A Novel Alloantigen-Specific CD8⁺PD1⁺ Regulatory T Cell Induced by ICOS-B7h Blockade In Vivo

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A Novel Alloantigen-Specific CD8⁺PD1⁺ Regulatory T Cell Induced by ICOS-B7h Blockade In Vivo

Atsushi Izawa,²* Kazuhiro Yamaura,²* Monica J. Albin,* Mollie Jurewicz,* Katsunori Tanaka,* Michael R. Clarkson,* Takuya Ueno,† Antje Habicht,* Gordon J. Freeman,* Hideo Yagita,§ Reza Abdi,* Todd Pearson,¶ Dale L. Greiner,¶ Mohamed H. Sayegh,³* and Nader Najafian*

Delayed ICOS-B7h signal blockade promotes significant prolongation of cardiac allograft survival in wild-type but not in CD8-deficient C57BL/6 recipients of fully MHC-mismatched BALB/c heart allografts, suggesting the possible generation of CD8⁺ regulatory T cells in vivo. We now show that the administration of a blocking anti-ICOS mAb results in the generation of regulatory CD8⁺ T cells. These cells can transfer protection and prolong the survival of donor-specific BALB/c, but not third party C3H, heart grafts in CD8-deficient C57BL/6 recipients. This is unique to ICOS-B7h blockade, because B7 blockade by CTLA4-Ig prolongs graft survival in CD8-deficient mice and does not result in the generation of regulatory CD8⁺ T cells. Those cells localize to the graft, produce both IFN-γ and IL-4 after allostimulation in vitro, prohibit the expansion of alloreactive CD4⁺ T cells, and appear to mediate a Th2 switch of recipient CD4⁺ T cells after adoptive transfer in vivo. Finally, these cells are not confined to the CD8-negative population but express programmed death 1, a molecule required for their regulatory function in vivo. CD8⁺PD1⁺ T cells suppress alloreactive CD4⁺ T cells but do not inhibit the functions by alloreactive CD8⁺ T cells in vitro. These results describe a novel allospecific regulatory CD8⁺PD1⁺ T cell induced by ICOS-B7h blockade in vivo. The Journal of Immunology, 2007, 179: 786–796.

The ICOS-B7h pathway has been shown to play a critical role in T cell activation and differentiation and the regulation of alloimmune responses (1). Unlike CD28, ICOS is not expressed on naive T cells; instead, its expression is up-regulated after T cell activation and persists on effector and memory T cells. This difference suggests that ICOS signaling may be more important for regulating activated T cells, whereas CD28 functions to prime naive T cells. In addition, several studies have demonstrated a unique role for ICOS in the generation of Th2 responses (1); this effect may be achieved by the enhancement of IL-4R-mediated signaling (2). Finally, the expression pattern of ICOS on T cells in germinal centers indicates a role for ICOS in T cell-B cell interactions (1).

ICOS-B7h blockade prolongs allograft survival in several transplantation models (3–9). Those studies indicate a key role for the ICOS-B7h pathway in acute and chronic rejection and demonstrate the potential use of anti-ICOS therapy in combination with other immunosuppressive agents or other T cell costimulatory blockade regimens to promote allograft survival (3–9). However, the exact mechanisms of action of ICOS-B7h blockade in vivo remain unclear. We have recently shown that delayed blockade of ICOS-B7h prolongs cardiac allograft survival (4). ICOS-B7h signal blockade results in the inhibition of alloreactive CD4⁺ T cell expansion and alloantibody production. In addition, the function of ICOS-B7h signaling was found to be independent of the presence or absence of a CD28 molecule in allograft recipients. Interestingly, the beneficial effect of the ICOS-B7h signal blockade was abrogated when CD8⁺ T cells were depleted in allograft recipients. It appears, therefore, that the effect of the ICOS-B7h signal blockade in preventing acute rejection is dependent on the presence of CD8⁺ T cells. Additional evidence supporting this hypothesis was the finding that cardiac allografts were promptly rejected in CD8-deficient recipients treated with ICOS-B7h blockade, suggesting that such a blockade may be generating regulatory CD8⁺ T cells that inhibit the alloimmune response in vivo (4).

The regulatory functions of CD8⁺ T cells have recently been highlighted in experimental models of autoimmune diseases (10–12), neonatal tolerance (13), and allergen-induced airway hypersensitivity (14). Several studies using tolerogenic APCs have demonstrated that regulatory CD8⁺ T cells play a key role in the mechanisms of either tolerance induction (15) or the suppression of pathogenesis of autoimmunity (16, 17). We have also demonstrated that the adoptive transfer of CD8⁺CD28⁺ T cells into CD8⁻/⁻ mice results in significant suppression of experimental autoimmune encephalomyelitis (12). A recent article described the regulatory functions of CD8⁺CD122⁺ T cells in vitro (18). Moreover, in human recipients of...
renal allografts minor histocompatibility Ag, HA-1-specific, regulatory CDB8+ T cells have been identified in the context of allograft tolerance (19). Also, regulatory CDB8+CD28- T cells were significantly expanded in patients without rejection of cardiac allografts as compared with patients with acute rejection (20). The relationship of ICOS and regulatory CD8+ T cells in general or in the context of allograft rejection has not been investigated.

In this study we report for the first time that an ICOS-B7h signal blockade results in the generation of allospecific regulatory CD8+ T cells as a novel mechanism of action mediating the prolongation of allograft survival, and we define the effector mechanisms of these cells in vivo in a murine model of cardiac transplantation.

Materials and Methods

Mice

BALB/c (H-2b), C57BL6 (H-2b), and C3H/He (H-2b) male mice were purchased from Taconic Farms. CD8-deficient (CD8+/H11002-/-) and CD82-deficient (CD8-/H11001-/-) B6 male mice were purchased from The Jackson Laboratory. All animals were used at 6–10 wk of age (20–25 g), and were housed in accordance with institutional and National Institutes of Health guidelines.

