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Blockade of CD28 During In Vitro Activation of Encephalitogenic T Cells or After Disease Onset Ameliorates Experimental Autoimmune Encephalomyelitis

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Previous studies have shown complex roles for the B7 receptors in providing both positive and negative regulation of experimental autoimmune encephalomyelitis (EAE). B7 blockade can ameliorate clinical EAE by indirectly interfering with CD28 signaling. However, B7 blockade can also result in disease exacerbation, presumably by interfering with regulatory B7:CTLA-4 interactions. Therefore, we have directly targeted T cell CD28 with specific mAbs both during initial Ag priming and after the onset of clinical signs of EAE. We found that CD28 blockade ameliorated EAE during the efferent and afferent limbs of the immune response. Disease amelioration at disease onset was associated with suppression of TNF-α production. Finally, Ab blockade of T cell CD28 during the first disease episode resulted in significant attenuation of the subsequent disease course, with no significant relapses. In contrast to previous studies targeting APC B7 with CTLA4-Ig, reagents targeting CD28 can block ongoing disease. Therefore, the present results suggest a clinically relevant therapeutic scenario for human diseases, such as multiple sclerosis. The Journal of Immunology, 1999, 163: 1704–1710.

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; LNC, lymph node cells.
repeated injections of Ab to differential regulation of Th1/Th2 differentiation (23). The effects of a single injection of Abs directed against B7-1 and B7-2 involve regulation of the encephalitogenic cytokine TNF-α (24). Blockade of B7-1 using Fab fragments during ongoing EAE prevents epitope spreading and subsequent relapse (26), whereas using the intact mAb exacerbates disease (27).

Given the complexity of B7-CD28/CTLA-4 interactions, B7-mediated costimulation is not necessarily equivalent to CD28-mediated costimulation. Indeed, previous studies predict that CTLA4-Ig therapy could prevent B7-CTLA-4 interaction and result in enhanced disease (21). Therefore, direct manipulation of CTLA4-Ig therapy could prevent B7:CTLA-4 interaction and mediated costimulation is not necessarily equivalent to CD28-mediated costimulation. This amelioration was associated with suppressed production of the proencephalitogenic cytokine TNF-α and decreased production of IL-2. Therefore, direct targeting of T cell CD28 is a potential strategy for treatment of autoimmune demyelinating disease.

Materials and Methods

Mice

Female (PL × SJL/J, mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All (PL × SJL/J) mice were 10 to 12 weeks of age when experiments were initiated. Transgenic bearing the Vβ8.2, Vε2.3 TCR specific for MBP Ac1–11 on the B10.PL background were provided by Dr. Joan Goverman (University of Washington, Seattle, WA). These mice were bred and maintained in our animal colony in compliance with the Animal Studies Committee (28, 29). In conducting this research, the investigators adhered to the Guide for Laboratory Animal Facilities and Care as promulgated by the Committee on the Guide for Laboratory Animal Resources (National Academy of Sciences, National Research Council, Department of Health and Human Services, Publication No. (NIH) 86-23).

Reagents

Whole MBP was prepared from guinea pig spinal cords (Rockland, Gilbertsville, PA) as previously described (30). Hamster anti-mouse CD28 hybridoma 37–51 was kindly provided by Dr. James F. Allison (University of California, Berkeley) (31), and mAb was purified from tissue culture supernatant. Hamster anti-mouse CD28 mAb PV-1 (32) was purified from tissue culture supernatant also. Control hamster IgG was purchased from Rockland. The endotoxin content of the Abs used in these studies was <0.4 EU/ml. Fab fragments were made using the ImmunoPure Fab Kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. The Fab fragments do not induce signal transduction and yet remain specifically block B7:CD28 interaction during EAE, as anti-CD28 Fab fragments do not induce signal transduction and yet remain capable of efficient blockade of B7 binding. Mice were immunized with MBP (400 μg) in CFA, and 10 days later draining lymph nodes were removed and cultured in the presence or absence of CD28 blockade. As shown in Fig. 1, in vitro activation of MBP-primed cells in the presence of anti-CD28 Fab fragments resulted in both lower disease incidence and decreased disease severity in animals developing clinical disease. In addition, the presence of anti-CD28 Fab resulted in a dramatic reduction in IL-2 production (Fig. 2), consistent with the known role of the B7-CD28 pathway in the production of this cytokine (39). These results demonstrate in the adoptive transfer model of EAE that encephalitogenic T cells require a costimulatory signal, delivered through CD28, to mediate significant disease-producing capability and secretion of inflammatory cytokines.

