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Rapid tolerance induction by hematopoietic progenitor cells in the absence of donor-matched lymphoid cells.☆

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ABSTRACT

Background: Donor specific hematopoietic cell transplantation has long been recognized for its potential in tolerance induction for subsequently transplanted organs. We have recently published that co-administration of Myeloid Progenitor (MP) and third party Hematopoietic Stem Cells (HSC) can induce MP-specific tolerance for subsequently transplanted organs [1].
Methods: Mice received an allogeneic HSC and third party MP transplantation simultaneous with placement of a MP-matched skin graft. Variants tested include time of graft placement, MP genotype and source of cells.
Results: Using B10;B6-Rag2$^{-/-}$Il2rg$^{-/-}$ mice, we demonstrate that specific tolerance can be induced by MP given simultaneous with the skin graft in the complete absence of MP-donor-matched lymphoid cells. Ex vivo expanded MP function as well as sorted cells in inducing tolerance. In addition we demonstrate that tolerance can be induced by MP in the context of autologous HSC transplantation.
Conclusions: Our results demonstrate that the previously observed expansion of organ donor matched Treg is not essential for tolerance, and that MP tolerance protocols can be envisioned in most clinical settings, including those involving deceased donor organs.

1. Introduction

Short-term organ survival following organ transplant is excellent but long-term survival remains limited. Only a little over 20% of transplanted lungs, e.g., remain functional ten years after transplantation [2]. Daily life is complicated by the morbidity associated with the diagnosis and treatment of frequent and debilitating rejection episodes. The long-term immunosuppressants necessary can be considered a complication, since the associated morbidities are numerous (including neural and renal toxicity and increased risk for cancer and opportunistic infections [3–6]).

Operational tolerance, the acceptance of the transplanted organ into an otherwise immunocompetent host, would greatly improve long-term outcome, patient quality of life, reduce the economic burden on society, and will especially benefit pediatric transplant recipients. Of the many approaches tested (reviewed in [7–14]), the success of hematopoietic cell transplantation (HCT), including rigorously purified hematopoietic stem cells (HSCs) [15], stands out. Effective methods of tolerance induction have involved lethal preconditioning regimens, limited by the associated morbidity and mortality. Sublethal preconditioning protocols for HCT and tolerance induction are being developed and tested clinically [16–20], including protocols that use total lymphoid irradiation [18]. Though these protocols are successful in inducing tolerance, limitations remain, especially in sensitized patients, and in crossing significant HLA disparity.

We focus on a unique population of cells called myeloid progenitor (MP) cells, which can generate all myeloerythroid lineages [21,22] and which we found to be capable of inducing specific tolerance. A potentially life-threatening complication of HCT is the resulting transient

Abbreviations: APC, Antigen Presenting Cell; ATS, AntiThymocyte Serum; DC, Dendritic Cell; GVHD, Graft Versus Host Disease; HCT, Hematopoietic Cell Transplantation; HSC, Hematopoietic Stem Cell; MDSC, Myeloid Derived Suppressor Cells; MP, Myeloid Progenitor Cells.

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severe neutropenia which leaves the host susceptible to infections. Purified MP can give a rapid but transient engraftment with myeloid cells (mostly granulocytes and monocytes) that protect from fungal and bacterial infections [23]. Key characteristics of myeloid progenitors include: specific tolerance induction potential in our experimental system [1]; clinical availability; lack of GVHD, allowing cells to be used unmatched; and the ability to be cryopreserved [24,25]. Initial clinical trials, aimed at reducing infectious complications in HCT recipients, are being conducted (clinical trials.gov, NCT01297543).

We have expanded upon our initial observations and find that MP do not require pre-administration, but can be given simultaneous with the organ (skin) graft. MP from mutant mice show that tolerance induction only requires graft matched myeloid cells. Tolerance induction does not require an accompanying allogenic HSC transplantation and can be performed using the mouse equivalent of human clinical grade MP, obtained after a short-term ex vivo expansion. These new observations expand the potential use to deceased donor grafts, the main source of organs for transplants.

2. Original hypothesis

Overall, we are addressing, in a preclinical model, the hypothesis that MP can be used to induce tolerance for organ transplant under clinically relevant conditions. In this study specifically we hypothesized that Treg, while present, do not play an essential role in the generation of specific tolerance in our transplant model and that it is possible to generate specific MP-matched tolerance by administrating these cells at the time of organ transplant. Furthermore, we speculated that organ graft matched MP, combined with host-autologous rather than allogeneic HSC, can be used to induce tolerance.

3. Materials and methods

3.1. Mice

Female BALB/cJ (H-2d, CD45.2), AKR/J (H-2k, CD45.2), FVB/nJ (H-2q, CD45.1) and C57BL/6 (H-2b, CD45.2) mice were purchased from Jackson Labs, B10;B6-Rag2$^{-/-}$Il2rg$^{-/-}$ mice from Taconics. The animals were maintained and used at the University of Missouri Kansas City LARC or the Medical College of Wisconsin BRC under IACUC approved protocols. Donor mice were five to eight weeks; recipients seven to twelve weeks old. BALB/c recipients received 8.5 Gy at 1.2 Gy/min in two doses three hours apart (Radsource, RS 2000 x-ray machine, UMKC) or 10 Gy at 82 Gy/min in two doses (Mark I Cesium Irradiator, MCW). The mice received antibiotic water (10^6 U/L polymyxin B sulfate and 1.1 g/L neomycin sulfate) for four weeks post-irradiation.