Abs and in vivo treatment protocol

The anti-ICOS mAb (clone 7E.17G9; rat IgG2b isotype) (4) and anti-programmed death 1 (PD-1) mAb (J43; hamster IgG) (21) have been recently described. All mAbs were manufactured and purified by ISC BioExpress. CTLA4-Ig is a human Ab IgG1 from Bristol-Myers Squibb.

Anti-PD-1 mAb was given i.p. according to the protocol of 0.5 mg of mAb on the day of transplantation and 0.25 mg of mAb on days 2, 4, 6, 8, and 10 after transplantation. Twenty-five milligrams of CTLA4-Ig is given i.p. on days 0, 2, 4, and 6. Delayed anti-ICOS treatment consists of 0.5 mg on day 4 and 0.25 mg on days 6, 8, and 10.

Murine cardiac transplantation

The vascularized intrathoracic heterotopic transplantation of cardiac allografts was performed using microsurgical techniques. In brief, donor and recipient mice were anesthetized with i.p. pentobarbital (70 mg/kg). Fully mismatched mismatched hearts were transplanted into the thoracic cavity of the recipient. Donor hearts were transplanted into B6 wild-type (WT), CD8-deficient (CD8+/H11002-/-), or CD8 deficient (CD8-/H11001-/-) recipients of BALB/c or C3H/He cardiac allografts. To study the effect of anti-ICOS treatment, recipients were administered 0.5 mg of anti-ICOS mAb on the day of transplantation and 0.25 mg of mAb on days 2, 4, 6, 8, and 10. Allografts were harvested at 14 days post-transplantation, cardiac contractility as determined by direct visualization. Graft survival is shown as the median survival time (MST) in days.

CD8+ T cell purification for adoptive transfer experiments and in vitro suppression assays

To obtain 100% purified CD8+CD28- and CD8+CD28+ T cells for adoptive transfer studies, we first prepared a single-cell suspension from spleens of naive WT B6 or CD8+/H11001-/- recipients of BALB/c cardiac allografts undergoing delayed treatment with anti-ICOS mAb (CD8ics) or appropriate isotype control Ig (CD8isos)14 days post-transplantation. CD8+ T cells were enriched (>95% CD8+ T cells) by MACS using a CD8a+ T cell isolation kit (catalog no.130-090-859; Miltenyi Biotec). To test whether regulatory CD8+ T cells were generated in recipients of cardiac allograft treated with ICOS blockade, CD8+ T cells were purified from C57BL6 or CD8+/H11001-/- recipients of BALB/c hearts 2 wk after transplantation as described, and 20 × 106 cells were transferred via tail vein into CD8+/H11001-/- recipients of either BALB/c or C3H/He cardiac allografts. To study the effect of adoptively transferred regulatory CD8+ T cells in the absence of alloreactive CD8+ effector cells, some WT recipients underwent transient depletion by a mAb to mouse CD8 (clone 2.43) (100 μg i.p. on days −6, −3, and −1 before transplantation) as previously described (12). To evaluate the role of the negative costimulatory molecule PD-1 in the function of the transferred CD8ics T cells, some CD8+/H11001-/- recipients of BALB/c hearts were treated with either anti-PD1 mAb after adoptive transfer (0.50 mg i.p. on day 0 and 0.25 mg i.p. on days 2, 4, 6, 8, and 10). In other experiments, CD8icos T cells were stained with PD-1 mAb (eBioscience) and depleted of cells expressing PD-1 (<1%) by using MACS LS columns before adoptive transfer. Next, we flushed the MACS column several times and collected the enriched CD8+PD1- T cells (>80%). Some of these cells were used for adoptive transfer experiments as described in detail above. Given the low number of this cell population, their regulatory activity was also tested in an in vitro MLR assay; spleenocytes from B6 CD8+/H11001-/- recipients of BALB/c allografts were used as responder cells to irradiated BALB/c spleenocytes. Purified CD8+PD1- and CD8+PD1+ T cells were then added to each well (regulatory to responder T cell ratios of 1:1, 1:2, and 1:4) either in direct contact with or separated from responder cells using 8-well strip inserts (Nalge Nunc International). To evaluate the suppressive activity of regulatory T cells on CD4+ and CD8+ effector cells in vitro, WT, CD4-deficient spleenocytes, or WT spleenocytes after CD8 depletion were also used as responders in the above MLR. The proliferation of responder cells (cpm) was then estimated by a [3H] thymidine incorporation in responder cells.

ELISPOT assay

The technique for ELISPOT analysis has been described recently by our group and others (4). ImmunoSpot plates (Cellular Technology) were coated with 4 μg/ml rat anti-mouse IFN-γ mAb or IL-4 mAb in sterile PBS overnight. The plates were then blocked for 1 h with sterile PBS containing 1% BSA-fraction V and washed three times with PBS. Spleenocytes (0.5 × 10^6) in 200 μl of HL-1 medium containing 1% fetal bovine serum (FBS) were then placed in each well in the presence of 0.5 × 10^6 irradiated (30 gray) syngeneic or allogeneic spleenocytes and cultured for 24 h at 37°C in 5% CO2. After washing with PBS followed by washing with PBS containing 0.05% Tween (PBST), 2 μg/ml biotinylated anti-mouse IFN-γ or IL-4 mAb was added and incubated for 24 h at 37°C. Plates were then washed four times in PBST followed by 2 h of incubation with HRP-conjugated streptavidin (DakoCytomation) diluted at 1:2000 in PBS with 1% BSA. After washing three times with PBST followed by PBS, the plates were developed using 3-aminoo-9-ethylcarbazole (Sigma-Aldrich). The resulting spots were counted on a computer-assisted, enzyme-linked ImmunoSpot image analyzer (Cellular Technology), and frequencies were expressed as the number of cytokine-producing spots per 0.5 × 10^6 spleenocytes.

