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Early Presence of Regulatory Cells in Transplanted Rats Rendered Tolerant by Donor-Specific Blood Transfusion

Hiroaki Kitade, Masaru Kawai, Omer Rutgeerts, Willy Landuyt, Mark Waer, Chantal Mathieu, and Jacques Pirenne

Mechanisms by which donor-specific blood transfusion (DSBT) promotes organ allograft acceptance are unclear. In a rat fully mismatched cardiac allograft model, we found that DSBT alone (without immunotherapy) induces the development of regulatory T cells (DSBT-Tregs) posttransplant, thereby shedding new light in the mechanisms of the transfusion effect. Compartments and timing of expansion, requirements, and phenotype of DSBT-Tregs are unknown. It is generally assumed that some time is necessary before Tregs develop. However, we show—by adoptive transfer from DSBT—tolerant into naive recipients: 1) the presence of DSBT-Tregs at 5 days posttransplant in spleen and lymph nodes; 2) their gradual expansion in these compartments; and 3) their presence in the graft 14 of 30 days posttransplant. DSBT-Tregs are donor specific and do not protect third-party allografts. Splenocytes from DSBT-treated nontransplanted recipients or from transplanted DSBT-untreated (rejecting) recipients do not transfer tolerance, indicating that both DSBT and graft are required for sufficient numbers of DSBT-Tregs to develop. Thymectomy (or splenectomy) before DSBT (not at transplantation) abrogate DSBT-Tregs generation and tolerance, showing that thymus (and spleen) are required for DSBT-Tregs generation (not for expansion/maintenance). In contrast with other Tregs models, DSBT-Tregs activity is not restricted to CD4<sup>+</sup>CD25<sup>+</sup> but to CD4<sup>+</sup>CD45RC<sup>−</sup> cells, whereas CD4<sup>+</sup>CD45RC<sup>+</sup> cells act as effector cells and accelerate rejection. In conclusion, DSBT alone induces—rapidly posttransplant—the development of alloantigen-specific Tregs in lymphoid tissues and in the graft. DSBT, graft, thymus, and spleen are required for DSBT-Tregs generation. DSBT-Tregs in this model are CD4<sup>+</sup>CD45RC<sup>−</sup> (identical to Tregs protecting from autoimmunity in rats). The Journal of Immunology, 2005, 175: 4963–4970.

Although effective in reducing acute rejection rates, immunosuppressive drugs have failed to prevent graft loss to chronic rejection. In addition, immunosuppressive drugs cause direct side effects (drug toxicity) and indirect side effects, e.g., infection and malignancies. Therefore, successful development of tolerance remains a major goal in transplantation medicine (1).

Recently, there has been increasing interest in the role of alloantigen-specific regulatory T cells (Tregs) in models of tolerance that involve the delivery of donor Ags in the context of an immune manipulation of the host (via anti-TCR Ab, costimulatory blockade, immunosuppression, and so on) (2–8).

Donor-specific blood transfusion (DSBT) is a clinically and experimentally proven method to induce hyporesponsiveness and does not necessarily require additional immunotherapy (9–14). However, the use of DSBT clinically has become less popular with the advent of calcineurin inhibitors and because a clear understanding of the mechanisms involved had remained elusive (11–12). Recently, our group demonstrated in a rodent transplant model (in which DSBT alone induces tolerance) that Tregs operate (15). These “DSBT-Tregs” are capable of transferring donor-specific tolerance in secondary naive animals (15). A similar observation was made by Bushell et al. (16) in a mouse model. These observations shed new light in the mechanisms of the blood transfusion effect. Further insight in the immunobiology of these DSBT-Tregs may provide new opportunities for the development of tolerance via DSBT in the clinic. The particular features of DSBT-Tregs are virtually unknown, and it is not known whether general features of Tregs also apply to DSBT-Tregs.

Therefore, using our previously described model of DSBT-induced tolerance (15), we searched to clarify the following points pertaining to DSBT-Tregs: in which compartments do DSBT-Tregs expand (graft and/or lymphoid tissues); what is the timing of this phenomenon; what are the requirements for DSBT-Tregs to develop (DSBT, graft, thymus, spleen); and what is the phenotype of DSBT-Tregs in this model?

