CD40-deficient mice generated by recombination-activating gene-2-deficient blastocyst complementation

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ABSTRACT  To study the role of the B-cell antigen CD40 in immune responses, mouse embryonic stem (ES) cells in which both copies of the gene encoding CD40 had been disrupted by homologous recombination were injected in RAG-2 (recombination-activating gene-2)-deficient blastocysts to generate chimeras in which all mature lymphocytes are derived from the CD40-deficient ES cells. T- and B-cell number and phenotype were normal in the CD40+/− chimeras. However, B cells failed to proliferate and undergo isotype switching in vitro in response to soluble CD40 ligand (sCD40L) with interleukin 4 (IL-4) but responded normally to lipopolysaccharide (LPS) with IL-4. CD40−/− chimeras completely failed to mount an antigen-specific antibody response or to develop germlinal centers following immunization with the T cell-dependent (TD) antigen keyhole limpet hemocyanin. In contrast, CD40−/− mutant mice responded normally to the T cell-independent (TI) antigens 2,4,6-trinitrophenyl (TNP)-LPS and TNP-Ficoll. The most noticeable alteration in the serum immunoglobulin levels of young (6-8 weeks old) CD40−/− animals was absence of IgE and severe decrease of IgG1 and IgG2a. These results confirm the essential role of CD40–CD40L interactions in the antibody response to TD antigens and in isotype switching.

CD40, a member of the tumor necrosis factor (TNF) receptor family of molecules, is expressed on all mature B lymphocytes (1). The ligand for CD40 belongs to the TNF family of molecules and is transiently expressed by activated CD4+ T cells in vitro and in vivo (reviewed in ref. 2). CD40 plays an important role in B-cell growth and differentiation. Anti-CD40 monoclonal antibodies (mAbs) deliver an antiapoptotic signal to immature B cells and synergize with phorbol esters, anti-CD20 mAb, anti-IgM antibodies, and interleukin 4 (IL-4) in causing proliferation of resting B cells (reviewed in ref. 2). Most importantly, CD40 engagement in the presence of cytokines induces class switching in B cells via deletional switch recombination (3-5). Interaction between CD40 and its ligand, CD40L, plays a critical role in T cell-dependent (TD) isotype switching in vitro and in vivo. Several groups have shown that the gene encoding CD40L is mutated and that CD40L expression is deficient in patients with chromosome X-linked hyper-IgM syndrome (HIGMX-1), who are unable to undergo isotype switching (6-10).

To study the role of CD40 in B-cell activation and differentiation in vivo, we generated CD40-deficient mice, using the recombination-activating gene 2 (RAG-2)-deficient blastocyst complementation strategy. RAG-2-deficient mice do not develop mature T and B cells (11). Injection of embryonic stem (ES) cells into RAG-2-deficient blastocysts generates chimeric mice in which all mature lymphocytes are derived from the injected ES cells (12). Thus, CD40−/− ES cells were injected into RAG-2-deficient blastocysts, and the chimeric mice were analyzed. T- and B-cell number and phenotype were normal in the CD40−/− chimeras, but their B cells failed to proliferate and undergo isotype switching in vitro in response to soluble CD40L (sCD40L)/IL-4. CD40−/− chimeras completely failed to mount an antigen-specific antibody response or to develop germlinal centers following immunization with the TD antigen keyhole limpet hemocyanin (KLH) but responded normally to the TI cell-independent (TI) antigens 2,4,6-trinitrophenyl-conjugated lipopolysaccharide (TNP-LPS) and TNP-Ficoll. These results confirm the essential role of CD40–CD40L interactions in the antibody response to TD antigens and in isotype switching.

MATERIALS AND METHODS

Construction of Mice with CD40-Deficient Lymphocytes. We isolated from a 129 Sv genomic library a 13-kb genomic DNA and prepared a targeting construct that eliminated exons 7, 8, and 9 (coding for the transmembrane and cytoplasmic regions) and allowed for both positive and negative selection (Fig. 1A). We transfected the linearized construct into ES cells of the J1 line and generated CD40−/− clones (Fig. 1B). We then selected homozygous CD40−/− (Fig. 1B) clones with increasing concentrations of G418 (12).

