Th2-Dependent B Cell Responses in the Absence of CD40-CD40 Ligand Interactions

Narendra Chirmule, John Tazelaar and James M. Wilson


http://www.jimmunol.org/content/164/1/248

---

**References**

This article cites 51 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/164/1/248.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Th2-Dependent B Cell Responses in the Absence of CD40-CD40 Ligand Interactions

Narendra Chirmule,*† John Tazelaar,*† and James M. Wilson2*‡‡

CD40 is thought to play a central role in T cell-dependent humoral responses through two distinct mechanisms. CD4+ T helper cells are activated via CD40-dependent Ag presentation in which CD80/CD86 provides costimulation through CD28. In addition, engagement of CD40 on B cells provides a direct pathway for activation of humoral responses. We used a model of adenovirus-mediated gene transfer of β-galactosidase (lacZ) into murine lung to evaluate the specific CD40-dependent pathways required for humoral immunity at mucosal surfaces of the lung. Animals deficient in CD40L failed to develop T and B cell responses to vector. Activation of Th2 cells, which normally requires CD40-dependent stimulation of APCs, was selectively reconstituted in CD40 ligand-deficient mice by systemic administration of an Ab that is agonistic to CD28. Surprisingly, this resulted in the development of a functional humoral response to vector as evidenced by formation of germinal centers and production of antiaadenovirus IgG1 and IgA that neutralized and prevented effective reeducation of vector. The CD28-deficient B cell response required CD4+ T cells and was mediated via IL-4. These studies indicate that CD40 signals to the B cells are not necessary for CD4+ Th2 cell-dependent humoral responses to be generated. The Journal of Immunology, 2000, 164: 248–255.

CD40 ligand (CD40L) is a T cell-specific, type II membrane glycoprotein that binds to its receptor CD40, which is a member of the TNF receptor family (1–3). The importance of CD40L in humoral immunity is best illustrated by its genetic deficiency which, in humans, causes hyper-IgM syndrome (4, 5). These patients develop recurrent infections due to abnormal humoral immunity, which is characterized by a failure to form germinal centers in lymph nodes and isotype switch to IgG, IgA, and IgE. A similar phenotype is seen in mice rendered CD40L-deficient by germline knockout or administration with a blocking Ab (6–8).

The receptor for CD40L, called CD40, is expressed on thymic epithelial cells, B cells, and a number of professional APCs (9, 10). The function of CD40 is best characterized in B cells, where it plays an essential role in T cell-dependent B cell responses (11). A number of in vitro and in vivo studies have implicated direct signaling of B cells by CD40 as a critical step at numerous stages of the B cell response including proliferation and clonal expansion, Ig production, germinal center formation, isotype switching, affinity maturation, and induction of B cell memory (12–17). Experiments in animals deficient in CD40L or CD40 suggest that these molecules also play a primary role in CD4+ T cell activation (18, 19). Although CD40 appears to deliver an important proliferation/differentiation signal to B cells, CD40 signal transduction also induces up-regulation of CD80/CD86 which provides costimulatory signals to the responding T cells. Which aspect of CD40 signaling is more important and whether both are required to generate a humoral response have not been established.

A murine model of adenovirus-mediated gene transfer to murine lung has been used in this and previous studies to define the role of CD40L-CD40 in Ag-specific responses. Instillation of E1-deleted adenovirus expressing β-galactosidase into lung of adult C57BL/6 mice results in activation of CTL, which eliminates the transduced lung epithelial cells. B cells are also activated and secrete IgA onto the airway surface to neutralize virus and to prevent a secondary administration of vector (20). CD4+ T cells, activated to both viral capsid proteins and β-galactosidase, are necessary for the CTL and B cell responses. Animals deficient in CD40L, by germline knockout or Ab-mediated inhibition, failed to mount CD4+ and CD8+ T cell responses to vector or to generate neutralizing Ab to virus (21, 22).