3.2. Flow cytometry

HSC were prepared as described (16). Briefly, bone marrow is flushed from femurs and tibiae, followed by ammonium chloride lysis of erythrocytes. The cells were enriched for CD117$^+$ (Macs column and anti-APC-microbeads, Miltenyi Biotec) and were stained for CD117^{APC} (2B8), CD90.1^{FITC} (HIS51), Lin^{PE} (CD3 (145-2C11), CD4 (L3T4), CD5 (53-7.3), CD8 (53-6.7), CD19 (ID3), B220 (RA3-6B2), CD11b (M1/70), Gr-1 (8C5) TER119 (TER119)) and Sca-1^{PECy7} (D7) (eBioscience, San Diego, CA). CD117$^+$, CD90.1^{lo}, Lin$^{neg/lo}$ and Sca-1$^+$ cells (KTLS) were sorted using a BDBiosciences FACSVantage Aria operated by the Flow Facility Core of Kansas University Medical Center. HSC from CD90.2 strains (BALB/c, C57BL/6, B10;B6) were sorted without CD90 staining as KLS cells. Short-term cultures to obtain MPc from sorted HSC were performed using established procedures [25].

MP cells included CMP, GMP, and MEP and were sorted from mouse bone marrow by enriching for CD117$^+$ cells as described above, and staining with CD117^{APC} (2B8), Sca-1^{PECy7} (D7) and Lin^{PE} (CD3 (145-2C11), CD4 (L3T4), CD5 (53-7.3), CD8 (53–6.7), B220 (RA3-6B2), Gr-1 (8C5) TER119 (TER119), CD90.1 (HIS51) and CD127 (A7R34)). Cells were sorted as CD117$^+$, Lin$^{neg/lo}$, Sca-1^{neg}, and CD90.1^{neg}. Except were noted otherwise 10^5 cells were used per animal.

For analysis (bone marrow, spleen, thymus or blood) erythrocytes were sedimented using 2% dextran (Pharmacia) in PBS (blood only), remaining erythrocytes were lysed with 0.15 ammonium chloride/0.01 M potassium bicarbonate. The cells were preblocked with rat IgG (Sigma), divided into aliquots and stained with antibodies as indicated, using MHC class I and CD45-allelic markers to distinguish origin. The cells were analyzed using an Attune flow cytometer. CBC counts were obtained using a ScilvetABC veterinary blood cell counter using mouse presets.

3.3. Skin transplants

Briefly, the mouse was anesthetized using xylazine/ketamine, shaved, prepped with hibiclens and placed supine. Eyes were protected using antibiotic eye ointment. A segment of tail skin was removed without subcutaneous tissues. Hemostasis was assured. The skin graft was placed and secured, slightly stretched, in an interrupted fashion with 6–0 Maxon or Biosyn. The graft was placed such that hair grows incongruous with surrounding hair. All fluid was decompressed from the graft. A 2 × 2 piece of gauze was then sewn over the graft with 4–0 Sofsilk. An analgesic, buprenorphine, was administered s.c. (0.05 mg/kg) twice daily as necessary. The graft was documented using regular digital photography using a Canon G2 camera and processed using Canvas X or Pixelmator. Post-acquisition processing was limited to change to grayscale mode, cropping and global use of the levels command to optimize contrast.

3.4. Statistical analysis

Data were analyzed using Graphpad Instat 3.0 and Graphpad Prism 4.0 (Graphpad Software Inc., San Diego, CA). Students *T*-test and Kaplan Meier with a Wilcoxon logrank test for groups was used. A $p < 0.05$ was considered significant. Nonparametric, Kruskal Wallis and Mann Whitney tests were used when the data were not normally distributed.

4. Results

4.1. Skin graft tolerance is not dependent on pre-established hematopoietic reconstitution

Fig. 1a shows our standard model and the variables tested. We switched to this model after establishing that mice given HCT and skin grafts at the same day readily accepted both host, MP-matched and HSC-matched skin grafts, while rejecting unrelated grafts (Fig. 1b). As expected, given the immunosuppressed state of the host at the time of transplant, unmatched skin grafts are rejected slowly. Untreated mice ([1], see also Fig. 3) or mice reconstituted two months earlier ([1], see also Fig. 4a), reject faster. In all conditions all unmatched grafts are lost.

4.2. Rag2$^{-/-}$Il2rg$^{-/-}$ bone marrow can induce tolerance in the absence of graft matched lymphocytes

Analysis of mice reconstituted with wild-type HSC and MP has demonstrated a marked over-representation (upto 20-fold) of host and MP-donor regulatory T cells (Treg) [1]. This raises the question whether these lymphocytes play an essential role. Due to breeding problems, attempts to address this using MP from FoxP3$^{-/-}$ mice [26,27] were limited to one BALB/c mouse reconstituted with 4000 AKR HSC and 100,000 FoxP3$^{-/-}$ MP and one mouse reconstituted with 8000 FoxP3$^{-/-}$ HSC and 100,000 AKR MP. Both mice readily accepted FoxP3$^{-/-}$-matched (C57BL/6) skin grafts (data not shown).

We subsequently used B10;B6-Rag2$^{-/-}$Il2rg$^{-/-}$ mice [28], which lack both the Rag2 gene and the common gamma-chain (part of the receptor complex for IL-2, 4, 7, 9, 15 and 21), and which lack all B, T and NK-cells. Simultaneous placement of HCT and skin graft allows us to test skin and hematopoietic cell grafts from the same donor, important with a mixed genetic background. Because Rag2$^{-/-}$Il2rg$^{-/-}$ bone marrow cells are incapable of producing lymphocytes we used both bone marrow as well as purified HSC or MP. Both resulted in tolerance for matched skin grafts (Fig. 2a,b). Reconstituted mice reject unmatched grafts.