such a system was not clear (33). Nevertheless, our system could provide further information about the role of CD28 in the regulation of CD40L in vivo. We found that, at 24 h after a single injection of anti-CD28, about 25% of the T cells (gated based on the expression of CD3) became positive for CD40L as detected by flow cytometry, while less than 1% of the T cells were positive for CD40L in control mice (Fig. 3). In addition, we also examined the expression of CD40L by Western blot analysis. After treatment with anti-CD28 or normal hamster Ig for 24 h, splenocytes were lysed, and the expression of CD40L was detected with anti-CD40L on Western blots. We found that anti-CD28 treatment increased the expression of CD40L detected as a band with the molecular mass of 32 kDa (Fig. 4). Therefore, in vivo ligation of CD28 alone could induce the expression of CD40L.

**Administration of anti-CD40L inhibits anti-CD28-induced B cell expansion in vivo**

It has been shown that ligation of CD40 alone provides potent mitogenic signals to B cells. We, therefore, investigated the role of CD40L expression in the induction of B cell expansion induced by in vivo CD28 ligation. We injected mice with a blocking Ab to CD40L together with anti-CD28. We found that coadministration of anti-CD40L dramatically reduced anti-CD28-induced enlargement of peripheral lymphoid organs, while coinjection of normal hamster Ig did not have such an effect (data not shown). The effect of anti-CD40L was also observed when splenocyte number was analyzed (Table I). On histology, we found that anti-CD40L also blocked the splenic expansion of the white pulp induced by anti-CD28 (Fig. 1). Therefore, in vivo CD28 ligation-induced CD40L expression appears to be largely responsible for the alteration of lymphocyte homeostasis.

**In vivo administration of anti-CD28 enhances the resistance of B cells to apoptosis**

Others and we have previously established that ligation of CD28 could inhibit activation-induced apoptosis in T cells (10, 28, 31).
It has been shown that CD40 ligation prevents the induction of apoptosis in B cells (20). Based on the observation that CD28 ligation could induce the expression of CD40L, we hypothesized that the effect of CD28 on B cells in vivo could be exerted by affecting the susceptibility of B cells to the induction of apoptosis, which might account for the splenic enlargement. Mice were injected i.p. with three doses of anti-CD28 at 48-h intervals. The splenocytes were harvested and treated with a high dose of anti-IgM (30 μg/ml), which has been shown to effectively induce apoptosis in primary B cells (29). Shown in Fig. 5, the splenocytes from anti-CD28-treated mice showed resistance to anti-IgM-induced apoptosis. In addition, under these culture conditions, about 20% of normal splenocytes undergo spontaneous apoptosis 12 h after culture, while no apoptosis was observed in anti-CD28 treated splenocytes.

We also tested the susceptibility of splenocytes to FasL-mediated apoptosis. Isolated splenocytes were cocultured with L cells transfected with FasL cDNA in either sense or antisense orientation for 12 h. Apoptosis was analyzed by DNA content analysis upon staining with propidium iodide. As shown in Fig. 6, FasL-expressing L cells induced significant apoptosis in splenocytes from untreated mice, while cells from mice treated with anti-CD28 were resistant to apoptosis induced by FasL. Anti-CD40L treatment diminished the survival effect induced by anti-CD28. This apoptosis is FasL specific, since L cells transfected with antisense FasL did not induce apoptosis, as determined by DNA content analysis.

There are two possible mechanisms for the increase in B cells mediated by anti-CD28-induced CD40L: 1) increase in proliferation; 2) reduction in apoptosis. As described above, we have shown that splenocytes from anti-CD28-treated mice are resistant to anti-IgM- or FasL-induced apoptosis. These cells are also resistant to spontaneous cell death in vitro. To test cell proliferation,
we isolated splenocytes from mice treated with anti-CD28, LPS, or both and cultured in vitro. As shown in Fig. 7, we found that anti-CD28-treated splenocytes exhibited low levels of cell proliferation at 6 h after culture. On the other hand, although LPS did not increase the size of spleens to the same extent as induced by anti-CD28 (data not shown), LPS-treated splenocytes showed a much higher degree of cell proliferation at 6 h after in vitro culture. Therefore, anti-CD28 treatment not only provides cell survival signals, but also induces some degree of cell proliferation, both of which are likely responsible for the accumulation of B cells mediated by enhanced expression of CD40L.

