Induction of alloantigen-specific tolerance by B cells from CD40-deficient mice

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ABSTRACT Interaction between CD40 on B cells and CD40 ligand molecules on T cells is pivotal for the generation of a thymus-dependent antibody response. Here we show that B cells deficient in CD40 expression are unable to elicit the proliferation of allogeneic T cells in vitro. More importantly, mice immunized with CD40+/− B cells become tolerant to allogeneic major histocompatibility complex (MHC) antigens as measured by a mixed lymphocyte reaction and cytotoxic T-cell assay. The failure of CD40+/− B cells to serve as antigen presenting cells in vitro was corrected by the addition of anti-CD28 mAb. Moreover, lipopolysaccharide stimulation, which upregulates B7 expression, reversed the inability of CD40+/− B cells to stimulate an alloresponse in vitro and abrogated the capacity of these B cells to induce tolerance in vivo. These results suggest that CD40 engagement by CD40 ligand expressed on antigen-activated T cells is critical for the upregulation of B7 molecules on antigen-presenting B cells that subsequently deliver the costimulatory signals necessary for T-cell proliferation and differentiation. Our experiments suggest a novel strategy for the induction of antigen-specific tolerance in vivo.

Two antigen nonspecific ligand receptor systems have been distinguished that mediate efficient activation of T and B cells. The first system is characterized by the interactions of CD28 and CTLA-4 on T cells with their physiological counter receptors, the B7 molecules, expressed on B cells and other antigen presenting cells (reviewed in ref. 1). CD28 is found both on resting and activated T cells, while CTLA-4 is expressed only after successful stimulation of T cells (2). The B7 family of receptors consists of present on two cloned molecules, B7.1 and B7.2, and a putative third member, B7.3 (3). Although crosslinking of CD28 alone does not deliver an activation signal to resting T cells, stimulation of preactivated T cells with anti-CD28 monoclonal antibodies (mAb) results in a vigorous proliferative response and marked secretion of multiple lymphokines (1). Conversely, prevention of signaling through CD28 in cognate T-B cell interaction using Fab fragments of an anti-CD28 antibody results in abrogation of antigen-specific proliferation and in failure to respond to a subsequent antigenic challenge (4, 5).

The second receptor ligand system important for lymphocyte activation involves the binding of the B-cell antigen CD40 to its physiological ligand, CD40 ligand (CD40L) expressed on activated T cells. Ligation of CD40 on B cells mediates a wide range of biological activities including homotypic adhesion, proliferation, expression of costimulatory molecules B7.1 and B7.2, and Ig isotype switching (6–9). CD40L is transiently expressed predominantly on CD4+ T cells after T-cell receptor (TCR) triggering and independently of CD28/CTLA-4 co-stimulation (7, 10).

The capacity of B cells to provide ligands for T-cell specific costimulatory molecules determines the outcome of T-cell activation. Resting B cells express only limited amounts of costimulatory molecules compared to activated B cells (11) and are thus ineffective as antigen presenting cells (12). This concept has also been demonstrated in vivo. For instance, monovalent rabbit anti-mouse IgD when presented by small resting B cells rendered mice tolerant to rabbit Ig (13). Polyclonal activation of antigen presenting B cells (e.g., by crosslinking with divalent rabbit anti-mouse IgD) prevented the induction of tolerance (13). Injection of resting allogeneic B cells has however failed to induce tolerance to allogeneic major histocompatibility complex (MHC) molecules, the principal T-cell targets in an allogeneic immune response (14). This may be due to the high frequency of allospecific T cells and/or to in vivo activation of the transferred B cells following their recognition by host T cells. If the latter explanation is correct, in vivo tolerance should be induced when allogeneic B cells are incapable of providing the costimulatory signal essential for initiation of T-cell activation. To test this hypothesis, we have determined the capacity of CD40-deficient B cells to elicit an allogeneic response in vitro and in vivo.

