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Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-κB-dependent pathway

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Abstract

Blocking T cell co-stimulatory signals by anti-B7-1/B7-2 mAb is an attractive approach to treat autoimmune diseases. However, anti-B7-1/B7-2 mAb treatment is known to exacerbate autoimmune diseases through mechanisms not fully understood. Tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) also play important roles in determining the clinical outcome of autoimmune diseases. In this study, we demonstrate that the anti-B7-1 and the anti-B7-2 mAbs activate macrophages for higher induction of TNF-α and other effector responses such as bacterial cytotoxicity and production of ROS. Nuclear factor-kappaB (NF-κB) was found to be increased with anti-B7-1/B7-2 mAb treatment. Inhibition of NF-κB activity by over-expression of phosphorylation-defective I-kappaB alpha in anti-B7-1/B7-2 mAb-treated macrophages decreased TNF-α production. These data indicate that anti-B7-1 and anti-B7-2 mAbs can trigger innate-effector responses in macrophages by activating NF-κB-signaling pathway. Our results suggest that the B7 molecules are not only essential for induction of adaptive immune responses but also play roles in activation of innate immune responses.

Introduction

The T cell activation is regulated by both the cognate peptide–MHC-driven stimulus (1, 2) and the non-cognate co-stimulatory signal (3–5). The B7 family of co-stimulatory molecules, the B7-1 and the B7-2 of the antigen-presenting cells (APCs), interact with CD28 receptors on T cells and provide major non-cognate co-stimulatory signals (6, 7). Under certain pathophysiological conditions, hyperactivation of T cells through B7-1 and B7-2 often leads to development of autoimmunity (8–11). Therefore, blocking the B7-1/B7-2 co-stimulatory molecules with antibodies against them is an attractive way to develop immunotherapeutics against autoimmunity (12, 13). However, in some models of autoimmune disease, anti-B7-1/B7-2 mAb therapy has yielded undesirable results (14–16). For example, administration of anti-B7-1 mAb resulted in accelerated onset of diabetes in the non-obese diabetic mice (15). Similarly, it has been demonstrated that injection of anti-B7-1/B7-2 mAb results in marked exacerbation of experimental allergic encephalomyelitis (EAE) incidence (16).

Mechanisms by which the anti-B7-1/B7-2 mAb treatment exacerbates autoimmune disease are not clear. Various groups have shown that tumor necrosis factor alpha (TNF-α) (17–25) and reactive oxygen species (ROS) (26, 27), produced by the activated APCs, are particularly involved in exacerbation of autoimmune disease. It has been found that mice with EAE when treated with combined anti-B7-1 and anti-B7-2 mAbs had increased levels of TNF-α (16). This observation raises the possibility that anti-B7-1/B7-2 mAb application can aggravate autoimmune disease possibly through production of higher amounts of TNF-α. However, it is not clear whether such higher level of the TNF-α production is a consequence of macrophage hyperactivation in response to anti-B7-1/B7-2 mAb treatment. Although some forms of autoimmunity are linked to T cell production of TNF-α (28), TNF-α and other inducible effector responses are known to be regulated mainly by the macrophages (29–32). Therefore, it is possible that these antibodies may activate

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TNF-α production by directly interacting with the B7-1/B7-2 molecules expressed on its surface. To test this hypothesis, we examined whether interaction of B7-1 and B7-2 molecules with respective mAbs could activate macrophages for higher induction of TNF-α and other inducible effector responses such as production of ROS, microbicidal activity and signaling pathways involved.

Methods

Mice

BALB/c mice were bred and maintained in the animal facility of Indian Immunologicals Ltd (IIL), Hyderabad, India. All mice were 6–12 weeks old. All experimental procedures were approved by the Institutional Review Committee for care and usage of animals of IIL.

Macrophage stimulation assay

Peritoneal exudate cells (PECs) from BALB/c mice were harvested by injecting 4% thioglycolate broth as described elsewhere (32). The RAW 264.7 macrophage cell line (H-2d) was obtained from National Centre for Cell Science, Pune, India. BMC2 macrophage cell line (H-2b) was a kind gift from Satyajit Rath, National Institute of Immunology, New Delhi, India. Both the macrophage cell lines were maintained in DMEM (Invitrogen, Grand Island, NY, USA) containing 10% FCS (Invitrogen) and antibiotics (DMEM-10). Macrophages were either left untreated or treated with various concentrations of either rat anti-mouse B7-1 mAb (clone 1G10, BD Biosciences PharMingen, San Diego, CA, USA) or rat anti-mouse B7-2 mAb (clone GL1, BD Biosciences PharMingen) and plated in 96-well tissue culture plate as 5×10^5 cells per well. Both anti-B7-1 mAb and anti-B7-2 mAb were of IgG2a isotype. Therefore, in control samples, isotype-matched antibody of IgG2a type (BD Biosciences PharMingen) was added. In some experiments, macrophages were activated with 3 μg ml^{-1} of LPS (Sigma–Aldrich, St Louis, MO, USA) and 3 ng ml^{-1} of IFN-γ (R&D Systems, Minneapolis, MN, USA) together (LPS + IFN-γ) in the presence of either IgG2a antibody or anti-B7-1/B7-2 mAb (BD Biosciences PharMingen). After 48 h, culture supernatants were harvested and assayed for TNF-α (BD Biosciences PharMingen) induction. Endotoxin contamination in various samples was removed by incubating samples with 10% v/v polymyxin B–agarose (Sigma–Aldrich; binding capacity, 200–500 μg of LPS from *Escherichia coli* serotype O128:B12 ml^{-1}) for 1 h at 4°C. After incubation, the agarose beads were removed by centrifugation and the supernatant was filter sterilized and used to stimulate macrophages. The $F(ab')_2$ fragments were prepared using a commercially available $F(ab')_2$ preparation kit (Cat. No. 44888) from Pierce Chemical Company (Rockford, IL, USA). In brief, the samples were cleaved using immobilized pepsin according to the manufacturer's protocol. Undigested Ig and Fc fragments were removed by affinity chromatography with protein A–sepharose. The fraction containing the $F(ab')_2$ fragments was collected and dialyzed, examined by SDS–PAGE to verify removal of non-digested IgG and Fc fragments, and used in the subsequent experiments.

