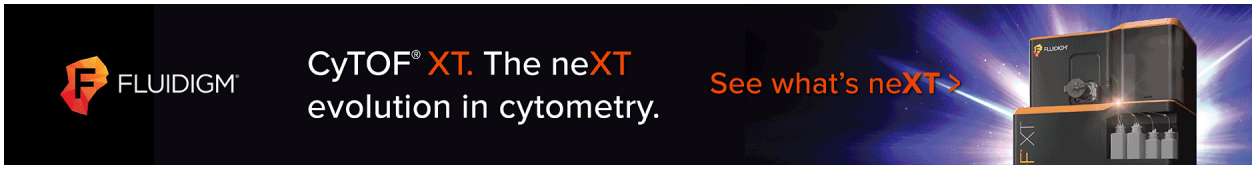

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Role of B7 in T Cell Tolerance[1]

Jens Lohr,[2] Birgit Knoechel,[2] Estelle C. Kahn, and Abul K. Abbas[3]

The induction of effective immune responses requires costimulation by B7 molecules, and Ag recognition without B7 is thought to result in no response or tolerance. We compared T cell responses in vivo to the same Ag presented either by mature dendritic cells (DCs) or as self, in the presence or absence of B7. We show that Ag presentation by mature B7-1/2-deficient DCs fails to elicit an effector T cell response but does not induce tolerance. In contrast, using a newly developed adoptive transfer system, we show that naive OVA-specific DO11 CD4$^+$ T cells become anergic upon encounter with a soluble form of OVA, in the presence or absence of B7. However, tolerance in DO11 cells transferred into soluble OVA transgenic recipients can be broken by immunization with Ag-pulsed DCs only in B7-deficient mice and not in wild-type mice, suggesting a role of B7 in maintaining tolerance in the presence of strong immunogenic signals. Comparing two double-transgenic models—expressing either a soluble or a tissue Ag—we further show that B7 is not only essential for the active induction of regulatory T cells in the thymus, but also for their maintenance in the periphery. Thus, the obligatory role of B7 molecules paradoxically is to promote effective T cell priming and contain effector responses when self-Ags are presented as foreign. *The Journal of Immunology,* **2004, 173: 5028–5035.**

The activation of naive T lymphocytes requires multiple signals, including Ag recognition by the TCR interacting with peptide-MHC molecules on APCs, and several costimulatory signals, the best characterized of which are B7-1 (CD80) and B7-2 (CD86) that interact with CD28 on the T cells (1–6). There is great interest in the consequences of Ag recognition without costimulation. Studies with T cell clones first showed that in vitro exposure to Ag in the absence of costimulation leads to clonal anergy, defined as a failure to proliferate and produce IL-2 upon secondary stimulation with Ag (7, 8). These findings led to the idea that signal 1 (MHC/peptide:TCR) without signal 2 (B7:CD28) results in the development of tolerance in CD4$^+$ T cells. In vivo blockade of B7:CD28 interaction with antagonists can result in prolonged graft survival and suppress autoimmunity (9, 10). However, these studies have not clearly shown whether Ag recognition with B7 blockade simply inhibits T cell activation or induces long-lasting tolerance. Nevertheless, such studies are the basis for developing B7 antagonists for treating autoimmune diseases and graft rejection in humans (11). The issue of the functional consequences of B7 antagonism has become even more complex with the recent recognition of a role of the B7:CD28 pathway in the development of regulatory T lymphocytes (12).

To definitively establish the effects of B7 deficiency on T cell responses, we have used knockout (KO)[4] mice lacking both B7-1 and B7-2 (B7KO) to ask how B7 costimulation influences T cell responses against Ags that are presented as foreign or self-Ag in vivo. The model for response to foreign Ag is the well-established system of adoptive transfer of DO11 TCR transgenic T cells followed by immunization with Ag-presenting dendritic cells (DCs) (13). To study responses to a bona fide self-Ag, we have developed a transgenic system in which a soluble form of OVA is expressed in the circulation on a wild-type (WT) or B7-deficient background. By adoptively transferring OVA-specific DO11 T cells into the Ag-expressing recipients, we show that anergy to the soluble self-Ag can be achieved in the presence or absence of B7 but it can be broken more easily by immunization if B7 is not present. We demonstrate that B7 not only enhances the development of Ag-specific CD4$^+$CD25$^+$ regulatory T cells in the thymus in response to self-Ag recognition but also maintains these cells in peripheral lymphoid organs. By comparing soluble with membrane-bound Ag, we demonstrate that, at least for the latter, B7 aids in the de novo generation of regulatory T cells, rather than simply increasing their survival. Because of its multiple roles, manipulating B7 expression and function may, therefore, have unexpected effects on tolerance induction in T lymphocytes.

Materials and Methods

Mice

All experimental mice were used at 6–12 wk of age. All mice were age and sex matched ± 1 wk. BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Transgenic mice expressing the DO11.10 TCR (DO11), specific for the chicken OVA peptide ($OVA_{323–339}$) in the context of the MHC class II molecule I-A^d, were obtained from Dr. K. Murphy (Washington University, St. Louis, MO). Mice deficient in B7-1 and B7-2 on the BALB/c background have been described and were provided by Dr. A. Sharpe (Harvard Medical School, Boston, MA) (4). Soluble OVA transgenic (sOVA Tg) mice expressing a soluble form of OVA in the serum under control of the metallothionein promoter were backcrossed onto BALB/c for >10 generations and crossed with DO11.10 TCR Tg mice (14).[5] RIP-mOva Tg mice expressing OVA in the β cells of pancreatic islets on a BALB/c background have been described (15, 16). All mice were bred and maintained in our pathogen-free facility in accordance with the guidelines of the Laboratory Animal Resource Center of the University of California (San Francisco, CA). All experiments were conducted with the approval of the Committee on Animal Research of the University of California, San Francisco. Peripheral blood of B7KO mice

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[2] J.L. and B.K. contributed equally to the work.

