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Original Article

The myth of the subclinical rejection: Is it real?

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Abstract

Introduction: Subclinical rejection (SCAR) of renal allografts refers to graft lymphocytic infiltration taking acute rejection histologic pattern despite stable renal function. There are no data to suggest that subclinical tubulointerstitial inflammation is regulatory or in any way beneficial to the graft. We have investigated whether C57BL/6 CD8 T cells home to long term engrafted (LTE) DBA/2 skin allografts and if it is protecting or rejecting. *Methods and results:* We transplanted two groups of B6 CD4 KO mice, 6 mice each, with MHC mismatched DBA/2 skin. Only the 1st group was treated with Rapamycin (RPM) as reported. After 100 days of LTE, we challenged RPM treated hosts with a 2nd DBA/2 skin graft. The 2nd but not the 1st graft was rejected. Then we investigated the functional effects of graft inflitrating CD8 T cells. DBA/2 skin grafts were harvested 100 days posttransplantation from (i) RPM treated B6 CD4 ko mice (N=5) and (ii) skin autografts (N=5) in DBA/2 recipients. LTE DBA/2 allografts or control DBA/2 autografts were then transplanted onto C57BL/6-Rag KO hosts, and peripheral blood lymphocytes (PBL) samples were collected 30 days post skin transplantation. CD8T cells can not be detected in PBL of naïve RAG-/- mice. 4.6 % CD8 T cells are detected in PBL of RAG-/- recipients of LTE allografts, but not in recipients of syngeneic grafts. To test the protective function of the graft homing CD8 T cells (from LTE RPM mice) that expanded by homeostatic proliferation and are present in PBL of the RAG-/-, 0.2 x 10^6 CD8 T cells from naive CD4KO mice were adoptively transferred into the RAG-/- hosts bearing the LTE DBA/2 allografts or DBA/2 autografts 30 days following skin transplantation. Survival of LTE DBA/2 skin allografts transplanted onto RAG-/- mice were significantly prolonged. *Conclusion:* Graft infiltrating CD8 T cells are regulatory and functionally active to protect allograft from rejection.

Introduction

The presence of lymphocytic infiltration in solid organ transplantation has been defined as acute cellular rejection when it is associated with graft dysfunction [1] and subclinical rejection (SCAR) when the graft function is quescient [1]. The assessment of allograft histology through prospective protocol biopsies was originally carried out for monitoring the advent of histopathologic lesions in clinically stable allografts [2]. The presence of asymptomatic tubulointerstitial cellular infiltrates has been defined as subclinical rejection [1]. The incidence of SCAR varies between 5% and 15% with current maintenance immunosuppressive drug regimens [3].

The only pervious single randomized clinical trial of biopsy and corticosteroid therapy demonstrated significantly improved early structural and functional outcomes, and a (nonsignificant) 17% risk reduction in 4-year graft survival [4]. Same authors, almost a decade later have reported that early protocol biopsies and corticosteroid treatment of subclinical rejection seem to have no benefit on short-term outcomes in renal transplant recipients

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being treated with tacrolimus, mycophenolate and prednisone [5].

In kidney transplant situations and despite many similarities between SCAR and clinical acute rejection exist, the inflammatory activated cell infiltrates are not completely identical while graft cytokine profiles and counteractive immune responses are characterized by subtle differences that could explain why SCAR is not accompanied by immediate graft dysfunction. Evidence that SCAR contributes to chronic allograft damage (interstitial fibrosis and tubular atrophy) and negatively affects graft outcome is counterbalanced by the scarcity of controlled data proving the beneficial effect of SCAR treatment [6-8].

To address this issue we used a skin transplant model in a CD4 KO mice (to study it in a single cell level) using a RPM treatment as it was reported to have a profound effect on CD8 T cells [9]. We investigated whether CD8 T cells home to LTE DBA/2 skin allografts and if these infiltrating lymphocytes have a functional effect (i.e. rejecting or protecting) in the skin allografts.

Material and methods

Animals: Male B6.CD4 knockout (KO; H-2^b), DBA/2 (H-2^d) mice, C3H/He (H-2^k) and C57BL/6J-Rag knockout (KO) mice 8–10 wk old, were purchased from Jackson Laboratory (Bar Harbor, ME). Animal use and care conformed to the guidelines established by the animal care committee at Beth Israel Deaconess Medical Center (Boston, MA).

