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# Pharmacological Immunosuppression Reduces But Does Not Eliminate the Need for Total-Body Irradiation in Nonmyeloablative Conditioning Regimens for Hematopoietic Cell Transplantation

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In the dog leukocyte antigen (DLA)-identical hematopoietic cell transplantation (HCT) model, stable marrow engraftment can be achieved with total-body irradiation (TBI) of 200 cGy when used in combination with postgrafting immunosuppression. The TBI dose can be reduced to 100 cGy without compromising engraftment rates if granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (G-PBMC) are infused with the marrow. T cell-depleting the G-PBMC product abrogates this effect. These results were interpreted to suggest that the additional T cells provided with G-PBMC facilitated engraftment by overcoming host resistance. We therefore hypothesized that the TBI dose may be further reduced to 50 cGy by augmenting immunosupression either by (1) tolerizing or killing recipient T cells, or (2) enhancing the graft-versus-host (GVH) activity of donor T cells. To test the first hypothesis, recipient T cells were activated before HCT by repetitive donor-specific PBMC infusions followed by administration of methotrexate (MTX) (n = 5), CTLA4-Ig (n = 4), denileukin diftitox (Ontak; n = 4), CTLA4-Ig + MTX (n = 8), or 5c8 antibody (anti-CD154) + MTX (n = 3). To test the second hypothesis, recipient dendritic cells were expanded in vivo by infusion of Flt3 ligand given either pre-HCT (n = 4) or pre- and post-HCT (n = 5) to augment GVH reactions. Although all dogs showed initial allogeneic engraftment, sustained engraftment was seen in only 6 of 42 dogs (14% of all dogs treated in 9 experimental groups). Hence, unless more innovative pharmacotherapy can be developed that more forcefully shifts the immunologic balance in favor of the donor, noncytotoxic immunosuppressive drug therapy as the sole component of HCT preparative regimens may not suffice to ensure sustained engraftment.

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## INTRODUCTION

Nonmyeloablative conditioning for allogeneic hematopoietic cell transplantation (HCT) is associated with lower regimen-related toxicities than myeloablative conditioning. This has made HCT a treatment option for a broader range of diseases, a well as older or medically infirm patients [1]. The clinical nonmyeloablative preparative regimen used at the Fred Hutchinson Cancer Research Center (FHCRC) has been developed in the preclinical canine model and relies on low-dose total-body irradiation (TBI; 200 cGy), and postgrafting immunosuppression with cyclosporine (CSP) and mycofenolate mofetil (MMF) [2]. Both TBI and pharmacological immunosuppression are required to prevent recipient T cell-mediated graft rejection. However, even 200 cGy TBI may increase the risk of late toxicity such as secondary malignancies [3,4], complications that need to be avoided in patients with nonmalignant diseases who may otherwise benefit from allogeneic HCT [5,6].

To address this issue, we proposed to further reduce the TBI dose by replacing its immunosuppressive function with additional immunomodulation strategies. In theory, this may be achieved by inducing recipient-antidonor tolerance before transplant, intensifying the immunosuppressive treatment after transplant, or enhancing engraftment-facilitating graft-versus-host (GVH) activities. In the current study,

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we asked whether any of the outlined strategies would permit TBI dose reduction to 50 cGy without compromising the rate of sustained engraftment. We had previously shown that sustained engraftment could be achieved in the majority of recipients prepared with 100 cGy TBI if they were given a combination of dog leukocyte antigen (DLA)-identical bone marrow plus granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cells (G-PBMC) followed by 5 weeks of CSP after transplant [7]. We found in the current study, however, that with 50 cGy TBI, immunosuppressive and/or tolerance-inducing strategies employed led to overall sustained engraftment in only 14% of recipients treated in 9 different experimental groups. These findings illustrate the difficulty to uniformly achieve sustained engraftment with pharmacological immunosuppression given in combination with TBI doses at or below 100 cGy.

## METHODS

### Dogs

The Institutional Animal Care and Use Committee of the FHCRC approved this study. Hematopoietic cell grafts were carried out using beagle or miniature mongrel-beagle crossbreeds, and standard care was provided as described previously [7-9]. Recipients (n = 42) were 6.8 to 27.5 (median, 8.6) months old and weighed 7.1 to 16.8 (median, 10.0) kg. The donors (n = 41) were 6.8 to 24.3 (median, 8.6) months old and weighed 7.0 to 19.0 (median, 12.7) kg. One dog served as the donor for 2 recipients. Forty-two donor/recipient littermate pairs were included in 9 HCT protocols that substituted minimally toxic immunomodulation for TBI. The pairs were DLA-identical on the basis of matching for highly polymorphic DLA-associated class I and class II microsatellite markers [10], which was confirmed by DLA-DRB1 allele sequencing [11].