Materials and Methods

Animals

Fully MHC-mismatched adult inbred male PVG (RT1<sup>+</sup>-RT1A<sup>+</sup>-B/D<sup>+</sup>) rats (200–250 g) and RA (RT1<sup>+</sup>-RT1A<sup>+</sup>-B/D<sup>+</sup>) rats (150–200 g) were used as recipients and donors, respectively. All animals received care in accordance with the guidelines of the Animal Care Committee of the Catholic University of Leuven.

Tolerance induction and transplantation

Under diethyl ether anaesthesia, heterotopic heart transplantation was performed using standard microsurgical techniques (17). The day of rejection was defined as the cessation of a palpable heartbeat. For the induction of tolerance, PVG recipients were transfused with 1.5 ml of heparinized (20 IU/ml) donor RA whole blood 12 days before transplantation, as described...
previously (15). No immunosuppression or other immune intervention was used posttransplant. Without DSBT, RA grafts were rejected 9.4 ± 0.7 days after transplantation (n = 8). With DSBT, RA grafts survived indefinitely (mean survival time [MST] > 90 days, n = 10). This tolerance was donor specific because long-term PVG recipients were tolerant to RA accepted secondary donor-type (RA) grafts but rejected third-party WKAH grafts (15). The donor-specificity of DSBT-induced tolerance is also shown by the observation that third-party (WKAH) DSBT does not induce tolerance to RA heart allografts in PVG rats (MST = 19.7 ± 3.9, n = 6, p < 0.001 vs controls).

Of note, DSBT-induced tolerance is strain specific. In a different strain combination (WKAH donor to PVG recipient), we found that WKAH graft survival is only modestly prolonged in PVG recipients pretreated with donor WKAH whole blood 12 days before transplantation: MST = 9.2 ± 2.3 vs 6.8 ± 0.4 days; p = 0.015).

Adoptive cell transfer experiments
In all adoptive cell transfer experiments, total body-irradiated (4.5 Gy; 18 MeV photons, linear accelerator) naive PVG rats were used as recipients. Cell injection was through the dorsal penile vein. Twenty-four hours after adoptive transfer, a RA heart was transplanted.

To determine in which compartments DSBT-Tregs expand and to define the timing of this expansion, splenocytes, lymph node cells (from the abdominal mesentery), thymocytes, and graft-infiltrating cells were procured in tolerant (DSBT-treated, transplanted) PVG rats at different time points: 5, 14, and 30 days and were adoptively transferred. For collecting graft infiltrating cells, grafts were incubated in the collagenase A (0.2 mg/ml) containing medium (RPMI 1640) for 1 h. Thereafter, cells were harvested through nylon mesh. In general, two or three grafts were necessary to obtain > 10^7 cells. For collecting thymocytes, lymph node cells, or splenocytes, cells were harvested through nylon mesh in RPMI 1640 medium without collagenase.

To test the specificity of DSBT-Tregs, third-party WKAH heart grafts were used.

Mixed lymphocyte reactions (MLRs)
MLRs were done at different time points: days 1, 4, 7, 14, 30, and 60. As responder cells, we used 5 × 10^5 spleen cells through nylon wool from tolerant (DSBT-treated, transplanted) PVG rats, rejecting (DSBT-ununtreated, transplanted) PVG rats, nontransplanted but DSBT-treated rats, and naive PVG rats. These cells were stimulated with 5 × 10^6 irradiated (20 Gy; 18 MeV photons, linear accelerator) RA or WKAH (third-party) splenocytes using standard techniques (18). Cells in 200 μl of complete medium per well (RPMI 1640 supplemented with 10% FCS, 5 × 10^-5 M 2-ME, and antibiotics) were incubated 4 days at 37°C in 5% CO2 incubator in flat-bottom 96-well microplates. During the last 16 h, 1 μCi of [3H]thymidine (Valeant Pharmaceuticals) was added. The cultures were harvested onto fiberglass filters, and the incorporation of [3H] was measured using a scintillation analyzer.

Statistical analysis
Data were analyzed using Student’s t test, Wilcoxon test, and nonparametric Mann-Whitney U test, as appropriate. A value of p < 0.05 was considered significant.