Because CD28 blockade suppressed pathogenicity of previously primed cells, we next assessed the role of CD28 during Ag priming in vivo. Animals were injected with 50 μg anti-CD28 Fab or hamster IgG Fab i.p. on days 2 and 4 after immunization with MBP. Although 5 of 5 animals in each group developed EAE, anti-CD28 Fab recipients developed a less severe disease (Fig. 3). The effect of anti-CD28 Fab injection lasted for the duration of the observation period (73 days). Thus, in the active model of EAE, a transient interruption of CD28 function results in long-term amelioration of clinical disease activity.

Effect of CD28 blockade on activation and encephalitogenicity of TCR transgenic T cells specific for MBP Ac1–11

The above results suggested that the likely mechanism of inhibition of disease was that encephalitogenic CD4+ precursors failed to produce adequate IL-2 and expand sufficiently during CD28 blockade to reach the threshold necessary to cause disease. To
address whether the effect of anti-CD28 merely reflected an inhibition of expansion of encephalitogenic precursors, MBP-specific TCR transgenic T cells were cultured at a very high precursor frequency in vitro with MBP and anti-CD28 blockade. Under these conditions, significant differences in the ability to induce EAE were not noted (Table I). In addition, no differences in IL-2, IL-4, or IFN-\(\gamma\) production in tissue culture supernatants were noted at 24 or 48 h after initiation of culture of these cells with Ag. (Fig. 4 and data not shown). These results, plus the ability of anti-CD28 to have such a long-lasting effect during Ag priming, strongly suggest that the effect mediated by anti-CD28 Fab in this model is due to inhibition of expansion of CD4\(^+\) encephalitogenic precursors that mediate EAE.

Effect of CD28 blockade at disease onset

Most studies of B7-mediated costimulation have focused on the initial immune cell interactions during Ag priming. While administration of anti-B7-1 Fab fragment can suppress established EAE (26), CTLA4-Ig injection does not alter established disease (25, 40). We therefore evaluated the ability of specific CD28 blockade to suppress EAE in the direct model after completion of initial Ag priming. As shown in Table II, anti-CD28 Fab injection just before disease onset can ameliorate disease.

In the experiment illustrated in Table II, we delayed injection of anti-CD28 Fab or control hamster Fab until after the first mice displayed clinical signs. Animals then received 10\(\mu\)g of anti-CD28 Fab or control hamster Fab every other day for 14 days beginning the day after the first animal displayed clinical disease signs. In this experiment, none of the anti-CD28 Fab recipients developed disease for 21 days. After the initial 21 days, the severity of disease eventually developed by the anti-CD28 Fab recipients was less severe than that developed by the control mice.

FIGURE 1. CD28 blockade suppresses the ability of MBP-primed cells to transfer EAE. A, LNC from (PL \(\times\) SJL)\(F_1\) mice obtained 10 days after immunization with MBP/CFA were cultured with 25 \(\mu\)g/ml MBP in the presence of either 5 \(\mu\)g/ml PV-1 (anti-CD28) Fab (six mice) or 5 \(\mu\)g/ml control hamster Fab (seven mice). Cells (30 \(\times\) 10\(^6\)) were injected into naïve recipients on day 0, and the clinical disease severity was assessed. The incidence of disease in animals receiving the cells pretreated with the anti-CD28 Fab was lower than that of the control animals \((p = 0.01)\), and the severity of disease from the time of disease onset until the end of the observation period (days 6 to 38) was reduced (Mann-Whitney sum of ranks, \(p < 0.01\)). B, LNC were cultured in the presence of either 10 \(\mu\)g/ml PV-1 Fab (five mice) or 10 \(\mu\)g/ml control hamster Fab (five mice). The severity of disease from the time of disease onset until the end of the observation period (days 8–36) was reduced in the animals receiving the cells pretreated with the anti-CD28 Fab (Mann-Whitney sum of ranks, \(p < 0.01\)).

