Prolonged acceptance of skin grafts induced by B cells places regulatory T cells on the histopathology scene

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Abstract

The participation of regulatory T (Treg) cells in B cell-induced T cell tolerance has been claimed in different models. In skin grafts, naive B cells were shown to induce graft tolerance. However, neither the contribution of Treg cells to B cell-induced skin tolerance nor their contribution to the histopathological diagnosis of graft acceptance has been addressed. Here, using male C57BL/6 naive B cells to tolerize female animals, we show that skin graft tolerance is dependent on $CD25^+$ Treg cell activity and independent of B cell-derived IL-10. In fact, B cells from IL-10-deficient mice were able to induce skin graft tolerance while Treg depletion of the host inhibited 100% graft survival. We questioned how Treg cell-mediated tolerance would impact on histopathology. B cell-tolerized skin grafts showed pathological scores as high as a rejected skin from naive, non-tolerized mice due to loss of skin appendages, reduced keratinization and mononuclear cell infiltrate. However, in tolerized mice, 40% of graft infiltrating $CD4^+$ cells were $FoxP3^+$ Treg cells with a high Treg:Teff (effector T cell) ratio (6:1) as compared to non-tolerized mice where Tregs comprise less than 8% of total infiltrating CD4 cells with a Treg:Teff ratio below 1:1. These results render Treg cells an obligatory target for histopathological studies on tissue rejection that may help to diagnose and predict the outcome of a transplanted organ.

Key words: Skin graft; Tolerance; B cells; Treg; Histopathology

Introduction

Induction of T cell tolerance has profound implications for treatment of autoimmune diseases and tumors and for controlling transplant rejection (1,2). Central T cell tolerance happens inside the thymus, where thymocytes that recognize with high affinity peptide-major histocompatibility complex antigens presented by thymic antigen-presenting cells are deleted or converted into natural regulatory T (Treg) cells (3,4). However, central tolerance is incomplete and harmful self-reactive T cells escape from deletion in the thymus. Therefore, additional mechanisms that regulate the immune responses at the periphery are needed, such as T cell anergy,

induction of Treg cells, and peripheral deletion (5). In fact, the outcome of antigen recognition at the periphery - tolerance or immunity - is highly dependent on the antigen- presenting cell type and on the signals that it provides to T cells. Although B cells can present antigens to T cells leading to priming and activation, they are also believed to induce tolerance (6). B cells have been shown to modulate dendritic cells, CD8 and CD4 T cells through secretion of soluble factors such as transforming growth factor-β (TGF-β) or interleukin-10 (IL-10), and to induce anergy or deletion of CD4 and CD8 cells directly (6). Naive B (nB) cells are potent inducers of Treg

cells *in vitro* and *in vivo*. They were shown to expand naive allospecific Treg cells *in vitro* without the need for exogenous cytokines (8) and to promote Treg-mediated tolerance *in vivo* in experimental models of gene therapy, autoimmune diabetes and experimental encephalomyelitis (9). Of particular interest, nB cells are able to induce tolerance to minor antigens in skin transplants (10). This evidence, together with the fact that B cells can generate allospecific Treg cells *in vitro* (8), led us to ask how Treg involvement in skin graft tolerance could impact the histopathological diagnosis of the graft.

We found that B cell-induced tolerance to skin transplants is in fact dependent on Treg cells. Surprisingly, the tolerized minor-mismatched skin graft showed significant mononuclear infiltration and loss of skin appendages, resulting in a high histopathological score in spite of graft acceptance. A refined analysis of the mononuclear infiltrate showed the presence of a large fraction of Foxp3$^+$ Treg cells that were virtually absent in the skin grafts of non-tolerized mice, strongly suggesting that T cell tolerance in skin grafts is an active process that requires the continuous surveillance of the graft by Treg cells. Thus, nB cells induce a state of tolerance that, upon histopathological examination, is not completely compatible with the definition of acceptance. We believe that the results shown in this report bring Treg cells into the scene of histopathological analyses and may help to avoid misleading diagnoses and therapeutics.

