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Break of Neonatal Th1 Tolerance and Exacerbation of Experimental Allergic Encephalomyelitis by Interference with B7 Costimulation

J. Jeremiah Bell,†‡ Booki Min,‖ Randal K. Gregg,* Hyun-Hee Lee,* and Habib Zaghouani3*

Ig-PLP1 is an Ig chimera expressing proteolipid protein-1 (PLP1) peptide corresponding to aa residues 139–151 of PLP. Newborn mice given Ig-PLP1 in saline on the day of birth and challenged 7 wk later with PLP1 peptide in CFA develop an organ-specific neonatal immunity that confers resistance against experimental allergic encephalomyelitis. The T cell responses in these animals comprise Th2 cells in the lymph node and anergic Th1 lymphocytes in the spleen. Intriguingly, the anergic splenic T cells, although nonproliferative and unable to produce IFN-γ or IL-4, secrete significant amounts of IL-2. In this work, studies were performed to determine whether costimulation through B7 molecules plays any role in the unusual form of splenic Th1 anergy. The results show that engagement of either B7.1 or B7.2 with anti-B7 Abs during induction of EAE in adult mice that were neonatally tolerated with Ig-PLP1 restores and exacerbates disease severity. At the cellular level, the anergic splenic T cells regain the ability to proliferate and produce IFN-γ when stimulated with Ag in the presence of either anti-B7.1 or anti-B7.2 Ab. However, such restoration was abolished when both B7.1 and B7.2 molecules were engaged simultaneously, indicating that costimulation is necessary for reactivation. Surprisingly, both anti-B7.1 and anti-B7.2 Abs triggered splenic dendritic cells to produce IL-12, a key cytokine required for restoration of the anergic T cells. Thus, recovery from neonatally induced T cell anergy requires B7 molecules to serve double functions, namely, costimulation and induction of cytokine production by APCs. The Journal of Immunology, 2003, 171: 1801–1808.

Neonatal exposure to Ags has always been considered tolerogenic because a later challenge with an immunogenic regimen of the same Ag does not elicit inflammatory reactions as in animals not exposed to Ag at the neonatal stage (1–3). Initially, T cell deletion/inactivation was considered the leading mechanism for such tolerance (4, 5), but recent investigations have shown that secondary T cell responses do indeed develop in animals exposed to Ag at the neonatal stage (6–9). However, the responses arise mostly in the spleen instead of the lymph node (7, 9) and are usually dominated by Th2-type cells that do not support inflammatory reactions (7, 9–17), thus preserving the notion that neonatal exposure to Ag induces tolerance. It is now well established that factors such as the type of APC (6, 16, 17), the adjuvant into which the Ag is emulsified (9, 16, 18), the dose (8, 16), form (19–21), in vivo availability (22), continuous supply (23), and the context of presentation (24) of Ag control the induction of neonatal immunity. Although Th2-type neonatal immunity would be beneficial for transplantation (25, 26) and suppression of autoimmunity (5, 14, 15, 27), the lack of Th1 cells puts the neonate at risk for microbial infection (3). A tremendous effort is being deployed to understand the lack of Th1 immunity in the newborn, and hopefully regimens will be defined that could induce Th1 cells effective against microbial infection in the neonate.

In previous investigations, we have demonstrated that genetic engineering and expression of peptides on Ig facilitate peptide delivery into APC through FcγRs, leading to efficient endocytic processing and increased peptide loading onto newly synthesized MHC class II molecules (28, 29). For instance, when the proteolipid protein-1 (PLP1) peptide corresponding to aa sequence 139–151 of PLP was expressed on an Ig molecule, the resulting Ig-PLP1 was presented to T cells 100-fold better than free PLP1 peptide (30). In addition, Ig-PLP1 given into mice in saline on the day of birth circumvented the use of IFA and conferred resistance against experimental allergic encephalomyelitis (EAE) induction (14–16). Indeed, mice recipient of Ig-PLP1 on the day of birth and induced for EAE with PLP1 peptide 7 wk later developed a mild initial phase of paralysis and recovered expeditiously without any relapse. In contrast, animals recipient of a control Ig backbone without any PLP peptide developed a severe initial phase of disease, which led to 40% death, and the surviving animals never recovered and displayed relapses for the 100-day period of clinical observation (14–16). Analysis of the responses mediating resistance to EAE in Ig-PLP1 neonatally tolerized mice indicated that the lymph nodes developed T cell responses that were deviated toward Th2, but the spleen displayed responses comprising non-proliferative T cells that could not produce IL-4 or IFN-γ, but secreted significant amounts of IL-2 (14). Surprisingly, these splenic cells could be restored to proliferate and even produce IFN-γ if assisted with exogenous IFN-γ or the IFN-γ-inducer.