Results

DSBT-Tregs capable of transferring tolerance are present among splenocytes and lymph nodes in tolerant rats at day 5 after transplantation

Fig. 1A shows the survival of RA hearts transplanted into naive PVG recipients following adoptive transfer of various cell types taken at day 5 from DSBT-tolerized rats. A total of 1 × 10^8 splenocytes and 1 × 10^8 lymph node cells partially transferred tolerance (33 and 40%, MST = 57.1 ± 30.1 days, n = 6, and 55.8 ± 28.5 days, n = 5, respectively, p < 0.001 vs control), whereas 1 × 10^7 thymocytes were not effective (MST = 25.0 ± 2.0 days, n = 4, not significant vs control) at this time point. At day 5, 1 × 10^7 graft-infiltrating cells—for methodological reasons, larger numbers of cells could not be obtained—did not transfer tolerance (MST = 26.2 ± 1.4 days, n = 5, not significant vs control). The adoptive transfer capacity of splenocytes depends upon the number of cells because 25 × 10^6 cells were not sufficient to transfer tolerance (MST = 23.0 ± 2.0 days, n = 4, p < 0.01, vs 1 × 10^8 cells).

At 2 and 4 wk posttransplant, DSBT-Tregs are present in all compartments tested: not only in the spleen and lymph nodes but also in the thymus and the graft

Compared with cells from tolerant rats at day 5, splenocytes (1 × 10^8) or lymph node cells (1 × 10^8) from tolerant rats 14 days after transplantation were highly efficient in transferring tolerance (100%) (Fig. 1, B and C). Of note, a reduced number of splenocytes (10 and 25 × 10^6) (which had failed to transfer tolerance when taken from tolerant rats at 5 days) transferred tolerance perfectly well when taken at 14 days, showing that DSBT-Tregs gradually expand. At day 14, both 1 × 10^7 graft-infiltrating cells and 1 × 10^8 thymocytes protected naive grafts from rejection (75%, MST = 73.3 ± 29.0 days, n = 4, and 80%, MST = 86.7 ± 7.5 days, n = 6, respectively, p < 0.001 vs control). At day 30, DSBT-Tregs were detectable in all compartments tested: spleen, lymph nodes, thymus, and graft-infiltrating cells, and results were similar to day 14 (Fig. 1C).

DSBT-Tregs are donor specific and require both DSBT and heart transplantation for their generation

The fact that DSBT-Tregs are donor specific is shown by the observation that 2.5 × 10^5 splenocytes from PVG recipients rendered tolerant to RA Ag (at day 30) did not protect third-party (WKAH) grafts from rejection (MST = 20.0 ± 5.9 days, n = 5, not significant vs control PVG recipients receiving WKAH grafts without cell transfer (MST = 21.0 ± 3.6 days, n = 5)) (Fig. 2).
planted. Controls (no cell transfer) are represented by irradiated (4.5 Gy) PVG rats. The following day, a RA heart is transplanted by the fact that 2.5 splenocytes (1.0 x 10^7) after transplantation. Single-cell suspensions of B cells (A), 14 procure in tolerant (DSBT-treated, transplanted) rats at 5 days (A), 22.8 days, (n = 4, not significant vs control). Contrary to CD8 cell subsets (2.5 x 10^7) transferred tolerance only modestly (MST = 33.3 ± 9.5, n = 7, p < 0.01, vs control). Second, regulatory activity of CD4^+ cells was found to be restricted to CD45^+CD45RC^- subset. Indeed, both CD45^- and CD45^-CD45RC^- subsets, but neither CD45RC^- nor CD45^-CD45RC^+ subset, transferred tolerance. CD45RC^+ cells

PVG rats at 30 days after transplantation did not protect naive PVG rats at all (MST = 9.6 ± 2.5 days, n = 5); interestingly, rejection was markedly accelerated in this group compared with control PVG rats receiving splenocytes from naive PVG rats (MST = 23.4 ± 1.5 days, n = 5, p < 0.01) or receiving no cell transfer (MST = 24.3 ± 3.0 days, n = 8, p < 0.01).