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[4] Abbreviations used in this paper: KO, knockout; DC, dendritic cell; WT, wild type; sOVA, soluble OVA.

[5] B. Knoechel, J. Lohr, E. C. Kahn, and A. K. Abbas. Signaling defects in anergic and regulatory T cells induced by systemic self antigen. *Submitted for publication.*

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was stained for the absence of B7-1 and B7-2, or typed by PCR as previously described (4).

Abs and flow cytometry

Purity of DCs was analyzed by staining with anti-IA^d (AMS 32.1), anti-B7-1 (CD80; 16-10A1), anti-B7-2 (CD86; GL1), and anti-CD11c (HL3). CD4$^+$ cells were stained with the clonotypic Ab KJ1-26 (Caltag Laboratories, Burlingame, CA), anti-CD4 (GK1.5, H129.19, and RM4-5), anti-CD25 (PC61 or 7D4), anti-CD69 (H1.2F3), or anti-CD62L (MEL-14). All Abs were obtained from BD Pharmingen (San Jose, CA) unless otherwise stated. Abs were used as FITC, PE, PE-Cy7, allophycocyanin, or PerCp protein conjugates. Fc-block (anti-CD16/CD32) was added before staining. Flow cytometric analyses were done on a FACSCalibur with CellQuest Software (both from BD Biosciences, San Jose, CA) or on a CYAN (DakoCytomation, Fort Collins, CO). Cells were sorted with a MoFlo cell sorter (DakoCytomation).

Cell preparations, purifications, and adoptive transfer

Bone marrow-derived DCs were generated by culturing bone marrow cells for 7–11 days in the presence of 10% supernatant from GM-CSF producing cell lines as described (17). Final maturation with 1 μg/ml LPS was done in the presence of 1 μg/ml OVA peptide for 24 h. CD4$^+$ cells for adoptive transfer were purified from spleen and lymph nodes using Dynabeads according to the manufacturer's protocol (Dynal Biotech, Oslo, Norway). CD4$^+$ cells were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) at 10×10^6 cells/ml for 10 min at 37°C and were washed before injection. A total of 5×10^6 CD4$^+$ DO11 T cells (purity >95%) were transferred into recipients by tail vein injection. One day after T cell transfer, recipients were immunized with 1×10^6 mature, OVA-pulsed WT DCs or 3×10^6 B7KO DCs by tail vein injection.

For restimulation assays, 4 days after T cell transfer, splenocytes of adoptive transfer recipients were enriched for CD4$^+$ cells by negative selection of B220$^+$ cells (Dynal Biotech), stained, and sorted for CD4$^+$KJ1-26$^+$ cells that had divided at least once by CFSE dilution or that were undivided from BALB/c recipients that were not immunized.

In vitro proliferation and cytokine assays

A total of 5000 sorted KJ1-26$^+$CD4$^+$ were cultured with 2.5×10^5 mitomycin C-treated BALB/c splenocytes in 200 μl of RPMI 1640 medium that contained 10% FCS in 96-well flat-bottom plates (Costar, Cambridge, MA). Cells were stimulated with 0–1 μg/ml OVA peptide. [^{3}H]Thymidine (1 μCi/well) was added on day 3 during the last 6 h of culture, and incorporation was measured by scintillation counting. Supernatants were collected after 48 or 72 h, and levels of IL-2 and IFN-γ were assayed by ELISA as previously described (18). In coculture assays, 25,000 sorted KJ1-26$^+$CD4$^+$CD25$^-$ T cells from DO11 mice were used as responders and stimulated on 25,000 APC with 1 μg/ml OVA. KJ1-26$^+$CD4$^+$CD25$^+$ T cells from double-transgenic mice were used as suppressors. [^{3}H]Thymidine (1 μCi/well) was added during the last 18 h of culture, and incorporation was measured by scintillation counting after 60 h of culture.

Results

B7 deficiency results in defective T cell activation but not tolerance in vivo

In the first set of experiments, we asked whether an immune response or tolerance develops against a foreign Ag in vivo if the Ag-presenting DCs are deficient in B7. Purified CD4$^+$ T cells from DO11.10 TCR transgenic (DO11) mice, specific for the chicken

