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B Cells Activated by Lipopolysaccharide, But Not By Anti-Ig and Anti-CD40 Antibody, Induce Anergy in CD8$^+$ T Cells: Role of TGF-β1[1]

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B cells recognize Ag through their surface IgRs and present it in the context of MHC class II molecules to CD4$^+$ T cells. Recent evidence indicates that B cells also present exogenous Ags in the context of MHC class I to CD8$^+$ T cells and thus may play an important role in the modulation of CTL responses. However, in this regard, conflicting reports are available. One group of studies suggests that the interaction between B cells and CD8$^+$ T cells leads to the activation of the T cells, whereas other studies propose that it induces T cell tolerance. For discerning this dichotomy, we used B cells that were activated with either LPS or anti-Ig plus anti-CD40 Ab, which mimic the T-independent and T-dependent modes of B cell activation, respectively, to provide accessory signals to resting CD8$^+$ T cells. Our results show that, in comparison with anti-Ig plus anti-CD40 Ab-activated B cells, the LPS-activated B cells (LPS-B) failed to induce significant levels of proliferation, cytokine secretion, and cytotoxic ability of CD8$^+$ T cells. This hyporesponsiveness of CD8$^+$ T cells activated with LPS-B was significantly rescued by anti-TGF-β1 Ab. Moreover, it was found that such hyporesponsive CD8$^+$ T cells activated with LPS-B had entered a state of anergy. Furthermore, LPS-B expresses a significantly higher level of TGF-β1 on the surface, which caused the observed hyporesponsiveness of CD8$^+$ T cells. Therefore, this study, for the first time, provides a novel mechanism of B cell surface TGF-β1-mediated hyporesponsiveness leading to anergy of CD8$^+$ T cells. ***The Journal of Immunology,*** **2003, 170: 5897–5911.**

B cells are known to play an important role in CD4$^+$ T cell activation and in the generation of the humoral immunity. B cells also exert regulatory effects on CD8$^+$ T cell responses; however, the precise nature of this phenomena has remained unclear. On the one hand, extensive literature suggests that B cells attenuate CD8$^+$ T cell responses, whereas, in contrast, they are known to potentiate CD8$^+$ T cell responses. The former is exemplified where B cells inhibit the induction of T cell-dependent tumor immunity in which class I-restricted CD8$^+$ T cells play a major role in tumor eradication (1). Although B cells express B7.1 and B7.2 costimulatory molecules, nonetheless, it has been documented that Ag recognition by CD8$^+$ T cells on such B cells induces tolerance in them (2). The B cell-induced tolerance is reported not only in naive CD8$^+$ T cells but also in CD8$^+$ T cell clones in a secondary in vitro culture (2, 3). In yet another study, it was shown that recognition of Ag on B cells directly tolerizes CD8$^+$ T cells, and this tolerance induction is not due to a lack of CD4$^+$ T cell help (4). Furthermore, studies in B cell-deficient mice indicate that B cells are incapable of inducing naive CD8$^+$ T cells and thus are not required for the initiation of the CTL responses (5, 6).

In contrast, B cells are also known to enhance CD8$^+$ T cell responses. Evidence suggest that B cells play an important role in induction (5) and maintenance of CD8$^+$ T cell memory responses (7). It was found that, in the absence of B cells, CTL memory responses to lymphocytic choriomeningitis virus became severely affected, and the viral-specific CTL response was exhausted (7). Moreover, studies in a nonobese diabetic model of autoimmune diabetes show that mice deficient in β_2-microglobulin (8) and B cells (9) had become resistant to insulin-dependent diabetes mellitus. These observations signify an important role of B cells in inducing autoreactive CD8$^+$ T cells that might cause β cell destruction. Recently, Sun et al. (10) also showed that CMV- and EBV-specific CTLs could be efficiently induced in vitro in the presence of a B lymphoblastoid cell line expressing specific Ag.

These discrepancies regarding the activating capabilities of B cells prompted us to reassess the role of B cells as accessory cells in CD8$^+$ T cell activation. Because resting B cells are known to express low levels of costimulatory molecules and can tolerize the T cells (5, 11, 12), we therefore used activated B cells as APC for our study. It is known that B cell activation may occur in response to two kinds of Ags, T-dependent and T-independent Ags (13). B cell activation in response to T-dependent Ags occurs when B cells capture Ags via their surface Ig and process it for presentation with MHC class II to CD4$^+$ T cells (14), resulting in an Ag-specific T:B cognate interaction. Moreover, interaction of CD40 ligand on T cells with CD40 on the B cells is an essential prerequisite for optimal B cell activation (15). Therefore, for T-dependent B cell activation, we used anti-mouse Ig as a model Ag in the presence of anti-CD40-activating Abs to mimic CD4$^+$ T cell help (16). In contrast, we used LPS, which is the best-studied T-independent

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Ag, to activate the B cells. LPS is a product of Gram-negative bacteria and is known to cause polyclonal B cell activation, proliferation, and production of cytokines, such as IL-1, IL-6, IL-8, and TNF-α (17). LPS also stimulates B cells to enhance their Ag-presenting capacity, which is accompanied by secretion of large amounts of LPS-neutralizing Abs (18). These cumulative effects of LPS are known to be accomplished by binding to its receptor, Toll-like receptor-4 (TLR-4)[3] (19).

Thus, in the present study, we used B cells that were activated with two different modes, i.e., either in the presence of anti-Ig and anti-CD40 Ab (16) or with LPS. These differentially activated B cells were then tested for their ability to activate resting CD8$^+$ T cells. Our results show that LPS-activated B cells (LPS-B) were less efficient in activating CD8$^+$ T cells as compared with anti-mouse Ig and anti-CD40 Ab-activated B cells (CD40-B). Furthermore, the CD8$^+$ T cells receiving signals from LPS-B enter into a state of anergy that can be rescued by exogenous IL-2. The observed hyporesponsiveness of CD8$^+$ T cells could be attributed to a higher expression of TGF-β1 on the surface of LPS-B. Therefore, the type of activation the B cell originally receives, either T independent or T dependent, seems to play an important role in deciding the fate of the CD8$^+$ T cell response. Thus, these results illuminate the immune evasion strategies adopted by both Gram-negative bacteria and retroviruses that specifically target TLR-4 signaling in B cells.

Materials and Methods

Mice

BALB/c and C57BL/6 mice (6–8 wk of age) used for this study were originally obtained from National Institute of Nutrition (Hyderabad, India) and maintained at our experimental animal facility under standard pathogen-free conditions.

Culture medium, cytokines, and Abs

T cells and B cells were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) medium containing penicillin (70 μg/ml), streptomycin (100 μg/ml), 2-ME (50 μM), sodium pyruvate (1 mM), HEPES (20 μM), and 10% FBS (Life Technologies). For TGF-β1 estimation, cells were cultured in low serum containing medium with 2% FBS and insulin-transferrin-selenium A (Life Technologies).

Anti-CD3 (145.2C11), anti-CD40 (HM40.3), anti-mouse Ig (187.1) and anti-CD16/CD32 (2.4G2), and anti-IL-10 (JES5-16E3) Abs and isotype control Abs rat IgG2b (R35-38), rat IgG1 (R3-34), and mouse IgG1 (107.3) were obtained from BD PharMingen (San Diego, CA). PE-labeled anti-CD8 (53-6.7), anti-CD25 (PC61), anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22), anti-ICAM-1 (3E2), anti-B7.2 (GL1), anti-B7.1 (16-10A1), rat IgG2a (B39-4), rat IgG2b (R35-38), rat IgG1 (R3-34), hamster IgG group-1 PE (A19-3), and hamster IgG group-2 (B81-3) Abs were obtained from BD PharMingen. FITC-labeled anti-CD8 (53-6.7), anti-CD45R (RA3-6B2), and rat IgG1 (R3-34) Abs were also obtained from BD PharMingen. Anti-IL-6 (MP5-20F3) Ab was linked to FITC (Sigma-Aldrich, St. Louis, MO) using standard protocol. Biotin-labeled chicken anti-TGF-β1 Ab, biotin-labeled goat anti-latency-associated peptide (LAP) (TGF-β1), monoclonal anti-TGF-β (1.2.3) (1D11) Ab, recombinant active TGF-β1, and recombinant latent TGF-β1 were obtained from R&D Systems (Minneapolis, MN), whereas normal chicken IgG and normal goat IgG were obtained from Bangalore Genie (Bangalore, India). Biotinylation of normal chicken IgG and normal goat IgG was conducted using *N*-hydroxysuccinimide-biotin (Pierce, Rockford, IL). Secondary reagent for biotin-labeled Abs used was PE-labeled streptavidin (BD PharMingen) and streptavidin HRP (Roche, Indianapolis, IN). Recombinant mouse cytokines IFN-γ, TNF-α, IL-6, IL-4, IL-2, IL-10, and human IL-15 were also obtained from BD PharMingen.

[3] Abbreviations used in this paper: TLR-4, Toll-like receptor 4; LPS-B, LPS-activated B cell; CD40-B, anti-mouse Ig and anti-CD40 Ab-activated B cell; LAP, latency-associated peptide; *DHFR*, dihydrofolate reductase; FasL, Fas ligand.

B cell purification and activation

The splenocytes from C57BL/6 mice were used for B cell purification. RBCs were depleted by hemolytic Gey's solution, and the resulting splenocytes were subjected to B cell enrichment mixture Abs (StemCell Technologies, Vancouver, British Columbia, Canada) and magnetically separated by negative selection according to the manufacturer's instructions. B cells obtained by this method were routinely >94% pure as analyzed by anti-B220 Ab staining. B cells were then activated either by LPS (*Salmonella typhosa*; Sigma-Aldrich) at a concentration of 20 μg/ml or with 4 μg/ml anti-mouse Ig and 10 μg/ml anti-CD40 (HM40.3) Abs at 2×10^6 cells/well of a 24-well plate. At the end of 72 h, activated B cells were harvested, and dead cells were removed by Histopaque (Sigma-Aldrich). The B cells were then repeatedly washed in large volumes of HBSS containing 2% FCS, especially, to remove traces of LPS before coculturing with the T cells. B cells obtained at this stage were 98% B220 positive.

T cell purification

The CD8$^+$ T cells were purified throughout the course of the study from alloantigen-primed mice to increase the frequency and yield of CD8$^+$ T cells in the spleen, as described earlier (18, 20). In brief, 1×10^7 freshly prepared splenocytes from BALB/c mice were injected i.p. into C57BL/6 mice. After 8–10 days, a single-cell suspension of spleen cells was prepared, and CD8$^+$ T cell purification was performed as described under *T cell proliferation assay*. RBC-depleted splenocytes were passed through a nylon wool (Robbins Scientific, Sunnyvale, CA) column, and eluted cells were treated with anti-CD4-coated magnetic beads (Dynal, Oslo, Norway) to remove CD4$^+$ T cells. Resulting cells were directly subjected to CD8$^+$ T cell enrichment system (StemCell Technologies) and were negatively sorted according to the manufacturer's protocol. Thus obtained, CD8$^+$ T cells were 95% pure with <0.1% CD4$^+$ T cell contamination as analyzed by anti-CD8 and anti-CD4 Ab staining. The CD8$^+$ T cells obtained by this method were found to exhibit a resting phenotype, as analyzed by their abilities to proliferate, to function as cytotoxic cells, and to express IL-2, IFN-γ, IL-2Rα, and CD69. It was found that these CD8$^+$ T cells do not proliferate in the culture, do not synthesize IL-2 and IFN-γ as analyzed by ELISA, and have very low cytotoxic ability, and 98% of them do not express IL-2Rα or CD69 on their surface. Such cells were considered as resting CD8$^+$ T cells (21, 22).

T cell proliferation assay

To study the role of B cells in the activation of CD8$^+$ T cells, we first fixed the B cells by gamma-irradiation at 2000 rads before culturing with the T cells. After irradiation, B cells were washed three times with HBSS containing 2% FCS. CD8$^+$ T cells were activated polyclonally in the presence of LPS-B or CD40-B in the presence of anti-CD3 Ab at a concentration of 1 μg/ml as a source of primary signal, in a U-bottom 96-well plate (Costar, Cambridge, MA) for 72 h. Proliferation of the cells was measured using [^{3}H]thymidine (NEN, Boston, MA; or BRIT, Mumbai, India) incorporation at 1 μCi/well for the last 12 h of the culture.

To determine the effect of soluble factors released by the coculture of CD8$^+$ T cells and LPS-B on the proliferation of CD8$^+$ T cells activated with CD40-B, dual-chamber Transwells were used. For this, 5×10^5 CD8$^+$ T cells or 2.5×10^5 irradiated LPS-B alone or 5×10^5 CD8$^+$ T cells together with 2.5×10^5 irradiated LPS-B in the presence of anti-CD3 Ab (1 μg/ml) were cultured in the lower Transwell chamber (Costar) in 800 μl of medium. The effect of soluble factors released by these cultures was assessed on the proliferation of 5×10^5 CD8$^+$ T cells activated in the presence of 2.5×10^5 paraformaldehyde (0.4%)-fixed CD40-B and anti-CD3 Ab (1 μg/ml) in the upper Transwell chamber. The cells were cultured for 60 h, and then 75 μl of medium containing the cells from the upper Transwell chamber were transferred to a 96-well plate for [^{3}H]thymidine incorporation for 8 h. Cells were harvested using cell harvester (Skatron, Norway), and radioactivity was counted in a liquid scintillation counter (Packard, Downers Grove, IL).

Cytotoxicity assay

CD8$^+$ T cells were activated in the presence of irradiated LPS-B or CD40-B and anti-CD3 Ab (1 μg/ml) in multiple wells of a 96-well plate. At the end of 6 days, cells were harvested, and the dead cells were removed by Histopaque (Sigma-Aldrich). The viable T cells were counted by the trypan blue exclusion method and checked for their anti-CD3-mediated cytotoxic ability in different E:T ratios against [^{3}H]thymidine-labeled P815 as target cells for 3 h, by JAM test (23).