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4 Abbreviations used in this paper: PLP, proteolipid protein; DC, dendritic cell; EAE, experimental allergic encephalomyelitis.
IL-12 (14). The splenic response was then referred to as IFN-γ-dependent anergy. These observations indicated that neonatal exposure to Ig-PLP1, although inducing a dominant Th2 response, mobilizes Th1 lymphocytes that could be made fully functional if assisted with exogenous cytokines. Further investigation demonstrated that these Th1 cells are unable to up-regulate CD40L and could not trigger cross-linking of CD40 on APC (31). Consequently, CD40-mediated IL-12 production by APC could not occur, leading to inability of the T cells to up-regulate IL-2Rα needed for absorption of IL-2 (31). Thus, growth (proliferation) of the cells and differentiation into IFN-γ-producing lymphocytes could not occur. Given these findings, we postulated that the residual EAE seen in Ig-PLP1 neonatally tolerized mice is due to restoration of the anergic splenic cells by the pathogenic challenge with PLP1 peptide in CFA. In an attempt to fully prevent EAE in Ig-PLP1 neonatally tolerized mice, we sought to block costimulation during EAE induction and inhibit both restoration of the anergic T cells and the residual disease. The results presented in this work indicate that engagement of either B7.1 or B7.2 with anti-B7 Abs during induction of EAE in Ig-PLP1 neonatally tolerized mice restores and exacerbates the disease rather than suppresses the residual paralysis. Moreover, the anti-B7 Abs used to interfere with costimulation were able to induce IL-12 production by dendritic cells (DC) and restored both proliferation and IFN-γ production by the anergic splenic T cells. It is therefore suggested that reversal of neonatally induced T cell anergy requires B7 costimulation and supply of IL-12 from APC to regain proliferation and IFN-γ production.

**Materials and Methods**

**Mice**

Six- to 8-wk-old SJL/J (H-2b) mice were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in our animal facility for the duration of experiments. For the generation of newborn mice, breeding sets, including an adult male and three females, were caged together, and when pregnancy was visible the females were separated and caged individually. Offspring were weaned when they reached 3 wk of age. All experimental procedures were conducted according to the guidelines of the institutional animal care committee.

**Ags**

**Peptides.** All peptides used in these studies were purchased from Research Genetics (Huntsville, AL) and purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLHGPDKF) encompasses an encephalitogenic sequence corresponding to aa residues 139–151 of PLP. PLP1 peptide is presented to T cells in association with I-A* MHC class II molecules.

**Ig chimeras.** Ig-PLP1 and Ig-W chimeras have been described previously (30). Briefly, construction of the chimeras used the genes coding for the L chain of the anti-aseocal Ab, 9iA3, as described (14, 30). The H chain CDR3 region was deleted and replaced with nucleotide sequences coding for PLP1 to generate the Ig-PLP1. Ig-W is the parental Ig (IgG2b κ), which does not encompass any peptide (14, 30). Both chimeras were expressed in the non-Ig-secreting myeloma B cell line SP20. Transfected cells were grown up in large-scale cultures of DMEM containing 10% iron-enriched calf serum (Intergen, Purchase, NY). The supernatants were purified from culture supernatant on affinity chromatography columns made of rat anti-mouse κ-chain coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NY). To avoid cross-contamination, separate columns were used to purify each chimera.

**Abs and cytokines**

Anti-B7.1 mAb, 1G10 (rat IgG2a), and anti-B7.2 mAb, 2D10 (rat IgG2b), specific for B7.1 and B7.2 molecules, respectively, were obtained from American Type Culture Collection (Manassas, VA). These Abs are directed against distinct, but closely related molecules of the B7 costimulatory family (32). Hybridoma 3/23 cells producing anti-CD40 Ab were kindly provided by M. Cancro (University of Pennsylvania, Philadelphia, PA). Anti-CD40, anti-B7.1, and anti-B7.2 mAbs were affinity purified from large-scale culture over a mouse anti-κ L chain (MAR185) column. The large-scale culture was carried out with bovine serum and medium that contain minimal amounts of endotoxin. Also, the Ig chimeras were purified by affinity chromatography, the preparations are free of endotoxins. Rat IgG used for control purposes were purchased from Sigma-Aldrich (St. Louis, MO). The following Abs were also used to detect cytokines by ELISA. The capture anti-cytokine Abs were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; rat anti-mouse IL-5, TRFK5; rat anti-mouse IL-12, 9A5 (specific for IL-12 p70); and rat anti-mouse IFN-γ, R4-6A2. Biotinylated anti-cytokine Abs were rat anti-mouse IL-2, JS66-5H4; rat anti-mouse IL-4, BV6D-24G2; rat anti-mouse IL-5, TRFK4; rat anti-mouse IL-12, C17.8 (specific for both IL-12p40 and p70); and rat anti-mouse IFN-γ, XMG1.2. All Abs were from BD PharmMingen (San Diego, CA). Recombinant mouse IL-2, IL-4, IL-5, and IFN-γ used to construct standard curves were also purchased from BD Pharmingen. Mouse rIL-12 was purchased from PeproTech (Rocksll, NJ).