DISCUSSION

Activation of costimulatory molecules is crucial for T cell activation and B cell proliferation and differentiation (34). CD40:CD40L and CD28/CTLA-4:CD80/CD86 are the best established costimulatory molecule systems. T cells provide help for B cell proliferation and Ab production by cell-cell contact and by releasing soluble factors. CD40L, which constitutes contact-dependent T cell help, is the predominant B cell costimulation protein expressed on activated T cells upon activation (35). On the other hand, B cells also express molecules such as CD80 and CD86 to provide costimulation to T cells via CD28 or CTLA-4 (36). Blocking CD40-CD40L interactions with specific Ab leads to severely impaired Ab production (37). CD40L-deficient mice exhibit defects in T cell-dependent B cell responses, which could be fully reconstituted by activating Abs to CD40 (38, 39). CD40-CD40L interaction induces the expression of CD86 on B cells. The fact that cross-linking CD40 on B cells promotes expression of the ligand (CD80) for CD28 suggests that T and B interactions may have a reciprocal amplification mechanism (40). We have demonstrated here that administration of anti-CD28 mAb in vivo leads to dramatic enlargement of peripheral lymphoid organs, due primarily to an increase in the number of B cells. These B cells are resistant to spontaneous and high dose anti-IgM-induced apoptosis. Furthermore, our data show that anti-CD28 induces the expression of CD40L on T cells. Anti-CD28-induced B cell expansion is blocked by inhibitory Ab to CD40L. Thus, CD28 ligation alone can induce the expression of CD40L, which is responsible for the expansion of B cells.

CD40L is present on activated, but not resting, T cells. The expression of CD40L on T cells is tightly regulated (41–43). The modulation of CD40L expression has been demonstrated in various systems. It has been shown that CD40L expression is induced, peaking at 5 h, after T cell activation (44). It has been shown that the induction of CD40L expression in vitro and in vivo on CD4+ T cells is dependent on the expression of CD80. When expressed on L cells, CD80 was found to be both necessary and sufficient for the induction of CD40L on normal CD4+ T cells (32). This is in agreement with the data presented herein. However, when normal accessory cell populations were used, only partial inhibition of induction of the CD40L was observed with reagents that inhibit B7/CD28 interactions (32). Furthermore, CD40L could be induced on CD4+ T cells from CD8-deficient mice (45). Therefore, non-B7/CD28 cellular interactions can also mediate the costimulatory signals needed for induction of CD40L expression. This may explain why in our experiment anti-CD40L only partially inhibited the B cell expansion resulted from anti-CD28 treatment.

More recent evidence indicates an expansion of the role of the CD40/CD40L in cellular interactions beyond Ab formation. Besides providing activating signals to B cells, CD40 also promotes B cell survival (20). It has been shown that activation of CD40 could inhibit anti-IgM-induced apoptosis in B cell lymphomas including WEHI231, Burkitt’s lymphoma, and Ramos, and in activated B cells. In contrast, in some lymphomas, ligation of CD40 could enhance the expression of Fas (46) and the susceptibility to Fas-induced apoptosis (47). Therefore, CD40-mediated protection of B cells from apoptosis is independent of the Fas pathway. However, most of the information concerning the role of CD40/CD40L in the regulation of apoptosis was derived from the in vitro system in combination with the signals from the B cell Ag receptor. The direct signals from CD40 are not clear. Nevertheless, it has been shown that CD40 ligation could induce the expression of Bcl-X (48). Recent studies have shown that CD40/CD40L interaction is also required for the formation of B memory cells and germinal centers, and signaling through CD40 prevents apoptosis of germinal center B cells. The increase in B cell number induced by repeated injection of CD28 is likely to be due to the induction of the expression of CD40L. The B cells from these mice showed prolonged survival in in vitro culture and resistance to anti-IgM- and FasL-induced apoptosis. Our data also show that the increase of B cells is due to the increase in B cell survival and cell proliferation (Fig. 7). This increase in cell survival may switch the balance between “life and death” toward more survival. Although, we have shown that the B cells bear the morphology of blasts, there is no significant increase in the amount of serum Ig- or hamster Ig-specific Abs. Therefore, CD28-regulated CD40L expression could play a critical in the maintenance of B cell homeostasis.

Lymphocyte costimulatory molecules are differentially expressed in different compartments of the lymphoid tissues, and at different stages of maturation and activation (49). Deregulated expression of CD40L has been associated with several autoimmune diseases, suggesting that selective immunotherapies to treat autoimmune disease and prevent graft rejection can be targeted on CD40L (50). One example is the recent demonstration of the effect of anti-CD40L in reducing atherosclerosis (51). Indeed, it has been shown that combination of CTLA-4-Ig with anti-CD40L resulted in long-lasting inhibition of murine lupus in NZB/NZW F1 (B/W) mice (52). Therefore, manipulation of the B7/CD28 and CD40/CD40L costimulation pathways could result in fundamental changes in the course of immune responses. The combination of anti-CD40L Ab and soluble CTLA-4-Ig has also been shown to abrogate the development of mercury chloride (HgCl2)-induced autoimmune disease in mice (53). Therefore, manipulation of lymphocyte accessory counterreceptor interactions may affect immune responses. We believe that further investigation of the molecular mechanisms governing CD28-mediated CD40L expression and

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**FIGURE 7.** Proliferation of splenocytes from mice treated with anti-CD28, LPS, or both and cultured in vitro for 6 h. Cell proliferation was measured by incubation with [3H]thymidine.
CD40L-delivered cell survival signal will provide important information for the understanding of the immune system and for the modulation of immune disorders.

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References