Cytokine analysis using ELISA and RT-PCR

Mouse IL-2, IFN-γ, IL-6, IL-4, and TNF-α were analyzed in the culture supernatants by sandwich ELISA using coating and detecting paired Abs obtained from BD PharMingen, while IL-15 was estimated using Abs (catalog nos. sc-8437 and sc-1296) obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The detection limit of IL-2, IFN-γ, IL-6, IL-4, and TNF-α was 30 pg/ml, whereas for IL-15, it was 100 pg/ml. Recombinant IL-2, IFN-γ, IL-6, IL-4, IL-15, and TNF-α were used to prepare the standard curves. For the estimation of IL-10 and IL-13, Quantikine M from R&D Systems was used. For TGF-β1 estimation, cells were cultured in the medium containing 2% FBS supplemented with insulin-transferrin-selenium A. Culture supernatants were treated with HCl to a pH of 2.5 for activation of pro-TGF-β1 to active TGF-β1. These supernatants were then neutralized with NaOH and estimated using an ELISA kit obtained from Promega (Madison, WI). The values obtained from the medium alone were subtracted from the test values.

Semiquantitative RT-PCR was used to detect cytokine mRNA synthesis from B cells. B cells were activated with LPS or anti-Ig plus anti-CD40 for 72 h. Cells were harvested, and total RNA was prepared by TRIzol reagent (Life Technologies) or by SNAP total RNA isolation kit (Invitrogen, San Diego, CA), according to the manufacturer's instruction. Total RNA (200 ng) was used to perform RT-PCR using Titan one tube RT-PCR system (Roche) in a 25-μl reaction. Housekeeping gene dihydrofolate reductase (*DHFR*) was used as a control to determine equal loading of RNA. The primer sets for DHFR, IL-2, TGF-β1, and IL-10 were obtained from Stratagene (La Jolla, CA). Other primers used were the following: IL-15 primers, 5′-CCATCTCGTGCTACTTGTG-3′ and 5′-CTGTTTGCAAGGT AGAGCACG-3′; TNF-α primers, 5′-GCGACGTGGAACTGGCAGA AG-3′ and 5′-GGTACAACCCATCGGCTGGCA-3′; IL-6 primers, 5′-TGGAGTCACAGAAGGAGTGGCTAA-3′ and 5′-TCTGACCACAGT GAGGAATGTCCAC-3′; and Fas ligand (FasL) primers, 5′-CGAAAC CAACCACTTGAGTGC-3′ and 5′-GAACACTAGTTGCTTTGACCC-3′. RT-PCR products were separated by electrophoresis in 1% agarose gel and were visualized using ethidium bromide staining.

Flow cytometric analysis of surface and intracellular markers

For surface staining, B cells were harvested from the culture and washed twice with FACS buffer (PBS containing 0.1% BSA and 0.05% sodium azide). Nonspecific binding of Abs to the cells was prevented by preincubation of cells with 30 μg/ml anti-CD16/32 Ab for 20 min and then stained with appropriate concentration of staining Abs for 45 min at 4°C. Cells were then washed twice with FACS buffer, followed by staining with streptavidin PE in the case of biotin-labeled Abs for 30 min at 4°C. Cells were again washed twice with FACS buffer and were fixed with 1% buffered paraformaldehyde until analyzed.

Surface staining of TGF-β1 was done using biotin-labeled chicken anti-TGF-β1 followed by streptavidin PE. To ensure the specificity of the surface staining, a competition experiment was conducted in the presence of rTGF-β1. For this experiment, anti-TGF-β1 Abs were titrated to a point where staining began to dilute out and then competition of anti-TGF-β1 Ab in the presence of rTGF-β1 was conducted to bind on the surface of LPS-B.

Intracellular cytokine staining was done as described elsewhere (24) with little modification. In brief, $CD8^+$ T cells were activated with nonirradiated LPS-B or CD40-B in the presence of 1 μg/ml anti-CD3 Ab. GolgiPlug (BD PharMingen) containing brefeldin A was added to block cytokine secretion after 2 h and was cultured for 10 more hours. Cells were harvested and washed twice with the FACS buffer. Cells were then surface stained with anti-CD8 staining Ab for 45 min, and after two washes with staining buffer, cells were fixed with freshly prepared 1% paraformaldehyde for 20 min at room temperature. For intracellular cytokine staining, cells were washed twice with PBS containing 0.1% BSA and 0.5% saponin (Sigma-Aldrich), and then cells were stained with anti-cytokine Abs for 30 min at room temperature. Cells were washed twice with PBS/BSA/saponin buffer followed by two washings with the FACS buffer. Samples were then analyzed on a FACSVantage (BD Biosciences, Mountain View, CA) flow cytometer.

Confocal microscopy

LPS-B or CD40-B were surface stained as described above, with biotin-labeled chicken anti-TGF-β1 Ab followed by streptavidin PE. Cells were mounted on the slide and viewed under confocal microscope at ×63 magnification (Zeiss, Jena, Germany).

Membrane preparation and Western blot analysis

The cell membrane preparation was done as described previously (25). In brief, LPS-B or CD40-B were harvested from the culture and washed three times with PBS before freezing overnight. Cells were then thawed and homogenized in homogenization buffer (20 mM Tris (pH 7.4), 0.25 M sucrose, and 1 mM EDTA) in the presence of a protease inhibitor mixture (Roche) and glass beads (106 μm; Sigma-Aldrich). The cell debris was removed by centrifugation at 700 × *g* for 15 min at 4°C. The supernatant obtained was then subjected to centrifugation at 110,000 × *g*. The pellet was solubilized in solubilization buffer (20 mM Tris, 20% glycerol, and 1% Triton X-100 (pH 7.5)) overnight at 4°C. The insoluble material was again removed by spinning at 100,000 × *g* for 1 h at 4°C, and supernatants containing the membrane proteins were collected.

The membrane proteins obtained were estimated by Bradford's reagent (Bio-Rad, Hercules, CA), and equal amounts of proteins from membrane preparations from the CD40-B and LPS-B groups were separated on 10 or 12% SDS-PAGE under nonreducing conditions (26) along with recombinant proteins and Rainbow markers (Amersham, Little Chalfont, U.K.). The proteins were then blotted on a nitrocellulose membrane and probed with biotin-conjugated anti-TGF-β1 or normal chicken IgG or anti-LAP (TGF-β1) Abs or its control normal goat IgG. The secondary reagent used was HRP-conjugated streptavidin. The blots were developed with chemiluminescent luminol substrate and visualized by exposing the blots to x-ray film.

Assessment of $CD8^+$ T cell anergy

Using the protocol described previously (27), purified $CD8^+$ T cells (1×10^6) were stimulated with immobilized anti-CD3 (2 μg/ml) Ab or with irradiated LPS-B or CD40-B (5×10^5) and (1 μg/ml) soluble anti-CD3 Ab for 2 days in a 24-well plate. The cells were then washed and rested for two more days in fresh RPMI medium containing 10% FCS. Unstimulated $CD8^+$ T cells were cultured with rIL-2 (2 ng/ml) to maintain the cell viability (2). The cells were harvested and subjected to Histopaque to remove the dead cells. The viable cells were counted by the trypan blue exclusion method, and 5×10^4 cells were restimulated in the presence of ionomycin and PMA with or without rIL-2 for 72 h. Proliferation of the cells was measured using [^{3}H]thymidine incorporation at 1 μCi/well for the last 12 h of the culture. In parallel cultures, the supernatant was harvested at 24 h after stimulation for IL-2 measurement by ELISA.

Statistical analysis

Statistical analysis was performed using the Student's *t* test.

Results

LPS-B are poor activators of $CD8^+$ T cells as compared with CD40-B

In the present study, we have made an attempt to elucidate the role of B cells in $CD8^+$ T cell activation. We used CD40-B and LPS-B as accessory cells to assess the activation of resting $CD8^+$ T cells. Our results show that, when CD40-B or LPS-B were used as accessory cells, $CD8^+$ T cells proliferated to a greater extent in the presence of CD40-B as compared with LPS-B in a dose-dependent manner (Fig. 1*A*). We consistently found that $CD8^+$ T cells activated with CD40-B, proliferated three to five times more than the $CD8^+$ T cells stimulated with LPS-B. In contrast, the proliferation in control experiments using irradiated B cells alone or autologous T cells activated with B cells without anti-CD3 Ab (28) was negligible. Because CD40-B induced higher $CD8^+$ T cell proliferation compared with LPS-B, we next examined the expression of the activation marker CD25 on $CD8^+$ T cells activated for 24 h in the presence of either of these B cells. We found that $CD8^+$ T cells activated in the presence of CD40-B expressed significantly higher levels (8- to 10-fold) of CD25 than $CD8^+$ T cells activated with LPS-B (Fig. 1*B*). We also tested the cytotoxic ability of the $CD8^+$ T cells activated either in the presence of LPS-B or CD40-B after 6 days of culture. Results of a cytotoxicity assay again showed that $CD8^+$ T cells activated in the presence of CD40-B were significantly more potent cytotoxic cells than $CD8^+$ T cells activated with LPS-B (Fig. 1*C*). The E:T ratio used for the cytotoxicity assay was based on the T cell number the LPS-B and CD40-B group, which was normalized after activation for 6 days of culture. This suggests that CTLs generated in the presence of LPS-B are defective in their cytotoxic effector function. These observations clearly

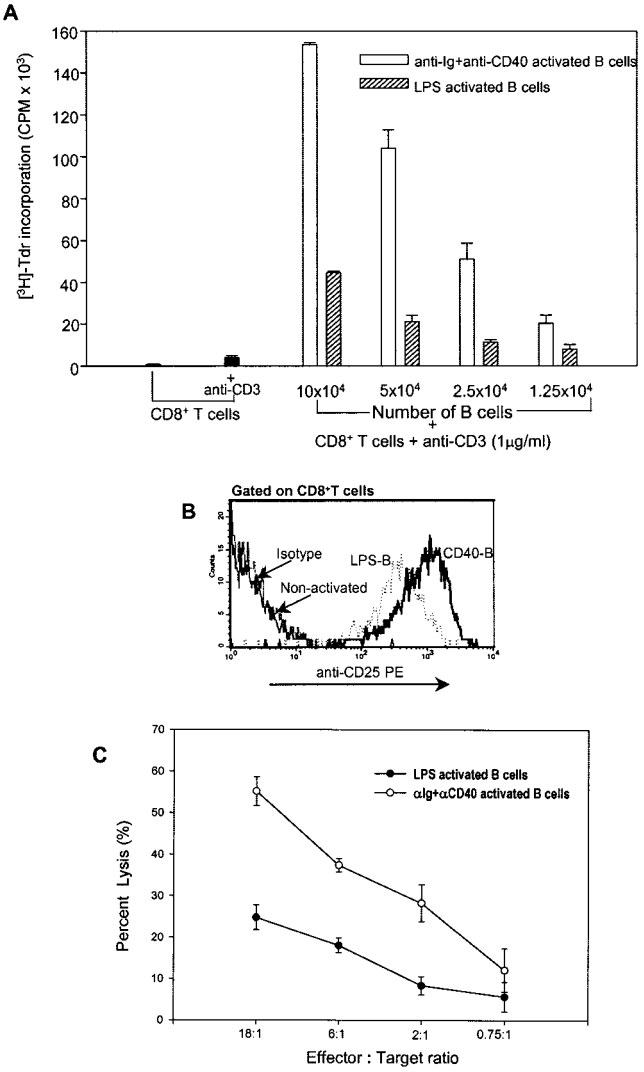

FIGURE 1. A. CD40-B are more potent stimulators of CD8$^+$ T cells than are LPS-B. Purified CD8$^+$ T cells (1×10^5) were cultured in the presence of anti-CD3 Ab (1 μg/ml) and various numbers of irradiated LPS-B or CD40-B per well of a 96-well plate. Cells were cultured for 72 h and T cell proliferation was assessed by [^{3}H]thymidine incorporation for the last 12 h of the culture. Background values of [^{3}H]thymidine incorporation of irradiated B cells alone at the highest cell number (1×10^5) used were 2452 $\pm$ 1098 and 2353 $\pm$ 875 for LPS-B and CD40-B, respectively. The counts of T cells cultured with LPS-B or CD40-B cells alone without anti-CD3 Ab were 3254 $\pm$ 568 and 3526 $\pm$ 875, respectively. The results shown are the means $\pm$ SD of triplicate wells and represent one of five individual experiments. *B*, CD8$^+$ T cells activated with CD40-B express a higher level of CD25 than do CD8$^+$ T cells activated with LPS-B. Purified CD8$^+$ T cells (1×10^5) were activated in the presence of anti-CD3 Ab (1 μg/ml) and nonirradiated LPS-B or CD40-B (5×10^4) for 24 h. Cells were harvested and stained with either anti-CD25 PE Ab or normal rat IgG2b PE as its isotype control. Expression of CD25 is shown on gated cells that were positive for anti-CD8 FITC. A representative of isotype control rat IgG2b PE staining on CD8$^+$ T cells activated with LPS-B is shown only, because its staining on those activated with CD40-B and nonactivated T cells was comparable. *C*, CTLs generated in the presence of LPS-B are poor cytotoxic cells in comparison with those generated in the presence CD40-B. Purified CD8$^+$ T cells (1×10^5) were activated with anti-CD3 Ab (1 μg/ml) and irradiated LPS-B or CD40-B (5×10^4) for 6 days in multiple wells of a 96-well plate. Cells were then harvested and washed, and dead cells were removed by Histopaque. The live cells were counted in the presence of trypan blue, and the cytotoxic ability of these cells was checked by incubating them for 3 h with anti-CD3 Ab and [^{3}H]thymidine-labeled P815 cells as targets in different E:T ratios. The results shown are the means $\pm$ SD of triplicate wells and represent one of three individual experiments.

demonstrate that LPS-B are poor activators of CD8$^+$ T cells as compared with CD40-B, resulting in decreased proliferation and maturation of CTLs into cytotoxic effector cells.