Reagents: All Abs used for cell surface staining were purchased from BD Pharmingen (San Diego, CA) unless indicated otherwise: FITC anti-mouse CD4 (clone RM4-5), CyChrome anti-mouse CD8 (clone 53-6.7), FITC anti-mouse CD8 and FITC-isotype control Abs. PE anti-CD3, PE anti-TCR. Rapamycin (RPM) (obtained from Wyeth-Ayerst) (Princeton, NJ) was prepared in carboxymethyl cellulose for i.p. injections.

Skin transplantation and immunosuppression protocol: Full-thickness tail skin grafts (1 cm^2) from donor mice were transplanted onto the thoracic wall of recipient mice. The skin grafts were secured with an adhesive bandage for the initial 7 days. One group of recipient mice was treated with RPM consisted of 3 mg/kg/day i.p. on days 0,1, and 2, followed by treatment every other day for 2 weeks as previously reported [9], while the second group was not treated. Graft survival was assessed by daily visual inspection. Rejection was defined as the complete necrosis and loss of viable skin tissue.

Skin allografts from long term engrafted (LTE) (>100 days) hosts were removed from the lateral thoracic wall of recipient mice and grafted onto the flank of C57BL/6J-Rag KO mice.

Preparation of purified T cell subsets and magnetic cell separation: CD8 T cells were purified as previously described [10]. After RBCs were lysed by hypotonic shock, lymph node and spleen cells were depleted of macrophages, granulocytes, B cells and erythrocytes cells by incubating them first with anti-CD11b (Mac-1) Ab, anti-GR1 (8C5) Ab, anti-CD4 (GK1.5) Ab, anti-CD45R/B220 and anti-erythrocytes Abs, and then with magnetic beads coupled to anti-rat Ig (Dynal). B cells were removed using magnetic beads coupled to anti-mouse IgG (Dynal).Purified CD8 T cells were negatively selected using MACS column (Miltenyi). The purity of the resultant CD8 T cells populations was determined by flow cytometry. A suspension of CD8 + T cells were resuspended in PBS/0.5% BSA (2 x 10^6/ml) and stained with FITC-conjugated anti-mouse CD8, on ice for 30 min, washed in PBS/BSA, and fixed in 1% formaldehyde before analysis. Purity was 95% in all experiments.

Cell staining and flow cytometry: Pooled lymph nodes and spleen were homogenized in PBS 0.5% FCS 0.2% NaN3 with a nylon cell strainer (Falcon), and distributed in 96-well U-bottom microplates (4 X10^6 cells per well). Staining was performed on ice for 30 min per step. Abs were obtained from BD Pharmingen unless otherwise indicated. To prevent unspecific binding of mAb, all samples were preincubated with blocking anti-Fc-RII/III mAb (2.4G2). The following Abs combinations were used: for surface phenotype analysis, anti-CD8-CyChrome, anti-CD4- FITC, anti-CD3-PE, anti-TCR-PE. All samples were analyzed using a FACSort equipped with CellQuest software (BD Biosciences, Mountain View, CA). Data were collected and analyzed by electronically gating on CD8 + populations. At least 100,000 events were collected for each sample.

In vitro suppression assay: Spleen cells from DBA/2 (H-2^d) or from C3H/He (H-2^k) mice were depleted of T cells by anti- CD4/CD8 beads (Miltenyi), treated with Mitomycin C (Sigma) at 50μg/mL for 30 min, and used as stimulators (4× 10^5) in round-bottomed 96-well plates. CD8 T cells were harvested as previously described [10] from CD4 KO mice either after long term engraftment of allogeneic skin transplant with RPM treatment or from naïve mice used as responders in a varying ratio (0, 1X10^5 and 2X10^5) in MLR culture for 3 days. Cells were pulsed with [3H] methylthymidine (0.5 μCi/well; NEN) for the last 12 h before

harvesting, and incorporated radioactivity of triplicate wells was counted. Data shown are representative of three separate experiments.