FIGURE 2. CD28 blockade suppresses IL-2 production by MBP-primed T cells. MBP-specific LNC were stimulated with 25 \(\mu\)g/ml MBP in the presence of 5 \(\mu\)g/ml of either PV-1 (anti-CD28) Fab or hamster IgG Fab. Supernatants were harvested after 24, 48, and 72 h. IL-2 was measured using the IL-2-dependent cell line, CTLL.EV. Isobars represent SEM. IL-2 production was significantly suppressed by CD28 blockade (Student’s \(t\) test, \(p < 0.05\)). Cells cultured in the absence of additional MBP did not produce detectable IL-2 (data not shown). Similar results were obtained in experiments using 10 \(\mu\)g/ml anti-CD28 Fab.

FIGURE 3. Anti-CD28 Fab injection suppresses the development of EAE. (PL \(\times\) SJL)\(F_1\) mice were immunized with MBP/CFA and received pertussis toxin on days 0 and 2. Animals received i.p. injections of either 50 \(\mu\)g anti-CD28 Fab or 50 \(\mu\)g control hamster IgG Fab on days 2 and 4 \((n = 5)\). Animals receiving the anti-CD28 Fab underwent a less severe disease than that of controls after the onset of disease until the end of the observation period (Mann-Whitney sum of ranks, \(p < 0.01\)).
These results demonstrate that blockade of B7:CD28 interactions just before the onset of disease can inhibit clinical signs of EAE. These results were surprising in that previous studies have shown that effector cells are relatively independent of costimulation.

We next assessed the effect of CD28 on the production of TNF-α, an important proinflammatory mediator of autoimmune demyelination (41). Animals were injected i.p. with 10 μg of anti-CD28 Fab or control hamster Fab every other day for 14 days beginning the day after the first animal displayed clinical disease signs. LNC were obtained 7 days after the final injection and were cultured in the presence or absence of MBP. TNF-α release into culture supernatants was assessed by ELISA. As shown in Fig. 5, CD28 blockade significantly reduced TNF-α secretion.

**CD28 blockade after the establishment of EAE ameliorates clinical disease**

Finally, we asked whether CD28 blockade, after the establishment of clinical disease, could attenuate the course of clinical EAE. We immunized (PL × SJL)F₁ mice and allowed them to develop EAE. After all of the mice had signs of EAE for at least 2 consecutive days, they were divided into two groups and injected with 10 μg of either anti-CD28 mAb 37–51 or control hamster IgG every other day. The mean maximal score achieved by both groups of mice before injection was 2.8. Each animal received six injections between days 20 and 30 postimmunization.

As shown in Fig. 6, anti-CD28 injection resulted in an attenuated disease course. The control group of animals had two episodes of relapsing disease, whereas the mice given CD28 blockade did not develop disease exacerbations. The therapeutic effect of CD28 blockade continued after the last injection until the end of the observation period on day 46 postimmunization. These results suggest that direct blockade of T cell CD28 represents a potential therapeutic approach for ongoing autoimmune demyelination.

**Discussion**

Previous studies examining the role of the B7:CD28/CTLA-4 pathway have used reagents directed against the B7 molecules (21, 23, 25–27, 42) or CTLA-4 (14, 15, 43). In these studies, we specifically targeted T cell CD28 to avoid interfering with regulatory B7-mediated interactions with the CTLA-4 molecule. We blocked CD28 during both the expansion of encephalitogenic T cells in vitro and the effector phase of the disease in vivo and found this approach feasible as a treatment of an ongoing, organ-specific autoimmune process.

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**Table I. Anti-CD28 Fab does not prevent adoptive transfer of EAE using TCR transgenic T cells specific for MBP**

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Mean Day of Onset</th>
<th>Mean Maximal Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Fab</td>
<td>6/6</td>
<td>12.67 ± 1.3</td>
<td>1.83 ± 0.4</td>
</tr>
<tr>
<td>Anti-CD28 Fab</td>
<td>4/4</td>
<td>12.75 ± 1.5</td>
<td>1.75 ± 0.25</td>
</tr>
</tbody>
</table>

*EAE was induced by activating TCR transgenic T cells specific for MBP Ac1-11 with MBP (25 μg/ml) for 48 h in the presence of either anti-CD28 Fab (PV-1) or control (hamster) Fab (10 μg/ml). Cells were then adoptively transferred into naive B10.P1 mice, which were observed daily for clinical signs of EAE. The incidence, disease onset, and severity of clinical disease did not differ statistically between the two groups.*