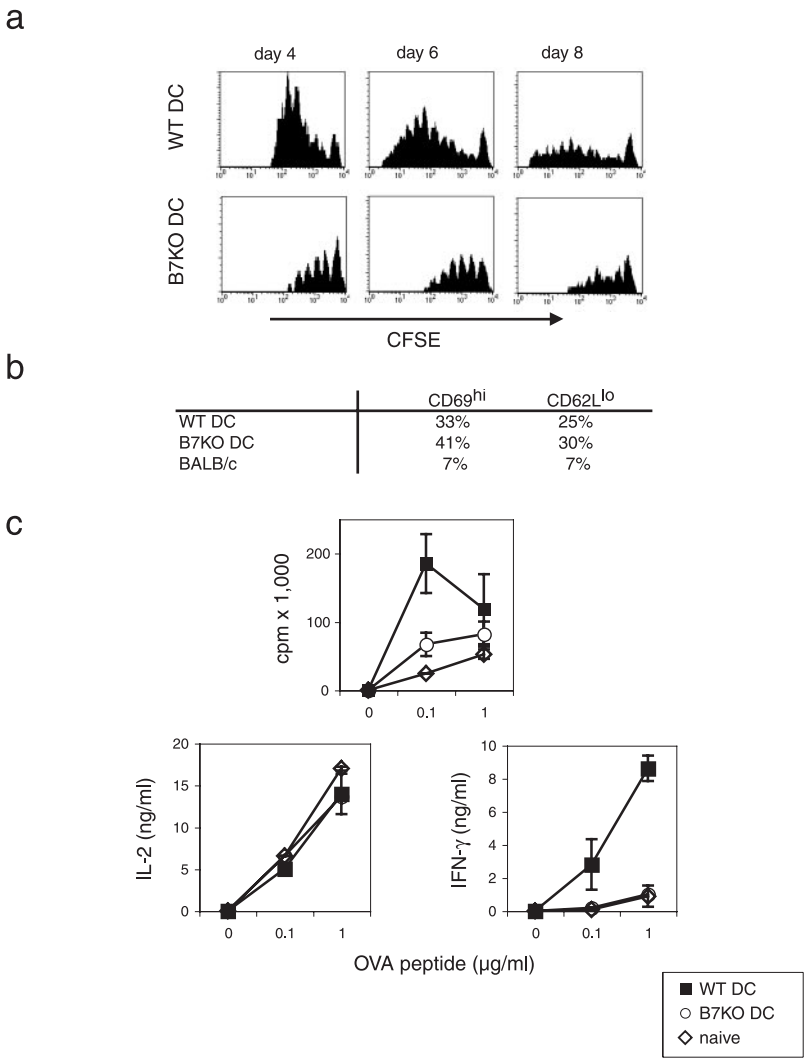

FIGURE 1. Absence of B7-1 and B7-2 on peptide-pulsed DCs results in inefficient activation but not tolerance in vivo. CFSE-labeled CD4$^+$ T cells from WT DO11.10 mice were adoptively transferred into WT BALB/c on day 0. On day 1, the mice were immunized i.v. either with 1×10^6 LPS-treated mature bone marrow-derived DCs from WT or 3×10^6 DCs from B7KO BALB/c mice, pulsed with OVA peptide for the last 24 h of culture. *a*, Spleen cells were recovered on days 4, 6, and 8, and CFSE profiles of gated KJ1-26$^+$CD4$^+$ cells are shown. All histograms were normalized to the KJ1-26$^-$CD4$^+$ population. Therefore, the area under the curve of the histogram is directly proportional to the absolute number of recovered KJ1-26$^+$CD4$^+$ cells. Results are from one representative experiment of two with two mice per group. *b*, Percentage of cells within the KJ1-26$^+$ fraction that up-regulate CD69 and down-regulate CD62L 1 day after immunization. *c*, On day 4, spleen cells were harvested and CD4$^+$KJ1-26$^+$ cells that had undergone at least one division by CFSE dilution were isolated by cell sorting and restimulated with Ag plus WT APCs. For naive cells, uncycled CD4$^+$KJ1-26$^+$ cells were sorted from BALB/c recipients. [^{3}H]Thymidine incorporation was assayed on day 3, supernatants were harvested from the restimulation cultures on day 2 for analysis of IL-2 concentration, and on day 3 for analysis of IFN-γ concentration by ELISA.

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OVA peptide (OVA peptide) + I-A^d, were labeled with CFSE and adoptively transferred into normal BALB/c recipients. The mice were immunized with LPS-treated mature bone marrow-derived DCs from normal or B7KO donors. The DCs had been incubated with the OVA peptide. The DO11 T cells were assayed 4, 6, and 8 days postimmunization for cycling and expression of activation markers. We have previously shown that under these conditions, the immunizing APCs are the Ag-pulsed DCs and there is no evidence of Ag carryover to host DCs (19). Strong T cell proliferation and expansion occurred only when the immunizing DCs expressed B7 (Fig. 1*a*). Increasing the numbers of Ag-pulsed B7KO DCs led to more T cell recruitment, but only a minimal increase in the number of completed cell divisions (data not shown). T cells primed with B7KO DCs at the indicated concentrations showed similar expression of CD69 and CD62L, therefore ruling out the possibility that the T cells simply ignore the Ag in the absence of B7 (Fig. 1*b*). Thus, Ag presentation without B7 costimulation elicits a weak T cell response in vivo.

To examine the functional responses of DO11 T cells activated by WT or B7KO DCs, cycled KJ1-26$^+$CD4$^+$ cells were purified from immunized mice by cell sorting and restimulated with OVA peptide ex vivo, and proliferation and cytokine production were analyzed. Naive cells were obtained by sorting uncycled KJ1-26$^+$CD4$^+$ cells from unimmunized BALB/c mice. T cells primed with B7KO DCs proliferated more than naive DO11 cells and produced comparable amounts of IL-2 (Fig. 1*c*). However, T cells primed in the absence of B7 did not develop effector function since there was no IFN-γ detectable. DO11 cells primed with B7KO DCs show ~3-fold less CD25 expression than DO11 cells primed with WT DCs (data not shown). This reduced IL-2 sensitivity may account for the reduced effector differentiation despite adequate production of IL-2. Thus, T cells primed with B7-deficient DCs in WT hosts are not tolerized, i.e., they retain their ability to respond to Ag, but less efficiently than T cells primed with WT DCs.