Cytotoxic T lymphocyte assay

Cell-mediated cytotoxicity was determined by 51Cr release assay. In brief, P815, a H2d tumor cell line, was maintained in our laboratory in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (HyClone), l-glutamine, and penicillin/streptomycin and served as the target cell. Target P815 cells were prepared by labeling with 10 μg/ml of 51Cr as described previously. Effector cells were seeded into triplicate wells at an E:T ratio of 100:1, 50:1, 25:1, and 12.5:1. Additionally, some wells received either either enriched CD8+PD1- or CD8+PD1+ T cells as indicated in the text. Total releasable radioactivity ([cpmmax], where max as maximum) was determined by incubating aliquots of target cells in a 5% solution of Triton X-100, and spontaneous radioactivity release ([cpmspont] where spont is spontaneous) was determined by incubating aliquots of target cells with medium alone. The results were expressed as the percentage of maximal release. Before incubation, cells were pelleted by centrifugation at 300 × g for 2 min and then incubated for 20 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. After incubation, 50 μl of supernatant was transferred to wells of a new microtiter plate containing 150 μl of OpTiiPhase SuperMix β-scintillation fluid (PerkinElmer) and counted ([cpm]) using a 1450 MicroBeta TriLux instrument (PerkinElmer). The percentage of specific lysis was determined using the raw cpm and the following formula: specific lysis (%) = ([cpmmax - cpmspont]/cpmmax) × 100.

Flow cytometry

To study the phenotype of purified CD8ics and CD8isos, cells were stained with CD8-FITC, CD4-FITC, and PE-conjugated mAb against CD1, PD-1, PD ligand 1 (PD-L1), PD-L2, CD27, CD70, glucocorticoid-induced TNFR (GITR), CD25, CD45RA, CD45RB, and CD103. To evaluate the CD28 expression on CD8ics and CD8isos, intracellular staining of CD28 was performed. Intracellular CTLA-4 staining was performed using the Cytofix/Cytoperin icellular staining kit. An anti-mouse Foxp3 staining set (eBioscience) was used to assess intracellular expression of Foxp3. Briefly, cells
Flow cytometry was performed using a FACSCalibur flow cytometer system and corresponding isotype controls were purchased from R&D Systems. Survival was achieved by CTLA4-Ig treatment (11; MST 10.5 days, n = 6, p = 0.001). CD8 T cell transfer from recipients treated with control Ig (CD8iso) (●) had no effect in CD8<sup>-/-</sup> recipients of CD8<sup>+</sup> T cells, which were similarly rejected as no treatment controls (○) (MST 10.5 days, n = 4 vs MST 10 days, n = 5, p = NS). B, Regulatory CD8icos are donor Ag-specific. Third party C3H/He heart grafts were promptly rejected in CD8<sup>-/-</sup>T cells. Although long-term allograft survival was achieved by CTLA4-Ig treatment (●, MST >100 days, n = 7), the adoptive transfer of CD8<sup>+</sup> T cell from B6 recipients of BALB/c hearts treated with CTLA4-Ig (△, CD8<sub>ctlA4Ig</sub>) into CD8-deficient recipients of the same allografts had no beneficial effect (MST 13 days, n = 6). s/p, Status post.

FIGURE 1. A, Delayed ICOS-B7h blockade induces regulatory CD8<sup>+</sup> T cells. Significant prolongation of allograft survival was observed when CD8<sup>-/-</sup> recipients of BALB/c hearts received CD8<sup>+</sup> T cells purified from B6 recipients of BALB/c hearts treated with delayed CD8icos (▼; MST 63.5 days, n = 6, p = 0.001). CD8 T cell transfer from recipients treated with control Ig (CD8iso) (△) had no effect in CD8<sup>-/-</sup> recipients of CD8<sup>+</sup> T cells, which were similarly rejected as no treatment controls (○) (MST 10.5 days, n = 4 vs MST 10 days, n = 5, p = NS). B, Regulatory CD8icos are donor Ag-specific. Third party C3H/He heart grafts were promptly rejected in CD8<sup>-/-</sup> recipients (MST 6 days, n = 6), suggesting that the regulatory function of CD8<sup>+</sup> T cells is donor-Ag specific. C, B7-CD28 pathway blockade does not induce regulatory CD8<sup>+</sup> T cells. Although long-term allograft survival was achieved by CTLA4-Ig treatment (●, MST >100 days, n = 7), the adoptive transfer of CD8<sup>+</sup> T cell from B6 recipients of BALB/c hearts treated with CTLA4-Ig (△, CD8<sub>ctlA4Ig</sub>) into CD8-deficient recipients of the same allografts had no beneficial effect (MST 13 days, n = 6). s/p, Status post.

Naive splenocytes (10<sup>6</sup>) of donor strain BALB/c were incubated for 30 min at 4°C with 50 μL of serially diluted sera obtained from naive B6 or BALB/c mice (controls) or B6 heart recipients adoptively transferred with CD8icos or CD8iso between days 10–14 after transplantation. Cells were washed twice, incubated with 50 μL of FITC-conjugated anti-mouse IgG1 or anti-mouse IgG2a (both from BD Pharmingen) at 4°C for 30 min, and analyzed by flow cytometry using a FACSCalibur flow cytometer system (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Measurement of serum alloantibodies

Naive splenocytes (10<sup>6</sup>) of donor strain BALB/c were incubated for 30 min at 4°C with 50 μL of serially diluted sera obtained from naive B6 or BALB/c mice (controls) or B6 heart recipients adoptively transferred with CD8icos or CD8iso between days 10–14 after transplantation. Cells were washed twice, incubated with 50 μL of FITC-conjugated anti-mouse IgG1 or anti-mouse IgG2a (both from BD Pharmingen) at 4°C for 30 min, and analyzed by flow cytometry using a FACSCalibur flow cytometer system (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). The percentage of donor cells stained at each serum dilution and the relative median fluorescence was determined and compared with that of control samples (4).