Cell Analyses. The number of lymphoid cells and their phenotype was determined in spleen, bone marrow, lymph nodes, and thymus. Southern blots and flow cytometry were performed with standard techniques. B- and T-cell surface markers were examined by double immunofluorescence using commercially available phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated mAbs to IgM, IgD, B220, CD43 (S7), CD4, and CD8 (PharMingen). Murine sCD40L, constructed as a fusion protein between the extra-cellular portion of CD40L and the extracellular portion of CD8a, was partially purified from the supernatant of a hybridoma secreting the chimeric protein (kind gift of P. Lane, Basel) (13). To detect CD40, B cells were incubated with sCD40L followed by anti-mouse CD8-FITC. CD8 positivity on B220+ cells demonstrates the presence of CD40 on the surface. The data were analyzed on a FACSscan instrument (Becton Dickinson) using the LYSYS software.

B-Cell Proliferation and Immunoglobulin Synthesis. Single-cell suspensions from spleen were examined for proliferation.

Abbreviations: CD40L, CD40 ligand; sCD40L, soluble CD40L; ES, embryonic stem; HIGMX-1, chromosome X-linked hyper-IgM syndrome; IL-4, interleukin 4; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; RAG-2, recombination-activating gene 2; TD, T cell dependent; TI, T cell independent; mAb, monoclonal antibody; TNP, 2,4,6-trinitrophenyl.
and immunoglobulin synthesis. Proliferation was measured as \(^{3}H\)thymidine incorporation after stimulation for 3 days of 10^5 cells per well. Immunoglobulin secretion in the supernatants of 5-day cultures was measured by ELISA.

**Immunizations.** Chimeric mice were immunized as follows. One group received 400 µg of an alum precipitate of KLH together with 10^9 Bordetella pertussis organisms. A booster dose of 30 µg of KLH was given after 14 days. A second group received 100 µg of TNP-LPS (gift of Dr. A. Abbas, Harvard University, Boston), and a third group received 250 µg of TNP-Ficol (gift of Dr. F. Finkelman, Uniformed Services University of The Health Sciences, Bethesda). All antigens were resuspended in saline and administered i.p. Serial dilutions of sera were analyzed for KLH or TNP isotype-specific antibodies by ELISA.

**ELISA Assays.** Immunoglobulin isotypes were measured by using polyclonal antibodies to IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnology Associates); anti-IgM and anti-IgG (Zymed); and two different mAbs to IgE (PharMingen). To detect specific antibodies, plates were coated with KLH or TNP-conjugated bovine serum albumin. Alkaline phosphatase-conjugated isotype-specific antibodies were used as revealing antibodies.

**Tissue Processing and Immunoperoxidase Technique.** Tissue samples from spleen, axillary and mesenteric lymph nodes, and small intestine were either fixed in formalin or frozen in OCT compound (Ames, Elkhart, IN). Frozen tissue sections, 4 µm thick, were stained with mAbs to CD3e, B220, IgM, IgD, and IgA by the avidin–biotin complex method (14). In addition, frozen sections were stained with biotin-labeled peanut agglutinin (Vector Laboratories) to demonstrate germinal centers.

**RESULTS**

**Southern Blot and Macroscopic Analysis of Lymphoid Organs in CD40^+/- Chimeras.** Complementation of RAG-2^-/- blastocysts with CD40^+/-, CD40^0/-, or CD40^-/- ES cells generated chimeras in which ES cell-derived B and T cells populated the lymphoid organs as confirmed by Southern blot analysis of thymus and spleen (Fig. 1C). The size and the cellularity of the lymphoid organs in the CD40^-/-, CD40^+/-, and CD40^0/- chimeras were equivalent (data not shown). As previously observed (12), ~20% of the injections lead to nonchimeras as evidenced by a RAG-2^-/- phenotype.