**Regimen for neonatal injections of Ig chimeras and adult immunizations with peptide**

Neonatal injections of 100 μg of either Ig-PLP1 or Ig-W were performed i.p. in 100 μl saline within 24 h after birth. When the mice reached 7 wk of age, they were immunized s.c. in the footpads and at the base of the limbs with 100 μg PLP1 peptide emulsified in 200 μl PBS/CFA (v/v). Ten days later, the mice were microwaved and their spleens were removed for analysis of T cell responses.

**Induction of EAE**

EAE was induced by s.c. injection in the footpads and at the base of the limbs with a 200 μl IFA/PBS (v/v) solution containing 100 μg free PLP1 peptide and 200 μg Mycobacterium tuberculosis H37Ra. Six hours later, 50 ng of Bordetella pertussis toxin (List Biologicals, Campbell, CA) was given i.p. After 48 h, another pertussis toxin injection was given to the mice. Mice were scored daily for clinical signs, as follows: 0, no clinical signs; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death. Where indicated, mice were given i.p. a 500 μl saline solution containing 200 μg of anti-B7.1, anti-B7.2, or rat IgG within 4 h of PLP1 peptide injection. Another injection was given 3 days later. In other experiments, the mice were a mixture of 200 μg anti-B7.1 and 200 μg anti-B7.2.

**Splenic T cell proliferation**

Spleen cells were incubated in 96-well flat-bottom plates at 1 × 10⁶ cells/100 μl/well with 100 μl of stimulator alone or mixed with anti-B7 Abs for 3 days. Subsequently, 1 μCi [³H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were then harvested on a Trilux 1450 Microbeta Wallac Harvester, and incorporated [³H]thymidine was counted using the Microbeta 270.004 software (EG&G Wallac, Gaithersburg, MD).

**Isolation of DC**

Splenic DC were purified according to a standard collagenase/differences adherence method, as described (33). Briefly, the spleen was disrupted in a collagenase solution, and isolated DC floated on a dense BSA gradient. Subsequently, the cells were allowed to adhere to petri dishes for 90 min at 37°C, washed, and incubated overnight. The floating DC were then harvested and used as bulk DC or separated into DC subsets. The isolation of DC subsets was performed, as described (34). Briefly, the bulk DC were incubated with anti-CD8α mAb-coupled microbeads (Miltenyi Biotec, Auburn, CA) and separated into CD8α⁺ and CD8α⁻ populations by MACS (Miltenyi Biotech). The CD8α⁻ fraction was repassed on magnetic columns following incubation with anti-CD8α mAb-coupled microbeads to eliminate any residual CD8α⁺ cells. Subsequently, the CD8α⁻ cells were further purified by positive selection using anti-CD11c mAb-coupled microbeads (Miltenyi Biotec). For preparation of CD8α⁺ CD4⁺ and CD8α⁻ CD4⁺ DC subsets, the CD8α⁻ fraction was labeled with anti-CD4 mAb-coupled microbeads, and the subsets were separated, as above. The CD4⁺ fraction was further purified by positive selection using anti-CD11c mAb-coupled microbeads (Miltenyi Biotec). Each fraction was assessed for purity by flow cytometry, and no population was used if contamination was greater than 5%.

**ELISA**

For measurement of IL-2, IL-4, IL-5, and IFN-γ, cytokines by ELISA. The capture anti-cytokine Abs were rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IL-4, 11B11; rat anti-mouse IL-5, TRFK5; rat anti-mouse IL-12, 9A5 (specific for IL-12 p70); and rat anti-mouse IFN-γ, R4-6A2. Biotinylated anti-cytokine Abs were rat anti-mouse IL-2, JS66-5H4; rat anti-mouse IL-4, BV6D-24G2; rat anti-mouse IL-5, TRFK4; rat anti-mouse IL-12, C17.8 (specific for both IL-12p40 and p70); and rat anti-mouse IFN-γ, XMG1.2. All Abs were from BD PharmMingen (San Diego, CA). Recombinant mouse IL-2, IL-4, IL-5, and IFN-γ used to construct standard curves were also purchased from BD Pharmingen. Mouse rIL-12 was purchased from PeproTech (Rocksll, NJ).
Results