Cytokine assay

The TNF-α, IL-10 and IL-12 cytokines in the macrophage culture supernatants were quantified by two-site sandwich enzyme-linked immunoassay (EIA) as described earlier (32, 33). Standard curve for the TNF-α/IL-10/IL-12 cytokine was obtained using recombinant standard protein.

Western blotting

Western blotting was used to examine nuclear factor-kappaB (NF-κB) levels in the nuclear extracts prepared from macrophages treated with 10 μg ml^{-1} of either IgG2a control antibody or anti-B7-1 mAb or anti-B7-2 mAb. Nuclear extracts prepared as described earlier (34) were separated in a 10% SDS–PAGE under reducing conditions. Following electrophoretic transfer to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK), the membranes were incubated with affinity-purified goat antibodies to the NF-κB family proteins p50 or p65 (Santa Cruz Biotechnology) followed by incubation with anti-goat Ig–HRP conjugate (Sigma–Aldrich). Blots were developed by chemiluminescence using the manufacturer's protocol (Amersham Biosciences).

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), 10 μg of the nuclear extracts prepared from various groups were incubated for 30 min at 37°C with 1 ng of ^{32}P end-labeled complementary oligo-deoxyribonucleotide containing the NF-κB (35)-binding region (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ and 5′-CCACGCCTCCCTGGAAAGTCCCCAGCGGAAAGTCCCTTGTAACAA-3′), 2 μg of poly (dI–dC) and a binding buffer (20 mM HEPES–KOH, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EDTA and 5% glycerol). The DNA-protein complexes were resolved on a 7% non-denaturing polyacrylamide gel in TGE-running buffer (50 mM Tris–HCl of pH 7.5, 40 mM glycine and 10 mM EDTA). The gel was dried and subjected to autoradiography.

Bactericidal assay

The bactericidal assay was performed as described (36). In brief, macrophages treated with IgG2a or anti-B7-1/B7-2 mAb were incubated with live *E. coli* at a ratio of 1:10. After 15 min, cells were washed and incubated at various time points in the presence of 10 μg ml^{-1} gentamycin to kill all extracellular bacteria. Cells were lysed with sodium deoxycholate and the number of viable bacteria at each time point for each group was determined by plating the lysates on LB agar. Internalized bacteria were defined as those macrophage-associated bacteria that were viable after a 15-min exposure and bacterial numbers, determined at subsequent time points for each group, were expressed as percentage of the internalized population.

Conditioned medium cytotoxicity on L929 cells

To assess biologic activity for the TNF-α produced by RAW 264.7 macrophages exposed to anti-B7-1/B7-2 mAb, L929 cytotoxicity assay was used (37). L929 cell line was a kind gift from Satyajit Rath, National Institute of Immunology, New Delhi, India. Cells were plated on 96-well plates at a density

of 30 000 cells per well. After 24 h, medium was changed and 50 μl fresh medium and 50 μl of the medium collected from RAW 264.7 macrophages exposed to IgG2a control antibody or anti-B7-1/B7-2 mAb were added in the presence of 1 μg ml^{-1} actinomycin D. After 18 h, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich) was added as 1 mg ml^{-1} and incubated further for 5 h. Cells were then lysed overnight using 100 μl of lysis buffer (20% SDS and 50% Dimethylformamide). Absorbance values were read at 550 nm. All assays were carried out in triplicate, and data were computed as mean ± SD of five different experiments. Results are expressed as the percentage of viable cells in relation to supernatants from IgG2a-treated RAW 264.7 macrophages.

Total RNA isolation and reverse transcriptase–PCR

For reverse transcriptase (RT)–PCR, total RNA was isolated from each sample using Trizol reagent as per the manufacturer's (Invitrogen) protocol. Reverse transcription was carried out using a GeneAmp RNA PCR core kit (Invitrogen). PCR was performed at an annealing temperature of 58°C with the following primers: TNF-α forward primer (5′-CAGGGGCCACCACGCTCTTC-3′) and reverse primer (5′-CTTGGGGCAGGGGCTCTTGAC-3′), which generates a product of 410 bp, and a house keeping gene β-actin forward primer (5′-GTGGGCCGCTCTAGGCACCA-3′) and reverse primer (5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′) which generates a product of 244 bp.

Dihydrorhodamine-123 fluorescence

Intracellular ROS level was measured with the use of fluorescence of rhodamine-123 produced from oxidation of dihydrorhodamine-123, as previously described (38). Briefly 5×10^5 cells were incubated at 37°C with 10 μg ml^{-1} of IgG2a or anti-B7-1/B7-2 mAb for 1 h, followed by incubation with 15 μM dihydrorhodamine-123 for 45 min. Finally, the cells were washed and embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Microscopy was performed on a Nikon fluorescence microscope (Nikon DX1, Japan).

Flow cytometry

For flow cytometry, macrophages were incubated with IgG2a or anti-B7-1/anti-B7-2 antibody for 60 min. Heat-aggregated rabbit sera were used at 1% concentration in the staining buffer to block Fc receptors. After washing in staining buffer, similar incubations were carried out for secondary reagent (anti-rat FITC, Sigma–Aldrich). Stained cells were analyzed on a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA, USA). Post flow cytometrical data analyses were carried out using CellQuest data analysis software (Becton Dickinson).

Transfection with phosphorylation-defective I-kappaB alpha ($^{\Delta}$I-κBα) plasmid construct

The phosphorylation-defective I-kappaB alpha (I-κBα) ($^{\Delta}$I-κBα) plasmid construct was a kind gift from Jürgen Heesemann (Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie, München, Germany). Transfections were carried out with 10 μg of the $^{\Delta}$I-κBα construct (39). Expression vector without any insert was used as negative control. The plasmid constructs were transfected into RAW 264.7 macrophages using the cationic lipid suspension lipofectin (Invitrogen). pSV-β-galactosidase (Promega, Madison, WI, USA) vector was used for transfection efficiency control. After 18 h, cells were stimulated with IgG2a control antibody or anti-B7-1/B7-2 mAb. Cells were either harvested after 1 h for the measurement of NF-κB activity in the nuclear extracts by EMSA or for quantitation of β-galactosidase protein expression in the cytoplasmic extracts by β-galactosidase ELISA (Roche Diagnostics, Penzberg, Germany) following the manufacturer's protocol or cultured for 48 h for estimation of TNF-α levels secreted in the culture supernatants by EIA.