Histological analysis

Cardiac allografts were harvested from CD8<sup>-/-</sup> recipients 14 days after operation. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Ventricular short axis sections were cut and stained with H&E. Immunohistological staining for CD4<sup>+</sup> and CD8<sup>+</sup> T cells were performed using mAb purchased from BD Biosciences. A representative field (×400 original magnification) was selected and the numbers of CD8<sup>+</sup> and CD4<sup>+</sup> cells were counted.

Real-time PCR

Cardiac grafts were removed from adoptively transferred CD8<sup>-/-</sup> recipients 14 days after operation. RNA extraction was performed following Polytron homogenization using TRIZol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol; RNA was then reverse transcribed to synthesize 60 μL of cDNA. Two hundred fifty nanograms of cDNA, 10 μL of SYBR Green master mix (Applied Biosystems), and 250 nmol of sense and antisense primer were used in a 20-μL quantitative PCR. Primers for IL-4 were designed with Primer Express software (sense, 5'-TCATCGGCATTTTGAACGAG-3'; antisense, 5'-CGTTTGGCACATCCATCTCC-3') Quantitative PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. For each reaction, emitted fluorescence was measured during the annealing/extension phase. The calculated number of copies was divided by the number of copies of the housekeeping gene GAPDH.

Statistics

Kaplan-Meier survival graphs were constructed and a log rank comparison of the groups was used to calculate p values. Student’s t test was used for comparison of means between experimental groups examined by ELISPOT assay. Differences were considered to be significant when p < 0.05.

Results

Delayed ICOS-B7h signal blockade results in generation of donor Ag-specific regulatory CD8<sup>+</sup> T cells

We have previously demonstrated that delayed ICOS-B7h blockade by mAbs significantly prolongs vascularized cardiac allograft survival in a fully mismatched mouse transplant model (4). However, the same therapy had no beneficial effect in CD8-depleted (by mAb therapy) or CD8-deficient recipients, suggesting the possible generation of regulatory CD8<sup>+</sup> T cells as a mechanism of prolongation of graft survival (4). In addition, whereas the transfer of naive CD8<sup>+</sup> T cells into CD8-deficient recipients of BALB/c
allografts led to acute rejection in a similar fashion as that of CD8-deficient mice with no transfer of such cells (MST 8 days vs MST 10 days, n = 5; p = NS), treatment of the former group with a delayed ICOS blockade demonstrated significant allograft prolongation (MST 15 days, n = 5; p = 0.002). These results demonstrate that naive or “conventional” CD8+ T cells alone (with no blockade of the ICOS-B7h pathway) have no effect on allograft survival; more importantly, the graft-prolonging effect of delayed ICOS-B7h blockade in WT recipients is dependent on the presence of CD8+ T cells.

To test this hypothesis, we have set up an adoptive transfer model where CD8+ T cells are purified 14 days posttransplantation from B6 recipients of BALB/c heart allografts (MST 10 days, n = 5; p = NS), treatment of the former group with a delayed ICOS blockade demonstrated significant allograft prolongation (MST 15 days, n = 5; p = 0.002). These results demonstrate that naive or “conventional” CD8+ T cells alone (with no blockade of the ICOS-B7h pathway) have no effect on allograft survival; more importantly, the graft-prolonging effect of delayed ICOS-B7h blockade in WT recipients is dependent on the presence of CD8+ T cells.

We then explored whether the generation of CD8+ regulatory T cells is unique to ICOS-B7h blockade. We have already demonstrated that the adoptive transfer of naive CD8+ T cells or CD8+ T cells purified from isotype-treated (rejecting) B6 recipients of BALB/c hearts were unable to prolong allograft survival of BALB/c hearts in CD8-deficient recipients (see above). Next we also compared the effect of the CD8icos anti-ICOS mAb with B7 blockade by CTLA4-Ig. In contrast to the anti-ICOS mAb, B7 blockade by CTLA4-Ig can achieve long-term allograft survival in CD8- T cells (MST >100 days, n = 7; Fig. 1C), suggesting that its effect is independent of the presence of CD8+ T cells. In addition, using the same adoptive transfer model described above we show that CD8+ T cells purified from CTLA4-Ig-treated B6 recipients of BALB/c hearts (that have long-term survival) were unable to prolong the allograft survival of BALB/c hearts in CD8- T cells (MST 13 days, n = 6; Fig. 1C). Taken together, these experiments demonstrate that the induction of regulatory CD8+ T cells is a unique feature of the ICOS-B7h blockade in our model.

**Regulatory CD8+ T cells mediate a Th2 switch of effector CD4+ T cells after adoptive transfer and localize to the graft**

We characterized the cytokine expression profile of CD8+ T cells used for adoptive transfer and isolated from engrafted animals in response to stimulation by donor or third party alloantigens by

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**FIGURE 2.** A and B, ELISPOT analysis of the cytokine profile of CD8icos and CD8iso after in vitro allostimulation. CD8+ T cells were purified from the B6 recipients of BALB/c heart allografts (allo) after receiving delayed treatment with CD8icos or receiving CD8iso 14 days posttransplantation. The frequency of IFN-γ-producing CD8icos (967.6 ± 20.4) and CD8iso T cells (782.6 ± 14.4) is enhanced in response to BALB/c donors as compared with third party Ags (520.5 ± 19 for CD8icos vs 315 ± 9.8 for CD8iso) due to allosensitization (A). Interestingly, although the frequency of IL-4-producing T cells in response to C3H alloantigen was similar in both groups (35 ± 2.8 vs 44 ± 0), there was a clear increase in the frequency of donor Ag-specific (BALB/c) IL-4-producing CD8icos cells as compared with CD8iso cells (97.8 ± 31 vs 50.2 ± 4.1, p = 0.02) (B). C and D, ELISPOT analysis of Th1 (IFN-γ) and Th2 (IL-4) cytokine profile (D) of splenocytes (s/p) derived from CD8-deficient mice on day 14 after adoptive transfer of CD8icos or CD8iso. C and D demonstrate a moderate decrease in the frequency of donor-Ag specific IFN-γ producing cells (227.5 ± 92.1 vs 100.5 ± 7.8, p = 0.06) and a significant increase in donor-specific IL-4-producing cells as compared with controls (340 ± 88.9 vs 93 ± 31.2, p = 0.003). The IFN-γ and IL-4 frequencies in response to donor Ag (BALB/c) or third party Ags (C3H) are determined by ELISPOT assay. Data are expressed as the mean ± SEM of triplicate wells. The results represent three independent experiments.