**Phenotypic Analysis of Lymphocytes in CD40^-/- Chimeras.** B- and T-cell surface markers were analyzed by cytofluorometry in the bone marrow, thymus, spleen, and lymph nodes in 6- to 8-wk-old chimeric and RAG-2-deficient mice. Table 1 shows the expression of CD4 and CD8 in thymus and spleen of CD40^-/- mice was equivalent to that of CD40^+/- mice; the expression of the B-cell surface markers B220, IgM, and IgD in the spleen and bone marrow of CD40^-/- and of CD40^0/- chimeras was similar. The same results were obtained in lymph nodes and peripheral blood of CD40^-/- and CD40^0/- chimeras (data not shown). Cell surface CD40 expression was examined on B220^+ cells in bone marrow, spleen, and peripheral blood. Fig. 2B shows

**Table 1. In vitro proliferation and immunoglobulin secretion of spleen cells**

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<thead>
<tr>
<th></th>
<th>IgM production, µg/ml</th>
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<tr>
<td></td>
<td>CD40^+/-</td>
<td>CD40^-/-</td>
<td>CD40^-/-</td>
</tr>
<tr>
<td>Proliferation, cpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>1,485</td>
<td>1,730</td>
<td>1.12</td>
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<tr>
<td>IL-4</td>
<td>6,250</td>
<td>9,705</td>
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<tr>
<td>LPS</td>
<td>121,895</td>
<td>136,279</td>
<td>80</td>
</tr>
<tr>
<td>+IL-4</td>
<td>107,100</td>
<td>124,355</td>
<td>50</td>
</tr>
<tr>
<td>CD40L</td>
<td>18,740</td>
<td>2,480</td>
<td>0.27</td>
</tr>
<tr>
<td>+IL-4</td>
<td>215,130</td>
<td>6,785</td>
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**Experiment 1**

**Experiment 2**

<table>
<thead>
<tr>
<th></th>
<th>IgG1 production, µg/ml</th>
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<tbody>
<tr>
<td>CD40^+/-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD40^-/-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD40^-/-</td>
<td>ND</td>
<td>ND</td>
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Similar results were obtained in two other experiments. ND, not detected (limit of detection was <0.007 µg/ml).
A

CD40+/+ RAG-2/- CD40/-

Thymus

CD4

CD8

Spleen

IgG

IgM

Bone marrow

B220

IgG

IgM

B

Spleen Bone marrow PBMC

CD40+/+

B220

CD4

CD8

CD40-/+

B220

CD4

CD8

Fig. 2. (A) Flow cytometry analysis of cells from thymus, spleen, and bone marrow of CD40+/+ chimeras, RAG-2/- mice, and CD40/-/ chimeras. (B) Flow cytometry analysis of CD40 expression in spleen, bone marrow, and peripheral blood mononuclear cells of CD40+/+ and CD40/-/ chimeras. Results are presented as two-dimensional dot-plots in which each dot represents an individual cell. Similar results were obtained in three experiments.

B

Spleen Bone marrow PBMC

CD40+/+

B220

CD4

CD8

CD40-/+

B220

CD4

CD8

Fig. 3. Serum antibodies in response to immunization with TNP-KLH, TNP-LPS, and TNP-Ficoll of CD40+/+ (○) and CD40/-/ (●) chimeras. Serial dilutions of serum were analyzed for KLH and TNP IgM- and IgG-specific antibodies. Results are expressed as OD at 405 nm of isotype-specific ELISA with KLH- and TNP-conjugated bovine serum albumin as capture reagents. Serum dilutions are indicated on the horizontal axes. Similar results were obtained in three experiments.

that B220+ cells from CD40/-/ chimeras completely failed to express CD40. Moreover, sCD40L caused up-regulation of major histocompatibility complex class II antigen and CD23 expression in CD40+/+ but not in CD40/-/ chimeras (data not shown).

