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Differential Effect of Anti-B7-1 and Anti-M150 Antibodies in Restricting the Delivery of Costimulatory Signals from B Cells and Macrophages

Javed N. Agrewala,^{1*} Susmit Suvas,* Rakesh K. Verma,* and Gyan C. Mishra[†]

B7-1 and M150 are potent costimulatory molecules expressed on B cells and macrophages. We have examined the capacity of Abs against B7-1 and M150 in differentially inhibiting the costimulatory signals delivered by macrophages and B cells to OVA-specific CD4⁺ T cells. The anti-B7-1 Ab significantly blocked the proliferation of Th cells, MLR, T cell help to B cells, and secretion of IFN- γ when B cells were used to provide costimulation, but not when macrophages were used. In contrast, anti-M150 Ab significantly decreased the proliferation of Th cells, MLR, and production of IFN- γ , when macrophages were utilized to provide costimulatory signals, but not when B cells were used as APC. However, when macrophages activated with IFN- γ were used as a source of costimulation, like anti-M150 Ab, Ab to B7-1 also down-regulated the activation of Th cells. The significance of this finding is that M150 is a potent first costimulatory signal for initiating proliferation and secretion of IFN- γ and providing cognate help for B cells by Th cells when the macrophage is used as an accessory cell. M150-induced IFN- γ production induces the expression of B7-1 on the surface of macrophages, which then delivers a second cosignal for Th cells. B7-1 works efficiently when B cell provides cosignal. Both of the molecules promote Th1 activity, as evidenced by the inhibition of the secretion of IFN- γ but not IL-4 by Th cells with anti-M150 and B7-1 Abs. *The Journal of Immunology*, 1998, 160: 1067–1077.

The optimum activation of Th cells requires not only TCR occupancy by presented MHC-Ag complex, but also a set of other costimulatory signals provided by APC (1–8). On the basis of lymphokine profile Th cells have been divided into Th1 and Th2 subtypes. Th1 cells secrete IL-2, IFN- γ , lymphotoxin, etc., and are mainly involved in the generation of cell-mediated immunity (CMI)² responses. Th2 cells secrete IL-4, IL-5, IL-6, etc., and are generally involved in humoral immunity (9–11). Both subsets recognize foreign Ags in association with MHC class II molecules. It appears that these two distinct Th cells are not only functionally different but also require discreet costimulatory signals for their optimum activation (12, 13). Although, the controversy still exists, it seems that B7-1 predominantly activates Th1 cells (14–16). Its other isoform, B7-2, stimulates Th2 cells (17–18).

B7-1 plays a major role in providing costimulation to T cells, leading to their proliferation, cytokine production, and development of effector functions. The fact that, in addition to B7-1, APCs are endowed with a large number of costimulatory molecules (19–25), raises a possibility of existence of multimolecular pathways for T cell activation and their clonal amplification (26, 27). The strongest evidence to date for the existence of non-B7-mediated costimulation has been reported from studies with CD28-deficient (CD28^{-/-}) mice (28, 29). Moreover, the resting macrophages do

not express B7-1. However, the expression of B7-1 is induced on the macrophages on activation with IFN- γ or LPS (30–32).

In our previous study, we provided evidence that a 150 kDa (M150) molecule associated with the membrane of macrophage activates Th cells leading to their proliferation and release of lymphokines representative of Th1 subtype (7). In the present study, we have compared the costimulatory activity of B7-1 and M150. By using Abs against B7-1 and M150, we have demonstrated that B cells activated with LPS primarily employ B7-1 and macrophages utilize predominantly M150 to costimulate Th cells to proliferate and secrete IFN- γ .

Materials and Methods

Animals

Female inbred BALB/c, C3He, and C57BL/6 mice, 8 to 10 wk old, were obtained from the Institute's Animals House Facility.

Ags, Abs, lymphokines, and reagents

OVA, phosphatidylcholine (PC), cholesterol, penicillin, gangliosides, and rabbit anti-hamster (Ham) Abs were purchased from Sigma Chemical Co. (St. Louis, MO). FCS was from Sera Lab (Crawley Down, U.K.); RPMI 1640 was purchased from Life Technologies (Grand Island, NY); and L-glutamine and streptomycin were from Serva (Heidelberg, Germany); rIL-4, rIFN- γ , rIL-12, and anti-IL-12 Abs were the products of Genzyme (Boston, MA); whereas Abs to IL-4 and IFN- γ were obtained from Texstar and Holland Biotechnology (Leiden, The Netherlands), respectively. Anti-IL-2 and anti-IL-2R Abs were used as culture supernatants (SN) from TIB 222 (PC 61.53), CRL 1698 (7D4), and HB 8794 (S4B6). Anti-B7-1 mAb was purchased from PharMingen (San Diego, CA); rabbit anti-mouse FITC-labeled Ab was procured from the Binding Site, Birmingham, U.K.

Cell lines and hybridomas

The cell lines and hybridomas used in this study, HT-2 (CRL-1841), TIB-222, CRL-1698, CRL 1878, TIB 217, and HB 8794, were procured from American Type Culture Collection (Rockville, MD). WEHI-279 and TIB 183 were gifts from Dr. S. Rath, National Institute of Immunology, New Delhi, India and 145.2C11 was from Prof. C. A. Janeway, Jr. (Yale University, New Haven, CT).

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² Abbreviations used in this paper: CMI, cell mediated immunity; PC, phosphatidylcholine; PEC, peritoneal exudate cells; BSS, balanced salt solution; SN, supernatant; Ham, hamster; Rt, rat; MFI, mean fluorescence intensity; M*, macrophages activated with IFN- γ .

Medium

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (50 $\mu\text{g/ml}$), streptomycin (50 $\mu\text{g/ml}$), and 2-ME (0.05 mM).

Anti-M150 Abs

Before immunization, the purity of each batch of M150 was established by two-dimensional gel electrophoresis as mentioned earlier (7). The isolated M150 contained only a single protein without any detectable contamination, demonstrated by the fact that in two-dimensional gel electrophoresis M150 appeared as a single spot (7). Purified M150 (100 μg) was emulsified in CFA. Syrian hamsters were immunized s.c. Later, 3 to 4 boosters of M150 (50 μg) emulsified in IFA were given after intervals of 21 days. The animals were bled for Ab when the titers were positive by ELISA and dot blot.

Purification of anti-M150 on protein A-Sepharose column

Protein A-Sepharose column was equilibrated with 10 mM Tris-HCl buffer, pH 8.0. Ham Ig obtained from precipitation of serum by ammonium sulfate was passed through the column. The column was washed extensively with 100 mM Tris-HCl buffer. The bound Ab was eluted with 100 mM glycine-HCl buffer, pH 3.0, and collected in tubes containing 1 M Tris-HCl to neutralize the pH.

Isolation and reconstitution of M150

The peritoneal exudate cells (PEC) were harvested from BALB/c mice inoculated 4 days previously with 2 to 3 ml of thioglycollate. The PEC were washed with cold balanced salt solution (BSS). The macrophages were obtained by adhering for 1 h at 37°C on plastic petri dishes, followed by washing at least three times in cold BSS and freezing overnight at -37°C. Membrane proteins were isolated as described elsewhere (33). Briefly, the cells were thawed and homogenized in the presence of 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA (pH 7.4) along with protease inhibitor cocktail. The cells debris was removed by spinning at 600 $\times g$ for 15 min at 4°C. The SN obtained was centrifuged at 110,000 $\times g$ for 2 h. The pellet was solubilized in 1% Triton X-100, 20% glycerol, 10 mM Tris-HCl (pH 7.5), and agitated overnight at 4°C. The insoluble material was removed by centrifugation at 100,000 $\times g$ for 1 h at 4°C. The proteins from SN were separated by SDS-PAGE (34) using 10% polyacrylamide gel.

After electrophoresis, M150 was located by staining a strip of the gel with Coomassie blue, and the appropriate unstained regions were crushed and eluted with 0.1% SDS, 0.05 M Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.15 M NaCl at 37°C for 24 h (15). After filtration and centrifugation to remove polyacrylamide particles. The protein content was estimated essentially according to Bradford (35). The SDS was removed from the eluted M150 by Extracti D gel column (Pierce, Rockford, IL). The column was equilibrated with 0.05 M Tris-buffer (pH 9.0). Before loading M150, 1 mg/ml BSA in elution buffer was passed through the column and then thoroughly washed to remove it. The M150 was loaded on the column and eluted. Positive fractions obtained were pooled and the protein content was estimated.