Histopathology: The skin graft was removed from recipient LTE RPM treated CD4/KO mice 100 days post- transplantation, snap frozen and stored at -80 C°. Serial tissue sections (5 μ) were prepared and mounted on Super Frost Plus glass slides (Fisher Scientific, Pittsburgh, PA), fixed in formalin for 5 min, and stained in H&E for histological evaluation.

Immunohistochemistry: The snap frozen tissue was cut into 5 micron sections, fixed in 2% paraformaldehyde, rinsed in PBS, interacted with 0.5% Triton X-100 treatment for 3 min., blocked 30 minutes in 2% BSA and interacted with 1μg/ml anti- CD4 (BD Pharmingen cat# 550278) and anti-CD8 (BD Pharmingen cat# 550281) monoclonal antibodies overnight at 4 C°.

Results

1- RPM short term treatment can induce long term engraftment:

It was reported that short term RPM can induce long term engraftment of a full mismatch allogeneic skin graft [9]. To examine if this LTE of skin graft have a functional (i.e. protecting vs rejecting) lymphocytic infiltrations, we transplanted two groups of B6 CD4 KO mice, 1st group was 12 mice and the 2nd group was 6 mice, with MHC mismatched DBA/2 skin. The 1st group was treated with RPM as described [9], while the 2nd group was not treated. After 100 days of engraftment and long following cessation of RPM treatment, we challenged RPM treated hosts with a second DBA/2 skin graft (n=6). Graft survival plotted. Both the initial and second DBA/2 grafts were rejected, but the newly implanted 2nd grafts were rejected faster than the original graft P=0.05 (figure 1a & b). Perhaps the 1st graft conducted in the context of RPM therapy harbored a CD8 T cells that are protective suppressor rather than rejecting.

2- Functionally active immunoregulatory CD8 T cells reside in donor skin allograft and help long term engraftment in RPM CD4KO treated model:

We next investigated whether the CD8 T cells home to LTE DBA/2 skin allografts and if they are functionally active protecting or rejecting the allografts. To test this hypothesis we transplanted a group with MHC mismatched DBA/2 skin (n = 15 mice). This group was treated with RPM as previously described [9]. After 100 days of engraftment, we 1st harvested 3 different grafts for histopathology as previously described. The histopathology revealed that only grafts from LTE RPM treated hosts harbor CD8 +T cells, but not the control (Naïve DBA/2 tail skin) (figure 2 a & b).

Then we investigated if the graft infiltrating CD8+ T cells are active in protecting rather than rejecting the LTE allograft. DBA/2 skin grafts were harvested 100 days post transplantation from (i) RPM treated B6 CD4 KO mice (n=5) and (ii) autologous DBA/2 (this is DBA/2 skin graft onto DBA/2 mice) recipients (30 days post-transplantation) (*n*= 5). As graft infiltrating CD8+ T cells emerge from the graft into the circulation of lympopenic hosts, LTE DBA/2 allografts or control DBA/2 autografts were then transplanted onto C57BL/6 RAG -/- hosts. A 3rd control group of naïve DBA/2 skin grafts were transplanted onto C57BL/6-Rag-/- hosts (n=6). Next peripheral blood lymphocytes (PBL) samples were collected 30 days post skin transplantation. CD8+T cells could not be detected in PBL of naïve RAG-deficient mice, but 4. 6 % CD8+ T cells are detected in PBL of C57BL/6-Rag -/- recipients of LTE allografts but not syngeneic grafts 30 days later p <0.01 (figure 3c & d). To test for protective function of graft homing CD8+ T cells (from LTE RPM treated mice) that expanded by homeostatic proliferation and are present in PBL of the C57 BL/6 Rag-/-, 0.2 x 10^6 CD8+ T cells from naive B6 CD4-deficient mice were adoptively transferred into the C57BL/6 Rag -/- hosts bearing the LTE DBA/2 allografts or DBA/2 synegeneic grafts (30 days after syngeneic transplantation). Survival of LTE DBA/2 skin allografts transplanted onto C57BL/6 Rag -/- mice were significantly prolonged as compared to controls p=0.02 (figure 3e). This indicates that RPM treated full mismatch skin graft in CD4-deficient recipients are protected from rejection by graft infiltrating and functionally active CD8 regulatory T cells.