MATERIALS AND METHODS

Animals. The CD40−/− mice were generated at the Center for Blood Research by a targeting construct eliminating exons 7, 8, and 9 of CD40 (15). Mutant embryonic stem cells from the J1 line heterozygous for CD40 gene-targeting were used for injection into C57BL/6 blastocysts. Chimeric founder mice were mated with C57BL/6 mice and inbreeding of the heterozygous mice resulted in mice homozygous for the CD40 deficiency. The phenotype of these knockout mice is identical to the one of CD40−/− mice obtained by recombination-activating gene-2-deficient blastocyst complementation (15). Wild-type C57BL/6 (H-2b), B10.BR (H-2h), and BALB/c (H-2d) mice were obtained from The Jackson Laboratory.

Cell Isolation. T and B cells were isolated from spleen and lymph nodes by magnetic cell separation using positive selection by Thy 1.2 or B220 (CD45R) magnetic beads, respectively (Milteny, Biotec, Sunnyvale, CA). Isolated cells were typically >97% pure as determined by flow cytometry and appeared viable by exclusion of trypan blue and by forward/side scatter analysis. Purified splenic B cells for in vivo experiments were obtained from single-cell suspensions after plastic adherence and two rounds of treatment with anti-Thy 1.2 mAb (clone 30H-12) at 4°C followed by an incubation with low TOX-M rabbit complement (Cedarlane Laboratories). B-cell purity was regularly >90%.

Abbreviations: APC, antigen presenting cell; CD40L, CD40 ligand; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TCR, T-cell receptor.

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Antibodies and Fusion Proteins. Antibodies against the following molecules were obtained from PharMingen: CD3-e (clone 145-2C11), CD8a, CD28 (37.51), CD40L (MR1), B200 (CD45R; RA3-6B2), B7-1 (CD80; 1G10), B7-2 (CD86; GL1). The expression of CD40 was detected by a CD40L-CD8α fusion protein (gift of P. Lane, Basel Institute for Immunology; ref. 16) followed by staining with anti-CD8α mAb. Soluble CD40 (sCD40), a fusion protein consisting of the extracellular domain of human CD40 and the Fc portion of human IgG1 (17) and murine CTLA-4/Ig (a generous gift from T. Strom, Beth Israel Hospital, Boston) were used for blocking studies in vitro.

Mixed Lymphocyte Reaction (MLR). Various numbers of irradiated (2000 rads) splenic B cells from wild-type or CD40−/− C57BL/6 mice (H-2b) were seeded into round bottom 96-well microtiter plates (Costar) as stimulators and cocultured with 1-2 × 10⁵ purified T cells from BALB/c mice (H-2b) in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma), penicillin, streptomycin, and 2-mercaptoethanol (50 μM). Where indicated, mAb and fusion proteins (1 μg/ml) of different specificity were added to the MLR at the onset of culture. For experiments with preactivated cells, purified B cells were stimulated for 24 hr with lipopolysaccharide (LPS) (20 μg/ml) prior to extensive washing, irradiation (2000 rads), and seeding to 96-well microtiter plates. Proliferation was measured by incorporation of [³H]-thymidine during the last 16 hr of a 5-day culture. The standard error for each data point was less than 15% of the mean.

Flow Cytometry. Purified B cells (5 × 10⁵/200 μl) were cultured in vitro for 48 hr in 96-well round bottom plates either in the presence of LPS (20 μg/ml; Sigma) or CD40L/CD8 chimeric protein (1 μg/ml), then analyzed by flow cytometry for the surface expression of B220 (fluorescein isothiocyanate-labeled RA3-6B2), B7.1 (PE-labeled 1G10), and B7.2 (phosphatidylethanolamine-labeled GL1) using a FACSscan and LYSYS software (Becton Dickinson).

In Vivo Immunization. BALB/c mice were injected intravenously with unstimulated or extensively washed LPS-stimulated B cells (40-50 × 10⁶) from wild-type and CD40−/− C57BL/6 mice. Recipients were sacrificed 6 days later and T cells were purified from spleens.

Cytotoxic T-Lymphocyte Activity. Cytotoxic activity was measured by a standard ⁵¹Cr-release assay using splenic T cells from in vivo primed BALB/c mice. T cells were mixed with 1 × 10⁴ ⁵¹Cr-labeled EL-4 (H-2b) or P815 (H-2b) target cells at different effector-to-target ratios. Specific lysis was calculated by comparison of ⁵¹Cr release in the supernatant of control and experimental cultures after 6 hr after onset. The standard error for each data point was less than 15% of the mean.