Reconstitution of M150 into lipid bilayers

Liposomal vesicles were prepared by dissolving L- α -PC (Sigma) in a 1:1 ratio of chloroform and methanol and evaporated under N₂ gas while rotating the vial to deposit a thin film. The lipids were dried under vacuum for 2 h and were then dissolved in 10 mM Tris-HCl, (pH 8.0), 0.5% Lubrol Px, 140 mM NaCl, and 0.1 mM EDTA. This was followed by sonication at 4°C in a bath-type sonicator for 30 min. A 500:1 ratio of vesicle preparation and M150 protein, isolated from SDS-PAGE, or control OVA, were mixed and vortexed properly (36). This mixture was then dialyzed at 4°C for 4 days against 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 0.1 mM EDTA with at least eight changes of buffer. The contents of the dialyzing bag were centrifuged for 2 h at 4°C at 178,000 $\times g$. The SN was discarded and the pellet was dissolved in 0.9% NaCl. The unbound protein was eliminated by passing the solution through Sephadex-G10 mini-columns. A 2-ml sample was layered on the top of the discontinuous gradient of 5 to 40% sucrose in 10 mM Tris-HCl (pH 6.8), 0.15 M NaCl, and 0.1 mM EDTA. The sample was centrifuged at 9800 $\times g$ overnight at 4°C. The 2-ml samples were collected from the 10% interface layer and washed in 0.9% NaCl at 178,000 $\times g$ for 2 h. The pellet was dissolved in 0.9% NaCl, passed initially through both 0.45- μm and 0.22- μm sterile filters, and stored at -20°C until further use. Similarly, all the 21 proteins of macrophage membrane visualized by Coomassie blue staining on SDS-PAGE were also purified.

Western blotting

Samples containing macrophage membrane were transferred from SDS-PAGE on nitrocellulose membrane. Electrophoretic transfer of proteins was carried out as described by Towbin et al. (37) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11.0) at 200 mA for 2 h at 4°C and stained in Ponceau S to ensure that the proteins were transferred. After blocking overnight in PBS (pH 7.4) containing 3% skimmed milk, the blots were exposed for 1 h at 37°C to hamster (Ham) anti-M150 Ab and control Ham Ig (1:10 dilution) with gentle rocking. The blots were then incubated with rabbit anti-Ham IgG Ab (1:1000 dilution) and later on with anti-rabbit IgG-peroxidase-labeled Ab (1:15,000). The bound Ab was visualized using substrate diaminobenzidine prepared in 50 mM Tris (pH 7.6) containing 0.01% H₂O₂. The usual steps of washings using PBS-Tween-20 were maintained after every incubation. The macrophage membrane lysate and m.w. markers were also run simultaneously in appropriate lanes and were stained with Coomassie blue.

Cell preparation

Ag-specific Th cells. Ag-specific Th cells were prepared as described earlier (38). OVA-specific Th cells were obtained from mice immunized s.c. in the footpad with 100 μg of Ag, emulsified in CFA. After 7 days, inguinal and para-aortic lymph nodes were removed and a single-cell suspension was cultured in 24-well plates. Th cells (5 $\times 10^5$ /well) were cultured with 6 $\times 10^6$ mitomycin C (50 $\mu\text{g/ml}$)-treated feeder cells in the presence of 1.5 ml RPMI 1640 containing 10% FCS and 100 μg OVA, in 7% CO₂ atmosphere at 37°C. On the fourth day, the cells were harvested and centrifuged on Ficoll-Hypaque at 22°C for 20 min at 1170 $\times g$. The cells at the interface were collected and recultured with syngeneic spleen cells treated with mitomycin C, as described earlier. This cycle of activation and resting was repeated at least three or four times before the cells were utilized for the experiments. The cells rested for 10 to 14 days were used in the study. These cells were of CD4⁺ Th phenotype, as revealed by FACS analysis, and produced IL-4, IFN- γ , and IL-2, and responded to the Ag OVA in a dose-dependent manner (data not shown).

B cells. A single-cell suspension of mice spleens was prepared in balanced salt solution (BSS). The RBCs were depleted by treatment with hemolytic Gey's solution. The adherent cells were removed by plating on the plastic petri plates (Nunc, Roskilde, Denmark) for 2 h at 37°C and 7% CO₂. The nonadherent cells were treated sequentially on ice for 45 min, each with a mixture of anti-Mac2 and 3 Abs, and a mixture containing anti-Thy1, anti-L3T4, and anti-CD8 Abs followed by complement-mediated killing. The cells were then incubated at a concentration of 4 $\times 10^7$ /10 ml/Petriplate with 10 $\mu\text{g/ml}$ LPS (from *Salmonella typhosa*) (Sigma) for 48 h at 37°C and 7% CO₂. The purity of cells stained with anti-IgM Abs by indirect immunofluorescence was over 85% as analyzed by FACS (Becton Dickinson, Mountain View, CA).

Macrophages. The PEC were harvested from BALB/c mice inoculated 4 days previously with 2 to 3 ml of thioglycollate. The cells were washed with cold BSS. The macrophages were obtained by adhering for 1 h at 37°C on plastic petri dishes, followed by washing several times in cold BSS. The purity of cells was >98%.

Biotinylation of anti-M150 Abs

Anti-M150 Ab was dialyzed against 0.1 M NaHCO₃ for 4 h. NHS-biotin (Pierce) (1 mg/ml) was dissolved in DMSO and added to proteins and mixed for 4 h at room temperature and then dialyzed against PBS containing 0.02% NaN₃.

Formation of complexes with M150

M150 (0.1 $\mu\text{g/ml}$) was incubated to form complexes with different concentrations (0.0001–10 $\mu\text{g/ml}$) of anti-M150 Ab, anti-IL-12 Ab, Ham Ig, and rat (Rt) Ig at 37°C for 1 h. Similarly, the complexes of IL-12 (10 pg/ml) were made using anti-IL-12 and anti-M150 Abs. These complexes were used in cultures to monitor the proliferation and secretion of IFN- γ by Th cells.

Expression of B7-1 and M150 on macrophages and B cells

One million thioglycollate-stimulated macrophages were incubated with rIFN- γ (100 U/ml) for 24 h at 37°C/7% CO₂. Both IFN- γ activated and nonactivated macrophages and LPS-activated B cells were incubated with the biotinylated anti-M150 (1:100 dilution) and anti-B7-1 Abs (1 $\mu\text{g}/100 \mu\text{l}$) diluted in PBS-FCS 2% for 1 h at 4°C. The cells were then washed three times with PBS-1% FCS. Streptavidin-FITC (1:10,000 dilution) was added and the cells were further incubated for 1 h at 4°C. The cells were then washed five times with PBS and fixed in 1% paraformaldehyde and analyzed by FACS for the expression of M150 and B7-1. As a control for

anti-M150 and anti-B7 Abs, the cells were also incubated with Ham Ig and Rt Ig.

The cells from each suspension were acquired on Lysis II software of FACScan (Becton Dickinson). Debris in the cell suspension was excluded from the analysis by suitable gating that allowed the collection of data only from those light-scattering events (i.e., cells) of a size consistent with macrophages or B cells. The analysis for the mean fluorescence intensity (MFI) was done on histograms in which the abscissa and the ordinate denote log FITC fluorescence and relative cell count, respectively.

The proliferation of Th cells

Inhibition of the proliferation of Th cells by anti-B7-1 and anti-M150 Abs when B cells and macrophages were used to deliver costimulatory signals. The 96-W microtiter plates (Costar, Cambridge, MA) were coated overnight at 4°C with 50 μ l of 10 μ g/ml of anti-CD3 Ab in carbonate-bicarbonate buffer, pH 9.2. The wells were washed three times with BSS. The OVA-specific CD4⁺ Th cells rested for 10 to 14 days and were used in the assays after separating the splenic feeder cells on Ficoll-Hypaque gradient. The LPS-activated B cells (1×10^5 /well) or thioglycollate-stimulated macrophages (1×10^5 /well) or macrophages activated for 24 h with IFN- γ (100 U/ml) were incubated with anti-B7-1 and M150 Abs for 1 h at 4°C. APCs preincubated with the Abs were then cultured with anti-CD3 Ab-stimulated Th cells (2×10^4 /well). Anti-M150 and B7-1 Abs were also present throughout culture conditions. In control wells, anti-M150 and anti-B7-1 Abs and control Ham and Rt Igs were also added to the cultures in which Th cells were cultured with anti-CD3 Ab only. The cultures were also set when Th cells were incubated with M150 without anti-CD3 Ab. Th cells incubated with only anti-CD3 Ab or in the presence of PMA (10 ng/ml); B cells unstimulated with LPS were also taken as controls. The cultures were incubated at 37°C in a humidified atmosphere containing 7% CO₂. After 48 h, the cultures were pulsed with 1 μ Ci of [³H]thymidine. After 16 h, the plates were harvested and incorporation of thymidine was determined by using an automatic cell harvester (Skatron, Tranby, Norway) and liquid scintillation counting.

The proliferation and secretion of IFN- γ by Th cells stimulated with M150 and IL-12 complexes. Anti-CD3 Ab-stimulated Th cells were incubated with M150 (0.01 μ g/ml) complexed with different concentrations (0.00001–10 μ g/ml) of anti-M150 Ab, anti-IL-12 Ab, Ham Ig, and Rt Ig. Similarly, Th cells were also cultured with the complexes of IL-12 (10 pg/ml) prepared with different concentrations of anti-IL-12 and anti-M150 Abs. The control cultures were also set where anti-CD3 Ab-activated Th cells were either incubated with medium only or control liposomes or OVA entrapped in liposomes. The cultures were incubated at 37°C in a humidified atmosphere containing 7% CO₂. After 48 h, the culture SNs were collected from the experimental and control wells to monitor IFN- γ . The cultures were pulsed 72 h later with 1 μ Ci of [³H]thymidine. After 16 h, the plates were harvested and incorporation of thymidine was determined using an automatic cell harvester (Skatron) and liquid scintillation counting.