3- CD8 T cells from long term engrafted RPM treated mice exert alloantigen specific hyporesponsivness:

To investigate if the CD8 T cells from LTE RPM treated CD4 KO recipients of allograft have donor specific hyporesponsivness. We did in vitro MLR as previously described. Using splenocytes from either donor specific DBA/2 (H-2^d) or from 3rd party C3H/He (H-2^k) mice as stimulators ($4X10^{5)}$). The responders were CD8 T cells from CD4 KO mice (harvested as previously described) either after long term engraftment of allogeneic skin transplant with RPM treatment or from naïve mice used in a varying ratios (0, $1X10^5$ and $2X10^5$) in MLR culture for 3 days. CD8 T cells from LTE mice were hypo-

proliferative in response to DBA/2 donor strain but not to third party C3H stimulator cells as compared to the control CD8+ T cells from the naïve CD4 KO mice (figure 3).
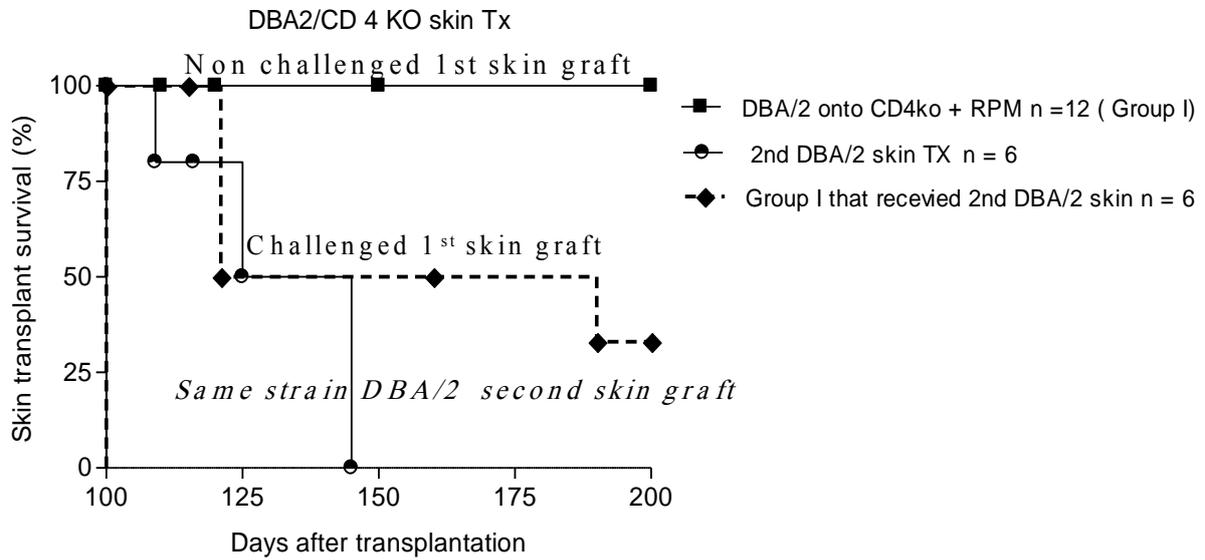

Fig. 1a. Challenging the RPM treated hosts harboring the 1st DBA/2 skin graft with a 2nd DBA/2 skin graft. The 2nd grafts were rejected faster than the original graft. Graft survival (days) was determined and presented as a Kaplan-Meier plot. P value between non Challenged 1st skin graft (◼) and challenged skin graft (◆) =0.004. P value between non Challenged 1st skin graft (◼) and same strain second graft (●) =0.006. P value between Challenged 1st skin graft (◆) and same strain second graft (●) =0.05.

