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The Roles of CD28 and CD40 Ligand in T Cell Activation and Tolerance

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Costimulation of T cell activation involves both the B7:CD28 as well as the CD40 ligand (CD40L:CD40) pathway. To determine the importance of these pathways in vitro and in vivo T cell activation, a direct comparison was made of the responses of TCR transgenic T cells lacking either CD28 or CD40L. In vitro, CD28−/− T cells showed a greater reduction in proliferative responses to Ag than did CD40L−/− T cells. The absence of CD28 resulted in defective Th2 responses, whereas CD40L−/− T cells were defective in Th1 development. In vivo, CD28−/− T cells failed to expand upon immunization, whereas CD40L−/− T cells could not sustain a response. These results suggest that CD28 is critical for initiating T cell responses, whereas CD40L is required for sustained Th1 responses. The different functional roles of these costimulatory pathways may explain why blocking B7:CD28 and CD40L:CD40 interactions has an additive effect in inhibiting T cell responses. The Journal of Immunology, 2000, 164: 4465–4470.

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he proliferation of naive T lymphocytes and their differentiation into effector cells require Ag recognition and additional signals provided by costimulators expressed on APCs. The best-defined costimulatory pathways are the B7:CD28 pathway and the CD40:CD40 ligand (CD40L) pathway (1, 2). The two known members of the B7 family, B7-1 (CD80) and B7-2 (CD86), are expressed on professional APCs, and their expression is enhanced by microbes and innate immune responses. Recognition of B7 by CD28, which is constitutively expressed on the majority of T lymphocytes, enhances Ag-stimulated cytokine production, clonal expansion of T cells, and their differentiation into effector cells (1). CD40 is constitutively expressed on APCs, and CD40L is induced on T cells upon Ag recognition. The interaction between CD40L and CD40 also enhances T cell responses (2, 3). Although the mechanism of this effect is unclear, it is currently believed that CD40:CD40L interactions serve to up-regulate the expression of B7 on APCs and to induce the production of cytokines, such as IL-12, which promote T cell differentiation (4).

There has been great interest in the roles of B7:CD28 and CD40L:CD40 interactions in T cell responses, largely because of the potential for targeting these ligand:receptor pairs for the prevention of graft rejection and the treatment of various immunological diseases (5–13). However, many fundamental issues about the roles of these two costimulatory pathways remain unanswered. For instance, it is not known if both B7:CD28 and CD40L:CD40L interactions are required for initiating and/or sustaining T cell responses, especially in vivo. Perhaps more importantly, it is not clear if the absence of these ligand-receptor interactions results in reduction in T cell expansion and differentiation, or conversely, in an increased susceptibility to tolerance induction. A much more detailed understanding of the relative contributions of these two costimulatory pathways to T cell responses to Ag is critical for optimizing clinical protocols that employ costimulator blocking agents in an effort to modulate immune responses.

To evaluate the roles of B7:CD28 and CD40L:CD40 interactions in T cell responses to a cognate Ag, we have exploited TCR transgenic mice lacking either CD28 or CD40L. T cells from these mice can be exposed to immunogenic and tolerogenic forms of the same peptide Ag, and proliferation and differentiation of these cells can be followed quantitatively. Our results show that CD28 and CD40L play distinct and complimentary roles in T cell activation, and suggest why blocking both these costimulatory pathways may have additive effects on T cell responses to Ag.

Materials and Methods

Mice

BALB/c mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Transgenic mice expressing the DO.11.10 TCR (DO.11), specific for the chicken OVA peptide (OVA323–339) in the context of the MHC class II molecule I-A, were obtained from Dr. Dennis Loh (Hoffmann-LaRoche, Nutley, NJ). Mice deficient in CD40 ligand (14) on the BALB/c background were obtained from Dr. Richard Flavell (Yale University, New Haven, CT) and were bred with DO.11 mice. Crosses of DO.11 mice with CD28 knockout mice were obtained from Dr. Jeff Bluestone (University of Chicago). All mice were bred and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Washington, DC). The mice were typed for the DO.11 TCR by staining peripheral blood cells with Abs against CD4 and Vβ8 (PharMingen, San Diego, CA). The CD40L-deficient mice were typed by genomic PCR for neomycin (5′-CATTTGACAAAGATGGATGCGACGGC-3′; 5′-CTCGATGCGATGTTTCGCTTGGTG-3′) and CD40L (5′-GGATCCTCAAAATGGACCAACACG-3′; 5′-CCGATGCGCTTGAGTACCCA-3′).