The critical role CD40L-CD40 plays in Ag-specific responses to adenovirus was demonstrated in a previous study which showed full reconstitution of CD4+ and CD8+ T cells and B cell responses to vector in CD40L-deficient (CD40LKO) animals that received an Ab that is agonistic to CD40 (22). Specifically, CTLs to viral Ags were restored, resulting in loss of transgene-expressing cells through direct killing, and neutralizing Abs to vector were formed, inhibiting readministration of virus; these effector responses required activation of both Th1 and Th2 subsets of CD4+ T cells. However, these previous studies did not delineate the specific CD40-dependent events that are necessary for CD8+ T cell and B cell effector responses to vector. Recent studies indicate that CD8+ T cells can be directly stimulated by APCs previously activated by CD4+ T cells through CD40 (23–25). This study attempts to dissect the specific role CD40 plays in T cell-dependent B cell activation. Specifically, what is the relative contribution of direct signaling of the B cells via CD40 vs activation of the CD4+ T cells through the APC in the CD40 dependent process?

Materials and Methods

Animals

CD40LKO animals obtained from The Jackson Laboratory (Bar Harbor, ME) were maintained in a specific pathogen-free environment on a...
C57BL/6-129 background. C57BL/6 mice were also purchased from The Jackson Laboratory. Animals (6–10 wk of age) were prepared for intratracheal instillation of adenoviral vector by exposing the trachea through dissection and injecting vector (10^{11} particles/100 μl) into the airway via a small incision. An E1-deleted lacZ-expressing vector was instilled on day 1 after which an E1-deleted alkaline phosphatase-expressing vector was instilled on day 28 (26). Mice received activating Abs to CD28 Ab (75 μg, clone 37.51, PharMingen, San Diego, CA), CD40 Ab (PharMingen), or control hamster Ig (75 μg, PharMingen) by i.p. injection on days −3, 1, and +3. Some animals also received depleting/blocking Abs to IL-4 (75 μg, clone 11B11, PharMingen) or CD4^{+} T cells (75 μg, GK1.5, American Type Culture Collection, Manassas, VA) on days −2, 1, 4, 11, 18, and 25 by i.p. injection.

Vectors

First generation adenovirus vectors, deleted of E1, were amplified on 293 cells and purified on cesium chloride gradients. The first vector expresses lacZ from the promoter of the immediate early gene of CMV, and the second vector expresses human alkaline phosphatase (ALP) from a CMV-enhanced β-actin promoter of chicken (27, 28).

Cellular and humoral assays

Splenocytes were harvested 11 days after vector delivery and were analyzed for activation to adenoviral Ags and cytotoxic activity as described (28). Serum and bronchoalveolar lavage (BAL) were recovered on day 28 and were evaluated for neutralizing Ab against adenovirus by measuring inhibition of in vitro transduction (28). Antivirus Abs in 1:500 dilution of the sera were also quantitated by isotype-specific ELISAs (28).

Transgene expression in lung

Three days after vector administration (day 3 for lacZ and day 31 for ALP), lungs were removed and inflated with 1:1 solution of PBS:OCT, frozen, cryosectioned, fixed in formalin, and histochemically stained (28, 29).

FIGURE 1. CD4^{+} T cell-mediated immune functions. CD40LK0 mice (A–C) were treated with control hamster IgG (Control), agonist CD40 Ab (CD40), or agonist CD28 Ab alone (CD28) or in combination with neutralizing IL-4 Ab (CD28 + IL-4) or depleting CD4 Ab (CD28 + CD4) and instilled intratracheally with Ad-lacZ. C57BL/6 mice (D–F) were treated with control hamster IgG (Control), agonist CD28 Ab (CD28), or depleting CD4 Ab (CD4) and administered vector as described above. The description of each of the Abs is provided in Materials and Methods. Splenocytes were harvested on day 11 for analysis. Lymphoproliferation (A and D). Triplicate cultures were incubated with either inactivated Ad-lacZ (multiplicity of infection = 10), or medium ( ■ ). After a 3-day incubation with Ag, proliferation was measured by a 16-h pulse label with [3H]thymidine. The uptake of isotope as 3H incorporation (cpm) into acid precipitates of cell lysates is presented. Cytokine release (IFN-γ, B and E; and IL-4, C and F). Lymphocytes were cultured with adenovirus ( □ ) or without Ags ( ■ ) for 48 h. Cell-free supernatants were collected and analyzed for the presence of IFN-γ and IL-4 by ELISA as described in Materials and Methods. Data are presented as concentration of cytokine in the supernatant (pg/ml). The results with C57BL/6 mice are representative of two separate experiments, whereas the CD40LK0 experiments are the mean ± 1 SD (number of experiments: n = control, CD28, 5; CD40, 3; CD28 + IL4, 2; and CD28 + CD4, 1). The cytokine responses were measured from spleens of four to six mice per group in each experiment.
Analysis of germinal centers