Role of B7 in tolerance induction in naive T cells transferred into self-Ag expressing mice

In the next set of experiments we asked how tolerance to a soluble self-Ag is affected by the presence or absence of B7. We have generated a transgenic mouse line that expresses sOVA in the circulation under the control of the metallothionein promoter (sOVA Tg),[5] and crossed these mice with B7KO mice. Purified CD4$^+$ T cells from DO11 TCR transgenic mice were labeled with CFSE and transferred into WT or B7KO sOVA Tg mice, and the recipients were followed for T cell expansion and activation. The majority of transferred DO11 T cells in WT sOVA Tg mice encountered the Ag, as demonstrated by the expression of the activation markers CD69 and CD62L, and almost all the cells were recruited into cycle. T cells transferred into B7KO sOVA Tg mice also started cycling and expressed activation markers, but recruitment into cycle was less and the total expansion was reduced compared with WT sOVA Tg recipients (Fig. 2, *a* and *b*). To analyze the

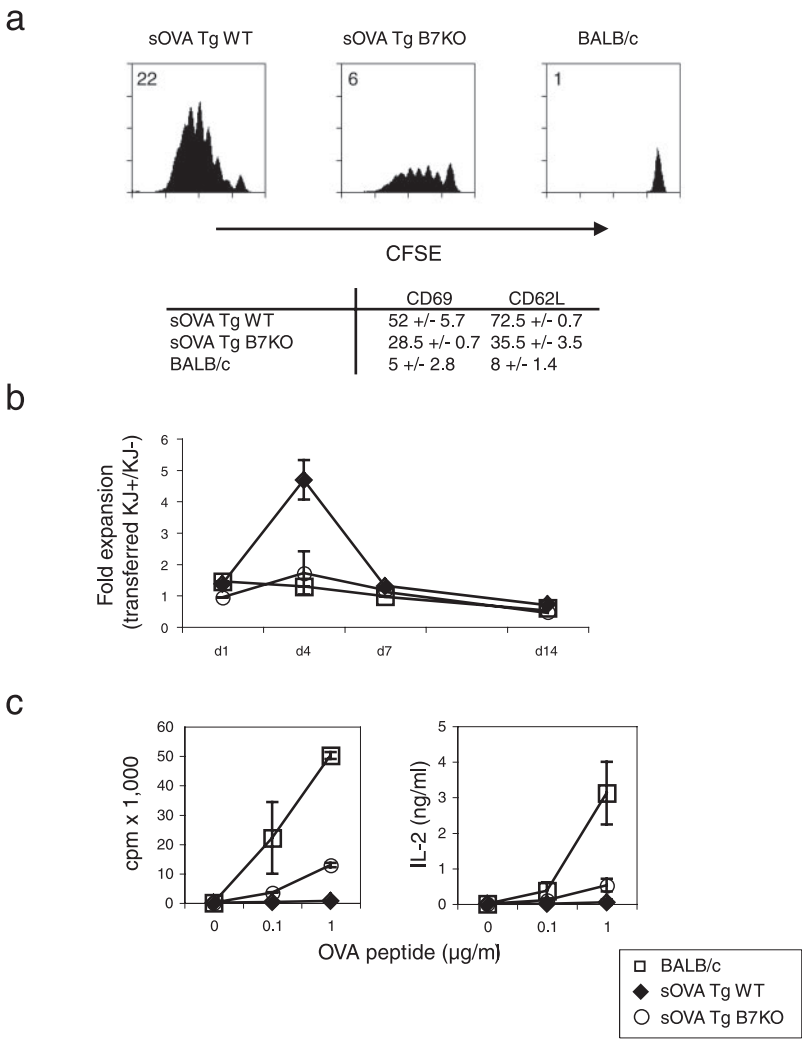

FIGURE 2. Tolerance to soluble self-Ag in the absence of B7. A total of 5 × 10^6 CFSE-labeled CD4$^+$ T cells from WT DO11.10 mice were adoptively transferred into sOVA Tg WT or B7KO mice and BALB/c controls on day 0. *a,* Lymph nodes were recovered on day 4, and CFSE profiles of gated KJ1-26$^+$CD4$^+$ cells are shown. The numbers indicate the proliferation index calculated as the ratio of cycled/uncycled KJ1-26$^+$ cells. Results are from one representative experiment of five. The table shows the percentage of cells within the KJ1-26$^+$ fraction that up-regulate CD69 and down-regulate CD62L. *b,* The numbers of KJ1-26$^+$CD4$^+$ were determined at the times indicated. Fold expansion refers to the increase of DO11 cells relative to CFSE$^+$KJ1-26$^-$CD4$^+$ cells. Each symbol represents the mean ratio from two individual mice. One representative experiment of three is shown. *c,* On day 4, lymph nodes were harvested, and CD4$^+$KJ1-26$^+$ cells that had undergone at least one division by CFSE dilution in the sOVA Tg mice and uncycled CD4$^+$KJ1-26$^+$ cells from the naive BALB/c mice, respectively, were isolated by cell sorting and restimulated with Ag plus WT APCs. [^{3}H]Thymidine incorporation was assayed on day 3, and supernatants were harvested from the restimulation cultures on day 2 for analysis of IL-2 concentration by ELISA.