Figure 2. Functionally active immunoregulatory CD8 cells reside in donor skin allograft and help long term engraftment in RPM CD4KO treated model
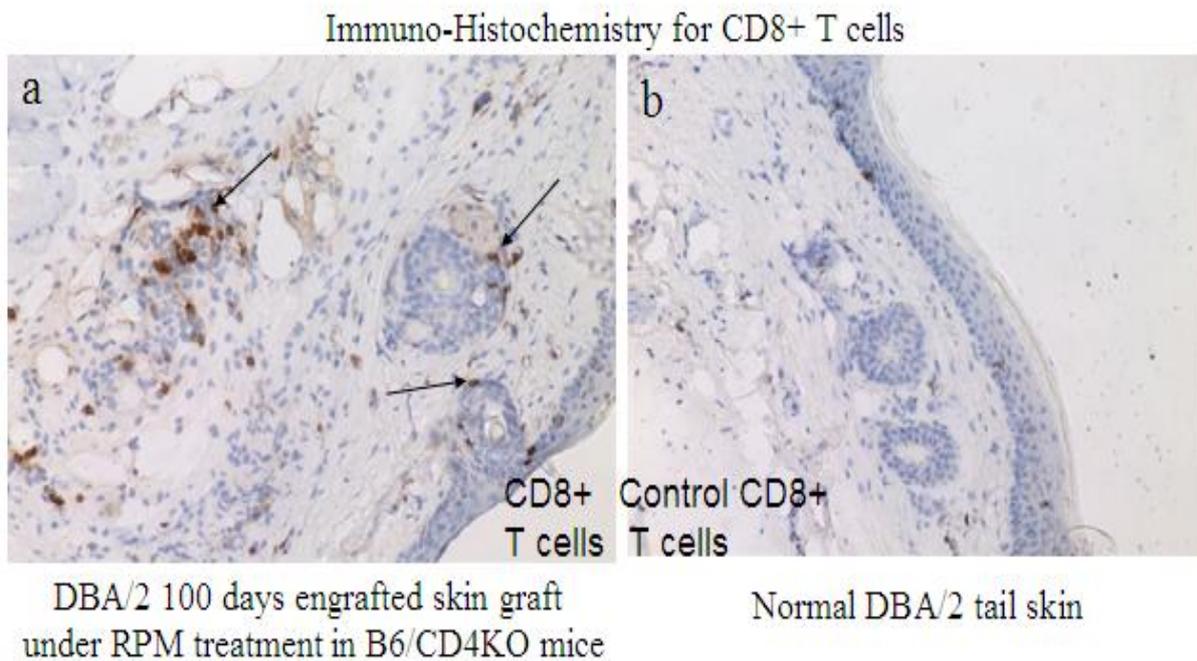

Fig. 2a & b. Immuno-Histochemistry for CD8+ T cells are homing in (1ba) RPM long term engrafted and not in (1bb) normal skin graft.

CD8 T cells repopulation in the peripheral blood of
C57 BL/6 Rag-deficient mice showing abundance of
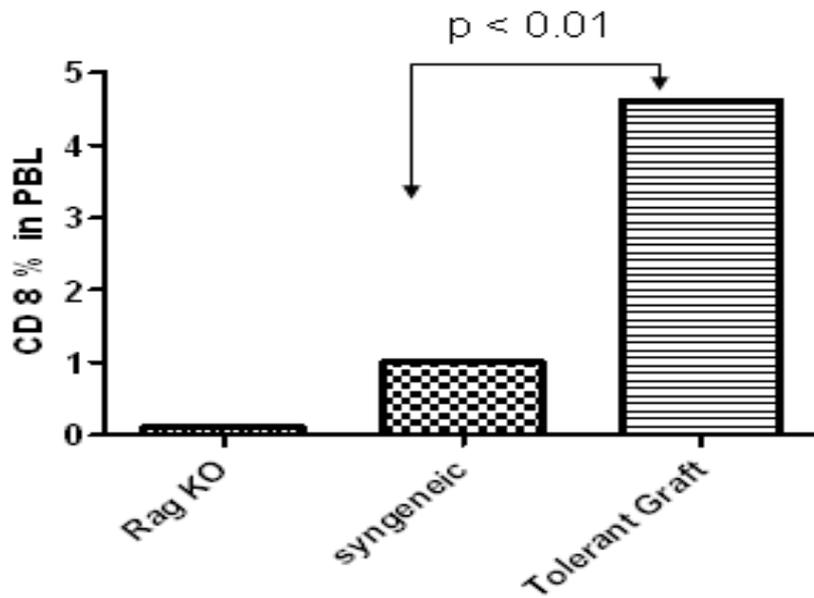CD8 T cells in the group bearing the tolerant graft .