The proliferation of Th cells using different proteins isolated from the membrane of macrophage. Twenty-one proteins were isolated from the SDS-PAGE and were tested for their ability to proliferate anti-CD3 Ab-activated Th cells. The culture conditions were the same as mentioned in the case of the proliferation of Th cells. Th cells were incubated with the isolated proteins (0.01 μ g/ml) and anti-M150 Ab (10 μ g/ml). The control cultures were also set where anti-CD3 Ab-stimulated Th cells were incubated either with control Ham Ig or with anti-M150 Ab. The cultures were incubated at 37°C in a humidified atmosphere containing 7% CO₂. After 72 h, the cultures were pulsed with 1 μ Ci of [³H]thymidine. After 16 h, the plates were harvested and incorporation of thymidine was determined by using an automatic cell harvester (Skatron) and liquid scintillation counting.

Lymphokines assays

The cultures were set as mentioned in Th cell proliferation using macrophages and B cells as a source of costimulation. The culture SN from the experimental and control wells were collected after 24 to 48 h and IL-4 was measured by its ability to induce the proliferation of HT-2 cells as described earlier (39). Briefly, 1×10^4 /well of HT-2 cells were cultured in 96-well plates containing medium and various concentrations of culture SN obtained from the control and experimental wells. For the selective inhibition of IL-2, Abs to IL-2 and its receptor (culture SN in 1:12 dilution of CRL 1698, TIB 222, and HB 8794) were used. The cells were incubated for 16 h at 37°C, pulsed with 1 μ Ci of [³H]thymidine, and harvested 8 h later. The [³H]thymidine incorporation was measured by liquid scintillation spectrometry. IFN- γ was assayed by its ability to inhibit the proliferation of WEHI-279 cells (40). WEHI-279 cells were cultured in 96-well plates at a density of 1×10^5 cells/ml with different dilutions of cultured SNs

harvested from control and experimental wells. [³H]Thymidine (1 μ Ci/well) was added and, after 24 h of incubation, the cells were harvested and counted 6 h later. For the specificity of the lymphokines, the inhibitory activity of IFN- γ and IL-4 was neutralized with anti-IFN- γ (4 μ g/ml) and 11B11 (1 μ g/ml) Abs, respectively. The lymphokine data were calculated from the mean cpm of triplicate determinations and expressed as picograms per milliliter in the culture SN as computed by comparison with the standard curve plotted using rIL-4 and rIFN- γ (Genzyme).

Mixed lymphocyte reaction

Thioglycollate-stimulated macrophages and LPS-activated B cells prepared from C57BL/6 mice were used as stimulator cells. These cells were treated with mitomycin C and were cultured in 1:1 ratio with 10^6 spleen cells of BALB/c mice in 3 ml complete medium. On day 4, T cell blasts were isolated by the Ficoll-density gradient method. The cultures for Th cell proliferation were set using different concentrations (1×10^3 – 10^5 cells/well) of stimulator cells (macrophages untreated or treated with IFN- γ and LPS-activated B cells) and T cells (2×10^4 /well) in the presence or absence of anti-M150 and B7-1 Abs. The cultures were also set using Igs of Ham and Rt as controls for anti-M150 and anti-B7-1 Abs. On day 5 of MLR cultures, [³H]thymidine was added and the cultures were harvested after 16 h and assayed for DNA synthesis by beta scintillation counting.

B cell proliferation

As described above, anti-CD3 Ab-stimulated and mitomycin C-treated Th cells (2×10^4 /well) were cultured with LPS-activated B cells (1×10^5 /well) either in the presence of M150 (0.01 μ g/ml) or Abs to M150 and B7-1 molecules at a concentration of 1 μ g/ml in a total volume of 200 μ l of RPMI 1640-FCS 10%. The cells were incubated for 48 h, pulsed with 1 μ Ci [³H]thymidine for a further 16 h, and then processed for beta scintillation counting. The suitable controls containing anti-CD3 Ab-stimulated Th cells incubated with control liposomes, Igs of Ham and Rt, or without Abs were also kept.

IgG1 and IgG2a isotypes

Cultures were set as mentioned in B cell proliferation assays. Supernatants were collected on day 6 from experimental as well as control wells and were analyzed for IgG1 and IgG2a by ELISA. Briefly, triplicate wells were coated overnight at 4°C with 5 μ g/ml of rabbit anti-mouse IgG in carbonate-bicarbonate buffer, pH 9.6. The unbound sites were blocked with 2% BSA and then log₁₀ dilutions of culture supernatants were added for 2 h at 37°C. IgG1 and IgG2a were detected using biotinylated goat anti-mouse IgG1 and IgG2a Ab, respectively. After incubating the plates at 37°C for 2 h, streptavidin-horseradish peroxidase was added. The usual steps of washings using PBS-Tween-20 were carried out at each step. Color developed due to the substrate OPD (orthophenylene diamine-2HCl) was visualized at 492 nm. The results are represented as the OD of a single dilution (1:100) of mean absorbance of triplicate wells after subtracting the absorbance obtained with control wells (i.e., SN obtained from LPS-stimulated B cells).

MHC restriction assay

Cultures were kept as mentioned in the case of the proliferation of Th cells. OVA-specific Th cells (2×10^4 /well) generated from BALB/c, C57BL/6, and C3He mice were stimulated with plate-bound anti-CD3 Ab and M150 derived from BALB/c mice. In control cultures, Th cells were incubated with M150 only or with anti-CD3 Ab and medium alone. After 72 h, 1 μ Ci/well of [³H]thymidine was added. The cultures were harvested after 16 h using an automatic cell harvester and beta emission were counted using liquid scintillation counting.

Results

Anti-M150 Ab recognizes M150 on Western blots

The Western blot analysis has revealed that the membrane of macrophages probed with anti-M150 Ab could specifically identify M150 only. Anti-IL-12 Ab, however, failed to recognize M150 (data not shown). The control Ham Ig also could not detect M150 (Fig. 1).

Expression of M150 and B7-1

The macrophages before and after treatment with IFN- γ were tested for the expression of M150 and B7-1 by FACS. LPS-activated B cells were also analyzed for the expression of M150. It

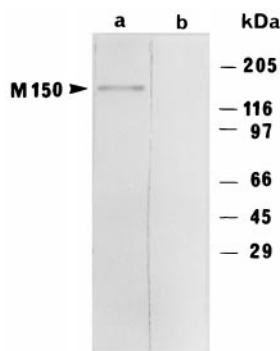


FIGURE 1. Anti-M150 Ab recognizes only M150 but not other macrophage membrane proteins. Western blots show membrane of macrophages probed with anti-M150 Ab (lane a) and control Ham Ig (lane b). The molecular masses indicated in kDa were estimated from standards electrophoresed in a parallel lane.

was observed that macrophages expressed a higher level of M150 (Fig. 2A) as compared with the B cells (Fig. 2C). In contrast, the expression of B7-1 was not observed on the macrophages (Fig. 2B). However, macrophages incubated with IFN- γ exhibited significantly higher expression of M150 as well as B7-1 (Fig. 2, A and B). M150 was detected on resident macrophages as well, but not on unactivated B cells (data not shown). The control Igs of Ham and Rt failed to show any shift in MFI in FACScan.

Anti-M150 and anti-B7-1 Abs neutralize costimulatory signals delivered by macrophages and B cells, respectively

In our earlier studies, we have reported that M150 costimulates Th cells resulting in their proliferation and lymphokine secretion (7). In the present study, we raised Ab against this molecule and analyzed the ability of this Ab to inhibit M150-induced proliferation of Th cells. Our study revealed that, compared with B cells, macrophages display a higher level of M150. Other workers have reported that unlike macrophages, B cells express a significant level

of B7-1. Therefore we next performed experiments to determine if there was selective utilization of costimulatory molecules by macrophages and B cells. Our assay system consisted of Th cells activated with anti-CD3 Ab and costimulated with either macrophages or LPS-activated B cells. A profound decline (82.70%) in the growth of Th cells was found in the cultures in which anti-M150 Ab was used to inhibit the costimulatory signals delivered by the macrophages (Fig. 3A). In a similar condition, the proliferative response of Th cells remained grossly unchanged when anti-B7-1 Ab was used. Like anti-M150 Ab, anti-B7-1 Abs could also significantly obstruct the proliferation of Th cells costimulated with macrophages activated with IFN- γ (or SN generated by culturing anti-CD3 Ab-stimulated Th cells with M150) (data not shown). It may be pointed out that it has been reported earlier that IFN- γ induces the expression of B7 on macrophages (30, 31). In contrast, when B cells were used as a source to provide costimulatory activity to Th cells, anti-B7-1 Ab significantly inhibited (54.38%) the proliferation of Th cells. Abs against M150, however, could only partially check the growth of Th cells (Fig. 3B). The Abs used in the study worked in a dose-dependent manner. To ascertain the specificity of anti-M150 Ab, Th cells were also costimulated with anti-CD3Ab and PMA. PMA-activated Th cell proliferation ($123,966 \pm 14,247$ cpm) could not be blocked with anti-M150 Ab ($142,031 \pm 6,539$ cpm). Furthermore, M150 could not induce the proliferation of PHA-activated human lymphoblasts (data not shown). The control cultures containing anti-CD3 activated Th cells incubated either with Ham Ig or Rt Ig, and M150 without anti-CD3 Ab did not show any effect on Th cell activity.