**Table II. Anti-CD28 Fab injection at the onset of clinical EAE**

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Mean Maximal Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 1–21 after first Fab injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Fab</td>
<td>3/6</td>
<td>1.8</td>
</tr>
<tr>
<td>Anti-CD28 Fab</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>Days 22–33 after first Fab injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Fab</td>
<td>6/6</td>
<td>2.6</td>
</tr>
<tr>
<td>Anti-CD28 Fab</td>
<td>4/5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*EAE was induced in 11 (PL to SJL)F₁ mice by immunization with MBP/CFA and two injections of PT as described in Materials and Methods. When the first mouse developed signs of clinical EAE, mice were randomly assigned to receive 10 μg of either hamster IgG Fab (six mice) or hamster PV-1 (anti-CD28) Fab (five mice) every other day for 14 days. The incidence of disease and mean maximal clinical score is noted for each time period of observation. Disease course was significantly less severe in the anti-CD28 group than in the control group using Mann-Whitney sum of ranks test during days 1–21 following the first Fab injection (p < 0.05) and days 22–33 following the first Fab injection (p < 0.05).
CD28 BLOCKADE AMELIORATES ESTABLISHED EAE

Most importantly, CD28 blockade using a blocking mAb attenuated established EAE. Mice receiving intact anti-CD28 mAb 37–51 underwent attenuated clinical disease and did not undergo significant subsequent relapses. Disease attenuation was also evident after the cessation of treatment, suggesting that short-term CD28 blockade may be sufficient to control expansion or effector functions of autoreactive effector T cells and thereby ameliorate the autoimmune response.

The ability of CD28 blockade to ameliorate established EAE is consistent with the ability of B7-1 Fab to prevent epiteope spreading and relapse (26). Conversely, CTLA4-Ig has not been effective at blocking ongoing EAE (25), even when using a homologous construct with the murine CTLA-4 extracellular domain (P. J. Perrin and C. H. June, unpublished observations). The present results, together with those of Miller et al. (26), suggest that CD28-targeted therapy may be a useful approach to autoimmune demyelinating diseases.

It is important to note that, although the encephalitogenic response is reduced, it is not completely eliminated, suggesting that encephalitogenic T cells, which may not be dependent on CD28, mediated costimulation. This observation would be consistent with recent findings in mice deficient in CD28. In an autoimmune myocarditis model, CD28−/− mice developed autoimmune heart disease, although it was less severe than that observed in heterozygous littermates (47). In addition, breeding NOD mice to CD28−/− exacerbated autoimmunity, suggesting that CD28 plays an important regulatory role in that disease model (48). However, the ability to suppress the ongoing immune response suggests that preactivated T cells may be, at least partially, dependent on T cell costimulation. Alternatively, CD28 blockade may prevent the further recruitment and activation of naïve T cells and interfere with the process known as epitope spreading (26, 49).

Collectively, our studies and those of others using CD28 Fab or other B7:CD28 antagonists in wild-type mice would suggest that the effect is primarily mediated by inhibiting the expansion of MBP-reactive T cells that mediate the effect resulting in clinical signs of disease (21, 24–26). However, it is interesting that when the encephalitogenic T cells are cultured at a very high precursor frequency, the effect of CD28 blockade may be, at least partially, dependent on T cell costimulation and relapse (26). Conversely, CTLA4-Ig has not been effective in blocking EAE or IL-2 production (Table I and Fig. 4). It is possible that at high Ag-specific T cell precursor frequency, inability to block all B7:CD28 interactions results in IL-2 production, which then overcomes the effect of the blockade, which is otherwise effective when the Ag-specific cells are at a low precursor frequency, as may be the case in wild-type mice. In addition, it is possible that B7 expression on the activated T cells themselves, particularly at high T cell precursor frequency, may be able to provide an additional costimulatory signal. However, B7 expression on T cells may also regulate the immune response in the setting of a high density of responder T cells. The presence of anti-B7-1 and anti-B7-2 Abs augments T lymphocyte proliferation in the presence of optimal stimulation by anti-CD28 Abs, suggesting that B7:CTLA-4 interactions may mediate inhibitory signals (50).