Statistical analysis

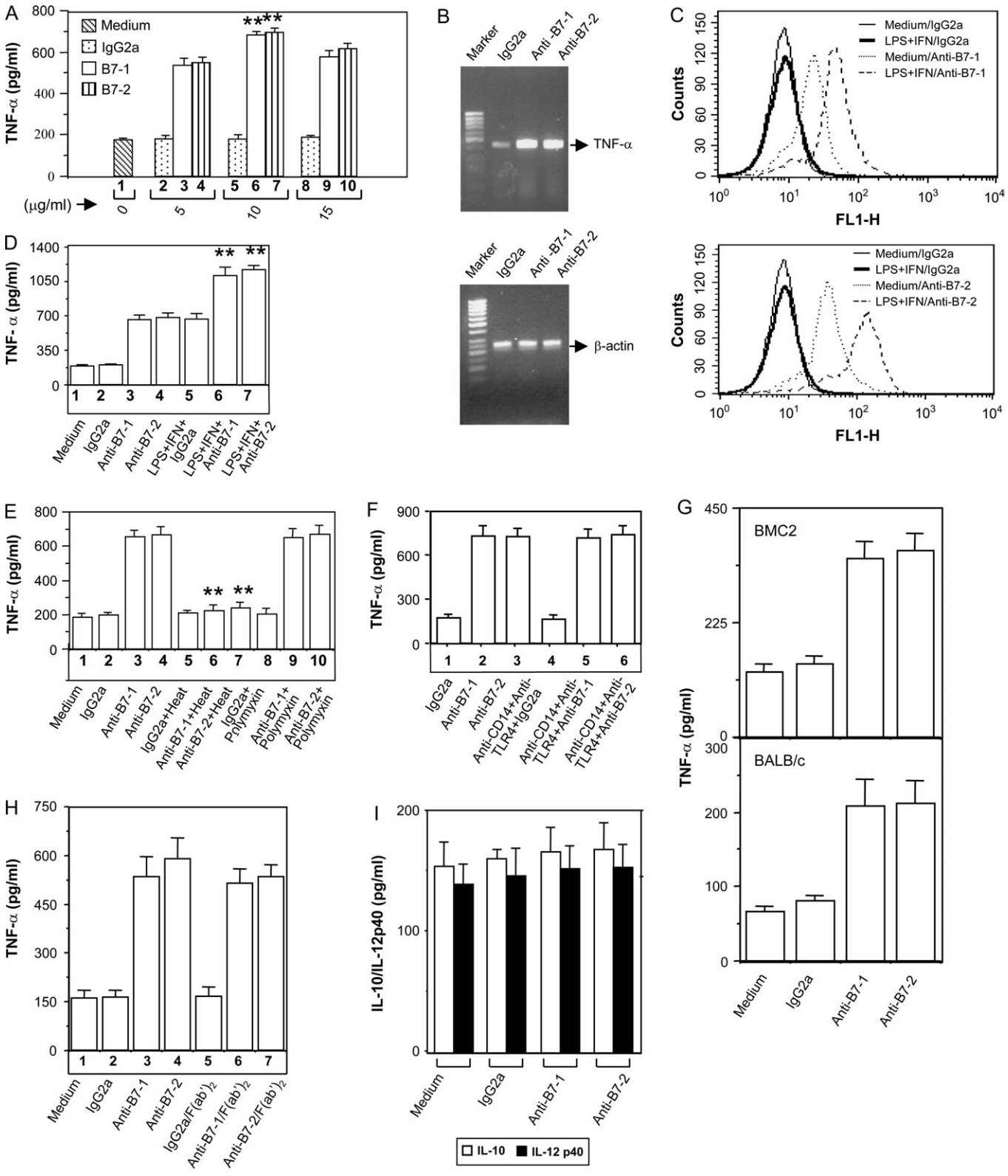
Data were expressed as mean ± SD. Statistical comparisons were made using Mann–Whitney *U*-test. The significance level was set at $P < 0.05$.

Results

Induction of TNF-α is higher in macrophages treated with anti-B7-1/B7-2 mAb

We first examined whether murine macrophages treated *in vitro* with mAb to either B7-1 or B7-2 produced higher levels of TNF-α in the cultures. Accordingly, RAW 264.7 macrophages were cultured with various concentrations (5, 10 and 15 μg ml^{-1}) of either isotype-matched control antibody (IgG2a isotype) or anti-B7-1 mAb or anti-B7-2 mAb and after 48 h, culture supernatants were tested for the levels of TNF-α produced by EIA. As opposed to the isotype control group, treatment with the anti-B7-1 mAb resulted in increased production of TNF-α for all treatment regimes (Fig. 1A). Similarly, the anti-B7-2 mAb also increased TNF-α production in RAW 264.7 macrophages (Fig. 1A). Since TNF-α production in the group treated with IgG2a isotype antibody even at 15 μg ml^{-1} concentration was low and similar to that produced by the group treated with medium alone (Fig. 1A, compare bar 8 with bar 1), it can be concluded that specific binding of B7-1/B7-2 molecules with its respective intact mAb can trigger macrophages to produce TNF-α. An antibody concentration of 10 μg ml^{-1} resulted in maximum TNF-α induction in both the cases (Fig. 1A). Therefore, all the following experiments were carried out using 10 μg ml^{-1} anti-B7-1/B7-2 mAb. Both anti-B7-1 mAb and anti-B7-2 mAb individually stimulated murine macrophages to preferentially express mRNA transcripts for TNF-α as compared with the IgG2a control group (Fig. 1B). We next examined whether induction profile of TNF-α was directly proportional to the number of B7-1/B7-2 molecules. Therefore, we stimulated macrophages with LPS + IFN-γ to increase B7-1 and B7-2 levels on macrophages as detected by flow cytometry (Fig. 1C). These cells were then treated with 10 μg ml^{-1} anti-B7-1/B7-2 mAb and TNF-α productions by these cells were compared with the levels produced from macrophages treated with anti-B7-1/B7-2 mAb only without LPS + IFN-γ. It could be seen that the TNF-α production was more in the LPS + IFN-γ-activated group (Fig. 1D) than those treated with anti-B7-1/B7-2 mAb alone. To confirm that the observed