Anti-B7 mAbs exacerbate EAE in neonatally tolerized animals

Previous studies have shown that free self peptide given to mice in IFA on the day of birth protects the animals against a later pathogenic challenge with the peptide and suppresses the development of EAE (5, 27). Furthermore, when the self peptide is delivered on Ig's, IFA is no longer required, and resistance against EAE still develops (14) (Table I). Indeed, Table I shows that Ig-PLP1 given to mice in saline on the day of birth protects against death, reduces the severity of the initial phase of EAE, and suppresses relapses, and the mice recover within 1 mo postdisease induction. These effects were not observed with Ig-W, the parental Ig backbone not harboring any PLP peptide, and the rate of mortality was 46%, the initial phase was severe with a mean maximal score of 4.0 ± 1.0, and the surviving animals were unable to recover and displayed relapses up to day 100 after disease induction.

Several regimens, including increase in the dose of Ig-PLP1, multiple injections of Ig-PLP1, or even administration of IFA with Ig-PLP1, have been applied at the neonatal stage to suppress the residual EAE, but these attempts failed and the initial phase of disease developed with all regimens (16). Analysis of the T cell responses in the mice given Ig-PLP1 at birth and challenged with PLP1 peptide in CFA at 7 wk of age indicated that the lymph node develops deviated T cells producing IL-4 instead of IFN-γ, and the spleen generates anergic T cells unable to proliferate or produce IFN-γ yet secrete significant amounts of IL-2 (14). Intriguingly, these splenic cells could be restored to proliferate if assisted with exogenous IFN-γ or the IFN-γ-inducer IL-12 during in vitro stimulation with PLP1 peptide (14, 15). In fact, exogenous IL-12 was able to restore disease severity when administered to Ig-PLP1 neonatally tolerized mice during disease induction at the age of 7 wk (15). The question then was whether the residual disease is due to restoration of these anergic splenic cells by the pathogenic challenge with PLP1 peptide in CFA. In such case, splenic T cell restoration could be blocked by anti-B7 Abs, and the residual EAE should be inhibited. To test this premise, newborn mice recipient of Ig-PLP1 in saline on the day of birth were induced for EAE with PLP1 peptide in CFA at the age of 7 wk, simultaneously given anti-B7 Abs, and monitored daily for clinical signs of EAE for 100 days. As can be seen in Fig. 1, the experiment produced results opposite to what we have expected, as injection of either anti-B7.1 or anti-B7.2 Abs restored the severity of disease instead of suppressing the residual paralysis in these neonatally tolerized mice. Indeed, the mice recipient of Ig-PLP1 on the day of birth and given anti-B7.1 Ab during induction of EAE at adult life developed severe paralysis, leading to death of all mice, while the animals injected with the rat IgG isotype control instead of anti-B7.1 Ab had a much less severe initial phase of disease and displayed rather expeditious recovery, a pattern similar to the animals who did not receive any Ab during EAE induction. Similarly, the mice treated with anti-B7.2 Ab had restored severity of disease and 29% of the animals died. Thus, anti-B7 Abs exacerbate the disease in previously tolerized animals.

Anti-B7 mAbs restore specific responses in anergic splenic T cells

If exacerbation of disease is due to restoration of the anergic T cells, then these lymphocytes should be able to regain proliferation and IFN-γ production when stimulated with PLP1 peptide in the presence of anti-B7 Abs. Fig. 2 shows that splenic T cells from Ig-PLP1-tolerized and PLP1 peptide-immunized mice restore their proliferation when stimulated in the presence of anti-B7.1 or anti-

Table I. Neonatal exposure to Ig-PLP1 supports resistance to EAE induction at adult agea

<table>
<thead>
<tr>
<th>Tolerogen</th>
<th>Incidencea</th>
<th>Day of Disease Onsetb</th>
<th>Mean Maximal Disease Severity</th>
<th>Mortality (%)</th>
<th>Day of Recoveryb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig-W</td>
<td>13/13</td>
<td>12.5 ± 1.6</td>
<td>4.0 ± 1.0</td>
<td>46</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Ig-PLP1</td>
<td>14/14</td>
<td>13.4 ± 2.5</td>
<td>2.5 ± 0.8</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>

a Groups of mice were given 100 μg of Ig-PLP1, or Ig W in 100 μl saline on the day of birth, and 7 wk later were induced for EAE with 100 μg free PLP1 peptide in CFA, as indicated in Materials and Methods.

b A mouse is considered to have EAE when it scores 1 (loss of tail tone) or above for a least 3 consecutive days.

The onset of EAE represents the day a mouse receives a score of 1 or above, and the indicated numbers represent the mean ± SD.