Material and Methods

Animals

C57BL/6 (B6), F1 (C57BL/6xBALB/c), C57BL/6 IL-10KO, and Marilyn T cell receptor (TCR) transgenic RAG$^{-/-}$ (specific for the male antigen H-Y + H-2^b) (11) transgenic mice were bred and maintained at the animal breeding facilities of Instituto Nacional de Câncer (Rio de Janeiro, RJ, Brazil). Ages ranged from 8 to 10 weeks. Animals were handled according to our institutional guidelines.

Flow cytometry and skin infiltrate analysis

Skins from tail to dorsum grafts were individually collected, infiltrated with HBSS solution with 20 mM HEPES, 10% fetal bovine serum (FBS), 0.5 M EDTA and 0.1 mM dithiothreitol (DDT, Sigma, USA), and incubated for 30 min at 37°C with shaking (80-100 rpm). The cells were then collected, washed with 10% Dulbecco's modified Eagle's medium (DMEM) containing FBS and analyzed by flow cytometry. Antibody staining was done according to manufacturer instructions. All antibodies were purchased from eBioscience® (USA). All cytometric data were acquired with a FACSCalibur® and analyzed with Cell Quest Pro® (BD, USA) or the Flowjo® (USA) software.

Naive B cell purification

nB cells were obtained from spleens of F1, B6, or IL-10KO male mice as previously described (10). Briefly, after a discontinuous Percoll gradient, cells were plated for 2 h in DMEM-10% FBS to remove adherent cells. Non-adherent cells were collected and T lymphocytes depleted using anti-CD4 + anti-CD8 antibodies (supernatants from GK1.5 and 53-6.72 hybridomas, respectively) followed by incubation with rabbit serum as a source of complement. B cell purity was 90 to 96% (Figure S1).

Skin transplants

Tail-to-tail or tail-to-dorsum skin transplants were performed in female B6 mice. Briefly, animals were anesthetized with 2,2,2,-tribromoethanol (0.58 mg/g body weight; Aldrich, USA), the dorsum was washed and a wound was created to be filled with the transplanted dorsal skin. A similar procedure was used for tail skin.

The graft was gently placed on the wound surface. For dorsal grafts, a skin fragment of approximately 1 cm^2 was used. For tail grafts, smaller skin fragments were used. After grafting, skin adhesive tapes were used to cover the grafts. Mice were maintained in individual cages for 1 week, after which skin adhesive tapes were removed. Scores were determined every 2 to 3 days until the end of the experiment. Rejection was considered to have occurred when macroscopic examination showed a 70% reduction of graft size, as shown in Figure S2.

Tissue histopathology

Transplanted skins were removed, processed for histopathology and stained with hematoxylin and eosin. Six to eight sections were analyzed per block. Skin histopathology scores were assigned according to Yi et al. (12). Briefly, each organ was evaluated for individual parameters, which received a value from 0 to 2 according to severity. The final score for each organ was then obtained by the sum of the following parameters: inflammatory infiltration, fibrosis, loss of appendages, epidermal changes, and ulceration.

Antibody treatment

Animals received 100 µg purified (13) anti-CD25, 7D4 monoclonal antibodies to deplete CD25$^+$ regulatory cells. Antibodies were injected intraperitoneally starting one day before nB cell injection and then weekly until the end of the experiment.

Statistical analysis

One-way ANOVA with the Bonferroni post-test and the log rank test for skin transplants were used under the Prism software (Graphpad Prism V5.0, USA).

Results and Discussion

B cell-induced skin graft tolerance depends on Treg cells

Based on previous data showing that nB are able to induce tolerance of skin grafts to the minor Dby male antigen (10), we asked whether this effect was dependent on Treg cells. nB

cells from F1 or B6 male mice were injected into B6 females. As expected, 80% of B6 female mice treated with nB cells from F1 males became tolerant and accepted male B6 skin grafts (Figure 1A). The induction of tolerance was dependent on B cell activation status. Indeed, lipopolysaccharide (LPS)-activated B cells (Figure 1A) could not induce skin graft tolerance in our system, although they have been shown to induce tolerance when used as vectors for gene therapy (9) or for *in vivo* prim-