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using an ELISPOT assay (Fig. 2). The frequency of IFN-γ-producing CD8icos (967.6 ± 20.4) and CD8iso T cells (782.6 ± 14.4) is enhanced in response to BALB/c donors as compared with third party (C3H) cells (520.5 ± 19 for CD8icos vs 315 ± 9.8 for CD8iso) due to allosensitization (Fig. 2A). Interestingly, while the frequency of IL-4-producing T cells in response to the C3H alloantigen was similar in both groups (35 ± 2.8 vs 44 ± 0), there was clear increase in the frequency of donor Ag-specific (BALB/c) IL-4-producing CD8icos cells as compared with CD8iso cells (Fig. 2B; 97.8 ± 31 vs 50.2 ± 4.1, p = 0.02).

We then performed ELISPOT analysis on the splenocytes of CD8-deficient recipients 2 wk after the adoptive transfer of CD8icos or control CD8iso cells. Fig. 2, C and D demonstrate a modest decrease in the frequency of donor Ag-specific IFN-γ-producing cells (227.5 ± 92.1 vs 100.5 ± 7.8, p = 0.06) and a significant increase in donor-specific IL-4-producing cells when the mice receiving CD8icos are compared with those adoptively transferred with CD8iso (340 ± 88.9 vs 93 ± 31.2, p = 0.003). These findings are intriguing because at this time point posttransplant CD8⁺ T cells are detected only in the allograft but cannot be detected in the spleen by flow cytometry (see below). Thus, regulatory CD8⁺ T cells inhibit IFN-γ-producing alloreactive CD4⁺ T cell expansion while promoting the development of CD4⁺ Th2 responses after adoptive transfer in vivo.

Although the H&E examination of all three groups on day 14 (CD8-deficient recipients of BALB/c allografts not adoptively
transferred vs those after adoptive transfer with CD8iso or CD8icos) demonstrate broadly similar degrees of cell infiltration, the immunohistological examination clearly demonstrate that the cell infiltrates found in the grafts of control CD8-deficient transplant recipients (that are rejecting) consists of CD4+ T cells only as expected (76 ± 23.5 per high power field (hpf)), whereas the cell infiltrates in both CD8iso (rejecting) and CD8icos (prolonged survival) recipients consist of similar degrees of both CD4+ (34 ± 3.3 and 33.2 ± 19.2 per hpf) and CD8+ T cells (47 ± 18.5 and 48 ± 9.3 per hpf) (Fig. 3A). Interestingly, there is significant up-regulation of intragraft IL-4 gene expression following the adoptive transfer of CD8icos as compared with that of CD8iso (0.0347 ± 0.0056 vs 0.0075 ± 0.0044 copies per copies of GAPDH, p = 0.0009) (Fig. 3B). Although the main difference seems to be the type and phenotype of infiltrating cells, we found no difference in the alloantibody production/titers (IgG1 and IgG2a) in the sera of CD8-deficient mice adoptively transferred with CD8icos or CD8iso T cells (Fig. 4). Taken together, the results indicate that an ICOS-B7h blockade generates regulatory CD8+ T cells that are characterized by donor Ag specificity, the production of IL-4 in response to donor Ag, the inhibition of alloreactive CD4+ T cells but not alloantibody production in recipients, and the ability to migrate to the target tissue that they protect from rejection.

Table I. Analysis of surface markers by flow cytometry

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Percentage of Marker-Specific CD8icos (%)</th>
<th>Percentage of Marker-Specific CD8iso (%)</th>
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</thead>
<tbody>
<tr>
<td>PD-1</td>
<td>13 ± 4.7%</td>
<td>7.5 ± 0.9%</td>
</tr>
<tr>
<td>PD-L1</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>PD-L2</td>
<td>&lt;1%</td>
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</tr>
<tr>
<td>CTLA-4</td>
<td>4 ± 0</td>
<td>4 ± 0</td>
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<td>CD27</td>
<td>&gt;95%</td>
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<tr>
<td>CD70</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>GITR</td>
<td>7 ± 1</td>
<td>7 ± 0.5%</td>
</tr>
<tr>
<td>CD25</td>
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<td>&lt;1%</td>
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<td>CD45RA</td>
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<td>CD103</td>
<td>40 ± 4.9</td>
<td>32 ± 6.3%</td>
</tr>
<tr>
<td>TGF-β/LAP</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

a The expression of a panel of regulatory markers on CD8icos and CD8iso (isolated from spleens on day 14 after transplantation) was studied by flow cytometry (data are means ± SD from four independent experiments).

b Latency-associated protein.

PD-1 is the phenotypic marker and the key molecule for the regulatory function of CD8+ T cells in vivo

To understand the mechanisms of action of the regulatory CD8+ T cells in vivo, we first compared the expression of a panel of known regulatory markers between CD8icos and CD8iso by using flow cytometry (Table I). We found that the expression of the inhibitory molecule PD-1 to be significantly enhanced on CD8icos (13 ± 4.7%) as compared with CD8iso (7.5 ± 0.9%, p = 0.04) (Fig. 5A) while there was no statistical difference in the expression of a
variety of other molecules including those previously reported to be associated with naturally occurring CD4⁺CD25⁺ regulatory T cells such as CD25, Foxp3, GITR, or intracellular CTLA4.