Mice were considered recovered when their clinical score was <0.5 for the duration of clinical observation.
FIGURE 2. Anti-B7 mAbs restore proliferation of splenic T cells from Ig-PLP1-tolerized mice. A group of newborn mice was injected i.p. with 100 μg Ig-PLP1 in saline on the day of birth and challenged with 100 μg PLP1 in CFA at the age of 7 wk. Ten days later, their splenic cells (1 × 10⁶ cells/well) were stimulated with 15 μg/ml PLP1 peptide in the presence of the indicated amount of anti-B7.1 (○), anti-B7.2 (triangles) mAb, or rat IgG (diamonds), and tested for proliferation by [³H]thyidine incorporation, as described in Materials and Methods. Stimulation with PLP1 peptide alone (●) is included to show the baseline proliferation upon stimulation with PLP1 peptide in the absence of anti-B7 mAbs. The data represent the mean ± SD of four individually tested mice.

B7.2 Ab. Indeed, when stimulation was conducted with no Ab, there was a low proliferative response. However, when the stimulation used anti-B7.1 or anti-B7.2 Ab, there was a significant proliferation that increased proportional to the amount of Ab used. Such an increase in proliferation was not observed when control rat IgG were used instead of anti-B7 Abs. Similarly, both anti-B7.1 and anti-B7.2 restored the production of IFN-γ, but the control rat IgG did not (Fig. 3a). As demonstrated previously, the splenic T cells produced IL-2 upon stimulation with peptide alone (Fig. 3b).

FIGURE 3. Anti-B7 mAbs restore IFN-γ production by splenic T cells from Ig-PLP1-tolerized mice. A group of newborn mice was injected i.p. with 100 μg Ig-PLP1 in saline on the day of birth and challenged with 100 μg PLP1 in CFA at the age of 7 wk. Ten days later, their splenic cells (1 × 10⁶ cells/well) were stimulated with 15 μg/ml PLP1 peptide in the presence of the indicated amount of anti-B7.1 (○), anti-B7.2 (triangles) mAb, or rat IgG (diamonds), and 24 h later tested for production of IFN-γ, IL-2, IL-4, and IL-5 by ELISA, as described in Materials and Methods. Stimulation with PLP1 peptide alone (●) is included to show the baseline cytokine production upon stimulation with PLP1 peptide in the absence of anti-B7 mAbs. The data represent the mean ± SD of five individually tested mice.

However, stimulation with anti-B7 Abs induced a decrease in IL-2 production proportional to the amount of Ab concentration. This is possibly due to reabsorption of IL-2 as the cells regained proliferation, as has been seen when the restoration was mediated by IL-12 (14). In support of this statement is the observation that the control rat IgG, which did not restore proliferation or IFN-γ production, has not reduced production of IL-2. The splenic T cells restored by the anti-B7 Abs did not deviate toward Th2, as no IL-4 or IL-5 was produced during such stimulation (Fig. 3, c and d).

To determine whether restoration of the splenic anergic T cells by anti-B7 Abs operates independently of Ag or requires stimulation with PLP1 peptide, experiments were performed in which the splenic cells were incubated with the anti-B7 Abs with or without addition of PLP1 peptide. As can be seen in Fig. 4, both anti-B7.1 and anti-B7.2 Abs induced, in the presence of PLP1 peptide, levels of proliferation and IFN-γ production similar to those seen with the positive control, peptide stimulation in the presence of IL-12. However, anti-B7 Abs without PLP1 peptide showed no significant change relative to PLP1 peptide alone, which in this neonatal system does not induce proliferation and IFN-γ production. As for IL-2 production, PLP1 stimulation turns into positive control and IL-2 production is significant. IL-2 production was not observed with either anti-B7.1 or anti-B7.2 Ab in the absence of PLP1 peptide. However, when PLP1 peptide was added, although some IL-2 was observed, its level was not nearly as significant as peptide stimulation without anti-B7 Abs. This is due to restoration of proliferation, which facilitates reabsorption of IL-2 for growth as did the positive control PLP1 and IL-12. Overall, restoration of splenic T cell responses by anti-B7 Abs requires Ag stimulation.