p < 0.01

Fig. 2c. P =< 0.01 between syngeneic & tolerant graft

Survival after adoptive transfer of $2x10^5$ naive CD8+ cells onto
Rag-/- bearing either the long term engrafted graft or the auto
graft

RPM treated DBA/2 Long term engrafted skin grafts transfered onto Rag-/- n=5

DBA/2 auto graft onto Rag-/- n=5
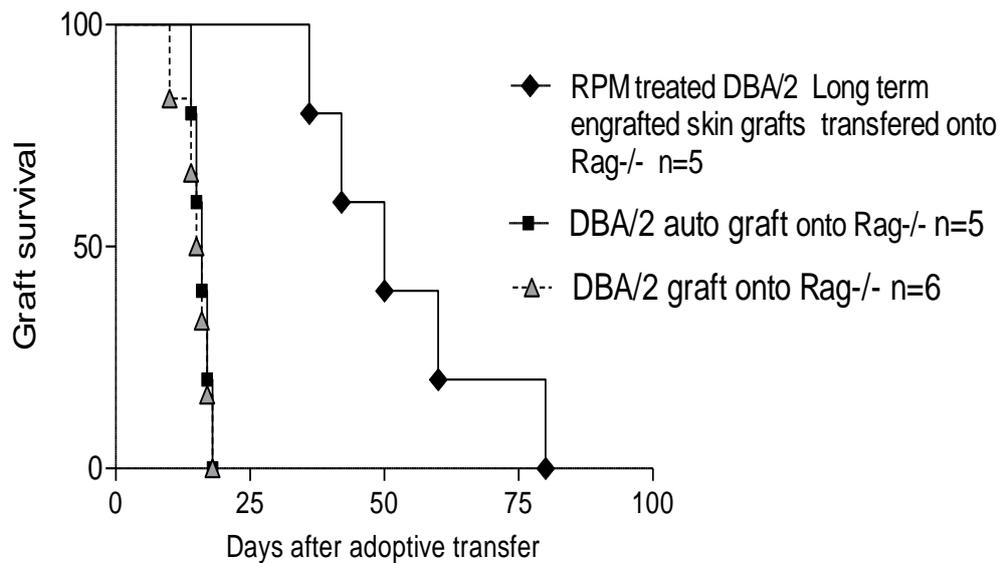
DBA/2 graft onto Rag-/- n=6

Fig. 2d. The graft survival is significantly longer in the C57 BL/6 Rag-deficient recipient bearing the LTE RPM treated graft compared to the control p = 0.024
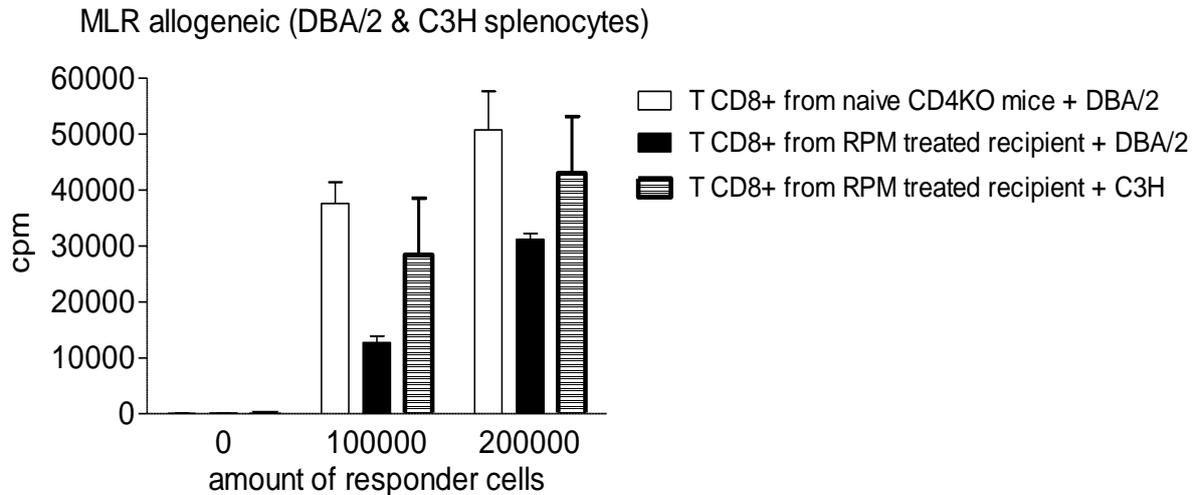
MLR allogeneic (DBA/2 & C3H splenocytes)