To evaluate the role of the PD-1 molecule in the regulatory effector function of CD8icos, we chose two different strategies. First, we made use of the same adoptive transfer model but treated the CD8⁻⁻⁻ recipients of the BALB/c hearts adoptively transferred with CD8icos with a blocking anti-PD-1 mAb as previously published by our group (21–23). The PD-1 blockade had no effect on allograft survival in the CD8⁻⁻⁻ recipients of BALB/c hearts (data not shown). Interestingly, we observed abrogation of the allograft-prolonging functions of CD8icos after PD-1 blockade (MST 17 days, n = 5, p = 0.008 compared with CD8icos group; Fig. 5B). Second, we show that the graft-prolonging effects of a delayed ICOS blockade in B6 recipients of BALB/c hearts (which we clearly have shown are dependent on the generation of CD8icos expressing PD-1) are completely abrogated after PD-1 blockade in vivo (delayed ICOS and PD1 blockade: MST 9 days, n = 5 vs delayed ICOS blockade alone: MST 16 days, n = 8, p = 0.001). This is important, because a PD-1 blockade in B6 recipients of BALB/c allografts by itself has no effect on allograft survival at all (PD-1 blockade: MST 9 days, n = 6 vs controls: MST 8 days, n = 10, p = NS). Taken together, these data support a mechanistic role of PD-1 in the generation/function of regulatory CD8⁺ T cells in vivo.

Next, we tested the effect of an adoptive transfer of CD8⁺ T cells after ex vivo removal of the PD-1-expressing subset of cells (Fig. 5A, CD8⁺PD1⁻ T cells). PD-1-depleted CD8icos cells lack any regulatory functions (MST 11 days, n = 4, p = 0.001) as compared with CD8icos (Fig. 5B).

To further validate these findings, we next wanted to test the suppressive activity of CD8⁺PD1⁺ T cells. Because obtaining the necessary number of these cells for adoptive transfer in vivo would require a very large number of transplanted and treated animals (20 million CD8icos contain ~3 million CD8⁺PD1⁺ T cells), we have performed adoptive transfer of 1.3 million (graft survival: 17 and 19 days) and 1.8 million CD8⁺PD1⁺ T cells (graft survival: 22 and 22 days) and found significant prolongation of graft survival (survival of controls: 8, 8, 9, 9 days, p = 0.04 and 0.04, respectively), demonstrating a sort of a dose response effect of regulation in vivo. Next, we compared the ability of enriched CD8⁺PD1⁺ T cells or PD1-depleted CD8⁺ T cells obtained from anti-ICOS-treated mice to suppress an MLR assay using CD8-deficient splenocytes as responder cells (Fig. 5C). In contrast to PD1-depleted CD8⁺ T cells, the enriched CD8⁺PD1⁺ T cells significantly suppressed the MLR at different ratios (percentage of suppression, 76.5 ± 8.5% for a regulatory-to-effector ratio of 1:1; >75% suppression at 1:2 and 1:4). These novel results are the first that clearly establish a key role for the inhibitory co-stimulatory molecule PD-1 as a phenotypic marker of regulatory CD8⁺ T cells in vivo. In addition, these data are consistent with the role of PD-1 in the suppressive functions of these cells.

To further elucidate the mechanisms of action of regulatory CD8⁺PD1⁺ T cells, we first characterized the cytokine expression profile of CD8⁺PD1⁺ as compared with CD8⁺PD1⁻ T cells in response to a donor alloantigen by using ELISPOT assay. The CD8⁺PD1⁺ T cells contained less IFN-γ-producing (236.7 ± 89.2 vs 589.3 ± 67.8, n = 3, p = 0.05) but significantly more IL-4-producing T cells (170 ± 12 vs 81 ± 5, n = 3, p = 0.009) after allostimulation when compared with CD8⁺PD1⁻ T cells.
Results in each experiment.

...significant, albeit biologically minor prolongation of allograft survival (MST 8.5 days, n = 6, p = 0.01) (A). CD8-depleted B6 recipients of BALB/c allografts demonstrated more enhanced prolongation of allograft survival (MST 11 days, n = 6, p = 0.0008), suggesting a lack of efficient suppression of alloreactive CD8\(^+\) effector cells by the currently used number of regulatory T cells in vivo. To evaluate suppressive effects of CD8\(^+\)PD1\(^+\) T cells on alloreactive CD4\(^+\) vs CD8\(^+\) effector cells, we used the in vitro suppression MLR assay by using either WT, CD8-depleted WT, or CD4-deficient splenocytes as responder cells (B). Although enriched CD8\(^+\)PD1\(^+\) T cells added at various ratios (1:1, 1:2, and 1:4) significantly suppressed the MLR of CD8-deficient splenocytes (s/p) (percentage of suppression, >75%, see above), they were significantly less efficient in suppression of WT B6 responders (1:1, 11528.3 ± 1649; p = 0.03; 1:2, 11015.3 ± 890, p = 0.06; and 1:4, 20097 ± 2118, p = NS vs 21251.6 ± 4506 cpm) or of CD4-deficient responders (1:1, 10799.3 ± 1400 vs 9437.3 ± 800, p = NS). However, there was a resumption of suppression after ex vivo depletion of CD8\(^+\) T cells from WT splenocytes used as responders (1:1, 7554.6 ± 349, p = 0.008; 1:2, 4576.6 ± 769, p = 0.007; and 1:4, 4159.3 ± 446, p = 0.007 vs 27226 ± 3031 cpm). Data are representative of three independent experiments and indicate the mean of triplicate results in each experiment.

All in all, these data suggest that regulatory CD8\(^+\)PD1\(^+\) T cells mediate their in vitro suppressive activity by regulatory cytokines in a cell contact-independent manner.