In vitro proliferation and cytokine assays

Naïve CD4+ T cells were purified using Dynabeads (Dynal, Oslo, Norway) from wild-type, CD28−/−, and CD40L−/− DO.11 mice. The percentage of CD4+ DO.11+ cells was determined by flow cytometry using a mAb to the DO.11 TCR (KJ1-26). To measure T cell proliferation, CD4+ cells containing 2.5 × 10⁴ KJ1-26+ T cells were cultured with varying numbers of mitomycin C-treated BALB/c splenocytes as APCs in 0.2 ml of RPMI.
CD28 AND CD40L IN T CELL RESPONSES

In vitro primary responses of wild-type (WT), CD28
transgenic mice lacking either CD28 or CD40L have normal numbers of T cells expressing the transgenic TCR in the thymus and peripheral lymphoid tissues, indicating that neither molecule is required for T cell maturation (data not shown; and Refs. 14 and 15). To compare the primary responses of costimulator deficient T cells with their wild-type counterparts, naive CD4+ T cells were purified from wild-type, CD28−/−, and CD40L−/− DO.11 mice and stimulated in culture with OVA peptide and mitomycin C-treated syngeneic APCs. Assays of T cell proliferation demonstrated that at all ratios of T cells:APCs and all Ag concentrations tested, CD28−/− T cells proliferated much less than wild-type T cells. In contrast, CD40L−/− T cells proliferated less than wild-type T cells at low Ag and APC concentrations, but they showed normal levels of proliferation when stimulated with high Ag concentrations and high APC:T cell ratios (Fig. 1). Thus, under most conditions of in vitro T cell stimulation, T cell proliferation is more dependent on CD28:B7 interactions than on CD40:CD40L interactions. Assays for IL-2 production during primary in vitro responses gave similar findings, with CD28−/− T cells showing reduced IL-2 production at all T cell:APC ratios, whereas
CD40L−/− T cells produced reduced levels of IL-2 in cultures with low numbers of APCs or low peptide concentrations (data not shown). None of the T cell populations produced IFN-γ or IL-4 upon primary stimulation (data not shown).

**Differentiation of T cells into effector populations**

To evaluate the roles of CD28 and CD40L in the differentiation of T cells into effector Th1 and Th2 populations, DO.11 T cells were primed with Ag and APCs without added cytokines and restimulated, and cytokine production was assayed. These experiments showed that in the absence of CD28, T cells did not produce detectable IL-2 or IL-4 but secreted significant levels of IFN-γ. In contrast, in the absence of CD40L the T cells had a selective defect in IFN-γ production, and often produced even more IL-4 than did wild-type T cells (Fig. 2). Thus, the CD28 and CD40L pathways are required for Th2 and Th1 development, respectively.

It is possible that the inability of CD40L−/− T cells to differentiate into Th1 cells is because these T cells fail to activate APCs to produce the essential Th1-inducing cytokine, IL-12 (4). To test this, wild-type and CD40L−/− T cells were primed with Ag and APCs with and without IL-12, and IFN-γ production was assayed in primary cultures and upon restimulation. The addition of IL-12 enhanced IFN-γ production by wild-type T cells, and restored the IFN-γ response of CD40L−/− T cells to normal levels even upon restimulation in the absence of added IL-12 (Fig. 3).