data of increased TNF-α production by anti-B7-1/B7-2 mAb was not due to endotoxin contamination in the antibody preparations, the anti-B7-1 mAb and the anti-B7-2 mAb were treated with heat (95°C for 10 min), incubated with polymyxin B–agarose or added to the macrophage cultures that were pre-treated with anti-CD14 plus anti-Toll-like receptor 4 (TLR4) antibody to block CD14 receptor and TLR4, specific for LPS binding (40). It was found that the activity of anti-



B7-1/B7-2 mAb on the production of TNF-α could be abrogated by heat but not by polymyxin B (Fig. 1E) or anti-CD14 plus anti-TLR4 antibody treatment (Fig. 1F). These results rule out contribution of endotoxin to the macrophage stimulatory property of anti-B7-1/B7-2 mAb.

We have shown earlier that the macrophage effector functions can be controlled at the level of MHC haplotype (35). Therefore, using macrophage of H-2b haplotype (BMC2), we examined whether the anti-B7-1/B7-2 mAb could enhance TNF-α production in H-2b macrophages (BMC2) to a similar extent in H-2d macrophages (RAW 264.7). The level of TNF-α produced in macrophages incubated with IgG2a or anti-B7-1/B7-2 antibody was compared. It was evident that the anti-B7-1 mAb and the anti-B7-2 mAb at 10 μg ml^{-1} concentration enhanced TNF-α secretion also in H-2b macrophages (Fig. 1G). This result suggests that anti-B7-1/B7-2 mAb induces TNF-α in macrophages irrespective of MHC haplotype. The anti-B7-1/B7-2 mAb at 10 μg ml^{-1} concentration induced higher level of TNF-α also in PEC-derived macrophages from BALB/c mice (Fig. 1G) indicating that results obtained with transformed mouse macrophage cell lines (RAW 264.7/BMC2) were equally true in primary cultures of mouse macrophages.

It is already known that signaling through the Fc receptors like CD32 and CD64 can activate macrophages for higher production of TNF-α (41). To rule out this possibility, we next treated anti-B7-1 and anti-B7-2 antibodies with pepsin to remove the Fc portion and used the purified $F(ab')_2$ fragments to cross-link B7-1 and B7-2 receptors. It was found that the $F(ab')_2$ fragments activated macrophages for increased TNF-α induction (Fig. 1H) in a similar manner as observed with the respective intact antibody (Fig. 1A). These results indicate that signaling through the B7-1/B7-2 activate macrophages to produce TNF-α. It was noticed that although TNF-α was increased, anti-B7-1/B7-2 mAb had little effect on the induction profile of IL-10 and IL-12 cytokines (Fig. 1I).

Increased production of TNF-α by macrophages treated with anti-B7-1/B7-2 mAb leads to enhanced cytotoxicity of L929 cells

The capacity of anti-B7-1/B7-2 mAb to stimulate RAW 264.7 macrophages to secrete TNF-α was further assessed by L929 cytotoxic bioassay. L929, a murine fibrosarcoma cell line is highly sensitive to the anti-proliferative and cytotoxic action of TNF-α (37). L929 cells were incubated with media harvested from the macrophage cultures treated either with IgG2a or anti-B7-1/B7-2 mAb in the absence or presence of LPS + IFN-γ. The MTT assay shown in Fig. 2(A) reveals that ~50% of the cells are dead when incubated with anti-B7-1/B7-2 mAb-treated culture supernatants. In cells co-treated with LPS + IFN-γ, the cytotoxicity effect was further increased (Fig. 2A) compared with anti-B7-1/B7-2 mAb treatment alone. Addition of 10 μg ml^{-1} of anti-TNF-α mAb in the culture assay inhibited the observed cytotoxicity for all treatment regimes (Fig. 2B) confirming that the cytotoxicity effect was due to the TNF-α. These results correlate with the concentration of TNF-α present in the supernatants (Fig. 1D) and additionally point to the biologic activity for this cytokine.

Anti-B7-1/B7-2 mAb increases induction of ROS in macrophages

TNF-α can augment ROS production in macrophages (42, 43). Intracellular ROS levels were therefore examined in the

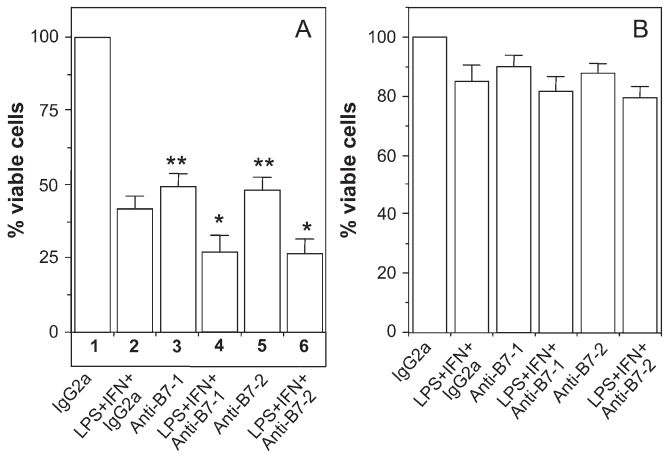


Fig. 2. Enhanced cytotoxicity of L929 cells by culture supernatants harvested from the macrophage cultures treated with anti-B7-1/B7-2 mAb. Centrifuged supernatants from RAW 264.7 macrophages treated as described in Fig. 1D were transferred onto cultures of L929 cells in the absence (A) or presence (B) of 10 μg ml^{-1} of neutralizing anti-TNF-α antibody. Cell viability was determined by MTT assay. The viability of macrophages in the wells that received culture supernatants from the IgG2a-treated group was considered to be 100% and viability of the test samples was monitored based on percentage of normalized control. Data are representative of five different experiments. The significance of the L929 cytotoxicity in (A) was determined by statistical analysis using Mann–Whitney *U*-test for the data shown in bar 3, bar 4, bar 5 and bar 6 by comparison with their respective controls. *indicates $P < 0.05$ and **indicates $P < 0.01$.