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functional responses of DO11 T cells exposed to the soluble self-Ag, we purified cycled T cells from either WT or B7KO sOVA Tg mice by cell sorting, restimulated the cells with OVA peptide ex vivo, and analyzed their proliferative and cytokine responses. Control cells were obtained from naive BALB/c mice by sorting on the uncycled KJ1-26$^+$ T cell population. T cells recovered from WT sOVA Tg mice were hyporesponsive to restimulation, as they showed reduced proliferation and a diminished IL-2 response (Fig. 2*c*) and lacked IFN-γ production when compared with T cells primed with OVA/IFA in BALB/c recipients (data not shown). T cells recovered from B7KO sOVA Tg mice also showed a defect in proliferation and IL-2 production and lack of effector cytokines. Typically, the T cells recovered from the B7KO sOVA Tg recipients were not as hyporesponsive as cells recovered from the WT sOVA recipients. Tolerance could also be achieved by adoptive transfer of DO11 Rag$^{-/-}$ cells into WT sOVA Tg mice, where defective IL-2 production could be observed after ex vivo restimulation, compared with naive cells (data not shown). Thus, tolerance to the transgene-encoded self-Ag develops even in the absence of B7, although less efficiently than in the presence of B7.

Immunization breaks tolerance to self-Ag only in B7-deficient hosts

In the experiments done so far, we assayed tolerance by ex vivo responses to Ag. Although this is an accepted method for studying tolerance (20–22), it is clearly important to show that tolerant T cells are also unresponsive to immunization in vivo. To do this, we transferred DO11 T cells into WT or B7KO sOVA Tg mice or control non-Tg BALB/c mice, and immunized all recipients with Ag-pulsed DCs. The DO11 T cells were purified 4 days later, and their responses to Ag were assayed. T cells that encountered the circulating Ag in WT sOVA Tg mice responded much less than T cells immunized in BALB/c recipients. Surprisingly, T cells recovered from the B7KO sOVA Tg recipients showed even greater IL-4 and IFN-γ production than cells recovered from control recipients (Fig. 3). Thus, although tolerance to the transgene-encoded self-Ag develops even in the absence of B7 (Fig. 2), this tolerance is readily lost when the mice are immunized with Ag-pulsed WT DCs. In contrast, tolerance cannot be broken by immunization in WT self-Ag-expressing mice.

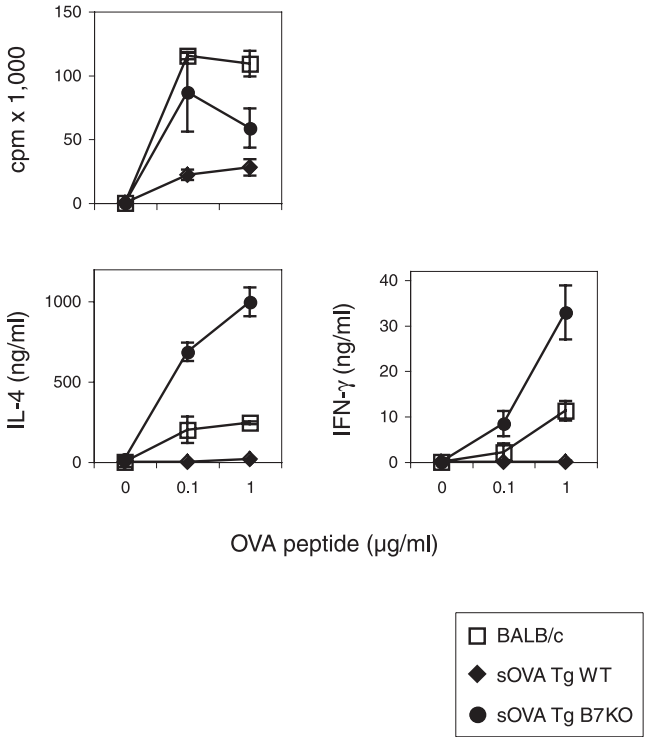

FIGURE 3. Tolerance to soluble self-Ag can be broken more easily in the absence of B7. 5 × 10^6 CFSE-labeled CD4$^+$ T cells from DO11.10 mice were adoptively transferred into WT sOVA Tg, B7KO sOVA Tg, or nontransgenic BALB/c recipients on day 0. On day 1, the mice were immunized with 1 × 10^6 mature bone marrow-derived DCs from WT BALB/c mice pulsed with 1 μg/ml OVA peptide. KJ1-26$^+$CD4$^+$ cells were isolated as in Fig. 2, and restimulated with Ag and WT APCs. [^{3}H]Thymidine incorporation was assayed on day 3. For cytokines, culture supernatants were harvested on day 2 and assayed for IL-4 and IFN-γ levels by ELISA. Data from one representative experiment of three are shown.

Role of B7 in generation of CD4$^+$CD25$^+$ cells and thymic deletion of self-reactive T cells