Inhibition of the secretion of IFN- γ by anti-M150 and anti-B7-1 Abs by Th cells, when macrophages and B cells were used as a source to deliver cosignals

Our experiments demonstrate that anti-M150 and anti-B7-1 Abs selectively blocked the costimulatory activity of macrophages and B cells. Literature also suggests that M150 and B7-1 molecules possibly participate primarily in the activation of Th-1 like cells (7, 14). Keeping these points in view, we next performed experiments

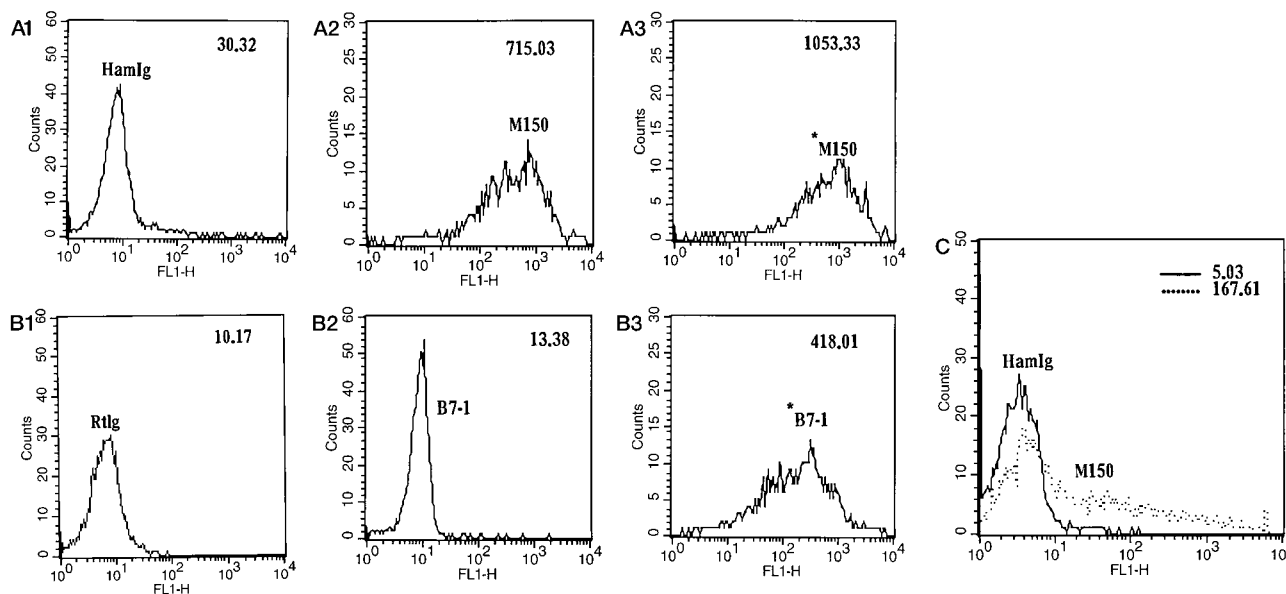


FIGURE 2. Expression of M150 and B7-1. Thioglycollate-stimulated peritoneal macrophages before and after IFN- γ treatment were stained with anti-M150 Ab (A), and anti-B7-1 Ab (B). The LPS-activated B cells were also stained with anti-M150 Ab (C) and analyzed by FACS. Igs of Ham and Rt were used as controls for anti-M150 and anti-B7-1 Abs. The asterisk (*) indicates macrophages activated with IFN- γ . The data shown in the figures represent MFI.

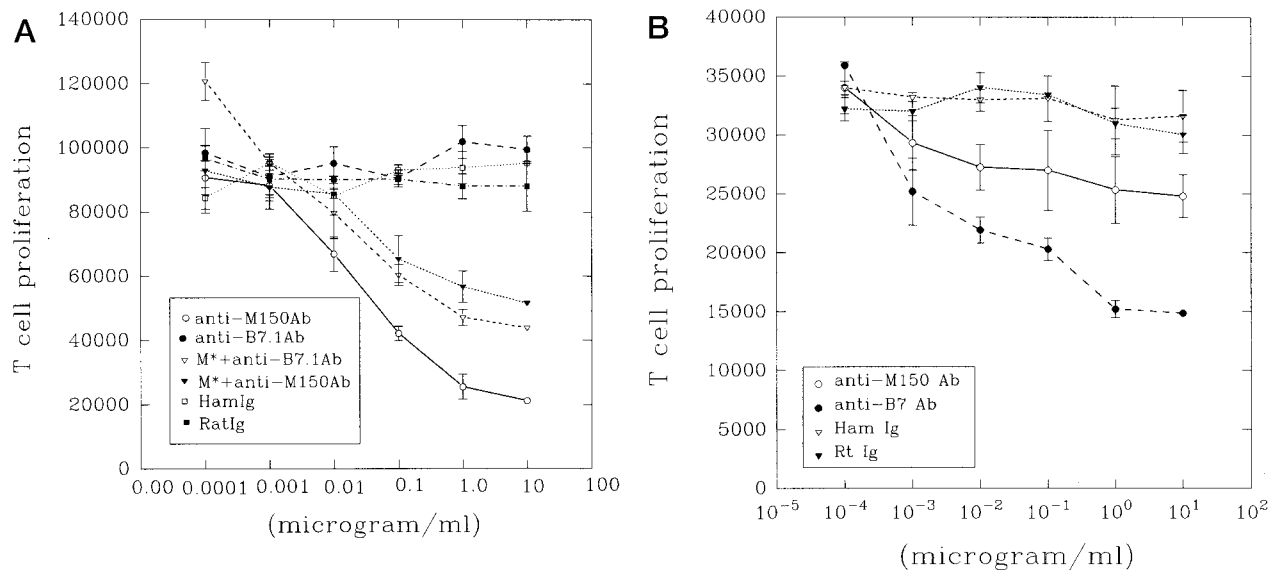


FIGURE 3. Differential capacity of anti-M150 and B7-1 Abs to block the costimulatory signals delivered by macrophages and B cells. *A*, The macrophages were incubated with different concentrations of anti-M150 and anti-B7-1 Abs at 4°C for 1 h and then cultured with Th cells stimulated with anti-CD3 Ab. The macrophages activated with IFN- γ (M*) were also used for costimulation. Anti-M150 and anti-B7-1 Abs were also present throughout the culture period. The proliferation was monitored by [³H]thymidine incorporation. As a control, anti-CD3 Ab-activated Th cells cultured either with thioglycollate-activated macrophages or M* showed $122,353 \pm 9,862$ and $82,937 \pm 3,352$ cpm, respectively. Anti-CD3 Ab-stimulated Th cells cultured with PMA and with or without anti-M150 Ab showed $142,031 \pm 6,539$ and $123,966 \pm 14,247$ cpm, respectively. Th cells cultured with anti-CD3 Ab generated <5000 cpm. Th cells cultured either with medium alone or macrophages without anti-CD3 Ab could not generate more than 2000 cpm. *B*, The B cells were cultured as mentioned in the legend to *A*. As a control, anti-CD3 Ab-activated Th cells cultured either with medium alone or with LPS-activated B cells showed $5,017 \pm 1,372$ and $32,568 \pm 3,715$ cpm, respectively. Th cells cultured with either anti-B7-1 or anti-M150 Abs or cultured with medium alone or with B cells in the absence of anti-CD3 Ab could not generate more than 2000 cpm. The Igs of Ham and Rt used as controls for anti-M150 and anti-B7-1 Abs, respectively, did not block the Th cell response. The data are the mean \pm SD of duplicate experiments.

to analyze the secretion of IFN- γ and IL-4 by Th cells costimulated by macrophages or B cells in the presence or absence of anti-M150 and B7-1 Abs. Interestingly, anti-M150 Ab could abrogate the secretion of IFN- γ by 97.28% when macrophages were used as APC but not when B cells were utilized to generate costimulatory signals. Anti-B7-1 Ab could only decrease the production of IFN- γ by 1.39%. In comparison, when macrophages stimulated with IFN- γ were used, the production of IFN- γ was inhibited by 46.25% and 88.72% by anti-B7-1 and anti-M150 Abs, respectively (Fig. 4A). In contrast, anti-B7-1 Ab nearly blocked (97.45%) the production of IFN- γ when B cells were used to costimulate Th cells. Anti-B7-2 Ab, however, inhibited the secretion of both IFN- γ and IL-4 when either macrophages or B cells were used as APC (data not shown). Blocking with both anti-M150 and anti-B7-1 Abs, however, enhanced the secretion of IL-4 by Th cells, costimulated with macrophages and B cells, respectively (Fig. 4B). The control cultures consisting of Igs of either Ham or Rt could not influence the secretion of lymphokines. The specificity of assays for monitoring IFN- γ and IL-4 was ensured by neutralizing the activity of these cytokines by their respective Abs.