**DSBT-Tregs propagate tolerance**

We then searched to clarify whether tolerant splenocytes (taken from PVG rats tolerant to RA) suppressed the rejecting activity of cotransferred naive PVG splenocytes. When 2.5 x 10^7 splenocytes from day 30 tolerant rats were cotransferred with 2.5 x 10^8 (n = 5) or 1.0 x 10^8 (n = 5) naive splenocytes, rejection normally mediated by naive splenocytes was prevented (MST >90 days, p < 0.01). In addition, 2.5 x 10^7 cells splenocytes taken from rats rendered tolerant by a first adoptive transfer (90 days after transplantation) prevented rejection of naive grafts in a second set of naive recipients (n = 5) (MST >90 days, p < 0.01).

**DSBT-Tregs are CD4^+CD45RC^-**

First, regulatory activity is mostly restricted to CD4^+ cells (Fig. 3). Indeed, CD4^+ cells (2.5 x 10^7) transferred tolerance in 100% of cases (n = 5), whereas CD4^- cells (2.5 x 10^7) prolonged survival only slightly (MST = 32.3 ± 21.9, n = 5, p < 0.01, vs control). Contrary to CD8^- cells (2.5 x 10^7), which transferred tolerance perfectly well (n = 5, 100%), CD8^- cells (2.5 x 10^7) prolonged graft survival only modestly (MST = 33.3 ± 9.5, n = 7, p < 0.01, vs control).

The fact that DSBT-Tregs require the presence of the graft is shown by the fact that 2.5 x 10^3 splenocytes from DSBT-treated but nontransplanted PVG rats (at day 30) did not protect naive rats (MST = 22.8 ± 0.8 days, n = 4, not significant vs control).

That DSBT-Tregs require DSBT is demonstrated by the observation that splenocytes from transplanted but DSBT-untreated
Interestingly, CD45RC− cells from spleen of tolerant PVG rats 30 days after transplantation are separated by using the MACS system. The cell number used is 2.5 × 10⁷ for CD4, CD8, and CD4CD25 cells and 1.0 × 10⁷ for CD45RC− and CD4CD45RC− cells. CD4⁺CD45RC−, CD8⁺CD45RC−, CD4+CD25−, and CD4+CD45RC− cells from spleen of tolerant PVG rats 30 days after transplantation are separated by using the MACS system. The cell number used is 2.5 × 10⁷ for CD4, CD8, and CD4CD25 cells and 1.0 × 10⁷ for CD45RC− and CD4CD45RC− cells. CD4⁺CD45RC−, CD8⁺CD45RC−, CD4+CD25−, and CD4+CD45RC− cells (●) and CD4⁺CD45RC− cells (○) are injected into total body-irradiated PVG rats. The following day, a RA heart is transplanted. Control (no cell transfer) is indicated by ●.

(1 × 10⁷) did not prevent rejection (MST = 22.0 ± 1.6 days, n = 5, not significant vs control), whereas 1 × 10⁷ CD45RC− cells (1 × 10⁷) transferred tolerance (MST > 90 days, n = 5, p < 0.01, vs control). Interestingly, CD4⁺CD45RC− cells (1 × 10⁷) shortened graft survival (MST = 17.3 ± 0.9 days, n = 3) compared with controls who received no cell transfer (MST = 24.3 ± 3.0 days, n = 8, p < 0.01) or who received (2.5 × 10⁷) naïve splenocytes (MST = 23.4 ± 1.5, n = 5, p < 0.01). On the contrary, CD4⁺CD45RC− cells (1 × 10⁷) perfectly protected naïve recipients from rejection (MST > 90 days, n = 6, p < 0.01, vs control).

The thymus and the spleen are required for the generation of DSBT-Tregs but not for their expansion

We examined the effect of thymectomy or splenectomy on graft survival and on generation of Tregs in DSBT-treated rats (Fig. 4, A and B). When thymectomy or splenectomy was performed 4 wk before DSBT, tolerance was abrogated (MST = 19.0 ± 8.1, n = 9, p < 0.01, and 36.5 ± 25.5 days, n = 6, p < 0.01, respectively, vs euthymic and eusplenic controls). However, when thymectomy or splenectomy were performed at the time of transplantation, tolerance was not abrogated (MST > 90 days, n = 4, and MST > 90 days, n = 4, respectively, not significant vs euthymic and eusplenic controls) (Fig. 4A).