A likely explanation for this paradoxical effect of B7 molecules in enhancing, rather than preventing, tolerance is that B7-deficient animals lack CD4$^+$CD25$^+$ regulatory T cells (19, 23). It has been demonstrated that Ag-specific CD4$^+$CD25$^+$ regulatory T cells can be generated in the thymus by high-affinity Ag recognition as an alternative pathway to negative selection (15, 24, 25). However, it is unclear whether B7-dependent costimulation plays a role solely in thymic generation of regulatory T cells or additionally in their peripheral survival or function. Further, it is unclear to what extent true induction or resistance to deletion contributes to generation of a peripheral pool of regulatory T cells. We have addressed these questions with two different double-transgenic mouse models by crossing DO11 mice either with sOVA Tg mice, in which OVA is secreted into the circulation (sOVA Tg), or with RIP-mOVA Tg mice that express membrane-bound OVA in the pancreatic islets (15). Both were backcrossed onto a B7-1/2-deficient background. Generation of Ag-specific T cells and CD4$^+$CD25$^+$ cells was analyzed in the thymus and in the periphery. As expected, lower total DO11 cell numbers were found in the thymus and lymph nodes of WT DO11 × sOVA Tg and DO11 × RIP-mOVA Tg animals, compared with single-transgenic DO11 animals (Fig. 4*a*). Deletion was more profound with soluble than with membrane-bound Ag, presumably due to higher concentration of the Ag in the thymus. Furthermore in both double transgenics, a substantial proportion of the surviving DO11 cells developed into CD25$^+$ cells (Fig. 4*b*). Interestingly, in lymph nodes and thymus of DO11 × sOVA Tg mice the relative percentage of CD4$^+$CD25$^+$ cells was much higher than in DO11 mice but the absolute cell number was only slightly increased. At least two possible explanations may account for this: 1) negative selection occurs independently of or before CD25 expression is actively induced; or 2) CD4$^+$CD25$^+$ cells accumulate more because they are more resistant to deletion and not because they are actively induced. While these possibilities cannot be easily distinguished in the DO11 × sOVA Tg model, the numbers of CD25$^+$ DO11 cells in the DO11 × RIP-mOVA Tg model were ~5-fold higher than in DO11 mice, suggesting that the CD25$^+$ cells are, indeed, actively induced by self-Ag. The efficiency of this induction and the susceptibility of CD25$^+$ to deletion may depend on the way the Ag is presented (soluble vs membrane-bound) and possibly its concentration.

In the absence of B7, deletion was reduced in both double-transgenic models, corresponding to a modestly increased percentage of KJ1-26$^+$CD4$^+$ cells in the thymus and the periphery (Fig. 4*a*). This trend was also reflected in total cell numbers. Importantly, in the absence of B7, there was a marked reduction in the

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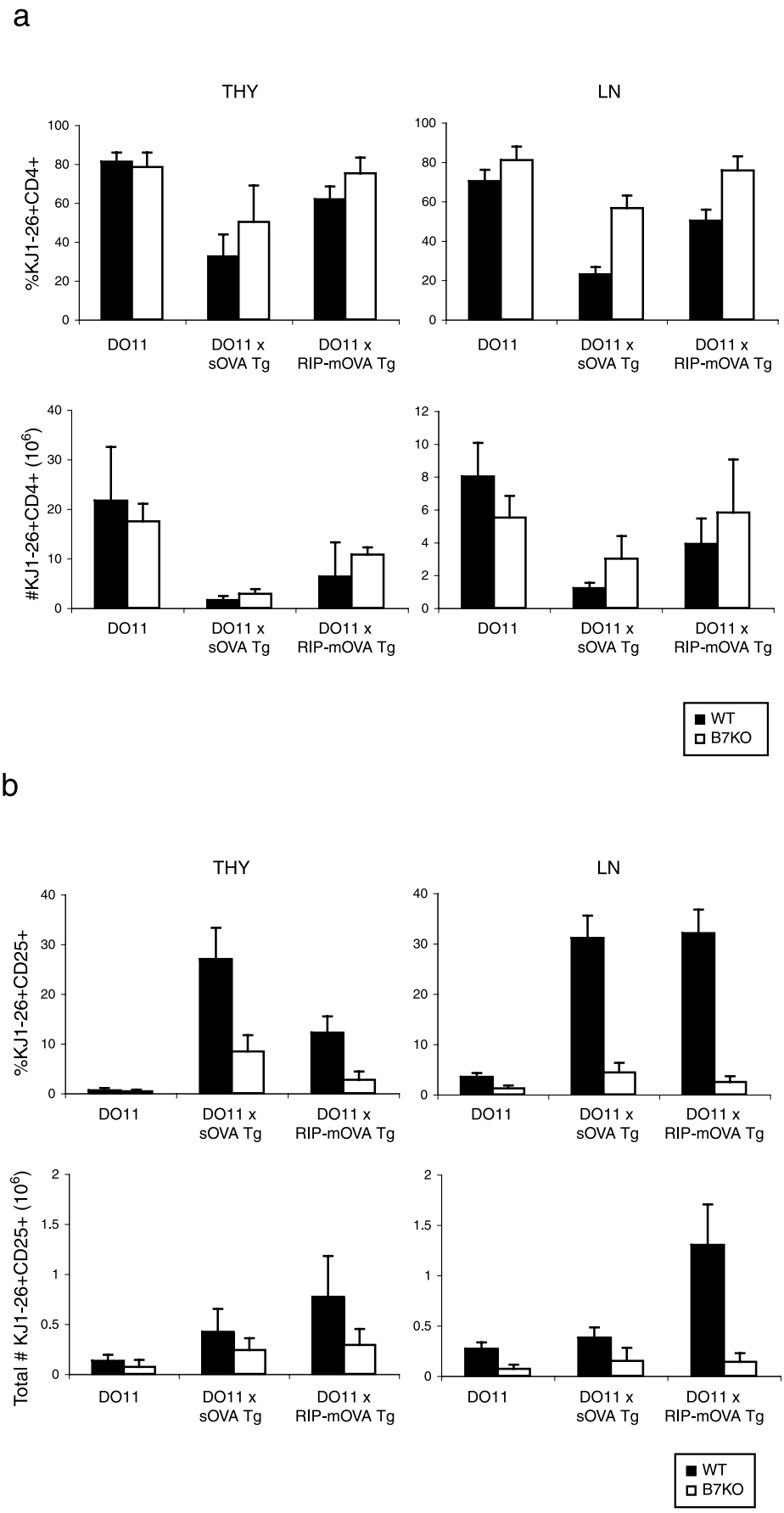

