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Cutting Edge: CD40 Ligation Prevents Neonatal Induction of Transplantation Tolerance

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To investigate the consequences of CD40 engagement on the neonatal induction of transplantation tolerance, BALB/c mice were injected at birth with (A/J × BALB/c) F1 spleen cells together with activating anti-CD40 mAb and grafted 4 wk later with A/J skin. Whereas A/J allografts were accepted in mice neonatally injected with F1 cells and control Ab, they were acutely rejected in mice injected with F1 cells and anti-CD40 mAb. Neonatal administration of anti-CD40 mAb resulted in enhanced anti-A/J CTL activity, increased IFN-γ, and decreased IL-4 production by donor-specific T cells in vitro. Experiments using anti-cytokine mAb and IFN-γ-deficient mice demonstrated that CD40 ligation prevents neonatal allotolerance through an IFN-γ and IL-12-dependent pathway. Finally, we found that newborn T cells express less CD40L than adult T cells upon TCR engagement. Taken together these data indicate that insufficiency of CD40/CD40L interactions contribute to neonatal transplantation tolerance.


Besides their critical roles in B cell-dependent immunity (reviewed in Ref. 1), CD40/CD40L interactions are also necessary for the induction of primary T cell responses (reviewed in Ref. 2). The importance of CD40/CD40L interactions for T cell costimulation is supported by observations showing that tolerance to alloantigens can be induced in adult animals by injection of blocking anti-CD40L Abs or CD40-deficient B cells (reviewed in Ref. 3).

In the classical model of transplantation tolerance induced by neonatal injection of semiallogeneic spleen cells (4), several mechanisms were found to be operative, including clonal deletion of donor-specific CTL (5) and immune deviation of helper T cells toward a Th2 phenotype (6–9). We reasoned that the ability of an adult spleen cell inoculum to silence Th1-type responses in newborn mice could be related to a defect in CD40 engagement on the injected cells, resulting in deficient T cell costimulation. To evaluate this hypothesis, we analyzed the consequences of perinatal administration of activating anti-CD40 mAb in BALB/c mice neonatally injected with (A/J × BALB/c) F1 spleen cells.

Materials and Methods

Mice

BALB/c (H-2d), A/J (H-2k), and C57BL/6 (H-2b) were purchased from IFFA CREDO (Brussels, Belgium) and (A/J × BALB/c) F1 hybrids were bred at our own colony. IFN-γ- and wild-type BALB/c mice (10) were bred under specific-pathogen-free conditions.

In vivo treatments

Neonatal tolerance was induced in BALB/c mice by injection of (A/J × BALB/c) F1 spleen cells (either 107 cells i.v. or 108 cells i.p.) within the first 24 h of life (day 0). Groups of mice were injected i.p. on days 1 and 3 with 10 µg of rat anti-mouse CD40 mAb (3/23, PharMingen GmbH, Hamburg, Germany) or control purified rat IgG (Sigma-Aldrich, Bornem, Belgium). When specified, mice were injected i.p. 2 h before injection of anti-CD40 mAb or control rat IgG with 100 µg of neutralizing rat anti-mouse IFN-γ mAb (R46A2) or rat anti-mouse IL-12 p40/p70 mAb (C15.1 and C17.8, kindly provided by Dr. G. Trinchieri, The Wistar Institute, Philadelphia, PA) or control isotype-matched anti-DNP rat mAb (LO-DNP-2 or LO-DNP-16, kindly provided by Dr. H. Bazin, Experimental Immunology Unit, Université Catholique de Louvain, Belgium), all in ascites form.

Skin grafting

Segments of (A/J × BALB/c) F1 tail skin were grafted onto the lateral thoracic wall of 4-wk-old BALB/c mice. Rejection was diagnosed when total epithelial breakdown occurred. Mice that retained their graft for more than 40 days were considered tolerant.

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Lymphokine production in MLC

MLC were prepared in complete RPMI medium between 2.5 × 10^6 lymphocytes from pooled axillary, inguinal, and mesenteric lymph nodes of experimental BALB/c mice and 5 × 10^6 irradiated (2000 rad) spleen cells from syngeneic BALB/c, donor-type (A/J × BALB/c) F_1, or third-party C57BL/6 mice. Culture supernatants were harvested after 72 h for IFN-g and IL-4 determinations using commercially available ELISA (Genzyme, Cambridge, MA).

**CTL assay**

MLC and 51Cr release assay were performed as already described (11). 51Cr release was measured with the Topcount 9912V counter (Packard Instrument, Meriden, CT) and results were expressed as percentages of specific lysis.