Anti-M150 and anti-B7-1 Abs blocks the signals delivered by macrophages and B cells in MLR

Another interesting feature we observed during the study was that anti-M150 Ab inhibited MLR by 69.89% when macrophages were used as stimulator cells (Fig. 5). Similarly, anti-B7-1 Ab blocked MLR by 72.66% when B cells were used as stimulator cells. Furthermore, Abs against M150 and B7-1 molecules did not significantly block the alloreactivity of B cells and macrophages, respectively. However, as the observation mentioned in Figure 3A, when macrophages activated with IFN- γ were used as allostimulators, anti-B7-1 Ab could also significantly inhibit (62.23%) the allo-

stimulation. The proliferation of Th cells remained grossly unchanged when control Igs of Ham and Rt were used.

Anti-B7-1 Ab, but not anti-M150 Ab, could inhibit the proliferation of B cells and the secretion of IgG2a Abs

Since it has been reported in the literature that LPS-activated B cells express B7-1 and since our FACSscan data also suggest the expression of M150 molecules, we therefore next determined whether blocking of these molecules would effect the proliferation and differentiation of B cells. For this, LPS-activated B cells were cultured with mitomycin C-treated and anti-CD3 Ab-activated Th cells. It was found that in such a culture system there was a significant proliferation of B cells. This proliferation was not inhibited by the addition of anti-M150 Ab (Fig. 6A). In contrast, anti-B7-1 Ab showed significant inhibition (75.86%) in the proliferation of B cells. We also evaluated the capacity acquired by anti-CD3 Ab- and M150-stimulated Th cells to provide help to B cells. It was observed that Th cells stimulated with M150 induced significant increase in the proliferation of B cells ($78,235 \pm 8,018$ cpm) as compared with the Th cells cultured without M150 ($22,304 \pm 6,032$ cpm). No proliferation of B cells was noticed in the control cultures containing Ham Ig, Rt Ig and liposomes incubated with Th cells.

We next measured the yield of IgG1 and IgG2a isotype during the coculture of T and B cells. We found that LPS activated B cells, when cultured with anti-CD3 Ab-stimulated Th cells, secreted both IgG1 and IgG2a isotypes. The secretion of IgG2a was only inhibited in the cultures when T and B cells were incubated with anti-B7-1 Ab. Anti-M150 Abs did not show any noticeable effect in the secretion of either IgG1 or IgG2a isotypes (Fig. 6B). Interestingly, when anti-CD3 Ab-stimulated Th cells were incubated with M150, there was augmentation in the secretion of

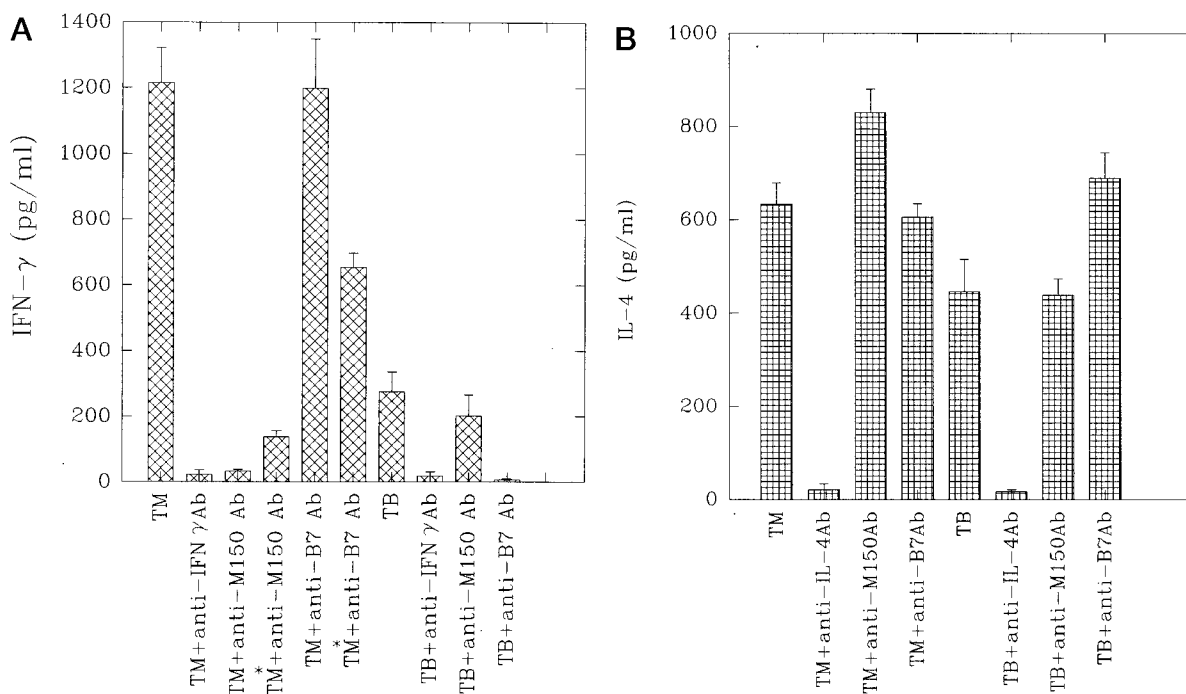


FIGURE 4. Anti-M150 and B7-1 Abs inhibits the secretion of IFN- γ but not of IL-4 when macrophages and B cells provide costimulus to Th cells. The cultures were set as described in the legend to Figure 3, A and B. The culture SN from the experimental and control wells were collected after 24 to 48 h for the estimation of IL-4 and IFN- γ , respectively. M* indicates macrophages activated with IFN- γ . A, IFN- γ was assayed by its ability to inhibit the proliferation of WEHI-279 cells. The specificity of the inhibition induced by IFN- γ was further established by the restoration of proliferation of WEHI-279 cells with anti-IFN- γ Abs (4 μ g/ml). B, IL-4 was measured by its ability to induce the proliferation of HT-2 cells as described in *Materials and Methods*. The specificity of IL-4 was established by blocking the proliferation of HT-2 cells by anti-IL-4 Ab (11B11, 1 μ g/ml). After 24 h of incubation, [3 H]thymidine (1 μ Ci/well) was added and the cells were harvested and radioactivity incorporated was counted 6 h later. Ham Ig and Rt Ig used as controls could not induce the secretion of lymphokines. All the data were calculated from the mean cpm of triplicate determinations.

IgG2a Abs. The control Igs of Ham and Rt used in the cultures could not influence any change in B cell activity.

Anti-M150 Ab, but not anti-IL-12 Ab, inhibits the proliferation of Th cells mediated by M150

We next monitored the specificity of the proliferation of Th cells induced by M150 by blocking its activity with anti-M150 Ab. M150 was complexed with different concentrations of anti-M150 and anti-IL-12 Abs. M150-induced proliferation of anti-CD3 Ab-stimulated Th cells was inhibited specifically with anti-M150 Ab but not with anti-IL-12 Ab (Fig. 7A). Ten micrograms of anti-M150 Ab complexed with 0.01 μ g/ml of M150 neutralized 90% of the M150-induced proliferation of Th cells. Anti-IL-12 Ab did not have any impact on the inhibition of the proliferation of Th cells. In contrast, anti-IL-12 Ab but not anti-M150 Ab, however, could efficiently block the proliferation of Th cells activated with anti-CD3 Ab in the presence of rIL-12. The Th cell activity was not affected by the control liposomes or OVA-entrapped liposomes. The Ham Ig and Rt Ig could not induce any change in M150-mediated proliferation of Th cells. Furthermore, M150 failed to induce the growth of Th cells in the absence of anti-CD3 Ab. Th cells activated with anti-CD3 Ab showed less than 5000 cpm.

Anti-M150 Ab, but not anti-IL-12 Ab, inhibits the secretion of IFN- γ by Th cells stimulated with M150

We also evaluated the role of anti-M150 and anti-IL-12 Abs in inhibiting the secretion of IFN- γ by Th cells activated either with M150 or rIL-12 (Fig. 7B). M150-induced secretion of IFN- γ was inhibited by anti-M150 Ab, but not with anti-IL-12 Ab. Similarly, IL-12-induced production of IFN- γ was blocked by anti-IL-12 Ab,

but not with anti-M150 Ab. The control cultures kept as mentioned in Figure 7A did not show detectable levels of IFN- γ .