FIGURE 4. PLP1 peptide is required for restoration of splenic T cell proliferative and IFN-γ responses by anti-B7 mAbs. SJL/J mice were injected with 100 μg Ig-PLP1 in saline within 24 h of birth. At the age of 7 wk, the mice were immunized with 100 μg free PLP1 peptide in CFA. Ten days later, the mice were sacrificed, and splenocytes (1 × 10⁶/well) were incubated with 40 μg/ml of anti-B7 mAb in the presence (+) or absence (−) of PLP1 peptide (15 μg/ml). Proliferation (a) was measured by [³H]thyidine incorporation after 72 h of cell culture. Production of IL-2 (b) and IFN-γ (c) was measured by ELISA after 24-h incubation. Peptide stimulation in the presence of IL-12 (10 U/ml) was included to serve as positive control. Each bar represents the mean ± SD of four individually tested mice.
Total blockade of B7 molecules prevents restoration of responses among anergic splenic T cells

Anti-B7 Abs incubated with the splenic cells encompassing both APC and T cells bind to B7 molecules on the APC and prevent interaction with CD28 on the T cells. It is not clear how this function would restore responses of the anergic splenic T cells. On a hypothetical basis, the B7 molecule is interacting with a down-regulatory ligand on the T cell (CTLA-4 for example), in which case blockade of such an interaction would inhibit negative signals and restore T cell responses. In this case, complete blockade of B7 molecules should inhibit costimulation, but still drive restoration of splenic T cell responses. Given the fact that the splenic response is polyclonal and TCR Vβ usage is diverse for PLP1-specific T cells, analyzing CTLA-4 expression on these cells is not feasible. Therefore, we elected to test the hypothesis by complete blockade of B7 molecules. As can be seen in Fig. 5, when peptide stimulation is conducted in the presence of a fixed dose of anti-B7.1 and graded amounts of anti-B7.2 Ab, restoration of splenic T cell proliferation is inhibited proportional to the increase in B7.2 concentration. Similarly, a fixed dose of anti-B7.2 combined with varying doses of anti-B7.1 leads to prevention of the restoration of T cell proliferation (Fig. 5b). Similar results were also observed for IFN-γ, and increase in anti-B7 Abs leads to a proportional decrease in cytokine production by the splenic T cells (Fig. 5, c and d). The effect of total B7 blockade was also tested at the disease level, and restoration of disease severity could not occur. Indeed, mice that were tolerantized with Ig-PLP1 on the day of birth, induced for EAE at the age of 7 wk with PLP1 peptide, and given mixture of anti-B7.1 and anti-B7.2 Abs had a pattern of disease that was similar to paralysis observed in animals given rat IgG isotype control instead of anti-B7 Abs (Fig. 6). Thus, total blockade of B7 molecules does not exacerbate the disease, as was observed when either Ab was given alone (Fig. 1). Overall, these findings indicate that costimulation through B7 molecules is required for reactivation of the anergic splenic T cells.

Anti-B7 Abs restore activation of anergic splenocytes through induction of IL-12 by APC

The experiments presented above demonstrate that anti-B7 Abs can assist the splenic cells for reactivation. The fact that such reactivation could not occur when optimal blockade is in place suggests that costimulation is required for T cell reactivation and anti-B7 Abs do not operate through inhibition of a negative signal. Thus, it is conceivable to consider the idea that anti-B7 molecules are rather triggering the production of cytokine that in conjunction with adequate peptide presentation rescues the splenic T cells for reactivation. Given the knowledge that the splenic anergic T cells of Ig-PLP1-tolerized and PLP1-immunized mice can be reactivated to proliferate and produce IFN-γ when assisted with IFN-γ or the IFN-γ-inducer IL-12 (14, 31), we sought to test APC for secretion of IL-12 upon incubation with anti-B7 Abs. Initially, we assessed bulk splenic DC, and the results indicate that both anti-B7.1 and anti-B7.2 trigger the production of IL-12 by these cells, as did the positive control anti-CD40 Ab (Fig. 7a). The isotype control rat IgG did not induce a significant amount of IL-12, indicating that anti-B7 Abs are operating through IL-12 production by binding to B7 molecules rather than FcγRs. Subsequently, experiments were performed to assess whether IL-12 induced in DC by anti-B7 Ab is involved in reactivation of the splenic T cells. Accordingly, splenic cells from Ig-PLP1-tolerized and PLP1-immunized mice were stimulated with PLP1 peptide in the presence of both anti-B7 and anti-IL-12 Abs and tested for reactivation. The results illustrated in Fig. 7b indicate that neutralization of IL-12 by anti-IL-12 Ab during stimulation of the splenic T cells with PLP1 peptide and anti-B7.1 or anti-B7.2 abrogates reactivation of the T cells. Indeed, addition of anti-IL-12 Ab to cell cultures containing PLP1 and anti-B7.1 or anti-B7.2 Ab produced proliferation equivalent to stimulation with PLP1 peptide alone, but much lower than proliferation observed with PLP1 and either anti-B7.1 or anti-B7.2 Ab. However, when anti-IL-12 was substituted with isotype control rat IgG, the proliferation increased significantly and reached levels comparable to the positive controls PLP1 and anti-B7.1 or anti-B7.2 Ab. Overall, these results indicate that IL-12 produced...
by DC upon incubation with anti-B7 Abs contributes to the restoration of splenic T cells. Despite the fact that B7.2, but not B7.1, is constitutively expressed on APC (35), anti-B7.1 Ab was much more potent than anti-B7.2 Ab in restoration of EAE in neonatally tolerized mice (Fig. 1), as well as in reactivation of the splenic anergic T cells of these mice (Figs. 2, 3, and 4). The question then was whether such a difference is related to the discrepancy in the induction of IL-12 production by DC. To address this issue, DC were fractionated into subsets, and production of IL-12 was measured upon incubation with anti-B7 Abs. The results presented in Fig. 8 show that splenic DC (20×10^3 cells/well) produce similar amounts of IL-12 upon incubation with anti-B7.1 and anti-B7.2 Abs. However, both CD4^+ and CD4^- subsets of the CD8^- population display significantly lower production of IL-12 upon incubation with anti-B7.2 Ab. It is well established that ~80% of the splenic DC population belongs to the CD8^- DC subset and the remaining 20% are CD8^+ (36). Thus, because the CD8^- population is much larger than the CD8^+ subset, and these cells produce less IL-12 upon stimulation with anti-B7.2 Ab, such discrepancy would remain apparent when whole splenic DC are stimulated with anti-B7 Abs.