FIGURE 4. Influence of B7 on thymic deletion and generation of $CD4^+CD25^+$ cells. $CD4^+$ single-positive thymocytes and lymph node cells from DO11 × sOVA Tg, DO11 × RIP-mOVA Tg, or DO11 Tg animals on a WT or B7KO background were analyzed for expression of CD4, CD25, and KJ1-26 by flow cytometry. KJ1-26^+CD4^+ within the single-positive $CD4^+$ population (*a*) or KJ1-$26^+CD4^+CD25^+$ within the KJ1-26^+CD4^+ cells (*b*) are shown in thymus (THY) and peripheral lymph nodes (LN). *Upper panels* show percentages; *lower panels* show absolute cell numbers which were calculated from total cell counts of thymocytes or lymph node cells and the percentage of the indicated populations as determined by flow cytometry. Each column indicates the mean of four to eight mice per group of three pooled individual experiments.

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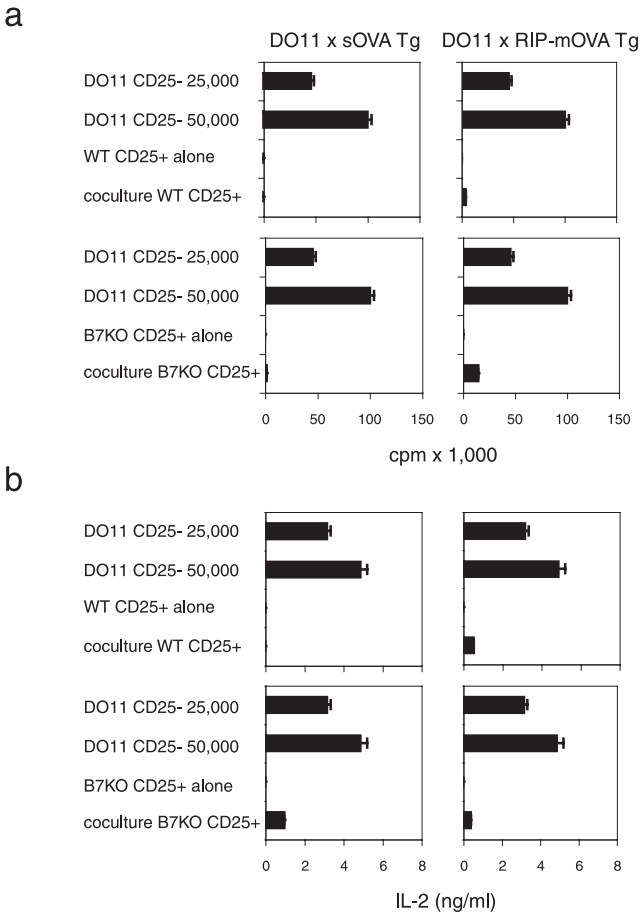

FIGURE 5. $CD4^+CD25^+$ cells from B7-deficient animals show regulatory function in vitro. KJ1-$26^+CD4^+CD25^+$ from lymph nodes of DO11 × sOVA Tg or DO11 × RIP-mOVA Tg mice either on a WT or B7KO background were purified by cell sorting, and 2.5×10^4 cells/well were cocultured with KJ1-$26^+CD4^+CD25^-$ cells from DO11.10 mice (2.5×10^4/well) and 2.5×10^4/well mitomycin C-treated BALB/c splenocytes and 1 μg/ml OVA peptide for 60 h. *a*, $[^3H]$Thymidine (1 μCi/well) was added during the last 18 h of culture. *b*, Supernatants for IL-2 were taken on day 2 of culture and assayed by ELISA. Data from one representative experiment of three with two individual mice per group is shown.

percentages and numbers of $CD4^+CD25^+$ cells induced by both forms of self-Ag (Fig. 4*b*), indicating that B7 plays an important role in the generation of $CD4^+CD25^+$ cells. Furthermore, the decrease in $CD25^+$ DO11 cells was even more profound in the lymph nodes than in the thymus suggesting that B7 molecules are required for both thymic generation of $CD4^+CD25^+$ cells and also for their maintenance in the periphery.

Finally, we asked whether the surviving Ag-specific $CD4^+CD25^+$ population in B7-deficient mice was functional as regulatory T cells. Therefore, we isolated DO11 $CD4^+CD25^+$ cells from WT and B7KO DO11 × sOVA Tg as well as from DO11 × RIP-mOVA Tg animals and analyzed their ability to suppress the response of naive $CD25^-$ DO11 cells in vitro. $CD4^+CD25^+$ cells from B7KO animals from both double-transgenic models showed suppressive function similar to $CD25^+$ cells recovered from WT double-transgenic mice, on a per-cell basis (Fig. 5). Thus, although B7 enhances the development and survival of regulatory T cells, the few $CD4^+CD25^+$ T cells that are generated in the absence of B7 remain functional.

Discussion

The goal of our studies was to examine the responses of $CD4^+$ T lymphocytes to immunization with a foreign Ag and exposure to a self-Ag in the absence of B7. The rationale for these studies is that it is virtually dogma that Ag recognition without B7 costimulation leads to T cell tolerance by some combination of anergy and enhanced apoptotic death (2). In fact, this idea has provided the impetus for developing B7 antagonists as therapeutic agents for treating pathologic immune reactions, as in autoimmune diseases and allograft rejection (9, 10). However, there are very few in vivo studies directly addressing the question of what happens to T cells if they see Ag in the absence of B7 (26–28). This is especially true for defined self-Ags recognized by T cells that can be identified and analyzed quantitatively. Therefore, we set out to address this question using transgenic and KO mouse models in which the T cells can be studied in detail, and their cognate Ag can be introduced as a "foreign" Ag presented by mature DCs or as a self-Ag produced constitutively and continuously.