Splenocytes (2.5 × 10⁷) from rats thymectomized 4 wk before DSBT failed to protect naïve grafts from rejection (MST = 19.0 ± 1.0, n = 5). On the other hand, splenocytes (2.5 × 10⁷) from rats thymectomized at the time of transplantation transferred tolerance normally (MST > 90 days, n = 5) (Fig. 4B).

In vitro hyporesponsiveness develops in tolerant rats and requires both DSBT and the graft

Fig. 5 shows the kinetic of MLR, using as responders, splenocytes from tolerant (DSBT-treated, transplanted) rats or from control-rejecting (DSBT-untreated, transplanted) rats, and as a target, donor-type irradiated splenocytes. MLR shows gradual hyporesponsiveness in tolerant rats up to 7 days posttransplant compared with naïve rats and progressive hyporesponsiveness thereafter. On the contrary, splenocytes from rejecting rats showed hyporesponsiveness at 1 and 7 days posttransplant and progressive hyperresponsiveness thereafter.

Hyporesponsiveness seen at day 30 in tolerant DSBT-treated heart transplant rats was donor specific and was not observed when using third-party WKAH-irradiated splenocytes. Hyporesponsiveness was not seen neither in DSBT-treated/nontransplanted rats nor in transplanted/DSBT-untreated rats, which is additional evidence that both DSBT and the graft are necessary for tolerance and hyporesponsiveness to develop (Fig. 6).

Discussion

Achieving graft acceptance with reduced or no immunosuppression is the ultimate goal of transplantation medicine (1). DSBT has been repeatedly shown—clinically and experimentally—to promote hyporesponsiveness (9–14). However, DSBT has been abandoned with the introduction of the calcineurin inhibitors and because the mechanisms of the “transfusion effect” were not clearly...
understood (1, 11–12). However, DSBT remains a promising strategy to establish tolerance clinically due to its proven efficacy, its simplicity, and the fact that no particular immunotherapy is necessarily required (9–14).

To study the mechanisms of DSBT-induced tolerance, we developed a rodent model in which tolerance is induced by DS BT alone without the need for additional immunotherapy (15). In our model and consistent with other reports, we observed earlier that DS BT, albeit eventually protective, causes an early immune activation: 1) tolerant splenocytes display antidoron hyperresponsiveness early posttransplant (18–19); 2) tolerized grafts contain dense cellular infiltrates (19–20); and 3) an early peak of IFN-γ is seen in grafts destined to become tolerant (15, 19). However, in vitro and in vivo hyperresponsiveness develop later posttransplant, suggesting that suppressor/regulatory mechanisms capable of suppressing this immune activation are taking place (10, 19).

The fact that Tregs are involved in DS BT-induced tolerance was then unequivocally demonstrated by our group by the capacity of splenocytes from rats rendered tolerant by DS BT alone to propagate tolerance into naive rats (15, 19). A similar observation was reported by Bushell et al. (16) in a mice model. That DS BT—on its own—is capable of inducing Tregs in unmodified and immunosuppressed recipients of an organ allograft is an innovative finding that provides new insight in the understanding of the mechanisms of the blood transfusion effect and that may open new avenues for the development of tolerance in the clinic.

Features of Tregs have been studied in experimental models where tolerance to organ allografts is induced by various immune manipulations of the recipient (Abs directed to TCRs or costimulatory signals, immunosuppressive drugs) together with the delivery of donor Ags (under various forms) (2–8). On the contrary, the particular features of Tregs induced by DS BT alone (“DS BT-Tregs”) have not been explored. Therefore, we used our previously established DS BT model to study the particular features of DS BT-Tregs.

Infectious tolerance, a general feature of Treg-based tolerance, refers to the observation that once tolerance is achieved, tolerance is maintained by Tregs having the capacity to inactivate naive cells and which can make subsequent naive recipients tolerant (7–8, 21–22). DS BT-Tregs prevented rejection mediated by cotransferred naive cells, and Tregs taken from naive recipients made tolerant by adoptive transfer from tolerant rats could perpetuate tolerance in a second generation of naive recipients. Although this is similar to the phenomenon of infectious tolerance described by other researchers, we cannot entirely exclude the contribution to regulation by original regulatory cells.