Fig. 1. The anti-B7-1/B7-2 mAb stimulates macrophages for increased TNF-α production. The RAW 264.7 macrophages were either left untreated or treated with various concentrations of either IgG2a or anti-B7-1/B7-2 antibody and after 48 h culture supernatants were tested for the levels of TNF-α produced by EIA (A). Total RNA from macrophages stimulated with 10 μg ml^{-1} of either IgG2a antibody or anti-B7-1 or anti-B7-2 mAb was subjected to RT–PCR analysis to determine induction of TNF-α mRNA with β-actin used as amplification control (B). RAW 264.7 macrophages were treated with LPS + IFN-γ for either flow cytometric analysis of B7-1 and B7-2 co-stimulatory molecules (C) or for measuring TNF-α production in the presence of IgG2a or anti-B7-1/B7-2 mAb (D). TNF-α induction was measured in RAW 264.7 macrophages incubated with native, heat boiled (95°C for 10 min) or polymyxin B-treated anti-B7-1/B7-2 mAb (E). In a separate experiment, anti-B7-1/B7-2 mAb-mediated TNF-α induction was measured in macrophages pre-treated with anti-CD14 plus anti-TLR4 antibody (F). Also TNF-α was measured in BMC2 or PEC-derived macrophages treated with IgG2a or anti-B7-1/B7-2 mAb (G). RAW 264.7 macrophages were treated with pepsin digested $F(ab')_2$ fragments of IgG2a or anti-B7-1/B7-2 antibody for 48 h and the levels of TNF-α produced in the cultures were measured by EIA (H). RAW 264.7 macrophages were treated with IgG2a or anti-B7-1/B7-2 antibody and after 48 h culture supernatants were tested for the levels of IL-10 and IL-12 cytokines by EIA (I). Data are representative of five different experiments. The significance of the results of anti-B7-1/B7-2 mAb-mediated induction of TNF-α at 10 μg ml^{-1} (A, bar 6 and bar 7) was statistically determined using Mann–Whitney *U*-test by comparison with the value of IgG2a control (bar 5). Similarly Mann–Whitney *U*-test was carried out for the data shown in bar 6 and bar 7 of (D and E) and compared with their respective controls namely bar 5 of (D) and bar 3 and bar 4 of (E). **indicates $P < 0.01$.

anti-B7-1/B7-2 mAb-treated macrophages using rhodamine-123 fluorescence assay based on the oxidation of dihydrorhodamine-123. It was evident that the anti-B7-1 mAb and the anti-B7-2 mAb increased intracellular ROS levels in RAW 264.7 macrophages as compared with the IgG2a control antibody (Fig. 3).

Macrophages treated with anti-B7-1/B7-2 mAb show enhanced microbicidal function

Induction of ROS is critically important for macrophage-mediated microbicidal activity (44, 45). The microbicidal function of macrophages treated with either anti-B7-1 mAb or anti-B7-2 mAb was therefore directly examined. Macrophages treated with either IgG2a (IgG2aM) or anti-B7-1 (B7-1M) or anti-B7-2 (B7-2M) antibody were incubated with live *E. coli* for 15 min and cultured for various time points before lysis. The numbers of surviving bacteria in the lysates were estimated by calculating the number of colony-forming unit on LB-agar plates as described (36). It was observed that the bactericidal ability of both B7-1M and B7-2M was significantly higher than that of IgG2aM (Fig. 4A, compare bar 2 and bar 3 with bar 1). After 3 h of incubation, the B7-1M and the B7-2M reduced bacterial viability by over 50% (Fig. 4A), while ~90% of the input bacteria were found to be alive in the macrophage treated with IgG2a antibody. By the end of 6 h, >90% of the input bacteria were killed in B7-1 mAb- or B7-2 mAb-treated macrophages (Fig. 4B), whereas ~55% of the input bacteria were alive in macrophages treated with IgG2a antibody. As expected, bactericidal activity of the macrophages treated with anti-B7-1/B7-2 mAb was still higher in the presence of LPS + IFN-γ when examined after 3 h (Fig. 4C). These results demonstrate the ability of anti-B7-1/B7-2 mAb-treated macrophages to display enhanced microbicidal function.

Macrophages treated with anti-B7-1/B7-2 mAb show increased induction of NF-κB

In the previous experiments (Figs 1, 2 and 4), we observed that anti-B7-1/B7-2 mAb increased TNF-α induction and up-regulated microbicidal activity of macrophages. The NF-κB family of transcriptional regulators is important for induction of TNF-α (32) as well as microbicidal activity (46). Therefore, we examined if macrophages treated with anti-B7-1/B7-2 mAb showed increased induction of NF-κB as measured by EMSA using a consensus NF-κB oligo-deoxynucleotide probe (35) labeled with [γ-^{32}P]-ATP. As compared with the IgG2a control group, intensity of the DNA-protein complex corresponding to the NF-κB proteins was apparently increased upon treatment of macrophages with either B7-1 mAb (Fig. 5A, compare lane 4 with lane 2) or B7-2 mAb (Fig. 5A, compare lane 6 with lane 2). As expected, LPS + IFN-γ treatment increased intensity of the complex (Fig. 5A, compare lanes 5 and 7 with 3). Homologous cold competition assay confirmed the specificity of the DNA-protein complex (Fig. 5A, lane 8). Since p50 and p65 NF-κB play important role in TNF-α gene activation (47, 48), we next examined expression profile of p50 and p65 NF-κB in macrophages treated with anti-B7-1/B7-2 mAb by western blotting. While control macrophages (incubated with IgG2a antibody) showed very little nuclear p50 and p65 NF-κB, LPS + IFN-γ-activated macrophages showed significant induction of these proteins (Fig. 5B and C, compare lane 2 with lane 1). Treatment with anti-B7-1 mAb (Fig. 5B) and anti-B7-2 mAb (Fig. 5C), as compared with the control