M150, but not other macrophage membrane proteins, induce the proliferation of Th cells

We did more experiments to establish the specificity of anti-M150 Ab. The SDS-PAGE analysis of the membrane of macrophage revealed 21 major bands when stained with Coomassie blue (7). All the proteins were isolated and tested for their ability to stimulate anti-CD3 Ab-incubated Th cells (Fig. 8). Only M150 induced a significant level of Th cell proliferation. The 59-, 75-, and 85-kDa proteins could induce only minor change in the proliferation of Th cells (<7100 cpm). The rest of the tested macrophage membrane proteins failed to stimulate Th cells (<5000 cpm). Anti-M150 Ab could block the proliferation mediated by M150 only, but not by other macrophage membrane proteins (Fig. 8). The control cultures containing Th cells and medium or anti-CD3 Ab-stimulated Th cells incubated either with medium alone or Ham Ig induced proliferation less than 5000 cpm.

M150 is not an alloantigen but a costimulatory molecule and works in an MHC-nonrestricted manner

OVA-specific Th cell lines were generated from BALB/c (IA^d), C57BL/6 (IA^b), and C3He (IA^k) haplotype of mice. M150 was isolated from the macrophages of BALB/c (IA^d) mice. Figure 9 shows that M150 induced significant proliferation of anti-CD3 Ab-activated Th cells generated from BALB/c, C57BL/6, and C3He mice. M150, however, did not exhibit any effect on Th cells of syngeneic or allogeneic strains without the occupancy of TCR. Anti-CD3 Ab-stimulated Th cells could not generate more than

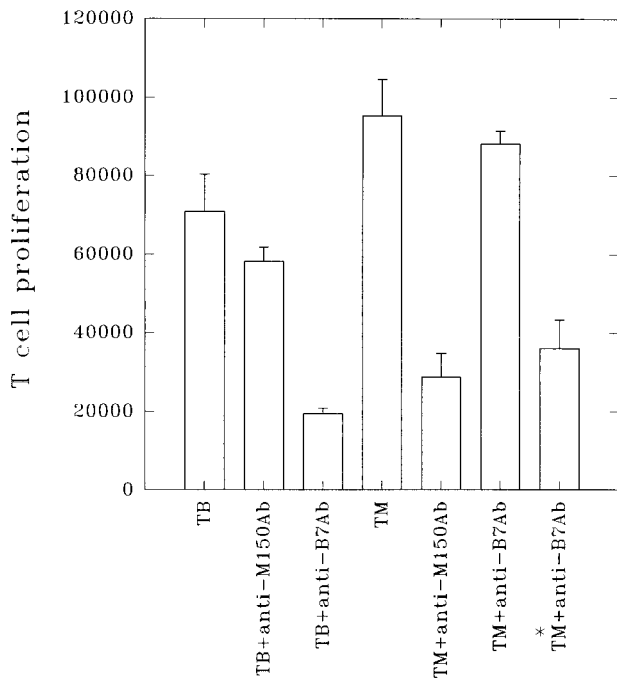


FIGURE 5. Anti-M150 and anti-B7-1 Abs block the signals generated by macrophages and B cells, respectively, for the proliferation of Th cells in MLR. Alloreactive T cells were generated against macrophages and B cells of C57BL/6 mice. The inhibition of the proliferation of T cells by anti-M150 and anti-B7-1 Abs was monitored in the presence of macrophages, M*, and B cells as stimulator cells. The control cultures containing T cells plus medium or mitomycin C-treated macrophages and B cells did not show any significant radioisotope incorporation (cpm < 1000). The control Igs of Ham and Rt did not show any decrease in T cell proliferation. Results represent the mean \pm SD of data from triplicate cultures.

5802 \pm 1641 background cpm as compared with 907 \pm 439 cpm in the case of unstimulated Th cells.

Discussion

CD4⁺ T cells need at least two stimuli provided by APC for their ultimate activation. The first signal is provided by the engagement of clonotypic TCR by a complex of MHC class II molecules and peptide. The second signal, known as costimulatory signal, is also accessory cell derived (1-3). The significance of accessory cell molecules in Th cell activation has gained considerable impetus following the observation that occupancy of TCR alone is generally inadequate for exerting complete T cell activation. Several reports are available in the literature highlighting the potential role of a number of stimuli necessary for the initiation of the differentiation events occurring during T cell-APC interactions. An array of costimulatory molecules, e.g., ICAM-1, LFA-3, VCAM-1, IL-1, heat-stable Ag CD40, M150, and B7 (4-8) are expressed on the surface of APC. The exact mechanism of sequence of signals provided by different costimulatory molecules in stimulating T cells is largely unknown.

The best defined costimulators to date are two structurally related proteins, B7-1 and B7-2, both of which have been well documented in providing a critical costimulatory signal for T cell activation by interacting with their specific receptor, CD28. Although one might assume that CD28^{-/-} mice should have absent T cell proliferation, cytokine production, and their responses to mitogen or alloantigens, instead, it has been reported that only 30% responses were reduced (29). Furthermore, significant levels of mRNA for IL-2, IL-4, and IFN- γ can be detected at early time points, presumably before the engagement of B7 and CD28, needed for T cell activation in vivo and in vitro (29, 41), and significant proliferative responses to soluble Ags can be detected.

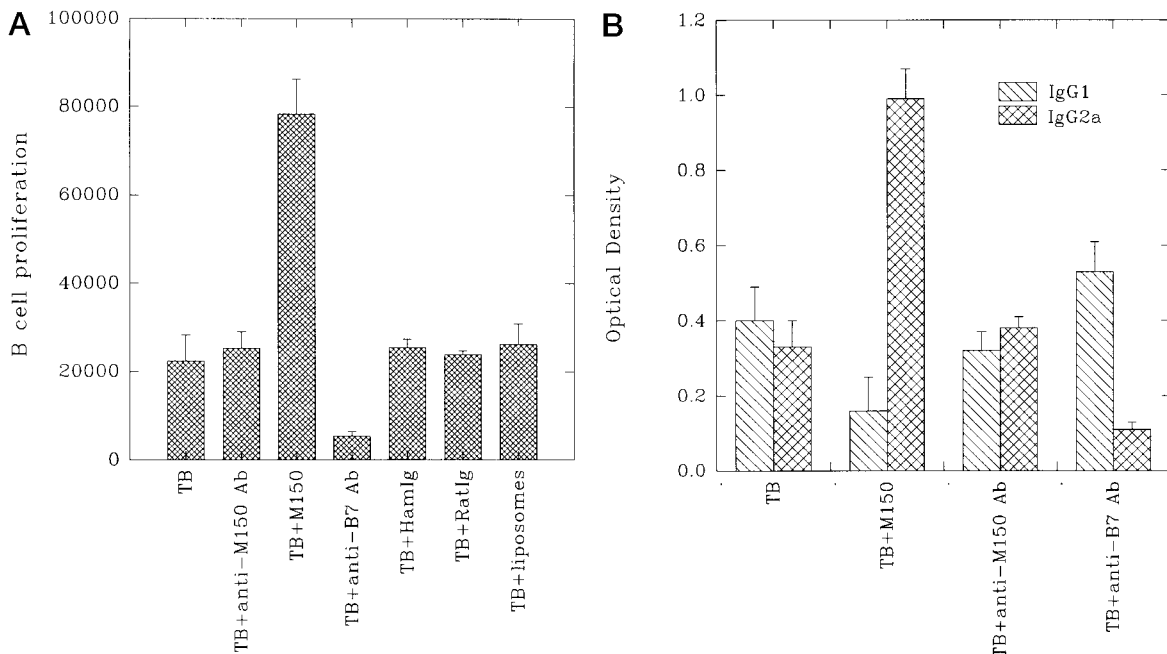


FIGURE 6. Anti-B7-1 Ab down-regulates whereas anti-M150 Ab shows no effect on B cell proliferation and IgG2a production. *A*, Mitomycin C-treated and anti-CD3 Ab-stimulated Th cells were cultured with LPS-activated B cells either in the presence of M150 (0.01 μ g/ml) or Abs to M150 and B7-1 (10 μ g/ml). The control cultures were also kept using Ham Ig, Rt Ig, and control liposomes. After 48 h, 1 μ Ci [³H]thymidine was incorporated. The plates were harvested 16 h later and processed for beta scintillation counting. *B*, For the estimation of IgG1 and IgG2a isotypes, the cultures were set as mentioned in the legend to Figure 6A. SN were collected from triplicate wells of control and experimental cultures on day 6, pooled, and analyzed for IgG1 and IgG2a by ELISA. The results are represented as OD of a single dilution (1:100) of mean absorbance of triplicate wells after subtracting the absorbance obtained with control wells (i.e., SN obtained from LPS-stimulated B cells). Results are expressed as mean \pm SD.