Expression of costimulatory molecules on B cells

The findings that different modes of activation of B cells influence their subsequent ability to stimulate CD8$^+$ T cells prompted us to investigate this further. We first examined the level of expression of costimulatory molecules like B7.1, B7.2, and ICAM-1 on both of these B cells. As shown in Fig. 2*A*, the expression of B7.1 and ICAM-1 on CD40-B and LPS-B was comparable. Surprisingly, LPS-B expressed much higher levels of B7.2 molecules as compared with CD40-B (Fig. 2*A*). B7.2 is known to play a central role in the CTL generation, alone and in combination with B7.1 (29, 30). Moreover, blocking the delivery of costimulation by B7.2 on LPS-B by neutralizing anti-B7.2 Ab further inhibited CD8$^+$ T cell proliferation (62%), whereas its isotype normal rat IgG2a did not block the proliferation significantly (8%) (data not shown). This suggests an important role of B7.2 in inducing CD8$^+$ T cell proliferation. Furthermore, the expression of FcR on both of the B cells was analyzed for the reason that anti-CD3 Ab-mediated activation of T cells is known to be dependent on the cross-linking of T cells by the FcRs on the accessory cells (31). Therefore, the differential levels of FcRs on B cells could also account for the observed difference in their CD8$^+$ T cell-activating capabilities. However, we observed that LPS-B and CD40-B express equal levels of FcRs (CD16/CD32) as examined by flow cytometry (Fig. 2*A*), suggesting that both the B cells provide equal levels of primary signal to T cells. Thus, the poorer ability of LPS-B relative to CD40-B, in terms of CD8$^+$ T cell stimulation, does not appear to be due to the deficiency at the primary signal level or at the level of costimulatory molecule expression.

Analysis of cytokines synthesized by CD8$^+$ T cells activated in the presence of LPS-B and CD40-B

To further investigate the molecular mechanism of the poor CD8$^+$ T cell-activating capabilities of LPS-B as compared with CD40-B, we analyzed the profile of cytokines secreted by CD8$^+$ T cells activated in the presence of B cells. We examined for cytokines such as IL-2, IFN-γ, TNF-α, IL-6, IL-10, IL-13, and TGF-β1 released in the culture supernatant, by sandwich ELISA. The results showed that IL-2, IFN-γ, TNF-α, IL-6, and IL-13 were detected at significantly higher levels in culture supernatants of CD8$^+$ T cells activated with CD40-B, as compared with CD8$^+$ T cells activated with LPS-B (Table I). In contrast, IL-10 and TGF-β1, which are T cell-inhibitory cytokines (32), were found to be much higher in the culture supernatant of CD8$^+$ T cells and LPS-B as compared with the culture supernatant of CD8$^+$ T cells and CD40-B (Table I). Apart from this, we also performed intracellular cytokine staining for IFN-γ, TNF-α, IL-2, and IL-6. Results of this experiment also suggested that LPS-B are much poor inducers of cytokine synthesis from CD8$^+$ T cells as compared with CD40-B (Fig. 3*A*), as indicated by the percentage of T cells positive for cytokine staining. Together, these results prove that, although LPS-B express sufficiently high surface costimulatory molecules, when used as accessory cells, their ability to induce CD8$^+$ T cells responses was severely compromised as compared with the ability of CD40-B. This was evident in terms of not only proliferation and maturation of CTLs, but also in terms of the synthesis of cytokines by the T cells. Moreover, it is worth noting that the differences between LPS-B and CD40-B were not only qualitative but also quantitative, as indicated by the higher levels of IL-10 and TGF-β1 secreted by

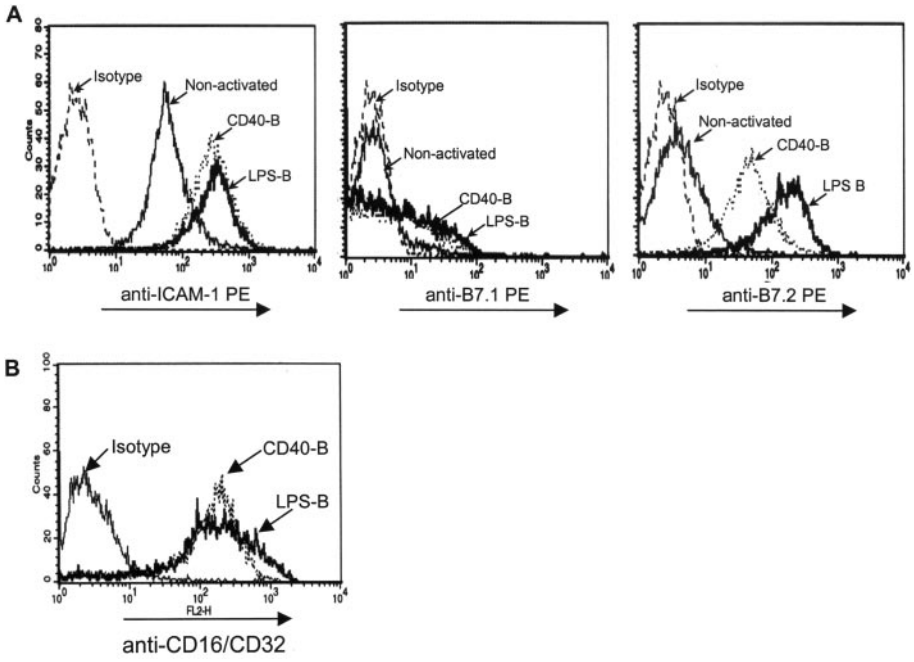

FIGURE 2. *A*, Expression of costimulatory molecules on B cells. Normal B cells or B cells activated with either LPS or anti-Ig plus anti-CD40 Abs for 72 h were stained with PE-labeled anti-B7.1 (hamster IgG, group 2) or anti-B7.2 (rat IgG2a) or anti-ICAM-1 (hamster IgG, group 1) Abs or their respective isotype controls. A representative of isotype control hamster IgG/group 1 PE or hamster IgG/group 2 PE or rat IgG2a PE staining on LPS-B is shown only, because its staining on normal B cells and CD40-B were comparable with the staining on LPS-B. The data shown represent one of the three individual experiments. *B*, Expression of FcRs on B cells. LPS-B and CD40-B were stained with biotin-labeled anti-CD16/CD32 or isotype control biotin-labeled rat IgG2b, followed by streptavidin PE as a secondary reagent. A representative of isotype control biotin-labeled rat IgG2b staining on LPS-B is shown only, because its staining on CD40-B was comparable with the staining on LPS-B. The data shown represent one of the three individual experiments.

$CD8^+$ T cells activated with LPS-B as compared with $CD8^+$ T cells activated with CD40-B. These higher levels of T cell-inhibitory cytokines such as IL-10 and TGF-β1 found in the culture supernatants could possibly explain the observed phenomena.

Previous reports suggest that activated B cells express functional FasL on their surface and are capable of killing Fas-sensitive target cells (33). Therefore, it is possible that higher expression of FasL on LPS-B could kill $CD8^+$ T cells and cause the observed reduction in the proliferation of T cells. Therefore, we analyzed the expression of FasL on LPS-B and CD40-B at the message level by RT-PCR and on the surface by staining cells with anti-FasL Ab (MFL4). We found no difference in expression of FasL on LPS-B and CD40-B, as analyzed at the message level by semiquantitative RT-PCR (Fig. 3*B*) and by flow cytometric analysis (data not shown). These results thus rule out the possibility that the differential capacity of the activated B cells to stimulate $CD8^+$ T cells is due to selective killing of T cells via Fas-FasL interaction.

LPS-B synthesize higher levels of TGF-β1 and IL-10 mRNA than do CD40-B

Results of the cytokine analysis suggested that inhibitory cytokines like IL-10 and TGF-β1 were the only cytokines that were detected

Table I. *Cytokine profile of $CD8^+$ T cells activated with B cells by ELISA*[a]

Cytokines	Culture Conditions			
	$CD8^+$ T cells + anti-CD3 + LPS-B (pg/ml)	$CD8^+$ T cells + anti-CD3 + CD40-B (pg/ml)	Irradiated LPS-B alone (pg/ml)	Irradiated CD40-B alone (pg/ml)
IL-2	155 ± 21.21	490 ± 28.28	ND	ND
		$p < 0.01$		
IFN-γ	1650 ± 70.7	3425 ± 318.19	ND	ND
		$p < 0.05$		
TNF-α	192.5 ± 24.74	1135 ± 190.19	ND	65 ± 7.07
		$p < 0.001$		
IL-6	205 ± 21.21	717.5 ± 38.89	ND	ND
		$p < 0.001$		
IL-13	187 ± 15.55	631 ± 26.87	ND	ND
		$p < 0.001$		
IL-10	570 ± 28.28	142.5 ± 10.60	100 ± 14.14	ND
		$p < 0.01$		
TGF-β1	780 ± 14.14	265 ± 49.49	190 ± 28.28	45 ± 7.07
		$p < 0.01$		

[a] Purified $CD8^+$ T cells (1×10^5) were cultured with anti-CD3 Ab and irradiated LPS-B or CD40-B (5×10^4) in a 96-well plate. Supernatants were harvested at 24 h for IL-2 and IFN-γ, 48 h for IL-6, IL-4, IL-15, TNF-α, IL-10, and IL-13, and 72 h for TGF-β1, after stimulation. Supernatants of T cells alone and T cells cultured with anti-CD3 Ab did not show detectable levels of these cytokines. Additionally, IL-15 and IL-4 were also not detected in any of these supernatants. The data shown are the means ± SD of duplicate wells assayed and represent one of at least three independent experiments. ND, Not detected.

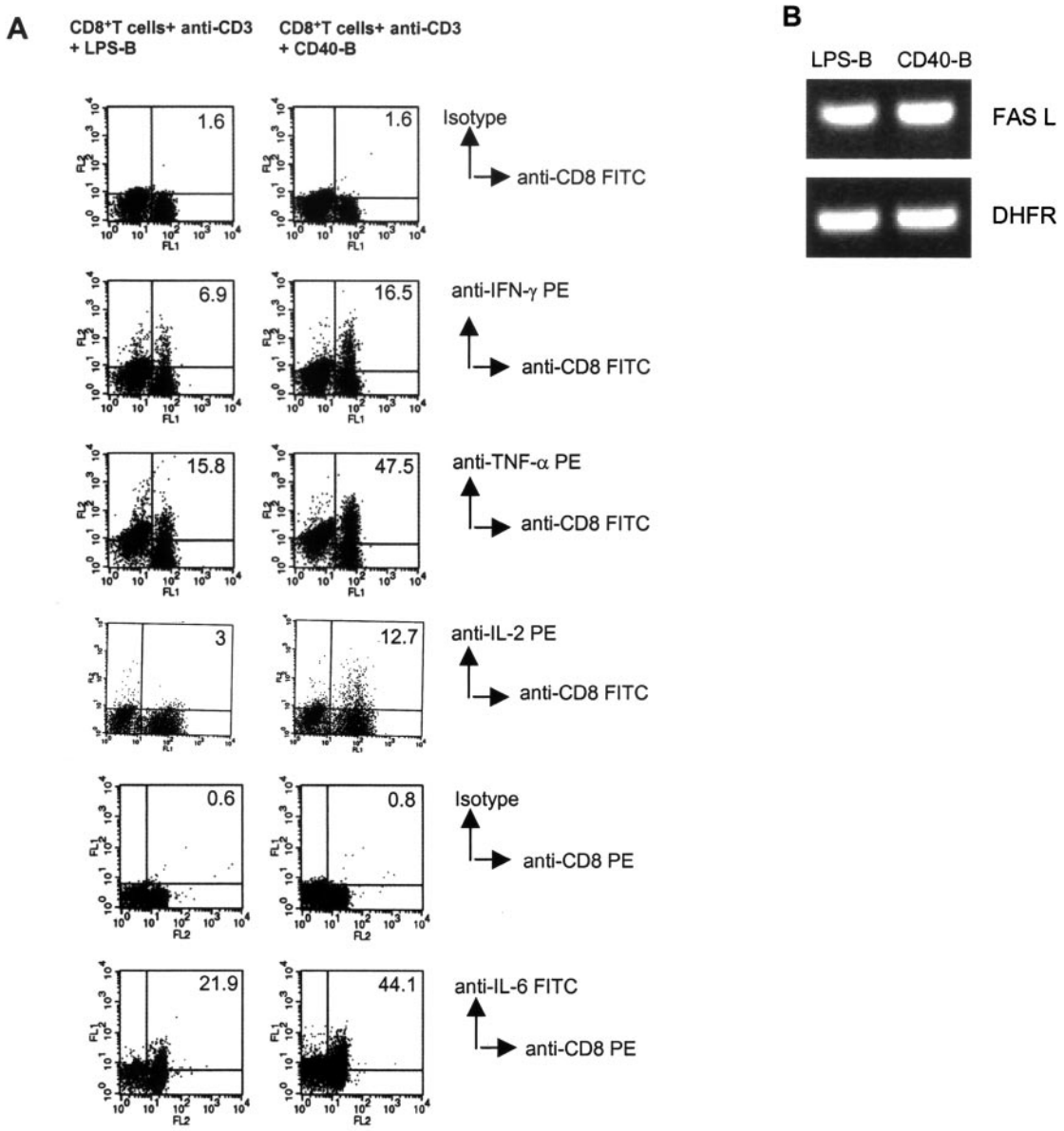

FIGURE 3. *A*, Intracellular flow analysis of cytokines. Purified CD8$^+$ T cells (1×10^5) were cultured with nonirradiated LPS-B or CD40-B cells (5×10^4) and anti-CD3 Ab for 2 h followed by 10 h in GolgiPlug. Cells were stained with PE- or FITC-tagged anti-CD8 Ab along with anti-IFN-γ (rat IgG1) PE or anti-TNF-α (rat IgG1) PE or anti-IL-2 (rat IgG2b) PE or anti-IL-6 (rat IgG1) FITC Ab or their respective isotype controls (i.e., rat IgG1 PE or rat IgG2b PE or rat IgG1 FITC), as described in *Materials and Methods*. A representative of the isotype control rat IgG1 PE is shown only, because the staining of isotype control rat IgG2b was comparable with the staining with rat IgG1 PE. The numbers shown in the quadrant are the percentages of CD8$^+$ T cells positive for cytokine staining. As the cocultures were done with T cells and B cells, the percentages were calculated by gating CD8$^+$ T cells only. The data are representative of three individual experiments. *B*, LPS-B and CD40-B cells express equal FasL mRNA. Purified B cells (2×10^6) were activated with LPS or anti-Ig plus anti-CD40 Abs in a 24-well plate. After 72 h, cells were harvested and total RNA was prepared and was used (200 ng) to perform RT-PCR, using gene-specific primers for FasL in a 25-μl reaction. The PCR products (5 μl) were separated on a 1% agarose gel and were visualized with ethidium bromide staining. The equal intensity of DHFR reflects the equal amount of RNA used for each RT-PCR. The data are representative of three individual experiments.