**In vivo responses of CD28−/− and CD40L−/− T cells to immunization**

The activation of T cells by Ag and APCs in culture may not accurately reflect their in vivo responses. The DO.11 TCR transgenic system is particularly useful for quantitative analyses of in vivo T cell responses (16). To examine the in vivo responses of CD28−/− and CD40L−/− T cells, 5 × 10^6 naïve DO.11 T cells were transferred into syngeneic BALB/c mice and the recipients were immunized by s.c. administration of OVA323–339 in IFA. Lymph node cells were isolated after 3 and 7 days, and examined for the expansion of DO.11 (CD4+ KJ1-26) cells by staining and flow cytometry. Three days after immunization, the CD40L−/− T cells increased in numbers to nearly the same extent as wild-type
cells. Conversely, the CD28<sup>−/−</sup> T cells exhibited markedly reduced expansion when compared with wild-type and CD40L<sup>−/−</sup> T cells. By day 7, the numbers of wild-type T cells remained 3- to 5-fold more than the numbers without immunization, but at this time even the CD40L<sup>−/−</sup> T cells were at baseline levels (Fig. 4). Thus, CD28 and CD40L play distinct roles in T cell expansion in vivo.

The T cells that had been exposed to Ag in vivo were recovered, restimulated with Ag in culture, and assayed for proliferation and cytokine production. These experiments showed that both CD28<sup>−/−</sup> and CD40L<sup>−/−</sup> T cells were defective in their responses to immunization. Thus, after immunization, wild-type DO.11 T cells showed increased proliferation and secretion of IL-2 and IFN-γ upon restimulation ex vivo, but all these enhanced recall responses were reduced in the CD28<sup>−/−</sup> and CD40L<sup>−/−</sup> T cells (Fig. 5).

**Induction of tolerance in CD28<sup>−/−</sup> and CD40L<sup>−/−</sup> T cells**

The role of costimulatory pathways in inducing tolerance in normal T cells can only be examined in vivo and is a major strength of the DO.11 T cell adoptive transfer system (17, 18). In the final set of experiments, recipients of DO.11 T cells were left untreated or given two doses of aqueous OVA<sub>323-339</sub> peptide i.v. (tolerogen, tol). Lymph node cells were recovered on day 3 or 7 after Ag administration, 5 × 10<sup>5</sup> cells were restimulated with Ag, and proliferation was assayed on day 3. Results of proliferation are corrected for numbers of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells added to cultures.

**FIGURE 6.** Induction of tolerance in wild-type (WT), CD28<sup>−/−</sup> and CD40L<sup>−/−</sup> DO.11 T cells. Adoptive transfer recipients, as in Fig. 4, were left untreated (naive), or given two doses of aqueous OVA<sub>323-339</sub> peptide i.v. (tolerogen, tol). Lymph node cells were recovered on day 3 or 7 after Ag administration, 5 × 10<sup>5</sup> cells were restimulated with Ag, and proliferation was assayed on day 3. Results of proliferation are corrected for numbers of CD4<sup>+</sup> KJ1-26<sup>+</sup> cells added to cultures.
or injected with two doses of aqueous peptide Ag i.v. The T cells were assayed 3 and 7 days later for proliferation responses to Ag stimulation ex vivo. Exposure to aqueous (tolerogenic) Ag inhibited subsequent responses of both wild-type T cells and CD40L−/− T cells (Fig. 6). It is not possible to examine the tolerance sensitivity of CD28−/− T cells by this method, because, as demonstrated above, even untreated CD28−/− T cells proliferate poorly in response to Ag.