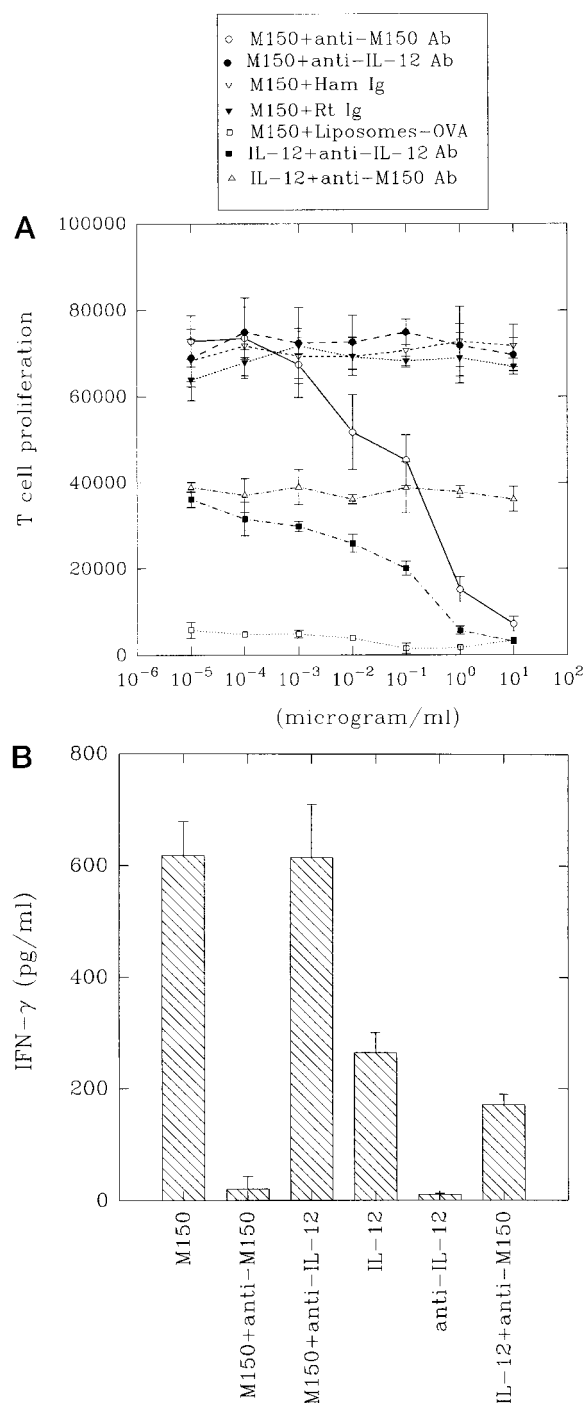


FIGURE 7. The induction of the proliferation of Th cells by M150 is specifically inhibited by anti-M150 Ab, but not by anti-IL-12 Ab. The complexes of M150 were prepared as described in *Materials and Methods*. The complexes were added to the wells containing anti-CD3 Ab-stimulated OVA-specific CD4⁺ T cells. The cultures were kept for 72 h. The proliferation of Th cells was monitored the last 16 h of 72-h cultures by [³H]thymidine incorporation. The OVA entrapped in liposomes was also used as a control. The control cultures containing either Th cells plus medium (1229 ± 1016 cpm) or Th cells stimulated with anti-CD3 Ab (6738 ± 569 cpm) and control liposomes, without M150 (6509 ± 1217 cpm), could not induce a significant level of Th cell proliferation. A, To measure IFN- γ , the cultures were set as described in the legend to A. The culture SN from the experimental and control wells were collected after 48 h and the secretion of IFN- γ was measured as mentioned in the legend to Figure 4A. The SN obtained from the control cultures did not induce the production of IFN- γ . The data are the mean ± SD of triplicate experiments.

Furthermore, T cells can also be activated in a CD28-independent manner (15). These findings, as well as studies demonstrating the failure to inhibit activation of CD4⁺ T cells in vitro (42) and in vivo (43) with anti-B7 Abs and the fact that CD28^{-/-}-deficient mice respond normally to nominal Ags, have raised the possibility to hypothesize the existence of undefined additional cosignals essential for the initiation of Th cell activation.

It is worth mentioning here that on the basis of their lymphokines profile CD4⁺ T cells have been divided into Th1 and Th2 subtypes. Th1 cells secrete mainly IFN- γ , IL-2, TNF- β , etc. and are responsible for CMI whereas Th2 cells chiefly produce IL-4, IL-5, IL-10, etc. and are responsible for humoral immunity (9, 10). It is still not clear how the differentiation and activation of CD4⁺ T cells into Th1 and Th2 occurs. It appears that cytokines and costimulatory signals delivered by APC may be the dominant factors in controlling the generation of Th1 and Th2 responses. To date, studies related to the biology of costimulation have largely been centered around activated B cells. Not many efforts have been made to analyze the ability of macrophages to mediate costimulation of T cells (12, 13, 18, 32). In this regard, we have recently identified a 150-kDa surface molecule of macrophage that possibly promotes Th1 cell activity (7). B7-1 also seems to be involved in the generation of a similar type of response (14, 15).

In the present study, we have attempted to analyze the relationship between anti-M150 and anti-B7-1 Abs in inhibiting the activation of Ag-specific Th cells. The following six major findings have emerged from this study. 1) Anti-M150 Ab significantly blocked the macrophage-mediated costimulation of Th cells but not of B cells; 2) anti-B7-1 Ab inhibited B cell-mediated costimulation but failed to check macrophage signals; 3) anti-B7-1 Ab could also obstruct the cosignal delivered by IFN- γ -activated macrophages; 4) anti-M150 and B7-1 Abs blocked the secretion of IFN- γ when costimulated with macrophages and B cells, respectively; 5) alloreactivity of macrophages was greatly checked when anti-M150 Ab was utilized but not by using anti-B7-1 Ab, whereas alloreactivity generated by B cells was abrogated by anti-B7-1 Ab but not by anti-M150 Ab; 6) anti-B7-1 Ab greatly inhibited B cell proliferation and IgG2a secretion.

It is noteworthy that macrophage-mediated costimulation of Th cells was inhibited by anti-M150 Ab, but not by anti-B7-1 Ab. However, anti-B7-1 Ab significantly obstructed the costimulation when macrophages were preactivated with IFN- γ . It may be mentioned that macrophages do not express B7-1, which is up-regulated later in immune responses upon stimulation with IFN- γ (30–32). IFN- γ appears to be secreted at a fairly late stage (48–72 h) of Th cell activation (44). Therefore, it is safer to assume that at the initial stage of macrophage-associated Th cell signaling, it might be M150 through which macrophages provide accessory help to Th1 cells. Macrophages do not express B7-1 but express a significantly higher level of M150. It is likely that the B7-1/CD28 pathway may not be very crucial in the initiation of early events of Th cell activation by macrophage; however, once the immune response is generated, and IFN- γ is available, it induces B7-1 and up-regulates M150 expression on the macrophages. Furthermore, the activation and clonal amplification of Th cells may then be controlled by both M150 and B7-1. The fact that, unlike normal macrophages, anti-M150 Ab could not sufficiently block Th cell activation of macrophages activated by IFN- γ , and the observation that anti-B7-1 Ab blocked costimulation only when IFN- γ activated macrophages were used, further strengthens this proposition.

LPS-activated B cells are known to express B7-1. Another interesting feature observed during the study was that anti-B7-1 Ab could significantly block the signals delivered by B cells for the proliferation of Th cells. M150 was, however, expressed to a lesser

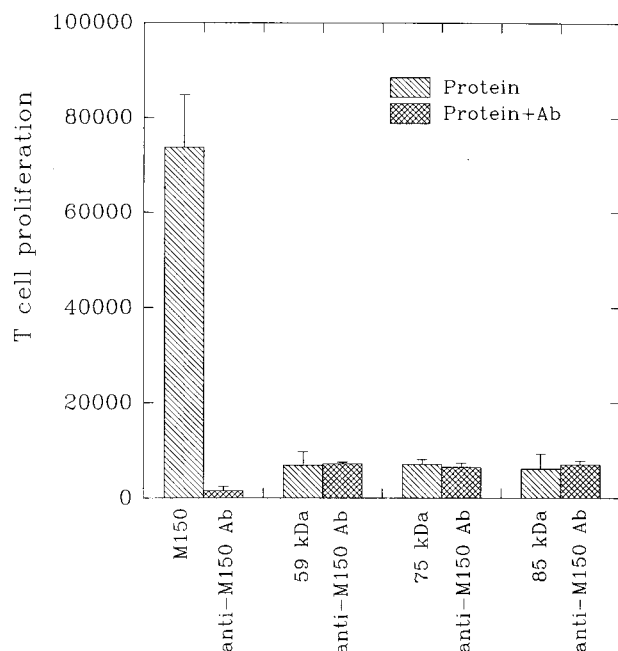


FIGURE 8. Anti-M150 Ab blocks the M150-induced proliferation of Th cells, but not of other macrophage membrane proteins. Anti-CD3 Ab-incubated Th cells were cultured with 21 different proteins (0.01 $\mu\text{g/ml}$) isolated from the membrane of macrophages. The proteins were also cultured with anti-M150 Ab (10 $\mu\text{g/ml}$). Except M150 and proteins of 59, 75, and 85 kDa, none of the proteins could enhance Th cell proliferation above the background level (i.e., anti-CD3 Ab + Th cells, 4937 ± 613 cpm). The induction of the proliferation of Th cells by M150 was blocked only by anti-M150 Ab. After 72 h, 1 $\mu\text{Ci/well}$ of [^3H]thymidine was added. The cultures were harvested 16 h later, and incorporated radioactivity was monitored. Data are expressed as mean \pm SD of three experiments.

extent on B cells. Furthermore, anti-M150 Ab could not significantly inhibit the proliferation of Th cells. Thus it can be inferred that at the initial stage of interaction macrophages and B cells use different costimulatory signals for the activation of Th cells. One may wonder why anti-M150 and anti-B7-1 Abs could not completely inhibit the signals delivered by B cells and macrophages. It could possibly be because of the involvement of other costimulatory molecules like B7-2 and CD40 in providing costimulation to T cells. It may be mentioned here that B7-2 is also reported on the resting macrophages (15, 16).