ing of the cytotoxic response to Dby (10). It is possible that the protocol used for gene transfer using LPS-activated B cells as vectors, alters LPS-activated B cell antigen presenting abilities. It is also possible that LPS-activated B cells are unable to prime a classical cytotoxic response measured *in vitro* (10), but, as shown here, do not inhibit the *in vivo* graft rejection response.
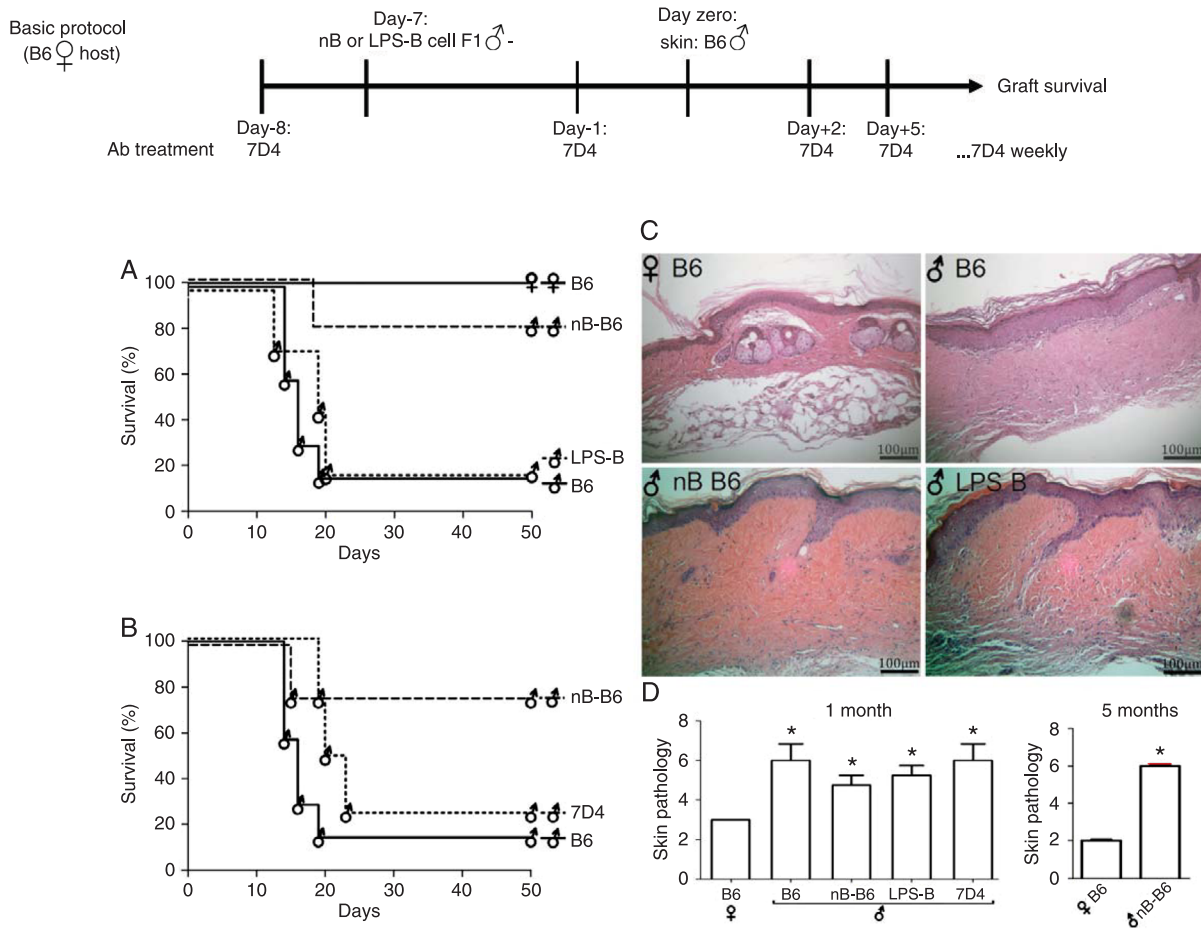
B cells have been shown to induce tolerance through two