Timing of expansion of DS BT-Tregs

Previous reports indicate that it may take weeks for Tregs to develop (2–8, 21). Accordingly, these reports emphasize the role of Tregs in the maintenance of tolerance rather than in its induction. One innovative finding in the present study is that DS BT-Tregs were detected in the spleen as early as day 5 after transplantation, suggesting that DS BT-Tregs play an important role in the induction phase of tolerance. However, only large numbers of splenocytes were capable of transferring tolerance at day 5. At 2 wk posttransplant, the number of splenocytes necessary to transfer tolerance was reduced by a factor of 10, and the efficiency of the adoptive transfer reached 100%, demonstrating that DS BT-Tregs, albeit present very early on, mature and gradually expand in the course of time.

Presence of DS BT-Tregs in the lymphoid tissues

In most experiments, splenocytes have been used as an easily obtainable source of Tregs for adoptive transfer experiments, leaving it unclear as to whether Tregs are present in other lymphoid tissues (2–8, 21, 23). We detected DS BT-Tregs not only in the spleen but also in the lymph nodes. Similar to the spleen, the regulatory properties evoked by lymph node cells increased from days 5 to 14. DS BT-Tregs were also detected in the thymus but only at later time points (2 and 4 wk), and the regulatory properties of thymic cells remained inferior compared with equivalent numbers of splenic or lymph node cells.
Studies on the trafficking of DSBT-Tregs are needed to determine whether these cells first expand in a particular lymphoid compartment and secondarily migrate to others or whether they mature and expand independently in distinct lymphoid sites.

After transplantation, APCs rapidly migrate to secondary lymphoid tissues where they activate naive T cells (24, 25). Our finding that significant numbers of DSBT-Tregs are present in the lymphoid tissues very early after transplantation makes it tempting to speculate that DSBT-Tregs act at this afferent site to block the initiation of the alloimmune response against the graft and actively participate at this site to the induction of tolerance. It should be clear that the nodal tissues we studied (mesenteric lymph nodes) do not represent the draining lymph nodes from the graft but the nodal tissue in general. Immune events in the true draining nodes from the graft are best studied in a skin transplant model.

**Presence of DSBT-Tregs in the graft**

The presence of Tregs in the spleen of tolerant rodents has been repeatedly demonstrated (2–8, 21, 26). However, a report by Graça et al. (27) in a model of tolerance induction via treatment with CD4 and CD8 mAb indicates the presence of Tregs in tolerant skin grafts. Consistent with this latter report, we found the presence of DSBT-Tregs among graft-infiltrating cells in tolerant rats at 2 wk posttransplant. An identical number of graft-infiltrating cells did not propagate tolerance at 5 days posttransplant in our model. This may indicate that DSBT-Tregs first mature and expand in the lymphoid tissues before infiltrating the graft. A second possibility is that Tregs were present ab initio in the graft but that we could not—for methodological reasons—demonstrate their presence. Indeed, we were limited to use $1 \times 10^7$ graft-infiltrating cells, which represents only 10% of the number of splenocytes required at the same time point to transfer tolerance. This possibility (e.g., the presence of Tregs ab initio in the graft) is strongly supported by the observation that grafts procured from tolerant rats at day 5 posttransplant continue to be tolerized after retransplantation in secondary naive recipients. In addition, these tolerized grafts can protect accompanying donor-specific but not third party naive grafts from rejection (Ref. 28 and data not shown).

The presence of Tregs in tolerated grafts strongly suggests that they have a direct protective role within the transplanted tissues (4, 27). Protection by Tregs has been reported to depend, among other factors, upon IL-4 (4, 7, 22, 29, 30). Certain Tregs (CD4$^+$CD45RC$^-$) have been shown to produce IL-4 (31). We previously reported a progressive accumulation of IL-4 in grafts tolerized by DSBT (15). This suggests that the protection conferred by DSBT-Tregs in the present model depends, at least in part, upon an in situ intragraft mechanism dependent upon IL-4. However, this needs to be studied in vitro and in vivo by using IL-4-neutralizing Abs. In addition, IL-4 may mediate alternative and more chronic forms of graft damage, and we previously reported in the same rodent model an association between IL-4 intragraft expression, Ab production, complement binding, and vasculopathy (15, 32).