**Discussion**

Recently, we have shown that neonatal exposure to peptides presented on Igs induces immunity rather than T cell deletion, as was observed for free peptides given to animals in IFA (14, 16). The use of Ig to vehicle the peptide overcomes the need for adjuvant, and T cell responses arose both in the spleen and lymph node (14, 16), while free peptide in IFA induces responses only in the spleen (7, 9). Intriguingly, the splenic response comprises Th1 cells that are not fully functional, but regain their proliferative and IFN-γ responses when assisted with exogenous IFN-γ or IL-12 (14). Moreover, this immunity conferred protection against EAE, and the neonatally sensitized mice had a mild initial phase of paralysis, with no relapses for the entire period of clinical observation (14) (Table I). Induction of splenic T cell anergy by neonatal exposure to Ag has also been observed in a different Ag system using a homogenous TCR transgenic animal model (37). Thus, this investigation came about to dissect the intriguing nature of this novel form of T cell anergy in the spleen. Our initial inclination was that the residual paralysis in neonatally sensitized mice stems from reactivation of the anergic splenic T cells by the active challenge with peptide in CFA. We then set up experiments to inhibit co-stimulation with anti-B7 Ab to prevent such reactivation and suppress the residual EAE. To our surprise, however, both anti-B7.1

FIGURE 7. Restoration of splenic T cell responses operates through anti-B7 Ab-mediated induction of IL-12 production by APC. a, Purified splenic DC (20×10^3 cells/well) were incubated for 24 h with 40 μg/ml anti-B7.1, 40 μg/ml anti-B7.2, or 80 μg/ml mixture (1 μg/1 μg) of anti-B7.1 and anti-B7.2 Abs. IL-12 (p70) production was then measured by ELISA. Rat IgG (40 μg/ml) were included for isotype control purposes. Anti-CD40 mAb is used as positive control for IL-12 production by DC. b, The splenic cells (1×10^6 cells/well) were stimulated with PLP1 peptide (15 μg/ml) and 40 μg/ml anti-B7.1 or anti-B7.2 in the presence of 10 μg/ml anti-IL-12 mAb or isotype control rat IgG. Proliferation was measured by [3H]thymidine incorporation, as described in Materials and Methods. Stimulation with PLP1 peptide alone is included to show the baseline proliferation. Stimulation with both PLP1 peptide and either anti-B7.1 or anti-B7.2 Ab without neutralization of IL-12 serves as positive control. Each bar represents the mean ± SD of triplicate wells.