**Evaluation of chimerism**

B cell chimerism in lymph nodes was assessed by flow cytometry using double staining with FITC-conjugated anti-donor MHC class II mAb (anti-I-Ad) and biotinylated anti-CD45R/B220 mAb plus phycoerythrin-conjugated avidin (PharMingen).

**CD40L expression**

For analysis of CD40L mRNA up-regulation upon in vivo T cell activation, lymph nodes from 4-day-old mice were harvested on day 30 or 90 min after i.v. injection of either 5 µg of anti-CD3 mAb (145-2C11), 10^7 T cell-depleted syngeneic BALB/c, or (A/J × BALB/c) F_1 spleen cells. Total RNA extraction, preparations of cDNA, and PCR for CD40L gene and for β-actin were performed using standard procedures. Reactions were incubated in a DNA thermal cycler for 35 cycles. PCR primers used for CD40L amplification consisted of the following: sense primer 5'-ACATACAGGCAACCTTCCCC-3' and antisense 5'-GTGCTGCAATTTGAGGATCC-3'. CD40L expression was also analyzed using flow cytometry on CD4^+ cells purified from lymph nodes by immunomagnetic isolation (Dynabeads, Dynal, Oslo, Norway) and stimulated in vitro in wells coated with 10 µg/ml anti-CD3 mAb (145-2C11). After 12 h incubation, cells were double stained with FITC-conjugated anti-CD4 mAb and biotinylated anti-CD40L mAb (MR1, PharMingen) plus phycoerythrin-conjugated avidin.

**Results and Discussion**

**CD40 ligation prevents the induction of transplantation tolerance after neonatal injection of allogeneic spleen cells**

BALB/c mice grafted with A/J skin at 4 wk of age were monitored for graft survival during 6 wk. Whereas all control un.injected mice rejected their grafts within 20 days, graft tolerance was observed in 78% of the mice that had been neonatally injected with (A/J × BALB/c) F_1 spleen cells in combination with control rat Ab. In contrast, all mice that had received the activating anti-CD40 mAb and F_1 cells rejected their graft with a kinetic similar to that observed in uninjected mice (Fig. 1A). Since ligation of CD40 on B cells of the neonatal inoculum might influence their survival in the host (12), we determined the influence of anti-CD40 mAb treatment on the level of B cell chimerism in lymph nodes at 2 wk of age. Similar percentages of B220^+ cells expressing donor I-A^k were found by FACS analysis after neonatal injection of F_1 cells and control Ab (3.6 ± 0.6%, mean ± SEM, n = 3) as compared with animals having received anti-CD40 mAb in addition to F_1 cells (3.4 ± 0.05%, n = 3). The lack of skin graft tolerance in anti-CD40 mAb-treated mice could therefore not be attributed to abrogation of B cell chimerism.

**Effects of neonatal anti-CD40 mAb treatment on donor-specific T cell activities**

To get insight into the mechanisms responsible for graft rejection in anti-CD40 mAb-treated mice, we analyzed IFN-γ and IL-4 production in MLC prepared between host lymph node cells and either donor-type (A/J × BALB/c) F_1 or third-party (C57BL/6) spleen cells. As shown in Table I, we found that coinjection of anti-CD40 mAb with F_1 cells resulted in up-regulation of IFN-γ secretion and down-regulation of IL-4 production by donor-specific T cells. Data obtained with third-party C57BL/6 stimulators demonstrated that these effects of anti-CD40 mAb were specific for A/J donor alloantigens (Table I). This cytokine shift might depend on IL-12, a cytokine that is known to be induced by CD40/CD40L interactions (1, 2). However, anti-IL-12 mAb treatment induced only a partial inhibition of the donor-specific IFN-γ production observed in mice inoculated at birth with F_1 cells and anti-CD40 mAb (not shown). Anti-CD40 mAb injection also prevented, in most cases, donor-specific CTL unresponsiveness induced by neonatal inoculation of F_1 cells (Fig. 2).
IFN-γ and IL-12 are involved in the abrogation of neonatal rejection induced by anti-CD40 mAb treatment

To determine the involvement of IFN-γ and IL-12 in the restoration of CTL responses, two groups of mice were coinjected with anti-CD40 mAb and neutralizing anti-IFN-γ or anti-IL-12 p40/p70 mAb. Anti-donor CTL were poorly detectable in those two groups (Fig. 2), indicating that the effects of anti-CD40 mAb on CTL responses were dependent on both IFN-γ and IL-12. These effects were specific for donor-type alloantigens, since CTL responses to third-party C57BL/6 mice were similar in all groups of mice. In parallel, we found that skin graft survival at day 30 was significantly enhanced in mice coinjected with anti-CD40 and anti-IFN-γ mAb (57%; n = 8) or anti-IL-12 mAb (55%; n = 9), as compared with mice injected with anti-CD40 mAb alone (0%; n = 9) (p < 0.05 using Fisher’s test for both anti-IFN-γ and anti-IL-12 mAb).