at higher levels in the supernatants of LPS-B cocultured with CD8$^+$ T cell. This was in contrast to CD8$^+$ T cells cocultured with CD40-B, which primarily secreted IFN-γ, IL-2, and TNF-α. Because LPS-B and CD8$^+$ T cells were cocultured together, one may argue that LPS-B might synthesize and secrete high levels of TGF-β1 and IL-10, which in turn induce more TGF-β1 and IL-10 production from T cells (34). To test this, we probed the status of TGF-β1 and IL-10 in both types of B cells using semiquantitative RT-PCR. We also analyzed expression of cytokines such as IL-15, IL-6, TNF-α, and IL-2. IL-15 is known to activate memory-type CD8$^+$ T cells (35), whereas IL-6 and TNF-α are reported to costimulate CD8$^+$ T cells (36). Therefore, these cytokines secreted by the B cells could also potentially play an important role in modulating CD8$^+$ T cell responses. We observed that the TGF-β1 and IL-10 message levels were, indeed, found to be much higher in LPS-B as compared with CD40-B (Fig. 4*A*). We also found that CD40-B expressed a higher level of IL-15 mRNA than did LPS-B, TNF-α and IL-6 mRNA levels were comparable, and IL-2 mRNA was hardly detected. To confirm the above-mentioned results, we also examined the levels of TGF-β1, IL-10, and IL-15 in the supernatants of LPS-B and CD40-B by sandwich ELISA. Indeed, we found that LPS-B comparatively secreted much higher levels of

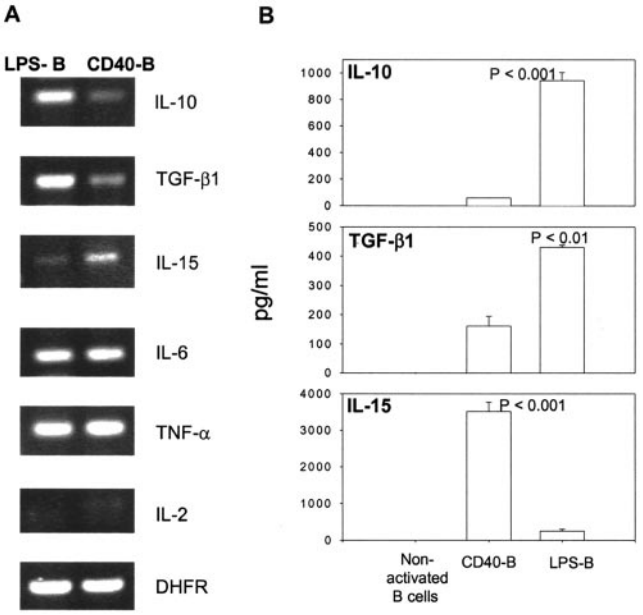

FIGURE 4. *A*, Analysis of cytokine gene expression of activated B cells using RT-PCR. B cells were activated, and total RNA was isolated as described in Fig. 3*B*. RNA (200 ng) from each sample was used to perform RT-PCR using gene-specific primers of TGF-β1, IL-10, IL-15, TNF-α, IL-6, and IL-2 in a 25-μl reaction. The PCR products (5 μl) were separated on 1% agarose gel and visualized with ethidium bromide staining. The equal intensity of DHFR reflects the equal amount of RNA used for each RT-PCR. The data are representative of three independent experiments. *B*, LPS-B secretes higher levels of TGF-β1 and IL-10, although low IL-15, as compared with CD40-B in the culture supernatant. Purified B cells (2×10^6) were activated either with LPS or anti-Ig plus anti-CD40 Abs in a 24-well plate, and supernatants were harvested at 70 h. Cytokine ELISA was performed as described in *Materials and Methods*. The data shown are the means $\pm$ SD of duplicate wells assayed and represent at least three independent experiments.

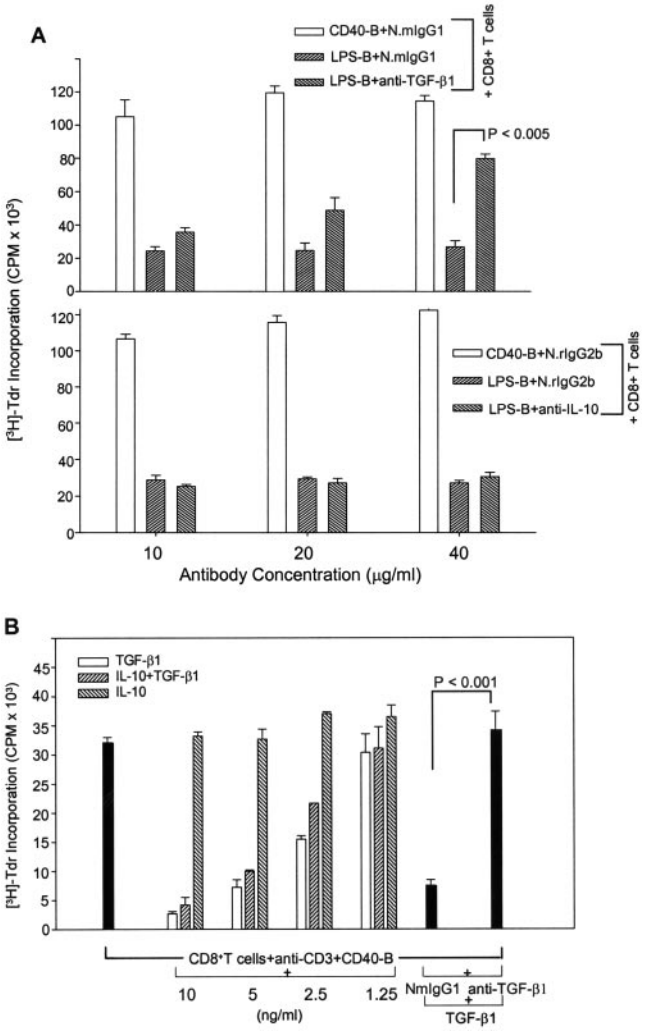

FIGURE 5. *A*, Anti-TGF-β1 neutralizing Ab significantly restores the proliferation of CD8$^+$ T cells activated with LPS-B. Purified CD8$^+$ T cells (1×10^5) were activated with anti-CD3 Ab (1 μg/ml) and irradiated LPS-B or CD40-B (5×10^4) in the presence of anti-TGF-β1 (1D11) Ab or its isotype control mouse IgG1 Ab or in the presence of anti-IL-10 Ab or its isotype control rat IgG2b. Cells were cultured for 72 h, with [^{3}H]thymidine incorporation (1 μCi/well) for the last 12 h of the culture. The results shown are the means $\pm$ SD of triplicate wells and are representative of one of the three individual experiments. *B*, Recombinant active TGF-β1, but not IL-10, inhibits CD8$^+$ T cell proliferation. Purified CD8$^+$ T cells (1×10^5) were activated with paraformaldehyde (0.4%)-fixed CD40-B (5×10^4) and anti-CD3 (1 μg/ml) in the presence of various concentrations of rTGF-β1 or IL-10 or a combination of TGF-β1 plus a fixed concentration of IL-10 (10 ng/ml). Cells were cultured for 72 h, with [^{3}H]thymidine incorporation (1 μCi/well) for the last 12 h of the culture. Note that recombinant active TGF-β1 completely inhibits CD8$^+$ T cell proliferation. This inhibition is rescued by preincubation of anti-TGF-β1 (1D11) Ab (10 μg/ml) but not by isotype control mouse IgG1, when 5 ng/ml recombinant active TGF-β1 was used to inhibit CD8$^+$ T cell proliferation. The results shown are the means $\pm$ SD of triplicate wells and are representative of one of the three individual experiments.

TGF-β1 and IL-10, whereas CD40-B secreted high IL-15 as compared with LPS-B (Fig. 4*B*). Therefore, it is possible that high TGF-β1 and IL-10 secreted by LPS-B could play a role in determining the decreased ability to activate CD8$^+$ T cells, as compared with CD40-B.

TGF-β1, but not IL-10, is responsible for the poor ability of LPS-B to activate CD8$^+$ T cells

We next addressed whether or not neutralizing the activity of TGF-β1 or IL-10 would restore the hyporesponsiveness of CD8$^+$ T cells activated in the presence of LPS-B as compared with CD8$^+$ T cells activated with CD40-B. We used anti-TGF-β1 and anti-IL-10 Abs to neutralize their activities in CD8$^+$ T cell and activated B cell cultures. The results show that anti-TGF-β1, indeed, significantly restored the proliferation of CD8$^+$ T cells activated with LPS-B, as compared with its isotype control normal mouse IgG1 (Fig. 5*A*). In contrast, anti-IL-10 (or anti-IL-10R Abs, not shown) had no effect on the proliferation of CD8$^+$ T cells as compared with its rat IgG2b isotype control (Fig. 5*A*).

To further prove the inhibitory role of TGF-β1, we also performed experiments in which CD40-B-activated CD8$^+$ T cells were cultured in the presence of recombinant TGF-β1, IL-10, and a combination of TGF-β1 and IL-10. For this, we chose to chemically fix CD40-B with paraformaldehyde, because such B cells do not proliferate at all and, unlike irradiation-fixed cells (Table I), they do not secrete any cytokines that might interfere with the effects of exogenously added cytokines on the proliferation of CD8$^+$ T cells. The results from such experiments show that, whereas recombinant active TGF-β1 completely inhibited the proliferation of CD8$^+$ T cells in a dose-dependent manner, IL-10 had no effect. Furthermore, in contrast to an earlier observation with CD4$^+$ T cells (32), no synergism between TGF-β1 and IL-10 could be observed. Moreover, addition of high-affinity mouse anti-TGF-β1 Ab was found to completely rescue the CD8$^+$ T cell

proliferation from the inhibitory effects of recombinant active TGF-β1 (Fig. 5*B*). These results link TGF-β1 to the hyporesponsiveness of CD8$^+$ T cells.

Hyporesponsiveness of CD8$^+$ T cells cultured in the presence of LPS-B is not mediated by secreted cytokines

We next addressed whether or not the soluble factors released by the coculture of LPS-B and CD8$^+$ T cells could induce hyporesponsiveness of CD8$^+$ T cells activated in the presence of CD40-B. This was prompted by our earlier observation of a significant decrease in the percentage of cytokine-positive CD8$^+$ T cells when stimulated with LPS-B as compared with stimulation with CD40-B, despite the fact that brefeldin A was added in these cultures to block the cytokine secretion (Fig. 3*A*). This raised the possibility that surface interaction between the T cells and the B cells play an important role in inducing the hyporesponsiveness of CD8$^+$ T cells activated in the presence of LPS-B. To test this possibility, we used the dual-chamber Transwell culture system to examine whether the soluble factors released by the stimulation culture of CD8$^+$ T cells with LPS-B could bring about hyporesponsiveness of the proliferation of CD8$^+$ T cells activated in the presence of CD40-B (indicator culture). We plated CD8$^+$ T cells activated in the presence of LPS-B in the lower Transwell chamber, and we assessed the effect of the soluble factors released by them on the proliferation of CD8$^+$ T cells activated with paraformaldehyde-fixed CD40-B cultured in the upper Transwell chamber. The results showed that CD8$^+$ T cells cultured with LPS-B in the lower Transwell chamber released no soluble cytokines that inhibited the CD40-B-activated CD8$^+$ T cells in the upper Transwell chamber (Fig. 6*A*). Rather, an augmentation in the proliferation of CD8$^+$ T cells was observed. The T cells alone or the B cells alone cultured in the lower Transwell chamber had no effect on the proliferation of CD8$^+$ T cells cultured in the upper Transwell chamber.