In Vitro B-Cell Response to CD40 Engagement. It has been shown (3) that normal B cells proliferate and undergo lymphokine-dependent isotype switching in response to CD40 ligation by sCD40L. Table 1 shows that spleen cells from 7-wk-old CD40+/+ (experiment 1) and 6-wk-old CD40+/+ (experiment 2) chimeras proliferated to sCD40L and secreted IgG1 and IgE in response to sCD40L/IL-4. In contrast, spleen cells from CD40/-/ chimeras failed to proliferate to

Table 2. Serum Ig levels

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Serum Ig levels, μg/ml</th>
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<tbody>
<tr>
<td>Pheno-</td>
<td>IgM</td>
</tr>
<tr>
<td>type</td>
<td>Age, wk</td>
</tr>
<tr>
<td>CD40+/+</td>
<td>5</td>
</tr>
<tr>
<td>CD40+/+</td>
<td>8</td>
</tr>
<tr>
<td>CD40+/+</td>
<td>8</td>
</tr>
<tr>
<td>CD40/-/</td>
<td>5</td>
</tr>
<tr>
<td>CD40/-/</td>
<td>8</td>
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<tr>
<td>CD40/-/</td>
<td>8</td>
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ND, not detected (limit of detection was <0.007 μg/ml).
sCD40L and failed to secrete IgG1 and IgE in response to sCD40L/IL-4. However, proliferation to LPS and secretion of IgG1 and IgE upon LPS/IL-4 stimulation were equivalent in CD40−/− and control chimeras.

**TD and TI Antibody Responses.** To investigate the role of CD40 in the antibody response to TD antigens, 6-wk-old mice were immunized with KLH on day 0 and administered a booster on day 14, and titers of IgM and IgG anti-KLH were determined on days 14 and 21. Fig. 3 shows that by day 21, CD40+/+ control chimeras had mounted both an IgM and an IgG response to KLH. In contrast, CD40−/− chimeras failed to respond with either IgM or IgG antibody to immunization with KLH. To examine the role of CD40 in the antibody response to TI antigens, 6-wk-old mice were immunized with the TI-type 1 antigen TNP-LPS and with the TI-type II antigen TNP-Ficoll. Fig. 3 shows that 14 days after immunization with TNP-LPS or TNP-Ficoll, CD40+/+ chimeras mounted IgM as well as IgG anti-TNP responses equivalent to those of CD40+/+ chimeras.

**Germinal Center Formation.** In control CD40+/+ mice immunized with KLH, lymphoid follicles with germinal centers were present in all lymphoid tissues examined (spleen, lymph nodes, and Peyer’s patches). The mantle zones (IgM+, IgD+) and germinal centers (IgM+, IgG+, or IgA+) showed characteristic staining patterns for immunoglobulins (Fig. 4 Left) and also stained intensely with peanut agglutinin. IgA+ cells were present in the spleen, Peyer’s patches, and lamina propria of the small intestine. In the CD40−/− mutant mice immunized with KLH, the lymphoid follicles were small with no alteration of the lymphoid architecture, and they resembled primary follicles with no recognizable germinal centers; virtually all of them contained exclusively B220+, IgM+, and IgD+ cells (Fig. 4 Right) and failed to bind peanut agglutinin. In occasional follicles, we observed faint staining of some cells for IgG and with peanut agglutinin (data not shown). Staining for IgA was not present in any of the lymphoid tissues examined.

**Serum Immunoglobulin Levels.** Deficiency of CD40L in humans (HIGMX-1) is characterized by elevated levels of IgM and low-to-undetectable levels of other immunoglobulin isotypes. The results in Table 2 show that 5- to 8-wk-old CD40-deficient chimeras have serum levels of IgM and IgG3 similar to those of CD40+/+ mice. The most noticeable alteration in serum levels of CD40−/− animals was absence of serum IgE and a severe decrease of serum IgG1 and IgG2a. Serum IgG2b and IgA levels were partially decreased.