Spleens from C57BL/6 or CD40LKO mice treated with control IgG, CD40 Ab, or CD28 Ab were harvested 11 days after intratracheal administration of E1-deleted lacZ adenovirus. Formalin-fixed paraffin sections were incubated with biotin-conjugated peanut agglutinin (PNA) and then with avidin-alkaline phosphatase. The Ag-Ab complex was localized by adding the following substrate: 0.165 mg 5-bromo-4-chloro-3-indolyl phosphatase and 0.33 mg/ml nitroblue tetrazolium in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl. Spleen tissue from four animals per group were examined for the presence of PNA clusters of cells, representing germinal centers. Morphological localization of each germinal center within a follicle was confirmed by adjacent sections stained with hematoxylin and eosin.

Results

Activation of a T cell-dependent B cell response to adenoviral vector by CD28 in the absence of CD40L

Our initial strategy was to characterize the immunologic response to vectors in CD40LKO mice while selectively reconstituting the T cell response by infusing an Ab that is agonistic to CD28. Consistent with our previous study, CD40LKO animals failed to generate T and B cell responses to vector in vivo. Lymphoproliferative responses to adenoviral Ags (Fig. 1A) were absent, and secretion of IFN-γ and IL-4 were reduced or absent (Fig. 1, B and C, respectively). CTLs were diminished (Fig. 2A), and transgene expression was prolonged over what is seen in C57BL/6 mice (Fig. 3,A, B, D, and E; Table I). Animals failed to form germinal centers (Fig. 4, A and B) or to elicit neutralizing Abs in serum and BAL (Table II), which resulted in efficient readministration of vector (Fig. 3, C and F; Table I). Systemic administration of an activating Ab to CD40 in the CD40LKO animals led to full reconstitution of T and B cell responses to vector at levels greater than or equal to that found in C57BL/6 mice (Tables 1 and 2; Figs. 1–4).

Systemic administration of CD28 Ab in immunized CD40LKO mice restored the proliferative responses of splenic-derived lymphocytes to vector-derived Ags (i.e., capsid proteins) and produced a slight decrease in IFN-γ secretion and a substantial increase in IL-4 secretion, consistent with a dominant Th2 response (Fig. 1). The activation of Th2 cells in this experiment was greater than what was observed in C57BL/6 mice or in CD40LKO mice who received CD40 Ab (Fig. 1), which is consistent with previous observations of a Th2 bias after strong CD28 signaling (30–32).

However, we were surprised to see that CD28 signaling in the absence of CD40L resulted in reconstitution of a functional humoral immune response. Neutralizing Abs were equal to (serum) or greater than (BAL) what was observed in C57BL/6 mice (Table II). Systemic administration of CD28 Ab in CD40LKO mice restored the proliferative responses of splenic-derived lymphocytes to vector-derived Ags (i.e., capsid proteins) and produced a slight decrease in IFN-γ secretion and a substantial increase in IL-4 secretion, consistent with a dominant Th2 response (Fig. 1). The activation of Th2 cells in this experiment was greater than what was observed in C57BL/6 mice or in CD40LKO mice who received CD40 Ab (Fig. 1), which is consistent with previous observations of a Th2 bias after strong CD28 signaling (30–32). However, we were surprised to see that CD28 signaling in the absence of CD40L resulted in reconstitution of a functional humoral immune response. Neutralizing Abs were equal to (serum) or greater than (BAL) what was observed in C57BL/6 mice (Table II). However, we were surprised to see that CD28 signaling in the absence of CD40L resulted in reconstitution of a functional humoral immune response. Neutralizing Abs were equal to (serum) or greater than (BAL) what was observed in C57BL/6 mice (Table II).