Figure 1. Naive and not lipopolysaccharide-activated B (LPS-B) cells induce regulatory T (Treg) cell-dependent skin tolerance to the male (Dby) antigen. The protocol used is shown at the top of the figure. B6 female hosts were treated or not with naive B (nB-B6) or LPS-B cells. When testing for the participation of CD25+ cells, antibody (Ab) treatment using anti-CD25 (7D4) was used. *A*, B6 female mice were left untreated, were treated with 10^7 male nB (nB-B6)- or LPS-B-activated cells from F1 mice. After 1 week, hosts were tail skin-grafted with male and female B6 as indicated by the symbols in the figure. *B*, B6 female mice received nB cells from F1 male animals (nB), and after seven days they were transplanted with B6 male skin. The experimental group was treated with anti-CD25 (7D4) monoclonal antibody on days -1, +2, +5, in relation to transplantation (day 0) and after that weekly until the end of the experiment. Non-nB cell-treated controls are shown (B6). N = 6-8 animals/group for two experiments each. P < 0.05 between nB/female and rejected groups. All the other groups are not statistically different. *C*, After 20 days the skins from experiments shown in *A* and *B* were removed and processed for H&E staining. Note the healthy appearance of the female graft, with the presence of skin appendages and absence of infiltrate. The male graft shows the absence of appendages, thinner keratin layer and mononuclear infiltrate. This is similar to nB cell and LPS-B cell grafts. *D*, Histopathology scores were obtained as described in Material and Methods (mean of 6 grafts/group).

different mechanisms: by inducing FoxP3$^+$ Treg cells or through the production of IL-10, which does not generate FoxP3$^+$ regulatory cells (6). The surface phenotype of the nB cell preparation used in this study was not compatible with regulatory B cells (Figure S1A). Moreover, nB cells from IL-10-deficient mice worked as tolerogens for the skin graft as well as nB cells from wild-type B6 animals (Figure S1B). In this case, skin survival was as prolonged as the one induced by control nB cells, lasting up to 150 days after transplantation.

To study the immune response of an nB cell-treated animal, 1 week after nB cell injection the animals were immunized in the footpad and 1 week later draining lymph nodes (DLN) were harvested and analyzed. The relative numbers of regulatory cells present in female B6 mice treated with male nB cells and immunized in the footpad with B6 male spleen cells were not different from those of untreated control mice (Figure S3A). However, the absolute number was much smaller. To confirm that this difference was really due to antigen-specific T cells, we repeated the experiment using Marilyn transgenic mice, which carry a specific anti-Dby TCR in the RAG KO background (11), as responders. As can be seen in Figure S2B, the absolute number of cells in the draining lymph nodes of Marilyn female mice pretreated with F1 male nB was smaller than that present in non-treated Marilyn mice, although the relative numbers of Treg was the same in both groups (Figure S3B). *In vitro*, total cells from DLN were assayed in mix experiments as a test for suppression function, since both DLN from nB cell-treated and -untreated mice have the same relative number of Treg cells. DLN cells from nB cell-treated female Marilyn mice were twice as potent as DLN cells from control mice in suppressing lymphocyte proliferation, suggesting a higher suppressor potency induced by nB cells (Figure S3C). *In vitro de novo* generation of Treg cells from CD25$^-$ cells by B cells has been reported (8,14) and it was also shown that allo-Treg cells preferentially expand in response to B cell stimulation at the expense of effector cells. We confirmed that *de novo* generation is taking part in Treg generation since naive Marilyn RAG KO mice are devoid of Treg cells.

Induction of Treg cells by B cells has been reported by several investigators, but so far there are no data on skin grafts. To definitely address the role of Treg cells in our model system *in vivo*, B6 female mice treated with male nB cells were injected with anti-CD25 monoclonal antibody 7D4 on days -1, +2, +5 (considering the transplantation day to be day 0) and after that weekly until the end of the experiment. Treatment with anti-CD25 inhibits the tolerance induced by nB cells evidenced by skin rejection (Figure 1B). These results established that skin graft tolerance induced by nB cells was indeed dependent on Treg cells.

The above results led us to ask if there were differences at the histopathological level between a syngeneic graft and a graft whose survival depends on an active suppressive environment. As expected, syngeneic grafts showed no mononuclear cell infiltrates and preservation of all skin appendages (Figure 1C). Surprisingly, this pattern was not observed in the grafts recovered from mice tolerized by treatment with male nB cells. These grafts showed absence of skin appendages and a significant mononuclear dermal infiltration, very similar to that of rejected grafts (LPS- or 7D4-treated groups).