**DISCUSSION**

Both the number and the phenotype of B and T lymphocytes were normal in CD40−/− chimeras (Fig. 2). The presence in the bone marrow and peripheral lymphoid organs of these chimeras of normal numbers of B220+ cells that express surface IgM and IgD indicated that B cells that lack CD40 are able to undergo normal maturation. In addition to B cells, CD40 is expressed on thymic epithelial cells (15), interdigi-
tating cells (16), dendritic cells (17, 18), and monocyte cells activated by cytokines (19). In our CD40−/− chimeras, CD40 deficiency is restricted to lymphocytes because the RAG-2−/− CD40−/− blastocyst makes the major contribution to nonimmune cells. Therefore, we cannot rule out a role for CD40 expressed on nonlymphoid cells in the development of B and T cells.

Inactivation of the CD40 gene abolished the capacity of the B cells to proliferate and to undergo isotype switching in vitro in response to CD40 engagement by its natural ligand (Table 1). This effect was restricted to CD40-mediated activation because B cells from CD40−/− chimeras proliferated normally to LPS and underwent normal isotype switching to IgG1 and IgE when stimulated with LPS/IL-4. These results strongly suggest that the LPS pathway of B-cell activation is CD40 independent.

It has been shown that CD40L can deliver a costimulatory signal for the proliferation of activated T cells (20). T cells from the CD40−/− chimeras proliferate normally in response to concanavalin A, anti-CD3e mAb, and KLH (data not shown), whereas T cells from patients with HIGMX-1 proliferate normally in response to antigens (21). From these observations, it appears that neither expression of CD40 nor of CD40L by T cells is critical for antigen-specific T-cell proliferation.

The most striking abnormality in the CD40-deficient chimeras was their complete failure to mount an antibody response to the TD antigen KLH. However, these mice mounted normal antibody responses to the type I and type II T1 antigens TNP-LPS and TNP-Ficol. It has been shown previously that treatment of mice with anti-CD40L reduces primary as well as secondary responses to exogenous TD antigens but does not affect antibody responses to TI antigens (22). Moreover, patients with HIGMX-1 make a poor antibody response to TD antigens but mount strong humoral responses to TI antigens (21). Taken together, these observations indicate that CD40−/−CD40L interactions play a critical role in T cell-driven antibody responses in vivo.

Germinal centers were absent from lymph nodes, spleens, Peyer’s patches, and lamina propria of CD40−/−−/− chimeras after immunization with KLH, pointing to a failure to develop memory B-cell responses. Lympoid organs revealed normal architecture, indicating that the inability to form germinal centers was not the result of a general disruption of lymphoid tissue. Patients with HIGMX-1 also fail to develop germinal centers (21). Together, these observations indicate that CD40−/−CD40L interactions are critical for the formation of germinal centers.

The serum immunoglobulin phenotype of young (5–8 wk old) CD40−/− chimeras (Table 2) did not completely parallel that observed in human CD40L deficiency—i.e., HIGMX-1. Serum IgM was normal in CD40−/− mice, whereas it is elevated in many but not all HIGMX-1 patients (21). This may relate to the recurrent infections in the patients, which have not occurred in our mice maintained in a pathogen-free environment. Serum IgE was absent, and serum IgG1 and IgG2a were severely decreased in CD40−/− mice. The presence of normal IgG3 levels and of only modestly decreased levels of IgG2b and IgA suggest that CD40-independent isotype switching exists. A large fraction of serum immunoglobulin is thought to be synthesized by CD5+Ly-1 B cells (23). Moreover, LPS-induced switching in vitro and the in vivo IgG response to the T1 antigens TNP-LPS and TNP-Ficol proceeded normally in CD40−/− mice, and we observed some IgG+ cells in the lymphoid follicles of CD40−/− mice. However, no IgA+ cells were detected in any of the lymphoid organs examined. Long-term follow-up is needed to eliminate the contribution of maternal immunoglobulin to the serum immunoglobulin levels in CD40−/− mice.

Taken together, our results show that CD40 expression on B cells is not required for their development and confirm the role of CD40 in B-cell activation, antibody response to TD antigens, isotype switching, and germinal center formation.

Note Added in Proof. While this manuscript was being processed, germline disruption of CD40 gene was reported by Kawabe et al. (24) with essentially similar findings.

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