### HCT

The following treatment applied to all experimental groups. The day of hematopoietic stem cell grafting was designated as day 0. Thirty days before HCT, marrow for transplantation was aspirated from the donors under general anesthesia through needles inserted into the humeri and cryopreserved. On days −5 to 0, donors were treated by subcutaneous injection with recombinant canine G-CSF (gift from Amgen, Inc., Thousand Oaks, CA) at 10 μg/kg per day. Leukapheresis were performed on day 0 via an intravenous catheter and a continuous flow blood separator (CobeSPECTRA, Cobe Laboratories, Lakewood, CO) [12]. On day 0, recipients were given a single dose of 50-cGy TBI delivered at 7 cGy/min from a high-energy linear accelerator (Varian CLINAC 4, Palo Alto, CA) followed by infusion of bone marrow and G-PBMC. Except for experimental group 1, which was treated with CSP alone, all experimental groups received a combination of CSP (15 mg/kg twice daily orally on days 1-35) and MMF (10 mg/kg twice daily subcutaneously on days 0-27) for immunosuppression after transplantation. Blood CSP levels were measured on days 7 and 21 and targeted at 400 to 800 ng/mL. Nine different immunomodulatory regimens were studied, and recipient treatments (n = 42) (Table 1) are summarized below:

- Group 1 (n = 5): Postgrafting CSP alone as outlined above. No further immunomodulation.
- Group 2 (n = 4): Postgrafting CSP and MMF as outlined above. No further immunomodulation.
- Group 3 (n = 5): Donor PBMC ($5 \times 10^6$/kg) infusions on days −5 and −3. Methotrexate (MTX) (0.4 mg/kg intravenously [i.v.]) was given on days −4 and −2 [13]. Postgrafting CSP and MMF.
- Group 4 (n = 4): Donor PBMC ($2 \times 10^6$/kg/day) infusions on days −7 until −1. rhCTLA4-Ig (4.0 mg/kg/day i.v.; provided by RepliGen, Waltham, MA) was given on days −7 until −1. Postgrafting CSP and MMF.
- Group 5 (n = 4): Donor PBMC ($2 \times 10^6$/kg/day) infusions on days −8 until −2. Denileukin diftitox (Ontak; 18.0 μg/kg/day i.v.; Eisai Pharmaceuticals, Teaneck, NJ) was given on days −7 until −1. Postgrafting CSP and MMF.
- Group 6 (n = 8): Donor PBMC ($2 \times 10^6$/kg/day) infusions on days −7 until −2. rhCTLA4-Ig (4.0 mg/kg/day i.v.) was given on days −7 until −2. MTX (0.4 mg/kg i.v.) was given on days −6, −4, and −2. Postgrafting CSP and MMF.
- Group 7 (n = 3): Ketorolac (0.5 mg/kg i.v.) was given on days −9 and −8 as thrombembolic prophylaxis before i.v. use of the 5c8 humanized monoclonal antibody (mAb) directed against CD154 (CD40 ligand) [9]. The 5c8 mAb (0.5 mg/kg i.v.) was then given on day −8. Donor PBMC ($2 \times 10^6$/kg/day i.v.) were given on days −7 until −3. MTX (0.4 mg/kg i.v.) was given on days −6, −4, and −2. Postgrafting CSP and MMF.
- Group 8 (n = 5): Recombinant human fetal Liver Tyrosine Kinase-3 (rhFlt-3) ligand (FL; 100 μg/kg/day subcutaneously; provided by Amgen, Seattle, WA) was given on days −10 until 0. Postgrafting CSP and MMF [14].
- Group 9 (n = 4): FL was given on days −10 until +10. Postgrafting CSP and MMF.

All dogs were given standard postgrafting care that included systemic enrofloxacin (Baytril [Shawnee Mission, KS, USA]), from day −5 until hematopoietic recovery from radiation nadirs occurred. The clinical status of the dogs was assessed twice daily. Upon completion of the study, dogs were euthanized, adopted, or

**Table 1. Duration and Level of Mixed Donor Chimerism Among Dogs (n = 42) Prepared with 50-cGy Total-Body Irradiation (TBI; Dose Rate, 7 cGy/min) and Different Immunomodulatory Regimens before DLA-Identical Marrow/G-CSF-Mobilized PBMC Transplantation**