RESULTS

CD40−/− B Cells Fail to Stimulate an Alloresponse in Vitro. Fig. 1 shows that splenic B cells from wild-type C57BL/6 mice (H-2b) stimulated the proliferation of BALB/c T cells (H-2b) in a dose-dependent fashion. In contrast, splenic B cells from CD40−/− mice failed to elicit an allogeneic response using an identical range of stimulator cells. The inability of CD40−/− B cells to stimulate an alloresponse is not restricted to the spleen, since B cells isolated from lymph nodes of CD40−/− mice also failed to stimulate the proliferation of allogeneic T cells (Fig. 1). Furthermore, mixing experiments with wild-type and CD40−/− B cells did not result in a suppression of an allogeneic T-cell response (data not shown), thus excluding the possibility of an inhibition of proliferation by mutant B cells.

Costimulation via CD28 Reverses the Inability of CD40−/− to Stimulate an Alloresponse. We first considered the possibility that the failure of CD40−/− B cells to evoke an allogeneic response was due to lack of CD40L engagement on T cells. Ligation of CD40L either by soluble CD40/Ig protein (sCD40) or by anti-CD40L mAb was unable to correct the deficiency of CD40−/− B cells to stimulate an allogeneic response (data not shown).

We next investigated whether co-engagement of CD28 by mAb can permit responder T cells to be activated by allogeneic CD40-deficient B cells. As shown in Fig. 2, addition of mAb against CD28 caused BALB/c T cells to proliferate in response to CD40−/− B cells. Importantly, in the presence of anti-CD28, the magnitudes of T-cell proliferation elicited by CD40−/− and wild-type B cells were equivalent. These results suggest that the failure of CD40−/− B cells to stimulate an allogeneic response may be due to deficiency in B7 expression and failure to engage CD28.

Induction of B7 Expression in CD40−/− B Cells Reverses Tolerogenicity. As previously reported (6), anti-CD40 mAb induced B7.1 and B7.2 expression on wild-type B cells that was comparable to that induced by LPS but did not induce B7.1 and...
B7.2 expression on CD40−/− B cells (data not shown). To rule out the possibility that CD40−/− B cells are inherently deficient in B7 expression we stimulated mutant B cells with LPS and examined B7 surface expression by flow cytometry. Fig. 3 shows that stimulation by LPS resulted in comparable expression of B7.1 and B7.2 in wild-type and CD40−/− B cells.

Because LPS induced the expression of B7 molecules on CD40−/− B cells, we determined whether pre-activation with LPS may overcome the deficiency of CD40−/− B cells to evoke an allogeneic response in vitro. Fig. 4 shows that CD40−/− B cells pre-activated with LPS induced an allogeneic response equivalent to that of wild-type B cells pretreated with LPS. Murine CTLA-4/Ig completely inhibited the MLR induced by LPS activated CD40−/−. Thus, the capacity of LPS to confer costimulatory activity on CD40−/− B cells was most likely due to the induction of B7 expression on these cells further confirming the importance of the B7/CD28/CTLA-4 interaction in T-cell activation.

**CD40−/− B Cells Induce Allogenic Tolerance in Vivo.** Engagement of the TCR in the absence of additional costimulatory signals via CD28/CTLA-4 molecules has been demonstrated to result in antigen-specific nonresponsiveness (18). We therefore examined whether the lack of CD40-mediated up-

![Fig. 3](image)

**Fig. 3.** B7 expression after stimulation of wild-type and CD40-deficient B cells with LPS. Purified B cells from wild-type and CD40−/− mice were stimulated for 48 hr in the presence of LPS (20 μg/ml). Expression of B7.1 and B7.2 on stimulated (filled curve) and unstimulated (open curve) B cells was then analyzed by flow cytometry.