FIGURE 8. Anti-B7.1 mAb is more potent than anti-B7.2 in induction of IL-12 production by APC. Subsets of DC (20×10^3 cells/well) including CD8^+ CD8^- CD4^+, and CD8^- CD4^- were incubated for 24 h with 40 μg/ml anti-B7.1, 40 μg/ml anti-B7.2, or 80 μg/ml mixture of anti-B7.1 and anti-B7.2 Abs. IL-12 (p70) production was then measured by ELISA. Rat IgG (40 μg/ml) were included for isotype control purposes. Anti-CD40 was used as positive control for IL-12 production. Each bar represents the mean ± SD of triplicate wells.
and anti-B7.2 mAbs used to block costimulation restored and even exacerbated the disease instead of inhibiting the residual paralysis (Fig. 1). Moreover, in vitro analyses showed that the splenic T cells regain the capability to proliferate and produce IFN-γ in an Ag-specific manner when the stimulation was conducted in the presence of either anti-B7 mAb (Figs. 2, 3, and 4). Although blockade with either Ab used high and possibly saturating concentrations, in both cases, one type of B7 molecule remains free and could mediate costimulation. When a mixture of anti-B7.1 and anti-B7.2 mAbs was used, restoration of proliferation and IFN-γ production was inhibited proportional to increase in Ab concentration (Fig. 5). Complete inhibition of such restoration was achieved with a saturating mixture containing 40 μg/ml of each mAb. In vivo, administration of a saturating mixture of anti-B7 mAbs did not exacerbate the disease, and the clinical signs were comparable to those observed in mice recipient of rat IgG isotype control. These in vitro and in vivo experiments indicated that reactivation of the anergic splenic T cells requires partial blockade of B7 molecules, and suggest that both B7-mediated costimulation and engagement with Ab are necessary in this process. A number of studies have previously suggested a regulatory interplay between IL-12 and B7-mediated costimulation in the activation of T cells (38, 39). We then sought to test whether engagement of B7 molecules with anti-B7 Ab triggers the APC to produce IL-12 that can serve for reversal of anergy. Indeed, the hypothesis proved right, and bulk splenic DC did produce IL-12 upon incubation with either anti-B7.1 or anti-B7.2 mAb (Fig. 7a). Moreover, such IL-12 was required for reactivation of the T cells because neutralization with anti-IL-12 Ab abolished the restoration of T cell proliferation (Fig. 7b). Thus, exacerbation of disease severity by anti-B7 Abs is most likely due to restoration of splenic T cell pathogenicity by IL-12. In fact, we have previously shown that administration of rIL-12 during induction of EAE in Ig-PLP1 neonatally tolerized animals restores disease to full severity (14). B7.1 and B7.2 have been shown to display discrepancies in T cell activation and modulation of autoimmunity (40–43). Anti-B7.1 and anti-B7.2 have shown quantitative differences in restoration of T cell proliferation and IFN-γ production (Figs. 2, 3, and 4), as well as exacerbation of EAE in neonatally tolerized mice (Fig. 1). Because DC may be the major presenting cells in neonatal immunity (44, 45), we fractioned the different subsets of DC and tested whether the anti-B7 Abs display quantitative differences in the induction of IL-12. The results indicated that the CD8α− subset produced similar amounts of IL-12 upon incubation with anti-B7.1 or anti-B7.2 Ab, but both the CD8α−/CD4+ and the CD8α+/CD4− secreted significantly more IL-12 when stimulated with anti-B7.1 Ab (Fig. 8). Given the fact that the CD8α− (comprising both CD8α+/CD4− and the CD8α+/CD4+) makes up ~80% of total splenic DC and the CD8α+ represents the remaining 20% (36), the amount of IL-12 that would be triggered by anti-B7.1 Ab in vivo would be much higher than that induced by anti-B7.2 Ab. It is likely that the increased severity of EAE observed with anti-B7.1 Ab is related to its superior IL-12 induction capability. IL-12 has previously been shown to reverse T cell tolerance (38), and the results presented in this work further support such an observation. It remains, however, unclear how IL-12 triggers reactivation of anergic splenic T cells. We have previously shown that the neonatal anergic T cells are unable to up-regulate IL-2Rα, but exogenous IL-12 assists in up-regulation of IL-2Rα and further facilitates both reabsorption of IL-2 and development of proliferative and IFN-γ responses (31). Overall, the study presented in this work demonstrates a dual role for B7 molecules serving both CD28 costimulation on T cells and IL-12 production by APC. These bidirectional functions revealed with a neonatal model of immunity may be operative in immune responses of adult mice, as B7 costimulation proves critical for T cell differentiation (46, 47) and effector functions (48–50). We should also mention that induction of IL-12 by anti-B7 Abs may be due to cross-reactivity with other members of the B7 costimulatory family (see Ref. 51 for review). If this is the case, then the discrepancy in IL-12 induction by anti-B7.1 and anti-B7.2 Abs could be related to variable cross-reactivity. The discrepancy among anti-B7.1 and anti-B7.2 mAbs in induction of IL-12 may account for the differential effects of B7.1 and B7.2 molecules in T cell differentiation (46) and manifestation of EAE (41, 43). Moreover, as neonatal DC seem to display deficiency in IL-12 production (52), cross-linking of their B7 molecules by CD28 from T cells may not trigger IL-12 secretion. Consequently, neonatal Th1 differentiation would be compromised, possibly leading to the novel form of splenic T cell anergy.

References