Discussion

The experiments described in this paper were designed to compare the responses of T cells lacking either CD28 or CD40L to Ag both in vitro and in vivo. Studies from numerous laboratories have shown that CD28 is required for initiating and/or sustaining T cell clonal expansion and differentiation in response to Ag recognition (19, 20). The CD40:CD40L pathway was first described for its role in T cell-dependent Ab production (reviewed in Refs. 2 and 21), but subsequent studies have shown that it is critically involved in T cell-mediated immunity, including macrophage activation and the generation of CTL (22–24). Few studies have directly examined the role of CD40L in T cell clonal expansion and differentiation. It has been suggested that in the absence of CD40L, helper T cells do not expand normally in response to Ag (6), but it is unclear whether this reflects defective proliferation, survival, or maintenance. In our experiments, analysis of primary T cell responses in vitro showed that CD28 is much more important for initiating the clonal expansion of T cells than is CD40L. Thus, CD28−/− naive T cells are deficient in proliferation (and IL-2 secretion) when stimulated with a wide range of Ag concentrations and APC numbers. In contrast, CD40L−/− T cells respond normally when stimulated in conditions of high Ag concentrations or high APC to T cell ratios, and show defects only when stimulated with low numbers of APCs or low concentrations of Ags (Fig. 1). These results are consistent with the hypothesis that B7/CD28 interactions are required for initiating T cell responses, whereas CD40L:CD40 interactions serve mainly to amplify such responses. This amplification function would be most apparent when costimulation is limiting; for instance, when T cells are stimulated with low numbers of APCs. The likely mechanism of CD40L-induced enhancement of T cell responses is that CD40L engagement of CD40 on APCs increases the expression of B7 on these APCs (25–26).

Our results also show that CD28 and CD40L serve opposing roles in the differentiation of CD4+ T cells into cytokine-producing effector cells. The absence of CD28 costimulation prevents Th2 differentiation when T cells are primed without exogenous cytokines, whereas the absence of CD40L prevents Th1 development (Fig. 2). The essential role of CD28 in Th2 differentiation has been described previously (27), although the operative mechanism is not known. It has also been suggested that the CD40 pathway is more important for Th1 differentiation (28), although there are few studies directly addressing this issue. The ability of IL-12 to overcome the lack of CD40L in Th1 differentiation (Fig. 3) is consistent with the idea that CD40L activates APCs to produce IL-12, and this is a mechanism by which it stimulates Th1 development (4). These results indicate that antagonists against B7:CD28 and CD40L:CD40 may have distinct effects on immune responses, and are therefore likely to be most useful for inhibiting different types of pathologic immunity. However, it should be pointed out that in vivo, CD28−/− T cells show little differentiation into any effector subset (Fig. 5). This is probably because under the limiting conditions of in vivo Ag exposure, CD28−/− T cells are unable to mount any functional responses.

The in vivo analysis of T cell responses also showed that CD28, but not CD40L, is required for initiating T cell expansion in response to immunogenic Ag. However, CD40L does play a role in sustaining the T cell response (Fig. 4). This is also consistent with an amplification function of the CD40L:CD40 pathway. Thus, with time after immunization, T cell expansion may be maintained by continuous stimulation by Ag released from its depot. As the quantity of available Ag decreases and the innate immune response to the adjuvant subsides, CD40L-mediated stimulation of APCs becomes increasingly important. In the absence of CD40L, T cell priming also fails to induce Th1 development in vivo (Fig. 5), as it does in vitro.

Finally, we have attempted to address the possibility that the absence of CD28 or CD40L increases the sensitivity of T cells to tolerance induction. Using an experimental system of tolerance induced by aqueous protein Ag, we find that CD40L-deficient T cells are as tolerance-sensitive as wild-type cells (Fig. 6). However, this experimental approach does not allow us to accurately and quantitatively compare the tolerance sensitivity of different cell populations, or to assay tolerance in CD28−/− T cells (because these cells normally fail to respond to Ag).

The results in this paper provide a framework for explaining the additive or synergistic effects of antagonizing both the CD28:B7 and the CD40L:CD40 pathways in various immune responses, such as graft rejection (11, 12). Blocking the CD28:B7 pathway will inhibit the primary T cell response, whereas blocking the CD40L:CD40 pathway will inhibit Th1 differentiation and the maintenance of the response. The distinct but complimentary roles of CD28 and CD40L may provide new avenues for developing therapeutic agents for different types of immunologic diseases.

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