C57BL/6 mice were infused with CD28 Ab at the time of vector to evaluate the role of this pathway in animals with normal

FIGURE 2. Cytotoxic T cell responses.
Splenocytes harvested at day 11 were stimulated in vitro with adenovirus-infected APCs and analyzed for cytolytic activity against MHC-compatible target cells (C57SV) in different E:T ratios. The percentage of cell lysis is plotted against E:T ratio. Each panel represents a different set of experiments, each of which has been repeated three times with similar results.
A, CD40LKO mice not treated ( ), treated with CD40 Ab ( ), or treated with CD28 Ab (○). B, CD40LKO mice not treated ( ), treated with CD28 (○) or with CD28 and IL-4 Abs (□). C, C57BL/6 mice not treated ( ) or treated with CD28 Ab (○). D, CD40LKO mice treated with CD28 Ab (○) or CD28 and CD4 Ab (△); and C57BL/6 mice not treated ( △) or treated with CD4 Ab (◆).
CD40L-CD40 function. Lymphoproliferative responses to vector Ags were enhanced 10-fold, concurrent with a 50-fold increase in IL-4 secretion and a 10-fold reduction of IFN-γ secretion (Fig. 1, D–F). Neutralizing Abs to vector doubled in serum and tripled in BAL (Table II), whereas the CTL response was diminished at higher E:T (Fig. 2C). These data indicate that enhanced CD28 signaling in an immune-competent mouse augments Th2 responses and increases the production of neutralizing Ab (Table I).

**Activation of B cells by CD28 in the absence of CD40L is T cell-dependent and requires IL-4**

One hypothesis to explain the effect of CD28 Ab on B cell activation is that it directly signals the CD4+ T cell to secrete cytokines (such as IL-4) during Ag presentation which, in sufficient quantities, can activate the B cell in the absence of CD40 signaling. The finding of CD28 on plasma cells suggests an alternative...
hypothesis of direct B cell signaling via the CD28 Ab (33). Experiments were performed to evaluate these potential pathways. We evaluated the role of the T helper cells in CD28 activation of B cells in immunized CD40LKO mice by Ab depleting CD4\(^+\) T cells at the time of CD28 activation. Lymphocytes from these animals failed to proliferate or secrete cytokines to adenovirus Ag (Fig. 1, A–C). CTLs were not activated (Fig. 2D) and transgene expression was prolonged (Table I; Fig. 3, O and P). The humoral response was restricted to the production of non-neutralizing IgM, which is similar to that seen in immunized CD40LKO mice not treated with CD4 Ab (Table II). These data confirm that the CD28-induced activation of B cells is mediated through the CD4\(^+\) T cell.

One mechanism by which the CD28-stimulated T cell could activate the B cell is through secretion of cytokines such as IL-4 (32, 34). This was evaluated in immunized CD40LKO mice by systemically depleting IL-4 with a blocking Ab when animals received CD28-agonistic Ab. Lymphocytes stimulated in vitro with adenovirus Ags proliferated and secreted IFN-\(\gamma\) (albeit diminished), but did not secrete IL-4, consistent with a suppression of Th2 subsets (Fig. 1, A–C). The animals did not form adenovirus-specific neutralizing Abs or anticapsid IgG2a, IgG1, and IgA (Table II), confirming the importance of IL-4 in the B cell response.