**CD8\(^+\)PD1\(^+\) T cells suppress CD4\(^+\) but not CD8\(^+\) alloreactive T cells**

Next we adoptively transferred 20 million regulatory CD8\(^+\) T cells derived from ICOS-treated WT recipients of BALB/c hearts into unmanipulated WT mice transplanted with BALB/c allografts. We found statistically significant, albeit biologically minor, prolongation of allograft survival (Fig. 7; MST 8.5 days, n = 6 vs MST 7 days, n = 6, p = 0.01). Based on these findings, we hypothesized that the regulatory T cells may be less effective in suppressing CD8\(^+\) effector cells. Thus, we repeated the adoptive transfer experiment but used CD8-depleted WT (by anti-CD8 mAb) rather than CD8-deficient mice as the recipients of the regulatory CD8\(^+\) T cell. This regimen has been previously shown to deplete CD8\(^+\) T cells for 10–14 days after which time the CD8\(^+\) T cells reappear gradually (12). Interestingly, as compared with unmanipulated B6 recipients, CD8-depleted B6 recipients of BALB/c allografts adoptively transferred with CD8icos demonstrated enhanced prolongation of allograft survival (Fig. 7; MST 11 days, n = 6, p = 0.0008), suggesting a lack of efficient suppression of alloreactive CD8\(^+\) effector cells by the currently used number of regulatory T cells. The shorter allograft prolongation induced by regulatory CD8icos in CD8-depleted vs CD8-deficient mice is not surprising, given that circulating anti-CD8 mAbs may also deplete some of the transferred regulatory T cells and also given the transient nature of CD8 depletion by mAb administration as compared with total deficiency in knockout mice.

Because adoptive transfer experiments of regulatory T cells into CD4-deficient mice would likely not be helpful (most CD4-deficient mice would accept BALB/c hearts anyway), we evaluated the suppressive effects of CD8\(^+\)PD1\(^+\) T cells on alloreactive CD4\(^+\) vs...
CD8⁺ effector cells using the in vitro suppression MLR assay by using either WT, CD8-depleted WT, or CD4-deficient splenocytes as responder cells (Fig. 7B). Although enriched CD8⁺PD1⁺ T cells added at various ratios (1:1, 1:2, and 1:4) significantly suppressed the MLR of CD8-deficient splenocytes (percentage of suppression, >75%; see above), they were significantly less efficient in the suppression of WT B6 responders (1:1, 45 ± 8.5%; 1:2, 46 ± 13.8%; and 1:4, 6 ± 3%) or CD4-deficient responders (1:1 7 ± 6%). However, there was a resumption of suppression after ex vivo depletion of CD8⁺ T cells from WT splenocytes (1:1 71.3 ± 3.2%; 1:2 82.6 ± 4.1%; and 1:4 84.3 ± 3.2%). The addition of purified CD8⁺PD1⁺ T cells to the CTL assay (E:T ratio 50:1) by 51Cr release (CD8⁺PD1⁺:effector ratio of 1:1) did not inhibit the killing of target cells by effector cells (percentage of lysis, 46.6 vs 27.6%). Indeed, the killing is increased by adding both CD8⁺PD1⁺ and CD8⁺PD1⁻ cells (percentage of lysis, 46.6 vs 45.6%) to approximately the levels seen in the E:T ratio of 100:1 (percentage of lysis, 35.1%). Taken together, these data suggest that CD8⁺PD1⁺ regulatory T cells are significantly effective in the suppression of alloreactive CD4⁺ but not alloreactive CD8⁺ T cells in vitro.

Regulatory function of CD8⁺ T cells is not confined to the CD28-negative population

Several studies have previously demonstrated the regulatory function of CD8⁺CD28⁻ T cells in autoimmunity and transplantation (12, 24). In addition, we and others have demonstrated that ~60% of CD8⁺ T cells have high expression and 40% have low or minimal expression of the CD28 molecule in WT mice (12) (Fig. 1). In the case of the adoptive transfer studies described above, this means that the 20 million CD8icsos cells derived from WT recipients of BALB/c allografts after the ICOS-B7h blockade contained roughly only 8 million CD8⁺CD28⁺ T cells. Because our own data show long-term allograft survival in CD28-deficient recipients after delayed ICOS-B7h blockade (4), we asked the question of whether the CD8⁺CD28⁻ subpopulation is primarily responsible for the regulatory functions of CD8icsos. Thus, we repeated the described adoptive transfer experiment except that we used CD8⁺ T cells isolated from CD28⁻/- mice transplanted with BALB/c hearts and treated with the CD8icsos anti-ICOS mAb or the CD8iso isotype control Ig. The adoptive transfer of 20 × 10⁶ CD8⁺CD28⁻ T cells effected only modest yet statistically significant prolongation of allograft survival (MST 20 days, n = 5 in ICOS-treated vs 13.5 days, n = 5 in isotype control Ig treated, p = 0.005), indicating that the regulatory function does not seem to be confined to CD8⁺ T cells lacking CD28. Further, we performed triple staining on CD8icsos T cells (with CD8, CD28, and PD-1) because we have demonstrated PD-1 as the key marker for our regulatory T cells and found that most CD8⁺PD1⁺ T cells are contained in the CD8⁺CD28⁺ (percentage of CD8 T cells expressing both CD28 and PD-1, 11.8 ± 5.6%) rather than the CD8⁺CD28⁻ (4.8 ± 2.9%) subset of T cells. Therefore, it is PD-1 and not CD28 that serves as the main marker of the regulatory CD8⁺ T cells in our model.