**Both graft and DSBT are required for DSBT-Tregs generation**

In certain models of tolerance, secondary grafts are rejected if the primary graft is removed, demonstrating that continuous exposure to graft-derived Ags is necessary for the maintenance of Tregs (7, 33). However, whether the graft is required for the induction of Tregs is unclear. In the present experiment, splenocytes from DSBT-treated but nontransplanted rats failed to protect naive grafts in vivo and failed to display hypersponsiveness in vitro, showing that the graft is necessary for DSBT-Tregs—in sufficient numbers to protect from rejection—to be induced.

The presence of the graft, albeit necessary, was not sufficient to induce enough Tregs to propagate tolerance in naive recipients. On the contrary, splenocytes from rejecting rats (not treated by DSBT) showed hyperresponsiveness in vitro and caused accelerated rejection in vivo in naive recipients, suggesting the predominance—among these splenocytes—of effector cells, an observation in accordance with a report from Schneider et al. (34).

As also hypothesized by Schneider et al. (34), we cannot exclude that Tregs develop in nontolerant recipients during the normal course of an alloimmune response and that these cells contribute to limit the extent of this response but are in too small numbers to prevent effector cells to destroy the graft. We are now examining this hypothesis by performing adoptive transfer of separated subsets of splenocytes (CD4$^+$CD45RC$^-$ see below) to determine whether small numbers of Tregs are detectable in nontolerant animals and whether they can be expanded ex vivo, a strategy which would open new avenues to achieve tolerance in the clinic.

**The thymus (and the spleen) are required for DSBT-Tregs generation**

Tregs capable of regulating autoimmunity are originating in the thymus, but the thymic dependency of Tregs induced after transplantation is less clear (2–8, 35–37). In particular, the thymic dependency of DSBT-Tregs has not been studied. In our model, we found that thymectomy at the time of transplantation did not affect tolerance, suggesting that the thymus is not required for the maturation of DSBT-Tregs. However, we performed thymectomy 6 wk before transplantation, and in these circumstances, we found that DSBT-Tregs did not develop in the periphery. This is consistent with the possibility that the thymus functions as a “supplier” of precursor cells (thymic emigrants), which mature into allogeneic-specific Tregs in the periphery after contact with donor Ags (36). Once the thymic emigrants have left the thymus, its physical presence would be no more required. We cannot rule out the possibility that central thymic deletion operates in this model, but this is unlikely because central deletion has been mostly described in models in which the recipients are first submitted to lymphoid depletion.

The fact that thymectomy at the time of transplantation did not influence long-term graft acceptance also indicates that the thymus is not necessary for the maintenance of DSBT-Tregs, a phenomenon in which the graft itself plays a more determinant role (7, 33). Therefore, the biological significance of the Tregs that we detected posttransplant in the thymus remains debatable. We cannot exclude that the thymus participates as a lymphoid organ to the expansion of Tregs, but this role is certainly not essential given that Tregs were detected only late posttransplant in the thymus and the regulatory function they evoked remained poor.

Interestingly, we found—similar to thymectomy—that splenectomy done 4 wk before DS BT, but not at the time of transplantation, abrogates tolerance. In addition, no regulatory activity was present in the lymphoid tissues of rejecting DSBT-treated and -splenectomized rats (data not shown), clearly showing that the spleen is essential for the generation of Tregs (35). Given that DSBT-Tregs were found early posttransplant and rapidly expanded in the spleen, we had speculated that the spleen was playing a key role in the expansion of these cells. However, the development of tolerance in rats splenectomized at the time of transplantation suggests that DSBT-Tregs amplification does not take place exclusively in the spleen.
DSBT-Tregs are CD4+ CD45RC

“Suppressor” cells were long thought to operate in various models of tolerance, including DSBT (10, 38), but this concept was abandoned because these cells could not be phenotypically characterized. Tregs regained interest when CD4+CD25+ cells were identified in the control of autoimmunity and thereafter in certain models of transplantation tolerance (2–8, 23, 29, 39).