Our experiments show that $CD4^+$ T cells that encounter their cognate Ag presented by mature DCs retain the capacity to proliferate and produce IL-2, much like naive T cells. There is no failure of the T cells to see Ag presented by B7-deficient DCs, because the T cells do express activation markers and undergo cycling. Thus, Ag recognition without B7 is an inadequate signal for T cell activation but it does not appear to be a tolerogenic signal. This result is consistent with findings that blockade of B7-1 and B7-2, e.g., by CTLA4-Ig, is successful in prolonging graft survival and alleviating autoimmunity, but eventual graft rejection or progression of autoimmune disease is often seen, indicating that permanent tolerance is not achieved (29–31).

A major goal of our studies was to develop a model for studying the role of B7 costimulators in responses to a true self-Ag. To do this, we have generated sOVA Tg mice and introduced DO11 T cells into these mice. T cells that encounter the self-Ag are stimulated to proliferate, but then they rapidly become anergic, as measured by lack of response to ex vivo restimulation with Ag or in vivo immunization with Ag-pulsed DCs. Strikingly, in the absence of B7, the T cells become hyporesponsive, as assayed by ex vivo restimulation. But immunization elicits a greater response even in the presence of the circulating self-Ag than is seen with naive T cells in the absence of self-Ag. Thus, the absence of B7 does not lead to tolerance, as is widely believed, but rather it results in less tolerance, or a decreased ability to maintain tolerance in the face of strong immunogenic stimuli.

This paradoxical role of B7 in maintaining the tolerant state was discovered during studies of the role of the B7:CD28 pathway in the autoimmune disease of NOD mice (23). It has subsequently been shown that in the absence of B7, even immunization with foreign Ag presented by DCs induces an exaggerated T cell response (19). A likely explanation for this role of B7:CD28 costimulation is that it is required for the generation and maintenance of $CD4^+CD25^+$ regulatory T cells (19, 32). To test this formally, we have used two transgenic systems in which exposure of DO11 T cells to their cognate Ag throughout development results in the generation of Ag-specific $CD25^+$ T cells that have the phenotypic and functional characteristics of regulatory T cells (15, 25, 33). We show that in the absence of B7, there is a marked reduction in thymic generation of Ag-specific regulatory T cells, and an even greater decrease in the numbers of these cells in peripheral lymphoid organs. Comparison of DO11 × sOVA Tg with DO11 × RIP-mOVA Tg mice reveals interesting differences. Deletion is greater in the DO11 × sOVA Tg mice, presumably because the

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concentration of Ag in the thymus is greater. Although the percentages of $CD25^+$ cells are increased compared with DO11 mice, the total numbers are increased very little. Thus, with the soluble self-Ag, there may be inefficient induction or alternatively regulatory T cells may be less susceptible to deletion. In DO11 × RIP-mOVA Tg mice, in contrast, there is less thymic deletion and clear induction of $CD25^+$ cells. Thus the form of Ag and its concentration may markedly influence the fate of T cells developing in the thymus. In both transgenic models, the absence of B7 has a profound effect on the number of $CD25^+$ DO11 cells in the thymus and the lymph nodes.

Our results suggest that in the absence of B7 there may be a small defect in the thymic negative selection consistent with previous published data (34–38), but by far, the major effect of B7 is in the generation and peripheral maintenance of $CD25^+$ regulatory T cells. Although the absence of $CD25^+$ regulatory cells in the B7-deficient mice is the likely explanation for the ease with which tolerance can be broken by immunization, it is difficult to prove this definitively, because regulatory T cells do not survive well in B7-deficient recipients. Therefore, we cannot correct the defects of these recipients by adoptively transferring $CD25^+$ Ag-specific T cells.

The B7:CD28 pathway may play additional roles in the maintenance of self-tolerance. In the absence of B7, T cells that encounter the circulating self-Ag cycle less than they do in the presence of B7. Cell cycling has been observed as a prelude to the development of anergy in many models of T cell tolerance (21, 39, 40). In fact, such results have led to the hypothesis that tolerance is a form of aborted activation, in which T cells begin to respond but their response is extinguished and they are silenced, perhaps permanently (41). Our results show that even the initial T cell response to self-Ag is increased by B7 costimulation. If the initial response is necessary for the subsequent development of tolerance, the reduced cycling of self-reactive T cells in the absence of B7 may also translate into reduced tolerance. It is unknown if this will be true of all forms of tolerance induced by all types of self-Ags.

Finally, a general concept is emerging that the immune system uses similar signals for the generation of effector and regulatory T cells. Two well-defined examples of such signals are the B7:CD28 pathway and IL-2. It follows that these signals can have opposing effects on immune responses to self- and foreign Ags (42). One of the challenges in the field is to predict which of the opposing actions will be dominant in different physiologic and therapeutic situations. Whether the ultimate effects of these signals are determined by other concomitant stimuli, such as innate immune responses, is another fundamental and important question.

In summary, the presented results may have important implications for the therapeutic potential of B7 antagonists. Although blockade of the B7:CD28 pathway may suppress acute pathologic T cell reactions, prolonged blockade may result in decline of self-Ag-reactive regulatory T cells and loss of self-tolerance. This could have potentially harmful consequences.

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