Table I. Morphometric analysis of transgene expression in the mouse lung

<table>
<thead>
<tr>
<th>CD40LKO Mice</th>
<th>ALP</th>
<th>C57BL/6 Mice</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>lacZ</td>
<td>CD28</td>
<td>CD28 + IL4</td>
<td>CD28 + IL4</td>
</tr>
<tr>
<td>Control Ig</td>
<td>d4</td>
<td>d4 + d8</td>
<td>d4 + d8</td>
</tr>
<tr>
<td>CD28</td>
<td>d4</td>
<td>d4 + d8</td>
<td>d4 + d8</td>
</tr>
<tr>
<td>CD4</td>
<td>d4</td>
<td>d4 + d8</td>
<td>d4 + d8</td>
</tr>
<tr>
<td>lacZ</td>
<td>CD28</td>
<td>CD28 + IL4</td>
<td>CD28 + IL4</td>
</tr>
<tr>
<td>Control Ig</td>
<td>d32</td>
<td>d32 + d32</td>
<td>d32 + d32</td>
</tr>
<tr>
<td>CD28</td>
<td>d32</td>
<td>d32 + d32</td>
<td>d32 + d32</td>
</tr>
<tr>
<td>CD4</td>
<td>d32</td>
<td>d32 + d32</td>
<td>d32 + d32</td>
</tr>
</tbody>
</table>

\(\text{a}\) Animals (C57BL/6 and CD40LKO) received lacZ vector on day 0 and ALP vector on day 28. Transgene expression was quantified by analyzing 20 bronchioles from each experimental group for the presence of transgene-expressing cells. Animals were analyzed for lacZ expression on days 4 and 28, or for ALP expression on day 32. The data are derived from CD40LKO animals that received intratracheal vector with i.p. control hamster Ig (“Control Ig”), Ab to CD28 (“CD28”), or Abs to CD28 and IL4 (“CD28 + IL4”) or to CD28 and CD4 (“CD28 + CD4”). In each case, four animals were analyzed. Transgene expression was measured using a Phase 3 imaging system and is presented according to the following criteria: 0, no transgene expression; 1–25%, transgene expression in 1–25% epithelial cells; and >25%, transgene expression in more than 25% of epithelial cells. The data are presented as the number of bronchioles expressing the indicated level of transgene from a total of 20 airways for each experimental group.

\(\text{b}\) Time after the initial vector administration in days.

FIGURE 4. Analysis of germinal centers. Spleen was harvested 14 days after vector, fixed in Formalin, embedded in paraffin, sectioned, and incubated with biotin-conjugated PNA and then with avidin-conjugated alkaline phosphatase. Representative germinal centers are presented.
Segregation of CD28-stimulated CTL and B cell responses to adenoviral vector in CD40LKO mice

Systemic modulation of CD28 signaling in the presence or absence of IL-4 provided a mechanism to evaluate the regulation of T helper cells in both the cellular and humoral responses to adenoviral vectors. Activation of CD40 completely reconstituted both cellular and humoral immunity. The Th2-predominant response that occurred after CD28 activation resulted in a suppression of Th1 responses to adenovirus in CD40LKO mice (Fig. 1B); the cytotoxic response remained low (Fig. 2, A and B) and transgene expression was stable (Table I; Fig. 3, I and J), although readministration of vector was inefficient due to neutralizing Abs (Table II; Fig. 3K). Inhibiting IL-4 function in these same animals enhanced CTL activity in vitro (Fig. 2B), leading to a diminution of transgene expression in vivo (Table I; Fig. 3, L and M) despite suppression of neutralizing Abs and efficient readministration of vector (Table II; Fig. 3N).