An analogous experiment was also conducted, in which CD8$^+$ T cells were cultured with irradiated LPS-B or CD40-B in the presence of anti-CD3 Ab for 60 h, and then the supernatants were harvested. Various concentrations of these supernatants were added to the coculture of CD8$^+$ T cells and paraformaldehyde-fixed CD40-B in the presence of anti-CD3 Ab, and the resultant proliferative response was measured. The results show that the supernatants of the cocultures of CD8$^+$ T cells activated with LPS-B and CD8$^+$ T cells activated with CD40-B had no inhibitory effects (Fig. 6*B*). On the contrary, stimulatory effects were observed, and the supernatants of cultures of CD8$^+$ T cells and CD40-B were more efficient than the supernatants of cultures of CD8$^+$ T cells and LPS-B in causing such effects. This could be due to a higher secretion of soluble costimulatory molecules (36), such as IL-6, TNF-α, and IL-2 secreted by the CD8$^+$ T cells, which are produced at significantly higher levels by the CD8$^+$ T cells activated with CD40-B, compared with those activated with LPS-B (Table I). In contrast, TGF-β1 secreted in the supernatant of coculture of CD8$^+$ T cells and LPS-B could not exert inhibitory effects, because it is secreted predominantly in its latent form. This was revealed by the fact that TGF-β1 estimation was done by acid activation of culture supernatants (Table I); without acid treatment, TGF-β1 was hardly detected. Thus, the results of both of these experiments clearly suggested that the hyporesponsiveness of CD8$^+$ T cells activated in the presence of LPS-B as compared with those activated with CD40-B is not mediated by secreted soluble factors.

LPS-B express higher levels of surface TGF-β1 than do CD40-B

The fact that anti-TGF-β1 significantly restored the proliferation of CD8$^+$ T cells activated with LPS-B to the level of those activated with CD40-B, while the secreted cytokines from such cultures do not play a role in causing the hyporesponsiveness of CD8$^+$ T cells, suggests that TGF-β1 is expressed on the surface of LPS-B. To examine such a possibility, LPS-B and CD40-B were

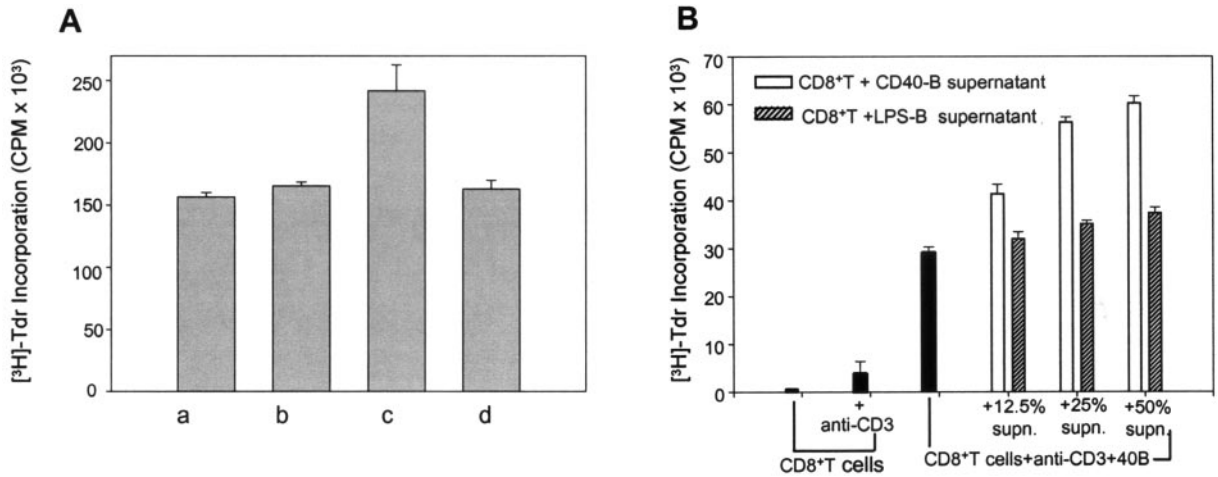

FIGURE 6. *A*, Hyporesponsiveness of CD8$^+$ T cells by LPS-B is not mediated by secreted cytokines. Medium alone (a), CD8$^+$ T cells alone (5×10^5) (b), CD8$^+$ T cells (5×10^5) with irradiated LPS-B (2.5×10^5) (c), and irradiated LPS-B alone (2.5×10^5) (d) were cultured in the lower Transwell chamber in 800 μl of medium in the presence of anti-CD3 Ab (1 μg/ml). The effect of the soluble factors released by this culture was monitored on the proliferation of CD8$^+$ T cells (5×10^5) activated with 2.5×10^5 paraformaldehyde (0.4%)-fixed CD40-B in the upper Transwell chamber in 250 μl of medium, in the presence of anti-CD3 Ab (1 μg/ml). Cells were cultured for 60 h, and then 75 μl of the medium containing the cell suspension from the upper chamber were transferred to a 96-well plate for [^{3}H]thymidine incorporation (8-h) assay. The results shown are the means ± SD of triplicate wells and represent one of three similar experiments. *B*, The supernatant obtained from the coculture of CD8$^+$ T cells and LPS-B does not cause the hyporesponsiveness of CD8$^+$ T cells. Purified CD8$^+$ T cells (1×10^5) were cultured with irradiated LPS-B or CD40-B (5×10^4) with anti-CD3 (1 μg/ml) Ab in multiple wells of a 96-well plate for 60 h, and the supernatants were harvested. The effect of this supernatant at different concentrations was examined on the proliferation of CD8$^+$ T cells (1×10^5) activated with paraformaldehyde (0.4%)-fixed CD40-B (5×10^4) and anti-CD3 Ab (1 μg/ml). Cells were cultured for 72 h, with [^{3}H]thymidine incorporation for the last 12 h of the culture. The data shown indicate means ± SD of triplicate wells and are representative of three independent experiments.

stained with chicken anti-TGF-β1 and goat anti-LAP (TGF-β1) Abs that recognize the active and latent forms of TGF-β1, respectively (37–39). We observed that LPS-B and CD40-B both expressed active TGF-β1 on the surface (Fig. 7*A*). To ensure the specificity of the surface staining obtained for TGF- β1, a competition experiment was conducted in the presence of rTGF-β1. The results showed that the staining of TGF-β1 on the surface of LPS-B was diminished by 8–10 times when stained with anti-

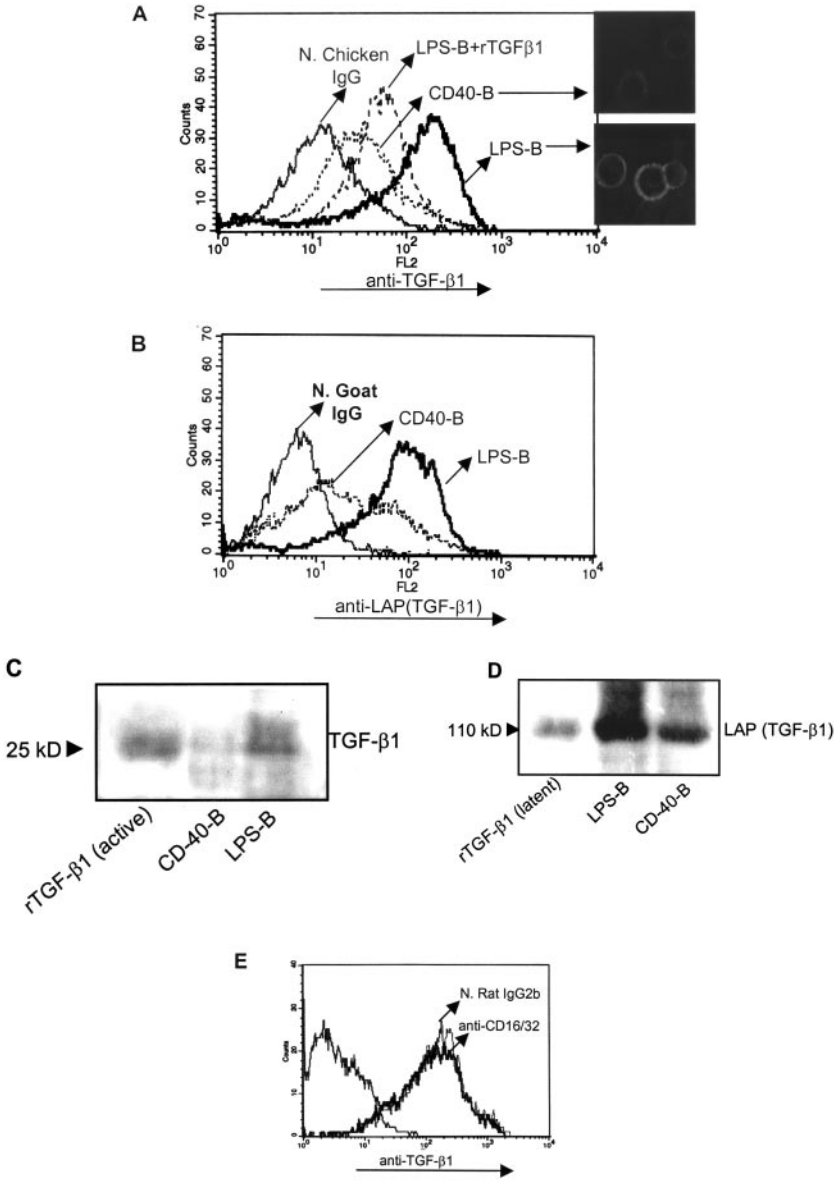

FIGURE 7. *A*, LPS-B express higher levels of TGF-β1 on the surface than do CD40-B. The biotin-labeled chicken anti-TGF-β1 Ab was titrated to a point where staining began to dilute out, and then LPS-B or CD40-B were stained with it or biotin-labeled normal chicken IgG Ab, followed by streptavidin PE. A representative control staining of normal chicken IgG on LPS-B is shown, because it was comparable with the staining on CD40-B and normal B cells. For competition, 2 μg/ml rTGF-β1 was incorporated during the staining with biotin-labeled chicken anti-TGF-β1 on LPS-B. Note that the staining of TGF-β1 on the surface was reduced 8–10 times in the presence of rTGF-β1. The *inset* presents images of confocal microscopy (63× magnification) showing the surface localization of TGF-β1 on CD40-B and LPS-B. Note the higher density of TGF-β1 expression on LPS-B than CD40-B. *B*, Expression of latent TGF-β1 on LPS-B and CD40-B surface. LPS-B or CD40-B were stained with biotin-labeled goat anti-LAP (TGF-β1) Ab or biotin-labeled normal goat IgG Ab, followed by streptavidin PE. A representative control staining of normal goat IgG on LPS-B is shown only, because it was comparable with the staining on CD40-B. Note the higher expression of latent TGF-β1 on the surface of LPS-B than CD40-B. *C*, Western blot analysis of active TGF-β1. Membrane proteins from 5×10^7 LPS-B or CD40-B were prepared as described in *Materials and Methods.* Equal amounts of membrane protein from both of the groups, along with 20 ng of recombinant active TGF-β1, were subjected to 12% SDS-PAGE and immunoblot analysis. Blots were probed with biotin-labeled chicken anti-TGF-β1 Ab (2 μg/ml) or normal chicken IgG. The blots probed with biotin-labeled normal chicken IgG did not show any band and therefore are not shown. The result shown is representative of at least three independent experiments. *D*, Western blot analysis of latent TGF-β1. Equal amounts of membrane protein from both of the groups along with 20 ng of recombinant latent TGF-β1 was subjected to 10% SDS-PAGE and immunoblot analysis. Blots were probed with biotin-labeled goat anti-LAP (TGF-β1) Ab (2 μg/ml) or normal goat IgG. The blots probed with biotin-labeled normal goat IgG did not show any band and therefore are not shown. The result shown is representative of at least three independent experiments. *E*, TGF-β1 on the surface of LPS-B is not bound to secreted IgG. Purified B cells at 2×10^6/well/ml of a 24-well plate were activated with LPS in the presence of 20 μg/ml anti-CD16/CD32 or its isotype control normal rat IgG2b Abs for 72 h. Cells were harvested and stained with biotin-labeled chicken anti-TGF-β1 or normal chicken IgG, followed by streptavidin PE. Note that there was no effect of anti-CD16/CD32 on the staining of TGF-β1 on the B cell surface. The data shown above represent at least three individual experiments.

TGF-β1 in the presence of recombinant active TGF-β1, suggesting that anti-TGF-β1 binding on the surface of LPS-B is specific to TGF-β1 (Fig. 7*A*). Comparing the level of expression of TGF-β1 on these B cells, it was found that LPS-B expresses 10-fold higher levels of TGF-β1 on the surface as compared with those of CD40-B (Fig. 7*A*). The presence of TGF-β1 on the surface of LPS-B was confirmed by confocal microscopy. As shown in Fig. 7*A* (*inset*), chicken anti-TGF-β1 Ab stained LPS-B much more intensely than CD40-B. We also analyzed the latent form of TGF-β1 on the surface using anti-LAP (TGF-β1) Ab and found that LPS-B express much higher levels of latent TGF-β1 than do CD40-B (Fig. 7*B*).

To further confirm the surface association of TGF-β1, Western blot analysis was done using membrane preparations of LPS-B and CD40-B. Membrane proteins were probed with chicken anti-TGF-β1 and goat anti-LAP (TGF-β1) Abs, and it was found that chicken anti-TGF-β1 Abs detected TGF-β bands at a molecular mass of 25 kDa, which corresponds to the active form of TGF-β1 (Fig. 7*C*). Probing with anti-LAP (TGF-β1) Abs yielded band at 110 kDa, corresponding to the small latent complex of TGF-β1 (Fig. 7*D*) (39, 40). Moreover, results with both of these Abs showed that LPS-B cells express much higher levels of TGF-β1 as compared with those of CD40-B. Collectively, we demonstrated higher expression of TGF-β1 on the surface of LPS-B compared with CD40-B using flow cytometry, confocal microscopy and Western blot analysis.