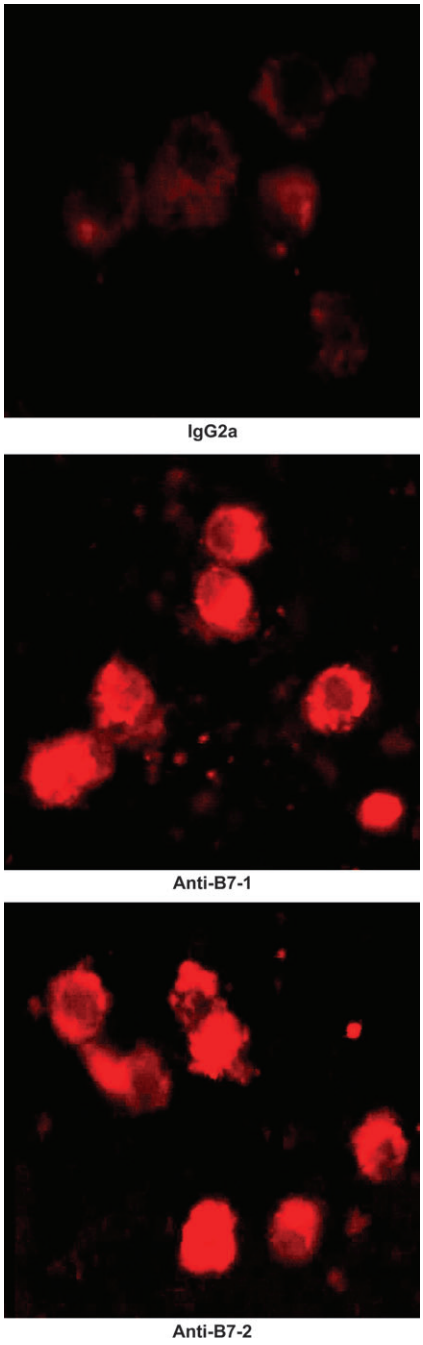


Fig. 3. The anti-B7-1/B7-2 mAb stimulates macrophages for the production of ROS. RAW 264.7 macrophages were treated with 10 μg ml^{-1} of either IgG2a or anti-B7-1 mAb or anti-B7-2 mAb for 1 h. Cells were then incubated with 15 μM dihydrorhodamine-123 for 45 min. Cells were washed and assayed for immunofluorescence on a Nikon fluorescence microscope. Data are representative of five different experiments.

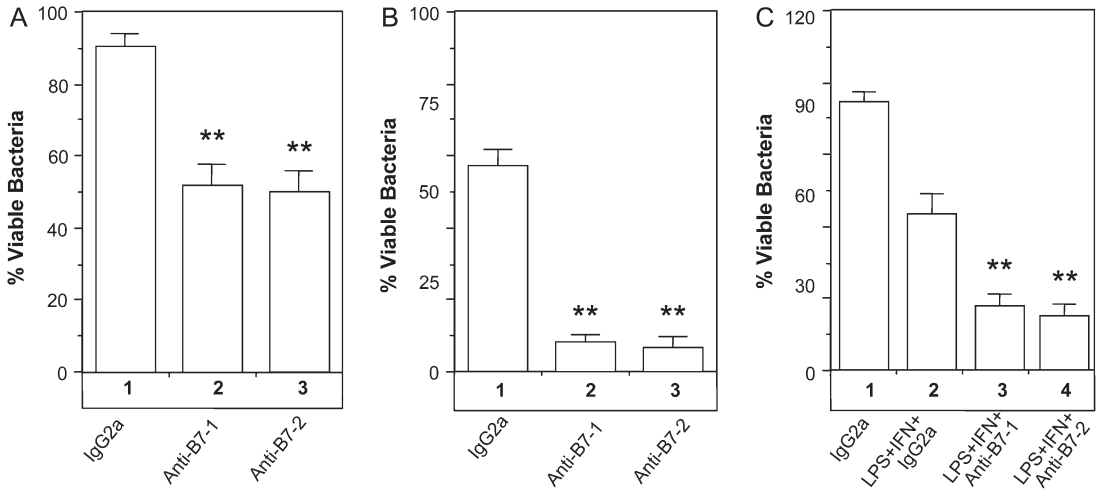


Fig. 4. Macrophages treated with anti-B7-1/B7-2 mAb show increased microbicidal activity. RAW 264.7 macrophages treated with 10 μg ml^{-1} of either IgG2a or anti-B7-1 or anti-B7-2 mAb were incubated with live *Escherichia coli* bacteria for 15 min. Bactericidal activity was monitored after 3 h (A) and 6 h (B). In another experiment, bactericidal assay was performed using macrophages treated with LPS + IFN-γ plus IgG2a or LPS + IFN-γ plus anti-B7-1/B7-2 mAb (C). Data are representative of five different experiments. Statistical significance of these data was determined using Mann–Whitney *U*-test by comparison with the respective controls. **indicates $P < 0.01$.

population, resulted in an increase in p50 and p65 NF-κB proteins (Fig. 5B and C, lane 3). As expected, LPS + IFN-γ-activated macrophages displayed very high expression of NF-κB proteins during treatment with anti-B7-1/anti-B7-2 mAb (Fig. 5B and C, lane 4). These results convincingly show that treatment of macrophages with anti-B7-1/anti-B7-2 mAb results in increased NF-κB activity as evident from EMSA and western blotting.