Discussion

The most surprising aspect of our study was the ability to generate effector humoral immune response to T cell-dependent viral Ags without signaling B cells through CD40. A number of experimental observations, including the immune deficiency disorder in humans associated with CD40L deficiency, have demonstrated the importance of CD40-CD40L in humoral immunity (6–13, 15–17, 35–38). Initial studies focused on the role of CD40 signaling of the B cell following engagement with CD40L from the T helper cell (11–17). A number of in vitro experiments have demonstrated the impact of CD40 signaling of the B cell, in combination with cytokines, on several steps along the pathway of B cell response, such as proliferation and Ig secretion (12–17). Other more complex steps in B cell development, such as germinal center formation, somatic hypermutation, and creation of memory, clearly require CD40L-CD40, as demonstrated by murine models in which these pathways are inhibited by germline mutations, infusion of blocking Abs, or soluble receptors (12–17). A few reports have demonstrated CD40-independent B cell responses in T cell-independent systems (9, 39, 40). However, the in vivo role of T cell-dependent responses in the absence of CD40L-CD40 interactions have not been studied.

An understanding of the role of CD40 in humoral immunity became more complex with the observation that APCs require CD40 signaling through the APC which up-regulates B7, thereby engaging CD28 on CD4+ T cells (21). In addition, CD40L-mediated signals to T cells have been suggested to contribute to T cell activation and cytokine secretion (19, 41). Therefore, inhibition of the CD40L-CD40 pathway in vivo cannot delineate the relative contribution of direct B cell signaling vs activation of T helper cells through the APC. In our studies, activation of the CD28 pathway with an agonistic Ab selectively reconstituted Th2 responses without directly affecting CD40 signaling pathways. Fully functional humoral responses to vector ensued, requiring both CD4+ T cells and IL-4.

The experiments in this study have attempted to manipulate in vivo immune responses using CD28 or CD40 molecule agonistic Abs. The response achieved following either anti-CD40 or anti-CD28 mAb stimulation in vivo resulted in equivalent humoral immune responses (as measured by neutralizing Abs and inability to readadminister vector) in CD40LKO mice. The T cell responses, on the other hand, were divergent in that the activation of Th2 cells is exaggerated by engagement of CD28 with the agonistic Ab. The ability of anti-CD28 mAb to induce Th2 shift in vivo has been extensively documented (31, 32); however, this may not necessarily be physiological because anti-CD28 mAb has 1000-fold greater affinity to CD28 than to B7 (C. June, unpublished observations). Further studies using physiological stimuli (e.g., B71/B72) need to be performed to confirm this phenomenon.

How does one reconcile the findings in this study with previous work suggesting an essential role of CD40 expressed in B cells? Activation of B cells requires the synthesis of signals mediated by cytokine receptors, and costimulatory pathways, such as CD40 (11). What is necessary in this process and what is redundant? Our studies show that IL-4 is necessary for the in vivo production of Th2-dependent Igs in the absence of CD40L-mediated signaling of CD40 which, presumably, occurs in part through the well-described Stat6 pathway (42). The dependence of CD4+ T cells on these responses suggests that activated Th2 cells are the source of IL-4. The cell signaling pathways linked to CD40-mediated activation are less well-defined. Early events involve stimulation of tyrosine kinase and phosphatase activity (43–46). More recently, several TNF receptor-associated proteins have been found to associate with the cytoplasmic domain of CD40 (47–49). Multi-merization of TNF receptor-associated proteins could contribute to NF-κB activation and regulation of apoptosis. Our data suggest
that these CD40 signaling pathways are not necessary for critical steps in B cell development if the other triggering events through the Ig complex and the T helper cell (e.g., cytokine production) are intact. Our studies also raise the possibility of alternate costimulatory pathways in the B cell. Several molecules have been suggested to be involved in B cell activation. In this respect, B lymphocyte stimulator receptor and ligand interactions (50), OX40L-OX40R, and CD70 receptor ligands (51, 52) have been shown to be involved in T cell-dependent B cell activation.

In summary, a better understanding of the signaling pathways required for a normal humoral response could help in the design of strategies for enhancing or inhibiting immune responses. This is particularly problematic for in vivo gene therapy where neutralizing Abs thwart attempts to readminister vector.

Acknowledgments

We thank the Vector, Cell Morphology, and Immunology (Ruth Qian, George Qian, and Parag Dhtagat) Cores of the Institute for Human Gene Therapy for scientific collaboration. Sarah Ehlen Haecker, Marcia Hous-...