Literature suggests that TGF-β1 has the capacity to bind to secreted IgG (41, 42). It has also been shown that this IgG-bound TGF-β1 can nonspecifically and powerfully suppress cytolytic T cell responses when injected into mice (41, 42). Moreover, the suppression of CTL responses mediated by IgG-bound TGF-β could be abolished by blocking the FcRs (42). Therefore, it is possible that this secreted IgG-bound TGF-β1 could bind the cells via FcRs on the B cell surface in our cultures and show surface staining of TGF-β1. To test this possibility, B cells were activated with LPS in the presence of a high concentration of neutralizing anti-CD16/CD32 Ab (20 μg/ml) and then checked for the level of TGF-β1 expression on the surface. It was found that the presence of anti-CD16/CD32 Abs had no effect on the staining of the TGF-β1 on the surface of LPS-B as compared with the control (Fig. 7*E*). Moreover, we also estimated the levels of secreted IgG in the supernatant of the B cells activated by LPS or anti-Ig and anti-CD40 Abs for 72 h. The results show that CD40-B secreted IgG (3880 $\pm$ 836 pg/ml) at a much higher level as compared with LPS-B (960 $\pm$ 358 pg/ml). These results rule out the possibility that the expression of TGF-β1 obtained in Fig. 7, *A* and *B*, was due to TGF-β1 binding to secreted IgG. Collectively, these results prove the presence of TGF-β1 on the surface, although the nature of its surface association remains unknown.

IL-2 completely rescues, whereas IL-6 and TNF-α only partially rescue, the hyporesponsiveness of CD8$^+$ T cells activated in the presence of LPS-B

The cytokine analysis of CD8$^+$ T cells showed that CD8$^+$ T cells activated with LPS-B secreted much lower levels of IL-2, IL-6, and TNF-α in the culture supernatants as compared with CD8$^+$ T cells activated with CD40-B (Table I). Therefore, we tested whether exogenous addition of recombinant IL-2, IL-6, and TNF-α to the cultures of CD8$^+$ T cells activated with LPS-B could rescue the hyporesponsiveness of CD8$^+$ T cells activated with LPS-B. Such experiments revealed that IL-2 completely restored the proliferation of CD8$^+$ T cells, whereas TNF-α and IL-6 were only partially effective (Fig. 8*A*). These results suggested that

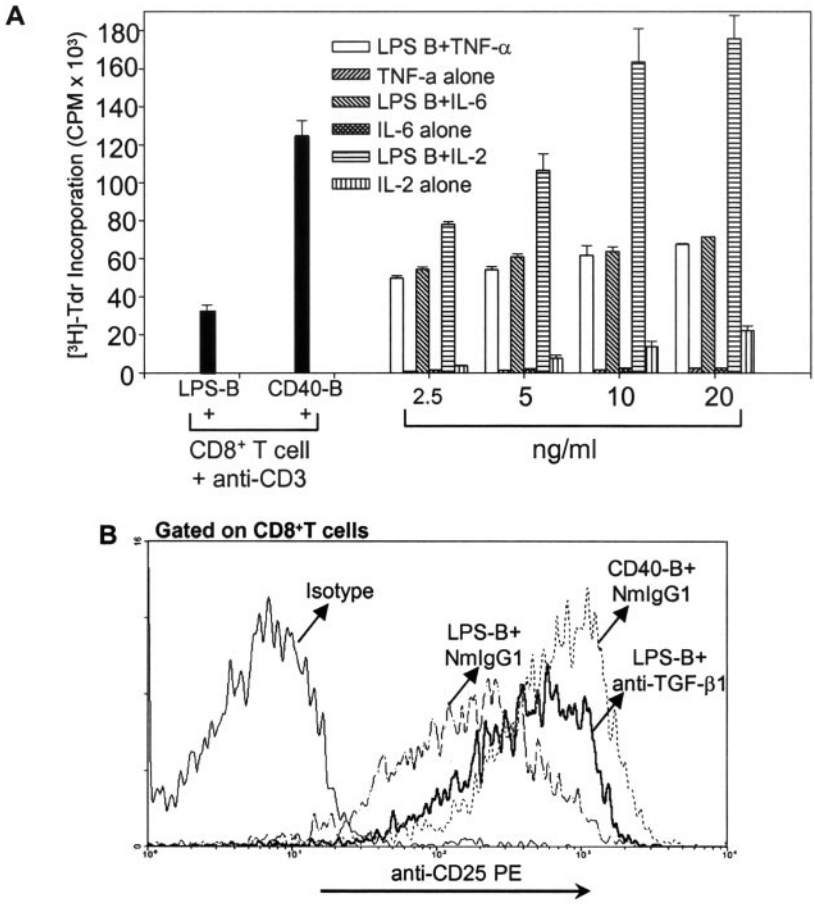

FIGURE 8. *A*, IL-2 completely restores the proliferation of CD8$^+$ T cells activated in the presence of LPS-B. Purified CD8$^+$ T cells (1×10^5) were activated with irradiation-fixed LPS-B (5×10^4) and anti-CD3 Ab (1 μg/ml). To restore the proliferation, recombinant IL-2, IL-6, and TNF-α were added, over the concentration range of 2.5–20 ng/ml to the CD8$^+$ T cells cultured with or without LPS-B, along with anti-CD3 Ab (1 μg/ml). Cells were cultured for 72 h with [^{3}H]thymidine pulse (1 μCi/well) for the last 12 h of the culture. Recombinant human IL-15 added to these cultures had no effect on the proliferation of CD8$^+$ T cells and is not shown. The proliferation of CD8$^+$ T cells activated with irradiated CD40-B is shown for comparison. The results shown are the means $\pm$ SD of triplicate wells and are representative of one of the three individual experiments. *B*, Anti-TGF-β1 Ab significantly restores the expression of IL-2Rα on CD8$^+$ T cells activated with LPS-B. Purified CD8$^+$ T cells (1×10^5) were activated with anti-CD3 Ab (1 μg/ml) and LPS-B or CD40-B (5×10^4) in the presence of anti-TGF-β1 Ab or its isotype control mouse IgG1 for 24 h. Cells were harvested and stained with either anti-CD25 PE Ab or normal rat IgG2b PE as its isotype control. Expression of CD25 is shown on gated cells that were positive for anti-CD8 FITC. A representative of isotype control rat IgG2b PE staining on CD8$^+$ T cells activated with LPS-B in the presence of anti-TGF-β1 is shown only, because its staining on CD8$^+$ T cells activated with LPS-B or CD40-B in the presence of normal mouse IgG1 was comparable.

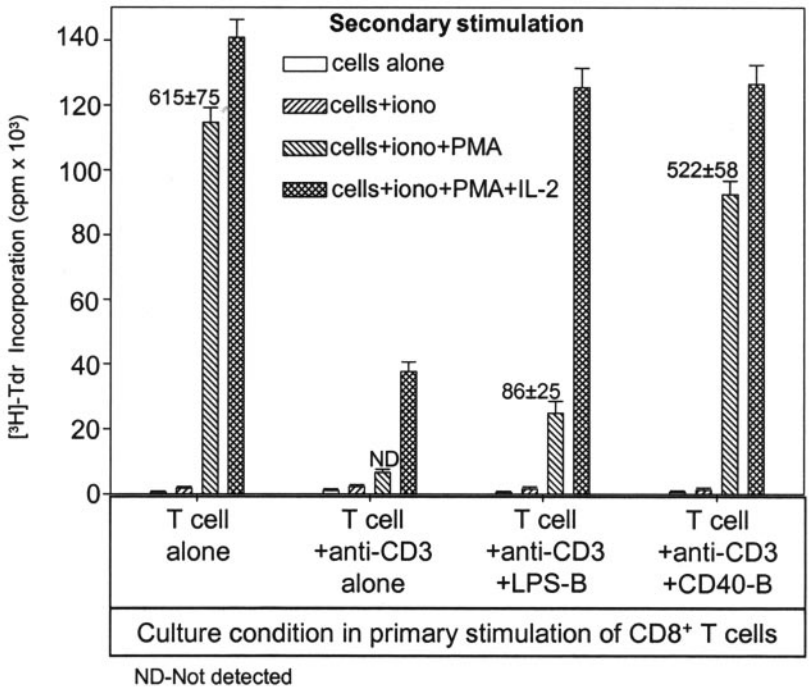

FIGURE 9. $CD8^+$ T cells activated with LPS-B go to a state of partial anergy. Purified $CD8^+$ T cells were cultured unstimulated or stimulated with immobilized anti-CD3 (2 μg/ml) or stimulated with irradiated LPS-B or CD40-B and soluble anti-CD3 (1 μg/ml) for 2 days in the primary stimulation. Cells were then harvested, washed, and rested in fresh medium for 2 more days. The cells were then subjected to Histopaque, and the viable cells were counted in the presence of trypan blue. T cells (5×10^4) were restimulated in the secondary culture with ionomycin alone or ionomycin and PMA with or without rIL-2 for 72 h in a 96-well plate. Proliferation of the cells was measured using [^{3}H]thymidine incorporation at 1 μCi/well for the last 12 h of the culture. The results shown are the means ± SD of triplicate wells and represent one of three individual experiments. In parallel cultures, supernatant was harvested at 24 h from T cells restimulated with ionomycin alone or with ionomycin and PMA for IL-2 measurement by ELISA. The inset values indicate the levels of IL-2 (picograms per milliliter) from the corresponding cultures and are the means ± SD of duplicate wells assayed. IL-2 in the supernatants of the T cells alone and those activated with ionomycin alone were not detectable and therefore not indicated. The data are representative of at least three independent experiments.

TGF-β1 on LPS-B surface induces hyporesponsiveness of $CD8^+$ T cells by preventing the up-regulation of IL-2 and IL-2Rα expression (Fig. 1*B*), because IL-2 is known to up-regulate IL-2Rα expression on $CD8^+$ T cells (43). To further verify this, anti-TGF-β1 Ab was added to the coculture of $CD8^+$ T cells activated with LPS-B, and we assessed whether neutralizing the activity of TGF-β1 could restore the expression of IL-2 and IL-2Rα by the T cells. We observed that anti-TGF-β1 completely restored the synthesis of IL-2 (NmIgG1, 145.5 ± 45; anti-TGF-β, 420 ± 25) from the T cells activated with LPS-B. Moreover, $CD8^+$ T cells activated with LPS-B significantly up-regulated the expression of IL-2Rα (Fig. 8*B*), which was found to be expressed at lower levels on $CD8^+$ T cells activated with LPS-B as compared with those activated with CD40-B (Fig. 1*B*). Because CD28 signaling to T cells is known to induce IL-2 production, we tested whether anti-CD28 agonist Ab would restore the proliferation of $CD8^+$ T cells activated with LPS-B. We found that anti-CD28 Abs also completely restore the proliferation of $CD8^+$ T cells activated with LPS-B when compared with the proliferation of $CD8^+$ T cells activated with CD40-B (data not shown). These results suggest that the TGF-β1-mediated hyporesponsiveness of $CD8^+$ T cells stimulated in the presence of LPS-B results from their attenuated ability to produce relevant cytokines, most importantly, IL-2.

$CD8^+$ T cells activated with LPS-B enter into a state of anergy

Previous reports have suggested a link between decreased T cell proliferation and lymphokine production and T cell anergy (44, 45). Therefore, additional experiments were conducted to investigate whether $CD8^+$ T cells activated with LPS-B enter a state of anergy, because they too showed decreased proliferation and lymphokine secretion (Figs. 2*A* and 4*A*). To assess this possibility, $CD8^+$ T cells were allowed to interact with LPS-B or CD40-B for 2 days and rested for 2 more days (27, 45). The viable cells obtained from these cultures were then examined for their ability to respond to restimulation with ionomycin and PMA with or without rIL-2. The immobilized anti-CD3 alone-mediated anergy of T cells was used as a positive control of the experiment (27, 45). The results demonstrated that T cells activated with LPS-B and those activated with anti-CD3 alone entered a state of anergy (Fig. 9). Exogenous addition of rIL-2 to these cultures completely restored the proliferation of T cells previously activated with LPS-B, although only partially in the case of T cells activated with immobilized anti-CD3 alone. However, $CD8^+$ T cells that were unstimulated and those activated with CD40-B in the primary culture responded normally to restimulation with ionomycin and PMA (Fig. 9). The IL-2 secretion in the supernatant of the T cells restimulated with ionomycin and PMA was also measured. We found that $CD8^+$ T cells activated with LPS-B in primary stimulation secreted much lower levels of IL-2 as compared with those of $CD8^+$ T cells that were unstimulated or stimulated with CD40-B, in response to ionomycin and PMA. Restimulation with ionomycin and PMA of T cells previously stimulated with immobilized anti-CD3 alone did not lead to IL-2 secretion, suggesting that these cells had entered a state of anergy (Fig. 9). These results clearly indicate that $CD8^+$ T cells activated with LPS-B enter a state of anergy, but are responsive to exogenous rIL-2.

Thus, in the present study, the role of TGF-β1 on LPS-B in causing $CD8^+$ T cell anergy was delineated. To increase the frequency and yield of purified $CD8^+$ T cells, mice were injected beforehand with allogenic cells. Although these T cells were found to have a resting phenotype, they might contain a mixture of the naive and memory T cell populations. Therefore, we obtained $CD8^+$ T cells from naive animals and activated them with LPS-B or CD40-B (data not shown). The results showed that LPS-B cells caused the hyporesponsiveness of $CD8^+$ T cells from naive mice to the same extent as $CD8^+$ T cells from alloantigen primed mice, and thus no significant difference in the response of $CD8^+$ T cells from naive and alloantigen-primed mice was observed.