Legend:
- T CD8+ from naive CD4KO mice + DBA/2
- T CD8+ from RPM treated recipient + DBA/2
- T CD8+ from RPM treated recipient + C3H

y-axis: cpm
x-axis: amount of responder cells

Fig. 3. CD8 T cells from LTE RPM treated mice are less proliferating (hyporesonsive) than naïve cell in antigen specific manner P (two way ANOVA) = 0.045

Figure 1a&b:- RPM short term treatment can induce long term engraftment:
Two groups of C57 BL 6/CD4-deficient mice, 1st group was 12 mice and the 2nd group was 6 mice, were transplanted with MHC mismatched DBA/2 skin. The 1st group was treated with Rapamycin (RPM; 3mg/kg) for 3 consecutive days then every other day for two weeks, while the 2nd group was not treated. After 100 days of engraftment, we challenged the RPM treated hosts with a 2nd DBA/2 skin graft (n=6). Graft survival (days) was determined and presented as a Kaplan-Meier plot. The 2nd grafts were rejected faster than the original graft. CD8+ T cells reside in donor skin allograft and perhaps help long term engraftment in RPM CD4KO treated model.

Figure 2:- Graft infiltrating CD8+ T cells home to the graft and are functionally active in protecting against rejection:
A group of CD4 KO were transplanted with MHC mismatched DBA/2 skin (n=15). This group was treated with RPM. After 100 days of engraftment, we 1st harvested 3 different grafts for histopathology as previously described. Immunohistochemistery was done for 3 different skin grafts harvested from LTE RPM treated mice and from naïve DBA/2 tails. CD 8+ T cells have proven to be homed in the graft from LTE RPM treated mice, but not the control (Figure 2a & b).
As graft infiltrating CD8+ T cells emerge from the graft into the circulation of lymphopenic hosts, LTE DBA/2 allografts (100 days posttransplantation N=5) or control DBA/2 autografts (30 days posttransplantation N=5) were harvested, then transplanted onto C57BL/6-Rag-/- hosts. A 3rd control group of naïve DBA/2 skin grafts were transplanted onto C57BL/6-Rag-/- hosts (n=6). Next peripheral blood lymphocytes (PBL) samples were collected 30 days post skin transplantation. CD8+T cells could not be detected in PBL of naïve Rag-/- mice, but 4.6 % CD8+ TCR + T cells are detected in PBL of C57BL/6-Rag-/- recipients of LTE allografts but not syngeneic grafts 30 days later (Figure 2c & d).
To test for protective function in graft homing CD8+ T cells (from LTE RPM treated mice) that expanded by homeostatic proliferation and are present in PBL of the C57 BL/6 Rag -/-, 0.2 x 10⁶ CD8+ T cells from naive B6 CD4KO mice were adoptively transferred into the C57BL/6-Rag -/- hosts bearing the long term engrafted DBA/2 allografts or DBA/2 syngeneic grafts (30 days after transplantation). Survival of LTE DBA/2 skin allografts transplanted onto C57BL/6-Rag -/- mice were significantly prolonged as compared to controls (figure 2c). RPM treated full mismatch skin graft in CD4 KO recipients are protected from rejection by graft infiltrating functionally active CD8 regulatory T cells.

Figure 3:- CD8 T cells from long term engrafted RPM treated CD4KO mice exert a donor specific hyporesponsivness p = 0.04.
In vitro MLR: Varying ratios of CD8 T cells either naive or from CD4 KO mice harboring LTE RPM treated DBA/2 allogeneic skin graft were stimulated with Mitomycin C-treated DBA/2 (H-2d) or C3H/He (H-2k) splenocytes for 3 days. T-cell proliferation in these cultures as measured by the mean values of incorporated thymidine of triplicate wells, is compared in both groups.
CD8+ T cells from RPM treated LTE mice are less proliferating (hyporesponsive) compared to the CD8+ T cells from the naïve CD4 KO mice when stimulated with DBA/2 splenocytes but not with C3H ones. Data represent three independent experiments.