![Fig. 4](image)

**Fig. 4.** Efficient allore cognition of CD40-deficient B cells is achieved by LPS pretreatment and can be blocked by CTLA-4/Ig. Purified B cells from wild-type (solid bar) and CD40−/− (lined bar) C57BL/6 mice were stimulated for 24 hr with LPS (20 μg/ml) prior to the seeding (2 × 10⁴) to microtiter wells for coculture with purified BALB/c T cells (5 × 10⁴/well). The cultures were performed in the presence or absence of CTLA-4/Ig (1 μg/ml) or isotype matched control Ig (1 μg/ml) (solid bar). This graph is representative of three independent experiments.

regulation of B7 molecules mediates also a state of allogeneic tolerance in vivo. B cells from wild-type or CD40−/− mice were injected intravenously into naive BALB/c recipients. Splenic T cells were obtained 6 days later and tested for their allogeneic proliferative and cytotoxic response to wild-type target cells. Fig. 5A shows that splenic T cells from BALB/c mice primed with wild-type B cells mounted a vigorous proliferative response to wild-type C57BL/6 splenic B cells in vitro. In contrast, T cells from BALB/c mice primed with CD40-deficient B cells were virtually unable to proliferate upon in vitro re-exposure to wild-type (CD40+/+) C57BL/6 B cells. This marked failure was alloantigen-specific because wild-type B10.BR B cells evoked a proliferative response by T cells of primed BALB/c mice that was the same regardless whether the mice were injected with wild-type or with CD40−/− C57BL/6 B cells. Similarly, T-cell cytotoxic activity of BALB/c mice primed with CD40−/− B cells was greatly reduced when compared with BALB/c mice injected with wild-type C57BL/6 B cells (Fig. 5B).

**Activated CD40−/− B Cells Induce a Normal Allogenic Response in Vivo.** Since the failure of CD40−/− B cells to stimulate an allogeneic response was overcome in vitro by pretreatment of B cells for 24 hr with LPS, we investigated whether LPS pretreatment would also prevent the in vivo induction of tolerance by CD40−/− B cells. As shown in Fig. 5C, T cells from BALB/c mice primed with LPS activated CD40−/− C57BL/6 B cells proliferated vigorously to wild-type C57BL/6 stimulator B cells. The magnitude of proliferation was equivalent to that of BALB/c T cells from mice primed with LPS activated wild-type B cells.

**DISCUSSION**

T-cell proliferation and their differentiation to various effector functions results from a combination of an antigen-specific, MHC-restricted first signal via TCR engagement and a second antigen-independent signal mediated by costimulatory surface molecules. In the absence of a second signal, T cells will enter a state of antigen-specific unresponsiveness, termed functional inactivation or clonal anergy (18, 19). Whereas the importance of the CD28/CTLA-4/B7 system for (allogenic and xenoge-
neic) transplantation could be demonstrated by blocking studies in vitro and in vivo, only limited knowledge has accumulated regarding the sequence of physiological events that result in the upregulation of B7 on APCs.

Resting B cells express low densities of B7.1 and B7.2 and are therefore not competent to serve as APCs. T-cell activation via TCR engagement results in the surface expression of CD40L, a contact-dependent process separate from B7/CD28/CTLA-4 pairing (10). Conversely, activated T cells induce normal B cells to increased B7 and CD54 (ICAM-1) expression via a process that also requires direct cell–cell contact (6). The mechanism of B7 induction and thus enhanced B-cell antigen-presenting cell activity is CD40/CD40L dependent since the addition of anti-CD40 mAbs or anti-CD40L to the cognate B/T-cell interactions inhibits significantly the increase in B7 expression (6, 20, 21). Moreover, in vivo co-administration of allogenic B cells and anti-CD40L mAbs prevented the induction of an in vitro proliferative and cytotoxic T-cell response further emphasizing the paramount importance of the CD40/CD40L axis for T-cell activation (22).