Although the data presented in this article suggest that the major effect of CD28 blockade is to reduce the expansion of encephalitogenic precursors, recent data published by our group suggest the possibility that CD28 blockade can produce qualitative effects on the ability to induce inflammation in the CNS. Although CD28−/− C57BL/6 mice develop EAE in response to myelin/oligodendrocyte glycoprotein immunization, CD28−/− mice develop experimental autoimmune meningesis (51). Experimental autoimmune meningesis is characterized by a polymorphonuclear neutrophil-dominated inflammatory response within the leptomeninges in the

The interactions involving B7-1 and B7-2 with either CD28 or CTLA-4 during EAE and other autoimmunity disorders, such as diabetes, are complex. Accessory signals delivered through the B7 receptors can either provoke or prevent clinical EAE (14, 21, 24–26). Injection of anti-B7-1 Ab suppresses the induction of EAE (24), and anti-B7-2 injection exacerbates clinical disease (21, 23, 24). Conversely, in the NOD mouse model of autoimmune diabetes, B7-2 administration was protective and anti-B7-1 administration exacerbated disease (20). Therefore, the contribution of the individual B7 molecules is dependent on the disease model as well as on the timing of administration of the various reagents.

Studies in which B7-1 and B7-2 are blocked simultaneously suggest further complications to approaches that target both B7-1 and B7-2. Injection of both anti-B7-1 and anti-B7-2 can either ameliorate (21) or exacerbate EAE (24), depending on the immunization protocol used for inducing EAE. In one model of EAE, CTLA4-Ig, which binds to both B7-1 and B7-2, can either suppress or enhance clinical disease, depending on the injection protocol used (21).

Therefore, it appears that blockade of the B7 ligands during periods when CTLA-4 regulatory interactions may predominate exacerbates autoimmunity. Although the dynamics of B7:CD28 and B7:CTLA-4 interactions might be defined in murine models of EAE, they may be more difficult to predict in patients undergoing less defined disease courses. Therefore, directly targeting CD28 costimulation represents an attractive alternative approach.

In the present studies, anti-CD28 Fab injection, either during Ag priming or later, before disease onset, significantly ameliorated EAE. This amelioration was associated with ablated TNF-α production. TNF-α has been associated with encephalitogenicity (41, 44, 45). T cell infiltration into the CNS, together with TNF-α blockade, results in the inhibition of clinical signs of EAE (45). On the other hand, recent studies have shown that EAE can be induced in mice deficient in lymphotoxin-α and TNF-α, suggesting that other cytokines can replace their central pathogenic role in EAE (46).

FIGURE 6. CD28 blockade after the onset of EAE ameliorates established clinical disease. (PL × SIL)F1 mice were immunized with MBP/ CFA and received pertussis toxin on days 0 and 2. Animals received i.p. injections every other day of either 10 μg anti-CD28 mAb 37–51 or 10 μg hamster IgG beginning on day 20, when all of the mice (n = 5) had clinical signs of EAE for at least 2 consecutive days. Each animal received six injections. Arrows indicate the days on which Ab was injected. Animals that received anti-CD28 underwent an attenuated disease course after the commencement of injections. Differences in groups were compared for the period of observation following the first anti-CD28 injection (days 20–45; Mann-Whitney sum of ranks, p < 0.01).
absence of classical perivascular infiltrates in the CNS characteristic of EAE. Thus, CD28 signaling may be important for T cells to be able to enter the CNS and induce encephalomyelitis, rather than merely meningitis. However, the present study demonstrates that interruption of the costimulatory signal delivered to encephalitogenic T cells through CD28 can result in inhibition of their expansion and important long-term clinical effects in an organ-specific model of autoimmunity.

Ag-specific tolerance is a goal for the treatment of autoimmune diseases. This could be accomplished by preventing the costimulatory signal, without interfering with presentation of Ag to the TCR. Furthermore, because activated cells are targeted with this strategy, the autoantigen does not need to be defined. This is particularly important when considering treatments for diseases such as multiple sclerosis, in which an immune-mediated process is very likely but the identity of the autoantigen unknown (52).

In conclusion, previous studies have examined the complex and varying roles of B7-mediated costimulation using reagents specific for the B7 molecules and/or CTLA-4. CTLA-4 Ig or B7 Abs could potentially interfere with B7-CTLA-4 interactions that are important for regulation of an immune response. Regulation of the CD28 signal represents a more direct approach to targeting the T cells that mediate EAE than does manipulation of B7. These studies establish the feasibility of this strategy.

References