Inhibition of NF-κB activity by expression of the phosphorylation-defective I-κBα ($^{\Delta}$I-κBα) results in inhibition of TNF-α production in macrophages exposed to anti-B7-1/B7-2 mAb

We next examined whether activation of NF-κB complex by anti-B7-1/B7-2 mAb was actually responsible for increased TNF-α induction in macrophages. The role of NF-κB pathway in the activation of TNF-α in anti-B7-1/B7-2 mAb-treated macrophages was confirmed by inhibiting NF-κB levels using phosphorylation-defective I-κBα ($^{\Delta}$I-κBα) plasmid construct. The RAW 264.7 macrophages were co-transfected with either backbone vector (PRC/CMV) or $^{\Delta}$I-κBα construct along with β-galactosidase vector (pSV-β-galactosidase). After 18 h of transfection, these macrophages were activated with 10 μg ml^{-1} of either IgG2a or anti-B7-1/B7-2 mAb. It could be seen that as compared with the control group (Fig. 6A, lane 2), both anti-B7-1 mAb (Fig. 6A, lane 3) and anti-B7-2 mAb (Fig. 6A, lane 4) increased NF-κB levels in macrophages (Fig. 6A, compare lanes 3 and 4 with lane 2). Transfection with $^{\Delta}$I-κBα inhibited nuclear levels of NF-κB in macrophages treated with either anti-B7-1 mAb (Fig. 6A, compare lane 6 with lane 3) or anti-B7-2 mAb (Fig. 6A, compare lane 7 with lane 4). This reduction in NF-κB activity in the $^{\Delta}$I-κBα transfected macrophages decreased TNF-α production in response to treatment with either anti-B7-1 mAb (Fig. 6B, compare bar 5 with bar 2) or anti-B7-2 mAb (Fig. 6B, compare bar 6 with bar 3). β-Galactosidase protein expression was found to be equivalent in all the groups indicating equal transfection efficiency (Fig. 6C). These data demonstrate that NF-κB plays an important role in the induction of TNF-α in response to anti-B7-1/B7-2 mAb treatment.

Discussion

Blocking the B7-mediated co-stimulation is a promising target for therapeutic intervention in autoimmune diseases (12, 13). Although in human rheumatoid arthritis disruption of the CD28 pathway with CTLA4–Ig is effective (49, 50), however, administration of the anti-B7-1 mAb or the anti-B7-2 mAb has been shown to exacerbate autoimmune diseases in various murine model (13, 51). The mechanism underlying this observation remains largely unknown. Therefore, understanding it is important. In the present *in vitro* study, we provide evidence that the anti-B7-1 mAb and the anti-B7-2 mAb but not the IgG2a isotype-matched antibody activate macrophages for higher production of inflammatory molecules, TNF-α and ROS. Since TNF-α and ROS are known to trigger autoimmune disease (17–27), it is possible that anti-B7-1/B7-2 treatment enhances autoimmune diseases by exacerbating TNF-α–ROS production. The observation that treatment of mice with anti-B7-1/B7-2 antibody results in increased TNF-α level (16) supports the possibility that TNF-α could be the causal factor for the EAE disease severity in mice treated with anti-B7-1/B7-2 mAb. This study indicates that in addition to provide non-cognate co-stimulatory signals to T cells, the B7-1 and the B7-2 molecules also provide signals to macrophages for induction of innate-effector responses, indicating that they are not only essential for induction of adaptive immune responses but also play crucial role in activating innate-effector responses.

The anti-B7-1/B7-2 mAb was found to affect the NF-κB signaling in that the nuclear p50 and p65 NF-κB levels were increased during cross-linking of the B7 ligands (B7-1 and

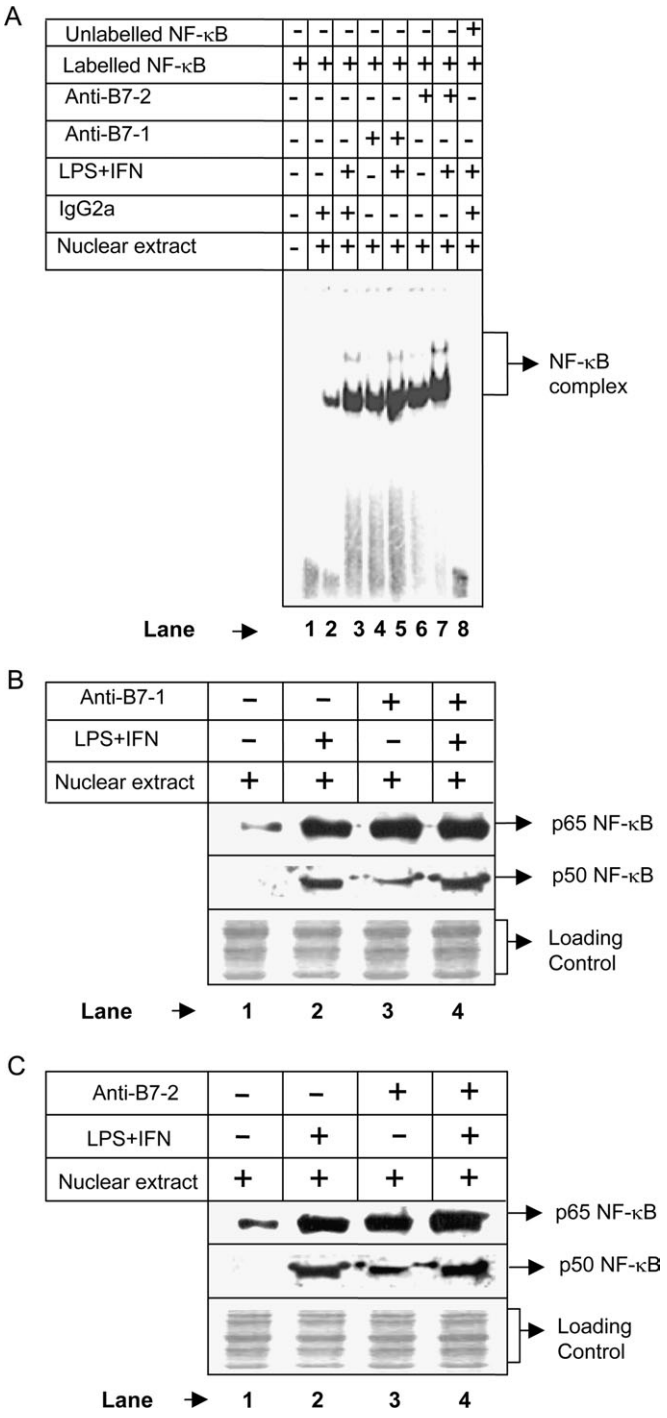


Fig. 5. Macrophages treated with anti-B7-1/B7-2 mAb show increased NF-κB activity. RAW 264.7 macrophages were treated with 10 μg ml^{-1} of IgG2a or anti-B7-1/B7-2 mAb in the absence or presence of LPS + IFN-γ. After 60 min, nuclear proteins were isolated and binding reactions were carried out using a consensus NF-κB oligonucleotide probe labeled with [γ-^{32}P]-ATP (A). Also nuclear extracts were used to examine p65 (B and C, upper panels) and p50 (B and C, lower panels) NF-κB levels by western blotting. The same membranes were stained with Ponceau S stain to confirm equal loading of proteins. Results shown are representative of at least five independent experiments.