In our model and in accordance with the majority of reports on Tregs (2–8), regulatory activity induced by DSBT was restricted to the CD4+ subset. However, in contrast with these reports and with Bushell et al. (16), regulatory activity induced by DSBT was not restricted to the CD4+CD25+ subset but was equally present in the CD4+CD25− subset. The fact that CD4+CD25+ is not an exclusive marker of DSBT-Tregs is consistent with data recently reported by Soulliol and colleagues (40): in a DSBT model similar to us, these authors found the existence of dominant regulatory function in the CD25+ T-cell subset. In addition, Graca et al. (26) also reported that tolerant mice contain CD4+CD25+ cells with regulatory potential.

We found that DSBT-Tregs activity is restricted to a distinct phenotype, the CD4+CD45RC+ subset. CD45RC molecules in rats are the counterpart of CD45RB in mice and CD45RA in man. CD4+CD45RC+ and CD4+CD45RC− cells are naive and memory cells, respectively (41). Ag stimulation normally switches resting CD4+CD45RC− naive cells into memory CD4+CD45RC+ cells capable of acutely rejecting grafts. However, DSBT may affect the function of primed CD4+CD45RC− memory T cells and alter their rejecting capacity (42). The regulatory properties of CD4+CD45RC− cells were identified previously in different experimental settings: 1) naturally occurring CD4+CD45RC− cells regulate autoimmune diseases in rats (30, 36); 2) CD4+CD45RC+ are present in increased numbers in accepted liver grafts (43); 3) CD4+CD45RC− cells function as Tregs in a model of tolerance involving administration of donor Ag in the presence of an anti-CD4 mAb (44); and 4) finally, Abs neutralizing CD4+CD45RB− but preserving CD4+CD45RB+ cells prolong graft survival in mice (45). However, one conflicting report with these data comes from Zhai et al. (46), who reported that CD4+CD45RC+ (and not CD4+CD45RC−) cells were hyporesponsive to alloantigen and able to suppress normal T cell function in coculture assays. In contrast with Zhai, we found that CD4+CD45RC− cells from tolerant animals early and late posttransplant were hyporesponsive to donor alloantigens and capable of inhibiting proliferation of CD4+CD45RC+ cells (data not shown).

In the present DSBT model, CD4+CD45RC+ cells functioned as effector cells capable of rejecting naive grafts in an accelerated tempo. Of note, we detected increased numbers of CD4+CD45RC− cells (DSBT-Tregs) and reduced numbers of CD4+CD45RC+ cells (effector cells) in infiltrates of tolerated grafts, whereas an opposite ratio was seen in rejected grafts (28). This predominance of intragraft CD4+CD45RC− Treg cells was already present at day 5 posttransplant (data not shown).

In conclusion, this study provides the first detailed description of the features of Tregs induced by DSBT alone, without immunotherapy (“DSBT-Tregs”). First, DSBT-Tregs are present very early after transplantation in both the spleen and the lymph nodes, suggesting that they block the initiation of the alloimmune response and participate to the induction of tolerance; these cells progressively expand in the lymphoid tissues; the presence of Tregs in the graft suggests that they can directly protect the transplanted tissues. Second, both DSBT and the graft are necessary for sufficient numbers of DSBT-Tregs to be induced and hyporesponsiveness to develop. Third, the thymus is required probably as a supplier of thymic emigrants, which transform in the periphery into alloantigen-specific Tregs after encountering donor Ags, a phenomenon which also requires the presence of the spleen; however, the thymus is not required at the time of transplantation for the maturation and afterward for the maintenance of DSBT-Tregs. Fourth, both CD4+CD25+ and CD4+CD25− cells evoke identical regulatory activity, whereas CD4+CD45RC− is an exclusive marker of DSBT-Tregs in this model (similar to Tregs protecting from autoimmunity in rats). Further investigations are required to delineate the trafficking of DSBT-Tregs between the thymus, the secondary lymphoid tissues, and the graft and the detailed mechanisms by which these cells suppress naive and effector cells in the lymphoid tissues and in the graft.

It is important to stress the strain-specific nature of the findings in this report. Caution should be used when extrapolating these data to different experimental settings and to clinical transplantation because the magnitude of the DSBT effect and the operating mechanisms may depend upon the strain combination and the species used.

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References


Lakkis, F. G. 2003. Where is the alloimmune response initiated?