Discussion

The ICOS-B7h is a complex T cell costimulatory pathway and its blockade results in different effects depending on the type of immune response, the timing of blockade, and/or the disease model. Early ICOS-B7h blockade on the day of transplantation led to a less impressive prolongation of a fully MHC-mismatched murine cardiac allograft survival as compared with late administration beginning on day 4 after transplantation (4). Interestingly, when using a minor mismatched murine cardiac allograft model, the early administration of the mAb accelerated the rejection while the delayed blockade clearly prolonged the survival (4). Similarly, early (day 0) but not late (day 4) targeting of the ICOS ligand by the ICOS Ig prolonged graft survival induced by CTLA4-Ig and donor-specific transfusion in a fully mismatched rat cardiac transplant model (25). In addition, the mechanisms of targeting this pathway in vivo remain unclear. In different species of rodents and humans there is compelling evidence that the regulation of the magnitude of protective immunity to foreign Ags as well as the control of autoaggressive immune reactions are ensured by multiple subtypes of regulatory CD4⁺ and CD8⁺ T lymphocytes that display anti-inflammatory and antiproliferative functions (26–28). Despite the long-held notion that manipulation of the immune system in vitro or in vivo can lead to generation of CD8⁺ regulatory T cells (29, 30), most recent papers have focused on the regulatory properties of CD4⁺ T cell subsets, mainly due to the absence of markers allowing their identification. We now provide evidence that the ICOS-B7h blockade results in the generation of a novel allospecific regulatory CD8⁺ T cell that is dependent on PD-1 for its suppressive functions in vivo. Interestingly, several studies previously showed that ICOS-B7h signaling is required for the generation and/or function of regulatory CD4⁺ T cells. ICOS-B7h signaling has been shown to be critical for the induction of Ag-specific regulatory CD4⁺ T cells producing IL-10 in a model of allergen-induced airway hyperreactivity (31). Other studies implicated ICOS signaling in the actual regulatory functions of CD4⁺ T cells (32, 33). In an autoimmune model of diabetes, CD4⁺CD25⁺CD69⁻ regulatory T cells operating directly in the autoimmune lesion were shown to be dependent on ICOS to keep it in a nondestructive state (34). In a rat cardiac transplant model, the combination of CD40-Ig and an anti-ICOS mAb led to the development of regulatory T cells (35), although the type, phenotype, and mechanisms of action of these cells were not further explored. Thus, our results are the first to clearly demonstrate that the ICOS-B7h blockade results in the generation of allospecific regulatory CD8⁺ T cells in vivo. The generation of regulatory T cells is unique to the blockade of the ICOS-B7h pathway, as neither the CD8⁺ T cells derived from isotype-treated (rejecting) or CTLA4-Ig-treated B6 recipients of BALB/c allografts (that have long-term survival) demonstrated suppressive function after adoptive transfer. Interestingly, there was not only a clear increase in the frequency of donor Ag-specific IL-4-producing CD8icsos cells as compared with CD8iso cells, but in addition these regulatory CD8icsos migrated to the allograft and inhibited the expansion of CD4⁺ alloreactive effector cells while promoting the development of Th2 responses in the host after adoptive transfer. These results support our previous data demonstrating an increased frequency of IL-4-producing cells after a delayed ICOS blockade and the need for an intact STAT-6 pathway for the graft-prolonging effect of this treatment (4).

To further evaluate the mechanisms of regulation, we compared the expression of a panel of regulatory markers on regulatory CD8icsos with that of control CD8iso. Previous studies demonstrated that the regulatory function is confined to the CD8⁺CD28⁻ subset of T cells in models of both transplantation and autoimmune (12, 24). Alloantigen-specific CD8⁺CD28⁻ suppressor cells were recently found to express Foxp3, GITR, and CTLA4 at levels similar to those observed in natural regulatory T cells even though they clearly differ from these cells both by their phenotype and mechanisms of action (36). Nevertheless, with the exception of PD-1 we found no difference in the expression of other regulatory markers. PD-1 is a new member of the CD28/CTLA-4 family that has been implicated in peripheral tolerance (37) and the regulation of alloimmune responses (23, 38–41). In fact, a recent study
clearly demonstrated that PD-1-PDL1 interaction is essential for induction of regulatory cells by the intratracheal delivery of al-
loantigen (42). Another study characterized CD4+ CD25+ PD-1+ T cells that uniquely produced large amounts of IL-4 and IL-10 in response to anti-CD3 and anti-CD28 mAb stimulation (43). Interest-
ingly, the same cells were able to inhibit the development of colitis induced by the adoptive transfer of CD4+ T cells into SCID mice in a CTLA4- but not PD1-dependent manner despite expressing the latter molecule on their surface. In this study we clearly demonstrate that PD-1 rather than CD28 is the main phenotypic marker of our regulatory T cells. It is also interesting that a PD-1 blockade abrogated the graft-prolonging effects of CD8+ PD1+ T cells in vivo. In contrast, CD8+ PD1+ T cells contain significantly more donor-specific IL-4-producing cells and promote a Th2 switch of host CD4+ T cells after adoptive transfer. Taken to-
gether, these data are consistent with a key role for the inhibitory costimulatory molecule PD-1 in the actual suppressive function in vivo and suggest that CD8+ PD1+ T cells mediate their suppressive ac-
ivity by the secretion of immunoregulatory cytokines. The fact that CD8+ PD1+ T cells can suppress in vitro in a contact-
-dependent manner may point to some differences in the mecha-
nisms of suppression by these cells in vivo and in vitro while still pointing to the importance of regulatory cytokines.

In sum, this paper describes a novel CD8+ PD1+ regulatory T cell induced by the blockade of a positive T cell costimulatory pathway, the ICOS-B7h pathway. The exact cellular and molecular interaction between ICOS-B7h and PD-1 in relation to the genera-
tion of regulatory CD8+ T cells requires further investigation. In fact, the therapeutic potential of a simultaneous stimulation of PD-1 and the blockade of ICOS-B7h costimulation in the preven-
tion of lupus nephritis has been recently demonstrated in a mouse model of the disease (44). Thus, our results are clinically relevant and have important implications in organ transplantation because they clearly support tolerance-inducing strategies that consist of blocking positive costimulatory pathways combined with enhanc-
ing inhibitory costimulatory signals.

Disclosures

The authors have no financial conflict of interest.

References

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