When the histopathological score was assessed, a significant difference was observed between female syngeneic grafts and nB cell-tolerized male grafts, but not between the latter and non-tolerized (rejected) grafts (Figure 1D). Importantly, after five months (150 days) the high histopathological score of the "tolerized" skin remained unchanged, suggesting that an active suppression was taking place to maintain the skin in place for long periods of time (Figure 1D and Figure S4).

Histopathological analyses of cells derived from nB-tolerized skin showed lymphocyte infiltrates of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells

Next we looked at the composition of the mononuclear infiltrate present in the "tolerized" grafts. We recovered cells from the skin grafts and analyzed them by flow cytometry. In this case, the grafts were recovered 20 days after transplant. At that point the graft area was similar among the groups studied. The relative numbers of lymphocytes recovered from syngeneic skin was less than 2% of all recovered cells while they increased to 30% in non-tolerized grafts. The tolerized group showed intermediate numbers, around 12% (Figure 2A). Differential analyses showed no differences in the relative numbers of B, T, NK, or NKT (Figure 2B). However, CD4$^+$CD25$^+$Foxp3$^+$ Treg cells represented 90% of the CD4$^+$-infiltrating cells in the syngeneic group and were virtually absent in the rejection group. In the tolerized test graft, Treg cells represented almost 50% of CD4 cells. We then looked at the effector (CD25$^+$Foxp3$^-$) population (Teff) and observed that these were less than 10% of the CD4 cells in the syngeneic and test groups while they were twice as many in the rejection group (Figure 2C). The Treg/Teff ratio was calculated, since this relationship is decisive for Treg-induced suppression of immunological responses (14). Figure 2D shows that this ratio is below 1 for the non-tolerized group while it is 6:1 for the tolerized group. This implies that effectors are actively maintained under control for a long time and that elimination of Treg cells impairs tolerance induction and leads to skin graft rejection, as shown in Figure 1B. Although we show that Treg cells induced by B cells are effective *in vitro* and *in vivo*, it would be interesting to transfer T cells from tolerized mice to evaluate the role of infectious tolerance (2). Treg infiltration of tolerized skin grafts has been shown in systems using immunocompromised hosts transferred with effectors and regulatory cells (15). However, in competent animals tolerized with nB cells, where Treg cells are in fact generated by the tolerization protocol used *in vivo*, we could
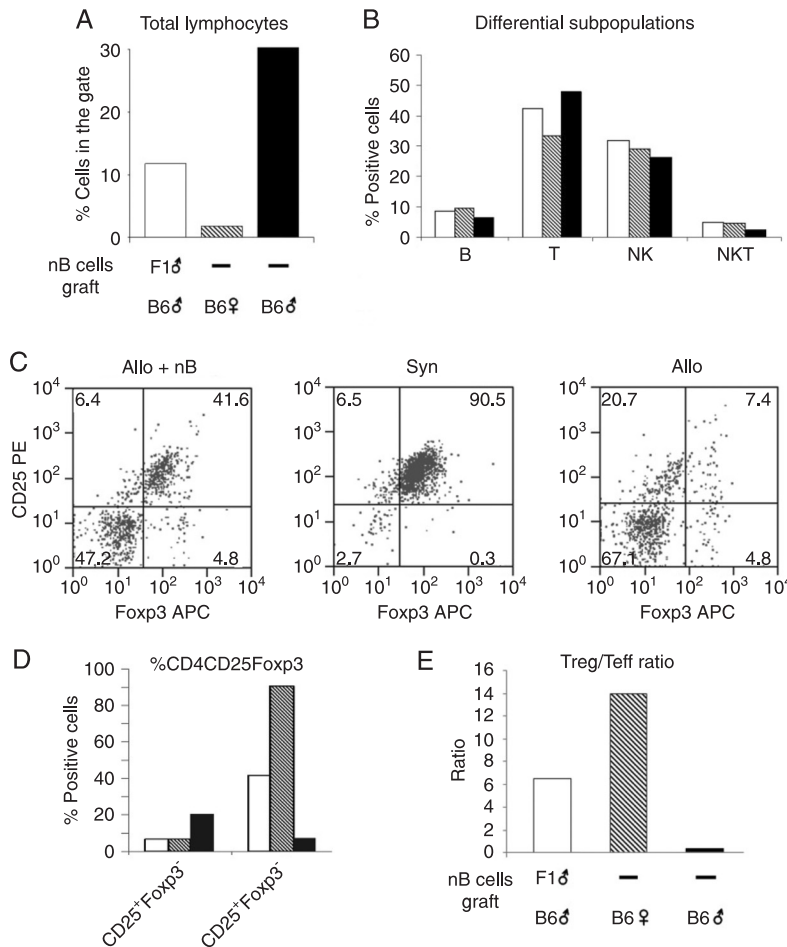
A Total lymphocytes