To further assess the role of IFN-γ in the development of graft rejection in mice injected with anti-CD40 mAb and F1 cells, experiments were performed in IFN-γ-deficient mice. First, we observed that adult unmanipulated IFN-γ-deficient mice rejected their grafts as efficiently as wild-type mice (Fig. 1B). In contrast with its effect in wild-type mice, neonatal injection of anti-CD40 mAb did not prevent the neonatal induction of transplantation tolerance (Fig. 1B) in IFN-γ-deficient mice. Moreover IFN-γ-deficient mice injected with F1 cells and anti-CD40 mAb were unable to develop donor-specific CTL responses, whereas they developed normal CTL responses against third-party C57BL/6 targets (data not shown). Thus, CD40 ligation in this model allows the differentiation of donor-specific CTL via an IL-12-dependent and IFN-γ-dependent pathway.

### Table I. Cytokine levels in MLCa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-A/J</th>
<th>Anti-C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 cells</td>
<td>mAb</td>
<td>IFN-γ (pg/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1283 ± 306</td>
</tr>
<tr>
<td>+ Anti-CD40</td>
<td>3275 ± 1384b</td>
<td>147 ± 84b</td>
</tr>
</tbody>
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a lymph node cells were prepared from control and experimental mice 40 days after grafting with A/J skin and incubated in MLC with either donor-type (A/J × BALB/c) F1 or third-party C57BL/6 irradiated spleen cells as stimulators. Cytokine levels in culture supernatants were determined as described in Materials and Methods and data were expressed as mean ± SEM of six to nine mice per group.

b p < 0.05 vs mice injected with F1 cells and control Ab (Wilcoxon’s test).

**FIGURE 2.** Effects of perinatal injection of anti-CD40 mAb on antidonor CTL. Control un.injected mice (square); mice injected with F1 cells and control rat Ab (circle); mice injected with F1 cells, anti-CD40 mAb, and anti-DNP mAb (triangle); and mice injected with F1 cells, anti-CD40 mAb, and anti-IFN-γ mAb (diamond) or anti-IL-12 (upside-down triangle) were tested for anti-AJ and anti-C57BL/6 CTL activities 40 days after grafting with A/J skin. Results were expressed as percentages of specific lysis at an effector:target ratio of 100:1. No CTL activities could be measured against syngeneic BALB/c targets in all groups of mice. In the anti-IFN-γ- and anti-IL-12-treated mice, each point represents a pool of two animals.

**FIGURE 3.** CD40L mRNA expression in lymph nodes of newborn mice. Lymph nodes were harvested from 4-day-old BALB/c mice before, and 30 and 90 min after injection of either 5 µg 145-2C11 anti-CD3 mAb or 10^7 T cell-depleted (A/J × BALB/c) F1 spleen cells. The F1 cell inoculum before injection is shown in lane 6.

**FIGURE 4.** Flow cytometry analysis of CD40L surface expression on adult and neonatal CD4+ cells. Unstimulated neonatal cells (left panel), anti-CD3 mAb-stimulated neonatal cells (middle panel) and anti-CD3 mAb-stimulated adult cells (right panel) were double stained with anti-CD4 mAb and anti-CD40L mAb. Values in parentheses represent mean fluorescence intensities.
cells are unable to efficiently engage CD40 on the few professional APC (i.e., dendritic cells) present in the donor spleen. This would be consistent with previous suggestions of Matzinger et al. (17) and their recent demonstration that dendritic cell preparations induce efficient CTL responses in newborn mice (18). The Th2 polarization of the newborn response to allogenic spleen cells might be related to deficient CD40/CD40L interactions, since blockade of CD40L in adult mice was shown to promote Th2 immune deviation (19, 20). Furthermore, neonatal T cells spontaneously produce IL-4 (21), which inhibits IL-12 synthesis by APC (22) and down-regulates the expression of the IL-12 receptor β2 subunit on T cells (23). Indeed, we and others found that perinatal neutralization of IL-4 abrogates neonatal tolerance (7, 24). In conclusion, we suggest that neonatal transplantation tolerance involves a down-regulation of IL-4 in activated neonatal T cells and their recent demonstration that dendritic cell preparations in-duce efficient CTL responses in newborn mice (18).

Acknowledgments
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References
19. Adkins, B., and K. Hamilton. 1992. Freshly isolated, murine neonatal T cells are unable to efficiently engage CD40 in CD40/CD40L interactions on one hand and the negative influence of IL-4 spontaneously produced by neonatal T cells on the other hand.

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