We next performed experiments to evaluate the role of anti-M150 and anti-B7-1 Abs in restricting the secretion of IFN- γ and IL-4 by Th cells when macrophages and B cells were used as APC. Interestingly, anti-M150 Ab, but not anti-B7-1 Ab, inhibited the secretion of IFN- γ when Th cells were costimulated with macrophages. However, when macrophages activated with IFN- γ were used for costimulation, anti-B7-1 Ab also inhibited the production of IFN- γ . In contrast, anti-B7-1 Ab, but not anti-M150, blocked the secretion of IFN- γ when Th cells were activated with B cells. None of the Ab could inhibit the secretion of IL-4. It may be concluded here that the activity of IFN- γ -producing Th cells can be differentially inhibited by both anti-M150 and anti-B7-1 Abs when macrophages and B cells, respectively, functioned as APC. Anti-B7-2 Ab nevertheless inhibited the secretion of both IFN- γ and IL-4 when macrophages as well as B cells functioned as APC (data not shown). Recent studies have suggested different conclusions about the relative roles of B7-1 and B7-2 in mediating costimulatory interactions with CD28/CTLA-4 and subsequent T cell differentiation. Some reports have suggested that IL-4 production

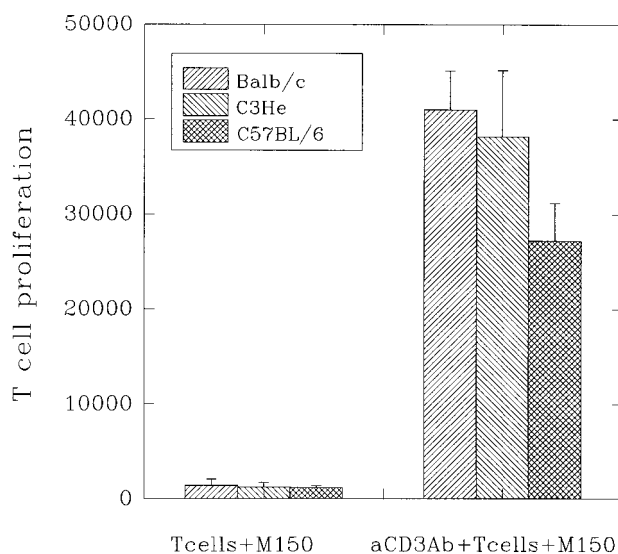


FIGURE 9. M150 is a costimulatory molecule and not an alloantigen. OVA-specific Th cells generated from BALB/c, C57BL/6, and C3He mice were cultured with M150 isolated from BALB/c mice in the presence or absence of anti-CD3 Ab. Anti-CD3 Ab-stimulated Th cells showed 5802 ± 1641 background cpm. After 72 h, 1 $\mu\text{Ci/well}$ of [^3H]thymidine was added. The cultures were harvested 16 h later, and incorporated radioactivity was monitored. Data are expressed as mean \pm SD of three experiments.

is particularly dependent on B7-2 signaling. Also, administration of anti-B7-1 and anti-B7-2 Abs during *in vivo* immune responses blocks primarily IFN- γ and IL-4 secretion, respectively (14, 24, 25). Furthermore, costimulatory molecules M150 and B7-1 may be playing a major role in selectively regulating the secretion of IFN- γ and in the development and differentiation of CD4 $^+$ T cells subsets. Thus, Th cell differentiation not only depends on the type of APC but also on the costimulatory molecules being displayed on its surface (45–49).

We carried out more experiments to generate further evidence in support of M150 and B7-1 providing costimulatory signals when macrophages and B cells, respectively, are associated with Th cell signaling. MLCs were set using macrophages and B cells as allo-stimulator cells. Our results demonstrate that anti-M150 Ab effectively blocked T cell alloreactivity when macrophages were used as a source of stimulation, but it blocked partially when B cells were employed. Conversely, anti-B7-1 Ab effectively blocked the alloreactivity of B cells but not of macrophages. These experiments further suggest that M150 is a primary costimulatory molecule on macrophages, while B7-1 functions chiefly from B cells.

We also looked into the role of these Abs in restraining the proliferation and IgG1 and IgG2a Ab secretion by B cells. We observed that anti-B7-1 Ab significantly blocked the proliferation of B cells and the production of IgG2a. This may be because B7-1 probably provides costimulatory signal to Th1 cells (14). Moreover, it is also known that the interaction between Th1 and B cells can lead to the secretion of IgG2a Ab, whereas cognate recognition between Th2 and B cells induces the production of IgG1 Ab (50). Since M150 is primarily expressed on macrophages, anti-M150 Ab could not significantly effect the secretion of IgG isotypes by B cells. However, when anti-CD3 Ab-stimulated Th cells were further stimulated with M150, the capability of Th cells to provide signals to B cells were dramatically improved, leading to enhanced secretion of IgG2a, whereas IgG1 response declined slightly. It may be recalled here that it has been reported that IFN- γ primarily activate B cells to secrete IgG2a and IL-4 induce the production of

IgG1 (51). As mentioned earlier, anti-CD3 Ab stimulated Th cells secrete IFN- γ when activated with M150, which may be stimulating LPS-activated B cells to produce IgG2a Abs.

The augmentations of the proliferation of Th cells and secretion of IFN- γ and IgG2a directly or indirectly have been reported for IL-12, which is produced at high levels by macrophages (52, 53). Although IL-12 (35–40 kDa) is not a 150-kDa protein, it was still of concern that M150 may be this cytokine. To address this question we confirmed that the proliferation of Th cells and the secretion of IFN- γ induced by M150 were specifically inhibited by anti-M150 Ab, but not by anti-IL-12 Ab. Similarly, IL-12-induced proliferation and production of IFN- γ was not blocked by anti-M150 Ab. Anti-IL-12 Ab also failed to recognize M150 on Western blot (data not shown). It may also be mentioned here that Th cells activated by M150 secrete IL-2 as well as IFN- γ (7), however, when stimulated with IL-12, they produce IFN- γ but not IL-2 (54). Moreover, mouse IL-12 (55) but not M150 can augment the proliferation of PHA-activated human lymphoblasts.

The specificity of anti-M150 Ab was further established by the fact that it recognizes only M150 but not other macrophage membrane proteins by Western blot analysis. Furthermore, only M150-mediated proliferation of Th cells was inhibited by anti-M150 Ab (Fig. 8). Nevertheless, the isolated M150 contained only a single protein and perhaps no minor contaminant was established by the fact that in two-dimensional gel electrophoresis M150 appeared as a single spot (7). The purity of each batch used in the experiments or immunization was always checked by two-dimensional gel electrophoresis.

The fact that M150 could not stimulate Th cells without TCR cross-linking by anti-CD3 Ab proves that the molecule is not an alloantigen. However, it works across the MHC barriers to provide a cosignal.

We conclude from our data that activation of CD4⁺ T cells is not a mere function of B7-1. Both M150 and B7-1 are potent costimulatory molecules for IFN- γ -producing OVA-specific Th cells. M150 plays a crucial role in Th cell-macrophage interactions for the early events of Th cell activation and IFN- γ secretion. It is reasonable to assume that IFN- γ produced from this signal then induces the expression of B7-1, which subsequently provides signals for further expansion of Th cells and secretion of lymphokines. Furthermore, M150 also helps in the augmentation of alloreactivity and enhances the ability of Th cells to help B cells and in the secretion of IgG2a Abs and, to a much lesser extent, in B cell-mediated activation of Th cells. B7-1 is an effective costimulatory molecule when B cells deliver signals to Th cells. Both of the molecules probably promote Th1-type of cells, as is evident in the secretion of IFN- γ and production of IgG2a Abs by B cells. Moreover, a possibility that those other costimulatory molecules, viz., B7-2, CD40, etc., also have an effect in activation of Th cells cannot be ruled out (Fig. 3, A and B).

The role of M150 and B7-1 in delivering costimulatory signals from macrophage and B cell, however, needs to be evaluated on the clonal Th1 cell population; and whether the costimulatory pathway is mediated by these molecules alone or in concert needs further investigation.

Acknowledgments

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