Discussion

Subclinical rejection defined as asymptomatic tubulo-interstitial infiltrate usually occurs within the first three months after transplantation, and might be followed by worsening of tubulointerstitial damage, late graft dysfunction and inferior graft survival [3]. The only controlled trial of treatment of SCAR, a decade ago, showed that pulse administration of corticosteroids significantly have a non significant trend to better 4-year graft survival (P=0.076). However the control group in this study was not biopsied which douptly question the solidity of this conclusion [4].
The prevalence rates of SCAR in participants (who were receiving ciclosporin – azathioprine – prednisone triple therapy) approximated 30%. This small, single-center study implied that protocol biopsies have a role in the detection and treatment of subclinical rejection [4]. A decade has passed since this study, and a second, multicenter trial from an expanded study group has now been published, showing a markedly different result: very low levels of SCAR (5.7–8.9%) and no

apparent benefit of protocol biopsy [5].

Currently, inconclusive results have been shown by several studies evaluating the predictive value of SCAR on graft outcome [1,11-14]. Furthermore, this controversy is even more evident when the renal effect of treating or not treating SCAR is analyzed [15,16,1,17].This fact would support the results of previous experiences [15-16] in which after not treating the so-called SCAR, neither increase of interstitial fibrosis nor progressive loss of graft function was observed.

In our study, we tried to study the functional effect of this skin allograft lymphocytic infiltration. Challenging the mice harboring a LTE RPM treated graft with same donor strain skin graft results in prolonged skin transplant compared to non treated same combination skin graft (Figure1a&b). Meanwhile, we noticed that the first skin graft can keep surviving longer than the challenging skin graft. One of the explanations is the effect of CD8 T cells that are residing in the first skin graft and they are functionally active suppressor and exert a protecting effect that could allow the LTE of the graft. Garca et al., 2002 and our laboratory already reported that the CD4 residing in the graft considered as regulatory as they were exerting anti-rejection protective effect that could help long term engraftment [18,19]. To test this hypothesis regarding the CD8 T cells, first immuno-histochemistry of LTE graft showed abundance of the CD8+ T cells compared to the control naïve skin graft (figure 3 a & b).

When we transplanted skin allografts from stable RPM LTE onto syngeneic immune-incompetent mice, graft-infiltrating T cells migrated from the graft site, expanded in the new host, and protected test allografts from acute rejection after transfer of naive syngeneic CD8 T cells (figure 3c, d & e). A similar finding concerning the CD4 regulatory T cells has been reported recently by Both Graca et al. [18] in a model of peripheral tolerance to minor histocompatibility Ag-mismatched skin allografts and by our laboratory [19] in a model in which skin allograft tolerance is achieved via BMT, RPM, and costimulation blockade. CD4 regulatory T cells are present in both secondary lymphoid organs and in the allograft itself, and are functionally active in protecting the grafts from rejection.

Our finding may strengthen the outcome of a recent study published by Bestard et al in 2008 [20] who found that the presence of Treg within asymptomatic cellular infiltrates in 6-mo protocol biopsies in kidney transplant patients may be a reliable biomarker for distinguishing an ongoing rejection/inflammatory process from a safe/ protective condition. This conclusion is supported by the better graft function evolution achieved at both 2 and 3 yr after transplantation in patients with Treg.

A potential mechanistic explanation that could clarify this process is that donor-antigen recognition by Treg directly in the graft would be necessary for developing a donor-specific hyporesponsive state, mediated by the suppressive activity of these CD8Treg as we have shown in (figure 3). In the in vitro MLR, the CD8 T cells from LTE RPM treated mice are hyporesopnsive in an antigen specific manner compared to the control. This finding go in concordance with what was reported in recent studies, which showed that presence of Treg within tubulointerstitial infiltrates in a group of stable renal transplant patients in 6-mo protocol biopsies was associated with peripheral donor-specific hyporesponsiveness, which was mediated by the antidonor suppressive activity of Treg [21-22].

So, we conclude that T lymphocyte home to the skin graft and actively exert a protective effect against rejection. This conclusion open the door to readdress the issue of the hazardous effect of graft lymphocytic infiltration defined as subclinical rejection in kidney transplant patients.

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