% Cells in the gate

30

0

nB cells   F1♂   —   —
graft   B6♂   B6♀   B6♂

B Differential subpopulations

% Positive cells

60
50
40
30
20
10
0
        B   T   NK   NKT

Figure 2. Tolerized skin grafts are infiltrated by regulatory T (Treg) cells. Skin grafts from B6 female mice treated or not with naive B (nB) cells were recovered after 20 days and cells were obtained as described in Material and Methods. Skin samples had an average size of 65, 75, and 45 mm^2 for tolerized male, syngeneic female and non-tolerized male, respectively. *A*, Frequency of lymphocytes in total cells obtained after enzymatic digestion of the skin. *B*, Frequency of different subpopulations present within the lymphocyte subpopulation obtained. *C*, Dot plots showing CD25 x Foxp3 staining of CD4$^+$ gated lymphocytes obtained from the indicated grafted skins. APC = antigen-presenting cells. *D*, Relative numbers of the indicated subpopulations in CD4$^+$ gated cells. *E*, Treg/Teff (effector T cell) ratio was calculated from the data shown in *C* (pool of 5 animals/group for 2 experiments).

C

Allo + nB
CD25 PE
10^4 6.4   41.6
10^3
10^2
10^1
10^0 47.2   4.8
        10^0 10^1 10^2 10^3 10^4
        Foxp3 APC

Syn
10^4 6.5   90.5
10^3
10^2
10^1
10^0 2.7   0.3
        10^0 10^1 10^2 10^3 10^4
        Foxp3 APC

Allo
10^4 20.7   7.4
10^3
10^2
10^1
10^0 67.1   4.8
        10^0 10^1 10^2 10^3 10^4
        Foxp3 APC

D %CD4CD25Foxp3

% Positive cells

100
80
60
40
20
0
        CD25$^+$Foxp3$^-$ CD25$^+$Foxp3$^-$

E Treg/Teff ratio

Ratio

16
14
12
10
8
6
4
2
0
nB cells   F1♂   —   —
graft   B6♂   B6♀   B6♂

see mononuclear cell infiltrates with half the CD4$^+$ cells expressing Treg markers.

Of note is the fact that, although the tolerized skin does not look 100% healthy, the transplanted skin is kept and is comparable to the area measured in the syngeneic graft with no loss in graft size. The lack of appendages in the tolerized skin could be the result of an inhibitory cytokine produced by the Treg cells themselves, like TGF-β (16), or of a direct action of effector cells. Detailed immunohistochemistry study allied to cytokine analysis needs to be done to dissect the physiopathology of the skin graft take in tolerized hosts. Similar to what was observed here for skin transplants, studies in kidney-grafted patients have shown that the Treg/CD3 ratio infiltrating the graft could discriminate the outcome of patients with subclinical rejection (17,18). The increased Treg/CD3 was associated with good renal function and graft survival in two-, three- and five-year studies. Although the immunosuppressive regimen suggests that patients on sirolimus and anti-thymocyte globulin have a significantly higher expression of Foxp3$^+$ Treg, some patients on calcineurin inhibitors also present Treg within graft infiltrates, which display a better evolution of graft function (17,18).

Taken together, these findings bring Treg cells into the pathological setting and make Treg cells an obligatory target when studying mononuclear infiltrates in doubtful situations. We believe that the present study can help pathologists and clinicians to better evaluate, diagnose and treat graft rejection.

Supplementary Material

Figure S1
Figure S2
Figure S3
Figure S4

Acknowledgments

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