Discussion

B cells have the capacity to present exogenous Ag through the MHC class I pathway (46), which can play a significant role in the regulation of CTL responses. In the present study, we examined the effect of various modes of activation of B cells on their ability to activate $CD8^+$ T cells. To activate the B cells, we used the combination of anti-Ig and anti-CD40 Abs, a condition expected to

be reminiscent of signals delivered during induction of a T-dependent humoral response. In contrast, we also used LPS—a known T-independent stimulus for the activation of B cells. The activated cells obtained from either of these procedures were then examined for their ability to engage and influence responses from resting CD8$^+$ T cells activated polyclonally in the presence of anti-CD3 Ab. As we have shown in this study, the mode of activation appears to have an important bearing, in both quantitative and qualitative terms, on the ability of these B cells to activate CD8$^+$ T cells. CD40-B proved to be excellent stimulators of CD8$^+$ T cells, whereas LPS-B in comparison were poor stimulators of CD8$^+$ T cells. Such hyporesponsive CD8$^+$ T cells activated with LPS-B was found to be due to high expression of TGF-β1 on the surface of LPS-B, which leads these cells to undergo a state of anergy.

The CD8$^+$ T cells costimulated with LPS-B secreted less IL-2 and also expressed less IL-2Rα in comparison with CD8$^+$ T cells costimulated with CD40-B. However, these anergized CD8$^+$ T cells retained their cytotoxic ability, but to a lesser degree than those stimulated with CD40-B. Several studies have demonstrated that, in addition to promoting proliferation and IFN-γ production (47), IL-2 is also required for the up-regulation of perforin gene expression in CD8$^+$ T cells (48). In contrast, TGF-β1 is known to inhibit IL-2Rα (49), perforin (49, 50), and granzyme (49) expression in CD8$^+$ T cells. It is these cumulative inhibitory effects of TGF-β1 that are probably responsible for the poorer ability of LPS-B-activated CD8$^+$ T cells to lyse the target cells. In addition, recently, CD8$^+$ T cells were also shown to be suppressed when activated with CD4$^+$CD25$^+$ regulatory T cells by T-T interaction (51), which is now known to exert its inhibitory effects by surface-bound TGF-β1 (52, 53). It had been also shown that these regulatory T cells inhibit the activation of the CD8$^+$ T cell response by inhibiting both IL-2 production and up-regulation of IL-2Rα (CD25) expression by them (51). Therefore, these previous observations have provided us with a framework for rationalizing our findings of the hyporesponsive nature of CD8$^+$ T cells activated with LPS-B. The surface-associated TGF-β1 described in the present study exerts its effects by preventing the up-regulation of IL-2 and IL-2Rα expression on CD8$^+$ T cells. Support for such a possibility is provided from our experiments demonstrating that exogenous addition of rIL-2 to the cocultures of CD8$^+$ T cells and LPS-B could completely rescue the T cells from the hyporesponsiveness induced by the latter cell population. This may have been accomplished by up-regulating IL-2Rα expression, because IL-2 is known to induce IL-2Rα expression on CD8$^+$ T cells (43). Moreover, treatment with anti-TGF-β1 Abs of the cultures of CD8$^+$ T cells and LPS-B completely restored the IL-2 production and significantly up-regulated IL-2Rα expression. These results thus provide evidence that TGF-β1 on LPS-B primarily prevents the up-regulation of IL-2 and IL-2Rα expression by T cells and, as a consequence, leads to inadequate production of molecules required for the effector function. In addition to the effects on IL-2 and IL-2Rα, the described decreased production of IFN-γ and TNF-α by LPS-B-activated CD8$^+$ T cells could also be significant for the CTL function. Both of these cytokines are also known to up-regulate granzyme B and perforin expression in CTLs (54, 55).

TGF-β1 is known to represent a multifunctional cytokine with growth-promoting as well as growth-inhibitory properties, depending on the cell type. Thus, although it exerts suppressive effects on the growth of hemopoietic progenitor cells (56), it has also been shown to costimulate proliferation of CD8$^+$ T cells (57). However, depending on the cellular context and activation status of the target cell, TGF-β1 has been also shown to have either stimulatory or inhibitory effects on the same cell type (58). TGF-β1 is synthesized as a large 110-kDa pro-TGF-β1 molecule, a disulfide-linked homodimer, which is also referred to as the small latent form of TGF-β1 (39, 40). Latent TGF-β1 is also known to associate with the TGF-β binding protein that is covalently linked to LAP, which is called the large latent complex (37, 38, 39). Cleavage of the LAP fragment is then required to generate the biologically active 25-kDa TGF-β1 molecule (59, 60), especially by thrombospondin-1, which is considered to be a major activator of TGF-β1 in vivo (37). In our study, LPS-B could be efficiently stained on the surface with the chicken anti-TGF-β1 Ab, an Ab that is known to recognize the active form of TGF-β1 and is also cross-reactive with latent TGF-β1 (52). We could also obtain surface staining with Abs generated against LAP. Moreover, in Western blot experiments, chicken anti-TGF-β1 could recognize a small fraction of the active form of TGF-β1 at 25-kDa molecular mass, while anti-LAP (TGF-β1) Ab recognized the band corresponding to the latent form of TGF-β1 at 110-kDa molecular mass. These results suggested that the surface-associated TGF-β1 on LPS-B cells exists predominantly in the latent form and partly as active form. The nature of membrane association of TGF-β1 and how the latent form of TGF-β1 on the surface of B cells is converted to its active form remain to be elucidated. However, Rowley and coworkers (40, 41, 42) showed that TGF-β could complex with IgG and that the binding of this complex on myeloid cells via the FcRs powerfully suppresses CTL responses. Furthermore, these effects could be abrogated by blocking the FcRs. Therefore, we argued that in our cultures also B cells, in principle, could bind to secreted IgG complexed with TGF-β (mediated by FcRs) and might falsely show surface staining of TGF-β1. To rule out this possibility, we activated the B cells with LPS in the presence of blocking anti-FcR Ab and then stained these cells with anti-TGF-β1 Ab. We found that anti-FcR Ab had no effect on the staining of TGF-β1 on the LPS-B surface. Moreover, we also measured the IgG secreted by B cells activated with LPS or anti-Ig and anti-CD40 Abs in the supernatant and found that CD40-B secreted markedly higher levels of IgG in the supernatant as compared with those of LPS-B. These results clearly rule out the possibility of TGF-β1 on the LPS-B surface being TGF-β-IgG complex. Literature suggests that latent TGF-β1, upon its secretion, first anchors on the membrane of the cell through mannose 6-phosphate receptors (61). Subsequent to this, LAP is cleaved by the membrane-associated plasmin to give rise to active TGF-β1 (62). Thus, it is possible that the latent form of TGF-β1 is activated on the surface of LPS-B only on contact with CD8$^+$ T cells, presumably through the effects of either plasmin or some other proteases present on the T cell membrane. It is this proximally formed TGF-β1 that then exerts its potent inhibitory effects on the T cells.

It is now widely accepted that the generation of CTLs occur by two distinct pathways. One of these is dependent on CD4$^+$ T cell help, whereas the other is Th cell independent. Ridge et al. (63) have proposed a dynamic model for the CD4$^+$ Th-dependent generation of CTLs. This proposal suggests that CD4$^+$ T cells first activate APCs to a state where they can now directly activate CD8$^+$ T cells. For this, the interaction between CD40 and CD40 ligand expressed on APCs and CD4$^+$ T cells, respectively, was suggested to be critical. Our present results demonstrating that B cells stimulated with the combination of anti-Ig and anti-CD40 Abs were potent stimulators of CD8$^+$ T cells is consistent with such a possibility. We also raised the question of whether CD40-B could synthesize a higher level of CD8$^+$ T cell stimulatory factor than LPS-B. Indeed, we found that CD40-B secretes markedly higher levels of IL-15 than does LPS-B. This result also signifies that the difference between LPS-B and CD40-B is not just quantitative, based on the levels of TGF-β1 and IL-10 synthesized, but

also qualitative as observed by high IL-15 levels synthesized by CD40-B. Therefore, B cells activated by T-independent or T-dependent modes induce distinct signaling pathways leading to differential gene expression. IL-15 is known to act through the IL-2Rβ chain (CD122), the high expression of which is restricted to memory $CD8^+$ T cells (35). We found no detectable levels of IL-15 in the culture supernatants of the coculture of $CD8^+$ T cells and both types of B cells, as the B cells were irradiation fixed, suggesting that the T cells do not contribute to IL-15 secretion. Moreover, in contrast to rIL-2 addition, exogenous addition of rIL-15 (2–20 ng/ml) to the cocultures of $CD8^+$ T cells and LPS-B had no effect on the proliferation. Collectively, these results suggest that, in our culture system, IL-15 does not play a role in inducing higher proliferation of $CD8^+$ T cells activated with CD40-B.

Although CD40-B proved to be effective stimulators of $CD8^+$ T cells, they were nevertheless also found to express some surface-associated TGF-β1. However, these levels were significantly lower than those present on LPS-B cells. Thus, the differences in the $CD8^+$ T cell-stimulatory capacities of CD40-B and LPS-B may be due to the different densities of surface-associated TGF-β1 expressed by these two subsets. The low concentrations on CD40-B may allow $CD8^+$ T cell activation, while the higher surface density of TGF-β1 would prevent LPS-B cells to optimally activate $CD8^+$ T cells. The inhibitory effects of TGF-β1 on $CD8^+$ T cells is consistent with the observation that mice deficient in TGF-β1 develop multifocal inflammatory disease and die at 3–4 wk of age (64). Depletion of $CD8^+$ T cells in vivo on a β_2-microglobulin-deficient background slows down the disease progression and improves survival in those mice (65). Recently, studies on immunosuppressive $CD4^+CD25^+$ regulatory T cells showed that such T cells express high levels of cell surface-associated TGF-β1 (52, 53). Therefore, our finding that surface-expressed TGF-β1 on LPS-B is responsible for the hyporesponsiveness of $CD8^+$ T cells could suggest an important mechanism underlying the induction of T cell anergy by diverse cell types. These observations may also provide an explanation of the earlier observation that LPS-B blast induces tolerance in CTLs in response to H-Y Ag and that LPS-B were three times more potent then resting cells in their capacity to induce tolerance in vivo (5). Additionally, for the same reasons, naive T cells that have a higher threshold of activation and are more dependent on costimulation for activation than are Ag-experienced T cells are known to be tolerized by B cells (66, 67) and cannot be activated by B cells as APCs (5).

Our results showed that $CD8^+$ T cells activated with LPS-B entered a state of anergy. Literature suggests that anergic T cells could act as suppressor cells in vitro as well as in vivo (27, 68). Therefore, B cells activated by T-independent Ags may play an important role in maintaining peripheral self-tolerance by inducing anergic or regulatory T cells. Support for such a possibility is provided by earlier reports that suggest a crucial role for B cells in the generation of oral tolerance, which also involves Ag-specific T cell anergy as one of its mechanisms (69). B cell-deficient mice were found to possess an altered cytokine environment in the gut-associated lymphoid tissue, with a defect in low-dose oral tolerance (70). Oral tolerance is mediated primarily by TGF-β1, as anti-TGF-β1 neutralizing Ab abrogates tolerance induction (69). Moreover, LPS-mediated signaling through the TLR-4 is thought to be obligatory for the induction of oral tolerance, because it was found to be absent in C3H/HeJ mice that are defective in LPS responses due to mutations within the *TLR-4* gene (71, 72).

Our findings of the hyporesponsive behavior of $CD8^+$ T cell in response to LPS-B may have important implications for bacterial infections. Thus, the recent observations by Shirai et al. (73) that persistent infection by *Helicobacter pylori*, a Gram-negative bacteria, down-modulates virus-specific CTL responses and prolongs viral infection may well be rationalized by our present findings. Moreover, these proposals could have intriguing implications for the initiation and sustenance of retroviral infections, many of which are known to target B cells through TLR-4. Retroviruses like mouse mammary tumor virus and Moloney murine leukemia virus activate the B cells by specifically binding via TLR-4, and this binding was found to be independent of viral attachment and fusion with the B cells (74). Therefore, C3H/HeJ mice, which have a mutant TLR-4, were found to have reduced incidence of mouse mammary tumor virus-induced mammary tumors compared with the normal C3H/HeN strain (75). Thus, activated B cells expressing the surface TGF-β1 and the viral Ags would then play a significant role in down-regulating virus-specific CTL responses.

In summary, we have demonstrated that the ability of activated B cells to influence responses from $CD8^+$ T cells depends significantly on the manner in which they were originally activated. B cells activated with a T-dependent mode of stimulus confer on $CD8^+$ T cells excellent stimulatory potential, whereas those activated with T-independent Ag cause anergy in $CD8^+$ T cells. These different properties of B cells to influence the $CD8^+$ T cell responses seem to be enforced by distinct levels of surface TGF-β1. Therefore, these findings provide helpful insight into immune evasion strategies adopted by both Gram-negative bacteria and retroviruses that specifically target TLR-4 signaling in B cells. Finally, the opposing consequences of the T-dependent vs T-independent modes of B cell activation observed in this study could also eventually be used to promote the development of therapeutic modalities for regulating the balance between tolerance and active immunity as required.