In this report we demonstrate that B cells deficient in CD40 expression fail to evoke an allogenic T-cell response both in vitro and in vivo and significantly diminish allospecific T-cell cytotoxic activity. The presented data: (i) the induction of B7 expression by anti-CD40 mAbs, (ii) the reconstitution of the CD40−/− B-cell antigen-presenting capacity in the presence of anti-CD28 mAbs and by treatment with LPS that induces B7 expression, and (iii) the inhibition by CTLA-4/Ig of the ability of LPS to rescue the alloantigen presenting capacity of CD40−/− B cells, suggest that CD40L/CD40 interactions play a major role in the surface expression of B7 during contact of T cells with allogenic B cells. Moreover, these results suggest that other receptor ligand pairs cannot substitute for the lack of CD40-mediated B7 expression and costimulation. By contrast, CD40/CD40L interaction is dispensable for maturation into IgM secreting B cells, although it is required for subsequent isotype switching (15). They also imply that TCR-mediated recognition and crosslinking of allo-MHC class II molecules in an immune response may not suffice for a substantial and prolonged upregulation of B7 on B cells to provide costimulatory signals. In this regard, antibody-mediated crosslinking of MHC class I molecules on B cells does not transduce signals that result in sustained and abundant upregulation of B7 (refs. 6 and 23; unpublished results). Thus, upregulation of B7 expression in the course of a cognate T-B cell contact appears to be predominantly dependent on CD40L/CD40 interaction.

The possible importance of B7 expression for early signaling events in the cognate T-B cell interaction in vivo was further underlined by our experiments with pre-activated B cells. Treatment of B cells with LPS in vitro caused the upregulation of B7 molecules on both CD40+/+ and CD40−/− B cells that were now equally competent to stimulate a proliferative allogeneic T-cell response. The critical importance of B7 for the regained antigen-presenting capacity of LPS-activated CD40−/− B cells was dem-

CD40+/+ B cells induce diminished cytotoxic T-cell activity in vivo. BALB/c mice were injected intravenously with unmanipulated, purified B cells from wild-type (■) and CD40−/− (open symbols) C57BL/6 donors. Recipient animals were sacrificed 6 days later and the cytotoxic T-cell activity was measured using 51Cr-labeled EL-4 (squares) and P815 (circles) target cells. The graph is representative of two experiments. (H) Pre-activation overcomes the deficit of CD40+/− B cells to stimulate an allogeneic response in vivo. Purified B cells from wild-type (■) and CD40−/− (○) C57BL/6 donors were stimulated for 24 hr with LPS, washed extensively, and injected intravenously into naive BALB/c mice. Recipient mice were sacrificed after 6 days and purified splenic T cells (1 × 105) were used for an MLR against wild-type C57BL/6 B stimulator cells. The graph is representative of three independent experiments.

Fig. 5. In vivo priming with CD40-deficient C57BL/6 B cells inhibits the allospecific proliferative and cytotoxic responses of splenic T cells from BALB/c mice. (A) CD40+/+ B cells induce antigenspecific tolerance in vivo as measured by MLR. BALB/c mice were injected intravenously with unmanipulated, purified B cells from wild-type (■) and CD40−/− (open symbols) C57BL/6 donors. Recipient animals were sacrificed 6 days later and the proliferative response of purified splenic T cells was measured in a 5-day MLR against irradiated wild-type C57BL/6 (squares) or B10.BR B cells (circles). The graph is representative of three independent experiments. (B)
onstrated by the complete inhibition of the MLR in the presence of CTLA-4/Ig. Moreover, the intravenous transfer of prestimulated B cells resulted in efficient priming of BALB/c mice indicating that LPS-activated B cells of CD40-deficient mice express all the costimulatory molecules necessary to elicit an allogeneic immune response. Thus, in vivo clonal anergy could be overcome by pre-activation of CD40−/− B cells. These findings parallel recent in vitro experiments, where costimulatory activity of LPS- or CD40L-stimulated allogeneic B cells was largely dependent on B7 expression (21, 24).

Our results that B cells deficient in CD40 expression induce clonal anergy in vivo are also interesting to regards with self-restricted T-cell recognition of processed alloantigens, a mechanism known as indirect alloantigen presentation (reviewed in ref. 25). Engrafted allogeneic cells shed MHC fragments that, when taken up and processed by host APCs, are presented as antigenic peptides by host-MHC to activate T cells in a self-MHC restricted fashion. Since the primed B-cell recipients in our experiments were entirely immunocompetent, one may have anticipated that indirect alloantigen presentation would circumvent tolerance induction by CD40−/− B cells. However, allore cognition by this alternative pathway must not have contributed significantly to the overall response.

Induction of tolerance by CD40−/− B cells has important implications for organ transplantation. In addition our results suggest that syngeneic CD40-deficient B cells may be used as “tolerogenic” APCs in the induction of clonal anergy to peptide antigens.

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