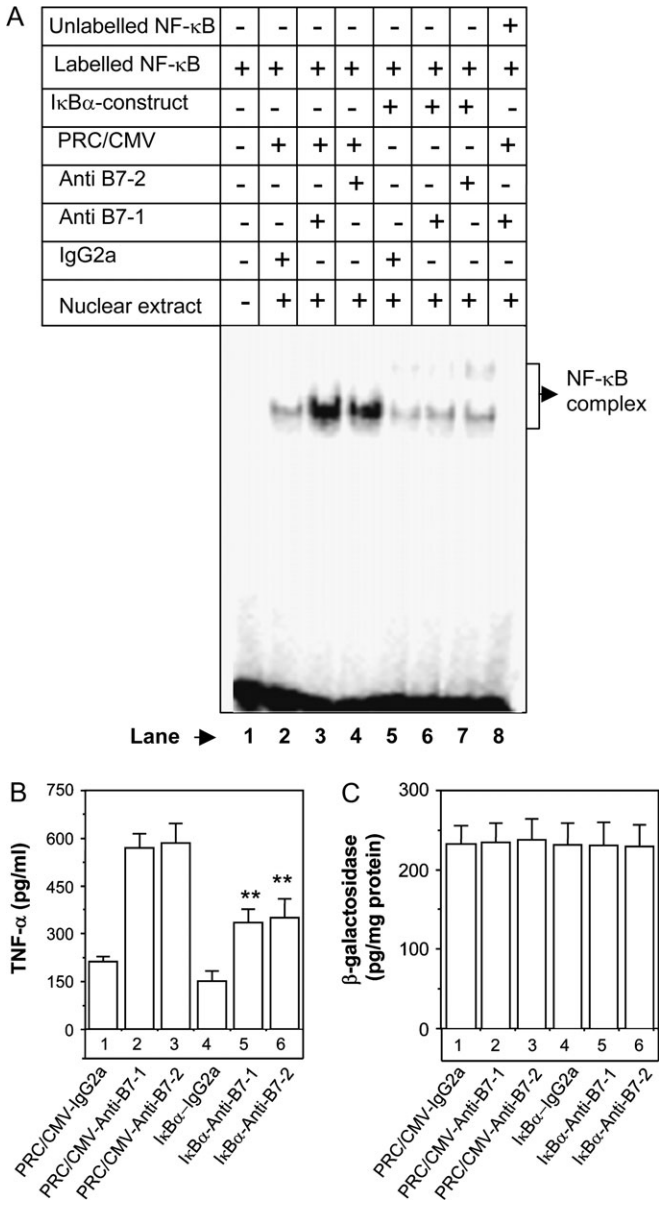


Fig. 6. Transient expression of the phosphorylation-defective I-κBα ($^{\Delta}$I-κBα) construct in the anti-B7-1/B7-2 mAb-treated macrophages results in inhibition of both NF-κB and TNF-α induction. The RAW 264.7 macrophages co-transfected with either $^{\Delta}$I-κBα or mock plasmid (PRC/CMV) along with β-galactosidase construct (pSV-β-galactosidase) were treated with IgG2a or anti-B7-1/B7-2 mAb. NF-κB levels were examined by EMSA (A) and TNF-α production by EIA (B). The β-galactosidase expression was measured following β-galactosidase ELISA (C). Data are representative of five different experiments. Statistical significance of the data shown in bar 5 and bar 6 of (B) was determined using Mann–Whitney *U*-test by comparison with the respective controls. **indicates $P < 0.01$.

B7-2) with the respective antibody. Induction of TNF-α by anti-B7-1/B7-2 mAb was found to be dependent mainly on NF-κB activation since inhibition of the NF-κB activity by expression of the $^{\Delta}$I-κBα resulted in inhibition of TNF-α induction in macrophages treated with anti-B7-1/B7-2 mAb.

Although B7-1 and B7-2 molecules induce differential responses in T cells (12, 52), our findings indicate that in macrophages, however, these molecules probably elicit similar responses. Since both anti-B7-1 and anti-B7-2 mAbs triggered equivalent levels of TNF-α and ROS in macrophages, it is likely that the downstream-signaling cascades triggered in response to stimulation through B7-1 molecules are similar to that of B7-2 molecules. This is further supported by the fact that both anti-B7-1 and anti-B7-2 mAbs could trigger NF-κB activation to almost similar extent in macrophages.

It has been proposed that activation of macrophage inflammatory responses, namely the TNF-α, is a 'danger signal' for induction of autoimmune diseases (17–22). Our studies suggest that anti-B7-1/B7-2 mAb therapy can prime signals for the higher production of TNF-α and ROS from macrophages and thus may contribute to disease severity in various models of autoimmunity. Since B7-1/B7-2 mAb activates induction of TNF-α as well as ROS and bactericidal activity in macrophages, anti-B7-1/B7-2 mAb treatment can be used to tailor immune responses to induce cytotoxicity against intracellular pathogens (53, 54) that reside inside the macrophages.

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Abbreviations

APC	antigen-presenting cell
EAE	experimental allergic encephalomyelitis
EIA	enzyme-linked immunoassay
EMSA	electrophoretic mobility shift assay
IIL	Indian Immunologicals Ltd
I-κBα	I-kappaB alpha
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor-kappaB
PEC	peritoneal exudate cell
RT	reverse transcriptase
ROS	reactive oxygen species
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor alpha

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