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References

1. Qin, Z., G. Richter, T. Schuler, S. Ibe, X. Cao, and T. Blankenstein. 1998. B cells inhibit induction of T cell-dependent tumor immunity. *Nat. Med. 4:627.*
2. Hollsberg, P., V. Batra, A. Dressel, and D. A. Hafler. 1996. Induction of anergy in CD8 T cells by B cell presentation of antigen. *J. Immunol. 157:5269.*
3. De Bruijn, M. L., J. D. Nieland, T. N. Schumacher, H. L. Ploegh, W. M. Kast, and C. J. Melief. 1992. Mechanisms of induction of primary virus-specific cytotoxic T lymphocyte responses. *Eur. J. Immunol. 22:3013.*
4. Bennett, S. R., F. R. Carbone, T. Toy, J. F. Miller, and W. R. Heath. 1998. B cells directly tolerize $CD8^+$ T cells. *J. Exp. Med. 188:1977.*
5. Fuchs, E. J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science 258:1156.*
6. Epstein, M. M., F. Di Rosa, D. Jankovic, A. Sher, and P. Matzinger. 1995. Successful T cell priming in B cell-deficient mice. *J. Exp. Med. 182:915.*
7. Thomsen, A. R., J. Johansen, O. Marker, and J. P. Christensen. 1996. Exhaustion of CTL memory and recrudescence of viremia in lymphocytic choriomeningitis virus-infected MHC class II-deficient mice and B cell-deficient mice. *J. Immunol. 157:3074.*
8. Kay, T. W., J. L. Parker, L. A. Stephens, H. E. Thomas, and J. Allison. 1996. RIP-β_2-microglobulin transgene expression restores insulitis, but not diabetes, in β_2-microglobulin-null nonobese diabetic mice. *J. Immunol. 157:3688.*
9. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig μ null mice. *J. Exp. Med. 184:2049.*
10. Sun, Q., K. E. Pollok, R. L. Burton, L. J. Dai, W. Britt, D. J. Emanuel, and K. G. Lucas. 1999. Simultaneous ex vivo expansion of cytomegalovirus and Epstein-Barr virus-specific cytotoxic T lymphocytes using B-lymphoblastoid cell lines expressing cytomegalovirus pp65. *Blood 94:3242.*
11. Eynon, E. E., and D. C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med. 175:131.*

12. Gilbert, K. M., and W. O. Weigle. 1994. Tolerogenicity of resting and activated B cells. *J. Exp. Med. 179:249.*
13. Liu, Y. J., J. Zhang, P. J. Lane, E. Y. Chan, and I. C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur. J. Immunol. 21:2951.*
14. Davidson, H. W., P. A. Reid, A. Lanzavecchia, and C. Watts. 1991. Processed antigen binds to newly synthesized MHC class II molecules in antigen-specific B lymphocytes. *Cell 67:105.*
15. Noelle, R. J., M. Roy, D. M. Shepherd, I. Stamenkovic, J. A. Ledbetter, and A. Aruffo. 1992. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc. Natl. Acad. Sci. USA 89:6550.*
16. Skok, J., J. Poudrier, and D. Gray. 1999. Dendritic cell-derived IL-12 promotes B cell induction of Th2 differentiation: a feedback regulation of Th1 development. *J. Immunol. 163:4284.*
17. Morrison, D. C., and J. L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med. 38:417.*
18. Cerottini, J. C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol. 18:67.*
19. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol. 162:3749.*
20. Denizot, F., A. Wilson, F. Battye, G. Berke, and K. Shortman. 1986. Clonal expansion of T cells: a cytotoxic T-cell response in vivo that involves precursor cell proliferation. *Proc. Natl. Acad. Sci. USA 83:6089.*
21. Cai, Z., and J. Sprent. 1994. Resting and activated T cells display different requirements for CD8 molecules. *J. Exp. Med. 179:2005.*
22. Curtsinger J. M., D. C. Lins, and M. F. Mescher. 1998. CD8$^+$ memory T cells (CD44^{high}, Ly-6C$^+$) are more sensitive than naive cells to (CD44^{low}, Ly-6C$^-$) to TCR/CD8 signaling in response to antigen. *J. Immunol. 160:3236.*
23. Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods 145:185.*
24. Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J. Exp. Med. 182: 1357.*
25. Prasad, D. V. R., V. V. Parekh, B. N. Joshi, P. P. Baneerjee, P. B. Parab, S. Chattopadhyay, A. Kumar, and G. C. Mishra. 2002. The Th1-specific costimulatory molecule M150 is a post-translational isoform of LAMP-1. *J. Immunol. 169:1801.*
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:680.*
27. Chai, J. G., I. Bartok, P. Chandler, S. Vendetti, A. Antoniou, J. Dyson, and R. Lechler. 1999. Anergic T cells act as suppressor cells in vitro and in vivo. *Eur. J. Immunol. 29:686.*
28. Scheinecker, C., K. P. Machold, O. Majdic, P. Hocker, W. Knapp, and J. S. Smolen. 1998. Initiation of the autologous mixed lymphocyte reaction requires the expression of costimulatory molecules B7-1 and B7-2 on human peripheral blood dendritic cells. *J. Immunol. 161:3966.*
29. McAdam, A. J., B. E. Gewurz, E. A. Farkash, and A. H. Sharpe. 2000. Either B7 costimulation or IL-2 can elicit generation of primary alloreactive CTL. *J. Immunol. 165:3088.*
30. Agadjanyan, M. G., J. J. Kim, N. Trivedi, D. M. Wilson, B. Monzavi-Karbassi, L. D. Morrison, L. K. Nottingham, T. Dentchev, A. Tsai, K. Dang, et al. 1999. CD86 (B7-2) can function to drive MHC-restricted antigen-specific CTL responses in vivo. *J. Immunol. 162:3417.*
31. Carpenter, P. A., S. Pavlovic, J. Y. Tso, O. W. Press, T. Gooley, X. Z. Yu, and C. Anasetti. 2000. Non-Fc receptor-binding humanized anti-CD3 antibodies induce apoptosis of activated human T cells. *J. Immunol. 165:6205.*
32. Zeller, J. C., A. Panoskaltsis-Mortari, W. J. Murphy, F. W. Ruscetti, S. Narula, M. G. Roncarolo, and B. R. Blazar. 1999. Induction of CD4$^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-β. *J. Immunol. 163:3684.*
33. Hahne, M., T. Renno, M. Schroeter, M. Irmler, L. French, T. Bornard, H. R. MacDonald, and J. Tschopp. 1996. Activated B cells express functional Fas ligand. *Eur. J. Immunol. 26:721.*
34. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature 389:737.*
35. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8$^+$ T cells in vivo by IL-15. *Immunity 8:591.*
36. Sepulveda, H., A. Cerwenka, T. Morgan, and R. W. Dutton. 1999. CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. *J. Immunol. 163:1133.*
37. Crawford, S. E., V. Stellmach, J. E. Murphy-Ullrich, S. M. Ribeiro, J. Lawler, R. O. Hynes, G. P. Boivin, and N. Bouck. 1998. Thrombospondin-1 is a major activator of TGF-β1 in vivo. *Cell 93:1159.*
38. Schultz-Cherry, S., S. Ribeiro, L. Gentry, and J. E. Murphy-Ullrich. 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-β in a chemically defined system. *J. Biol. Chem. 269: 26775.*
39. Lyons, R. M., L. E. Gentry, A. F. Purchio, and H. L. Moses. 1990. Mechanism of activation of latent recombinant transforming growth factor-β1 by plasmin. *J. Cell Biol. 110:1361.*
40. Beck, C., H. Schreiber, and D. Rowley. 2001. Role of TGF-β in immune-evasion of cancer. *Microsc. Res. Tech. 52:387.*
41. Stach, R. M., and D. A. Rowley. 1993. A first or dominant immunization. II. Induced immunoglobulin carries transforming growth factor-β and suppresses cytolytic T cell responses to unrelated alloantigens. *J. Exp. Med. 178:841.*
42. Rowley, D. A., and R. M. Stach. 1998. B lymphocytes secreting IgG linked to latent transforming growth factor-β prevent primary cytolytic T lymphocyte responses. *Int. Immunol. 10:355.*
43. Nakajima, H., X. W. Liu, A. Wynshaw-Boris, L. A. Rosenthal, K. Imada, D. S. Finbloom, L. Hennighausen, and W. J. Leonard. 1997. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor α chain induction. *Immunity 7:691.*
44. Groux, H., M. Bigler, J. E. de Vries, and M. G. Roncarolo. 1996. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4$^+$ T cells. *J. Exp. Med. 184:1.*
45. Chai, J. G., and Lechler, R. 1997. Immobilised anti-CD3 mAb induces anergy in murine naïve and memory CD4$^+$ T cells. *Int. Immunol. 9:935.*
46. Ke, Y., and J. A. Kapp. 1996. Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. *J. Exp. Med. 184:1179.*
47. Cousens, L. P., J. S. Orange, and C. A. Biron. 1995. Endogenous IL-2 contributes to T cell expansion and IFN-γ production during lymphocytic choriomeningitis virus infection. *J. Immunol. 155:5690.*
48. Zhang, J., I. Scordi, M. J. Smyth, and M. G. Lichtenheld. 1999. Interleukin-2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat) 5 activation of two enhancers. *J. Exp. Med. 190: 1297.*
49. Inge, T. H., K. M. McCoy, B. M. Susskind, S. K. Barrett, G. Zhao, and H. D. Bear. 1992. Immunomodulatory effects of transforming growth factor-β on T lymphocytes: induction of CD8 expression in the CTLL-2 cell line and in normal thymocytes. *J. Immunol. 148:3847.*
50. Smyth, M. J., S. L. Strobl, H. A. Young, J. R. Ortaldo, and A. C. Ochoa. 1991. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8$^+$ T lymphocytes: inhibition by transforming growth factor-β. *J. Immunol. 146:3289.*
51. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of CD8$^+$ T cell activation by CD4$^+$CD25$^+$ immunoregulatory cells. *J. Immunol. 167: 1137.*
52. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4$^+$CD25$^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor-β. *J. Exp. Med. 194:629.*
53. Annunziato, F., L. Cosmi, F. Liotta, E. Lazzeri, R. Manetti, V. Vanini, P. Romagnani, E. Maggi, and S. Romagnani. 2002. Phenotype, localization, and mechanism of suppression of CD4$^+$CD25$^+$ human thymocytes. *J. Exp. Med. 196:379.*
54. Liu, C. C., S. V. Joag, B. S. Kwon, and J. D. Young. 1990. Induction of perforin and serine esterases in a murine cytotoxic T lymphocyte clone. *J. Immunol. 144: 1196.*
55. Fitzpatrick, L., A. P. Makrigiannis, M. Kaiser, and D. W. Hoskin. 1996. Anti-CD3-activated killer T cells: interferon-γ and interleukin-10 cross-regulate granzyme B expression and the induction of major histocompatibility complex-unrestricted cytotoxicity. *J. Interferon Cytokine Res. 16:537.*
56. Goey, H., J. R. Keller, T. Back, D. L. Longo, F. W. Ruscetti, and R. H. Wiltrout. 1989. Inhibition of early murine hemopoietic progenitor cell proliferation after in vivo locoregional administration of transforming growth factor-β1. *J. Immunol. 143:877.*
57. Lee, H. M., and S. Rich. 1993. Differential activation of CD8$^+$ T cells by transforming growth factor-β1. *J. Immunol. 151:668.*
58. Wahl S. M. 1994. Transforming growth factor-β: the good, the bad, and the ugly. *J. Exp. Med. 180:1587.*
59. Assoian, R. K., A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn. 1983. Transforming growth factor-β in human platelets: identification of a major storage site, purification, and characterization. *J. Biol. Chem. 258:7155.*
60. Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel. 1985. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature 316:701.*
61. Dennis, P. A., and D. B. Rifkin. 1991. Cellular activation of latent transforming growth factor-β requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc. Natl. Acad. Sci. USA 88:580.*
62. Odekon, L. E., F. Blasi, and D. B. Rifkin. 1994. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-β to TGF-β. *J. Cell. Physiol. 158:398.*
63. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell. *Nature 393: 474.*
64. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, et al. 1992. Targeted disruption of

the mouse transforming growth factor-β1 gene results in multi-focal inflammatory disease. *Nature 359:693.*
65. Kobayashi, S., K. Yoshida, J. M. Ward, J. J. Letterio, G. Longenecker, L. Yaswen, B. Mittleman, E. Mozes, A. B. Roberts, S. Karlsson, and A. B. Kulkarni. 1999. β_2-Microglobulin-deficient background ameliorates lethal phenotype of the TGF-β1 null mouse. *J. Immunol. 163:4013.*
66. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
67. Luqman, M., and K. Bottomly. Activation requirements for $CD4^+$ T cells differing in CD45R expression. *J. Immunol. 149:2300.*
68. Lombardi, G., S. Sidhu, R. Batchelor, and R. Lechler. 1994. Anergic T cells as suppressor cells in vitro. *Science 264:1587.*
69. Krause, I., M. Blank, and Y. Shoenfeld. 2000. Immunomodulation of experimental autoimmune diseases via oral tolerance. *Crit. Rev. Immunol. 20:1.*
70. Gonnella, P. A., H. P. Waldner, and H. L. Weiner. 2001. B cell-deficient (μ MT) mice have alterations in the cytokine microenvironment of the gut-associated lymphoid tissue (GALT) and a defect in the low dose mechanism of oral tolerance. *J. Immunol. 166:4456.*
71. Wannemuehler, M. J., H. Kiyono, J. L. Babb, S. M. Michalek, and J. R. McGhee. 1982. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J. Immunol. 129:959.*
72. Kiyono, H., J. R. McGhee, M. J. Wannemuehler, and S. M. Michalek. 1982. Lack of oral tolerance in C3H/HeJ mice. *J. Exp. Med. 155:605.*
73. Shirai, M., T. Arichi, T. Nakazawa, and J. A. Berzofsky. 1998. Persistent infection by *Helicobacter pylori* down-modulates virus-specific $CD8^+$ cytotoxic T cell response and prolongs viral infection. *J. Infect. Dis. 177:72.*
74. Rassa, J. C., J. L. Meyers, Y. Zhang, R. Kudaravalli, and S. R. Ross. 2002. Murine retroviruses activate B cells via interaction with Toll-like receptor 4. *Proc. Natl. Acad. Sci. USA 99:2281.*
75. Outzen, H. C., D. Corrow, and L. D. Shultz. 1985. Attenuation of exogenous murine mammary tumor virus virulence in the C3H/HeJ mouse sub-strain bearing the Lps mutation. *J. Natl. Cancer Inst. 75:917.*