| | Immunosuppression | | | | Mean TNC Dose per kg Recipient Weight ($\times 10^8$/kg) | | Peak Chimerism in Granulocyte Fraction within First 12 Weeks after HCT (%) | |
|---|---|---|---|---|---|---|---|---|
| | Before HCT | | | | | | | |
| Experiment Number | Drug | Donor PBMC | After HCT | N | BM | G-PBMC | Duration of Mixed Chimerism (Weeks) | Stable Mixed Chimerism > 26 Weeks after HCT, n (%) |
| 1 | — | No | CSP | 5 | 3.9 | 8.4 | 35, 25, **20**, 15, 15<br>10, 9, >36, 11, 9 | 1 (20) |
| 2 | — | No | CSP/MMF | 4 | 4.6 | 6.9 | 40, 30, **20**, 10<br>14, 14,>36, 7 | 1 (25) |
| 3 | MTX | Yes | CSP/MMF | 5 | 4.5 | 6.8 | **75**, 55, 55,10,10<br>>36,12, 6, 9, 8 | 1 (20) |
| 4 | CTLA4-Ig | Yes | CSP/MMF | 4 | 5.2 | 7.3 | **35**, 28, 22, 13<br>>36, 9, 8, 12 | 1 (25) |
| 5 | Ontak | Yes | CSP/MMF | 4 | 4.7 | 7.9 | 52, 49, 16, 9<br>12, 11, 12, 12 | 0 (0) |
| 6 | CTLA4-Ig/MTX | Yes | CSP/MMF | 8 | 4.5 | 8.2 | 100, 89, **45**, 34, 30, 23, 20, 15<br>14, 12, >36, 8, 8, 9, 10, 9 | 1 (12) |
| 7 | 5c8/MTX | Yes | CSP/MMF | 3 | 4.7 | 4.6 | 25, 21, 13<br>11, 9, 19 | 0 (0) |
| 8 | FL | No | CSP/MMF/FL | 5 | 5.2 | 7.5 | 14, 9, 7, 7, 5<br>10, 9, 8, 8, 7 | 0 (0) |
| 9 | FL | No | CSP/MMF | 4 | 5.8 | 8.5 | **15**, 15, 10, 5<br>>36, 5, 15, 10 | 1 (25) |
| Total | | | | 42 | | | | 6 (14) |

MTX indicates methotrexate; FL, rhFlt3 ligand; CTLA4-Ig, Cytotoxic T-Lymphocyte Antigen 4; Ontak, denileukin diftitox; 5c8, humanized monoclonal antibody directed against CD154 (CD40 ligand); HCT, hematopoietic cell transplantation; Donor PBMC, infusion of donor peripheral blood mononuclear cells; TNC, total nucleated cells; BM, bone marrow; G-PBMC, G-CSF–mobilized peripheral blood mononuclear cells. The bolded percentage values in the category "Peak granulocyte chimerism within 12 weeks after HCT" indicate recipient dogs with sustained mixed donor/host chimerism.
The different immunomodulatory regimens (Experiments 1-9) are detailed in the Methods section. Except for recipients in experimental group 1 that were treated with cyclosporine (CSP) alone, recipients in all other groups received a combination of CSP (15 mg/kg twice daily orally on days 1-35) and mycophenolate mofetil (MMF; 10 mg/kg twice daily subcutaneously on days 0-27) for immunosuppression after transplant.

transferred to other studies. When euthanized, they underwent complete necropsies with histological examinations of tissue samples.

### Assessment of Chimerism

The presence of donor cells among PBMC and granulocytes after HCT was assessed by fluorescent variable number tandem repeat polymerase chain reaction (PCR) assays using an ABI Prism 310 Genetic Analyzer and Gene Scan 3.1 software (Applied BioSystems, Foster City, CA) as described [15,16]. The endpoint of the study was stable mixed donor/host hematopoietic chimerism beyond 26 weeks after HCT. This time point was chosen because historically, no graft rejections were seen after week 26.

## RESULTS AND DISCUSSION

Our previous studies in the DLA-identical HCT model demonstrated that the addition of G-PBMC to DLA-identical marrow grafts allowed for the reduction of TBI dose from 200 cGy to 100 cGy and required only CSP for postgrafting immunosuppression to achieve stable mixed chimerism in 5 of 8 dogs transplanted [7]. In this model, graft rejection, if it occurs, is typically completed within the first 26 weeks after HCT. The engraftment-facilitating effect associated with G-PBMC in this model was mediated by the relatively large number of additional T cells because only 1 of 7 recipients had sustained mixed donor chimerism when G-PBMC products were T cell depleted. Based on these findings, we hypothesized that the TBI dose sufficient for sustained engraftment in the majority of recipients in this model (100 cGy) could be further reduced by (1) tolerizing or killing rejection-mediating host T cells or (2) enhancing engraftment-facilitating GVH reactions.

In preparation for testing these 2 hypotheses, we first showed that a TBI dose reduction from 100 cGy to 50 cGy was not sufficient to ensure sustained mixed chimerism in the majority of recipients (Experiment 1: rate of sustained engraftment, 1 of 5 dogs, 20%) (Table 1). We then showed that intensifying the posttranplant immunosuppressive regimen by adding 4 weeks of MMF did not significantly improve this result (experiment 2: rate of sustained engraftment, 1 of 4 dogs, 25%) (Figure 1).

After having established 100 cGy TBI as a critical threshold dose for sustained engraftment in this

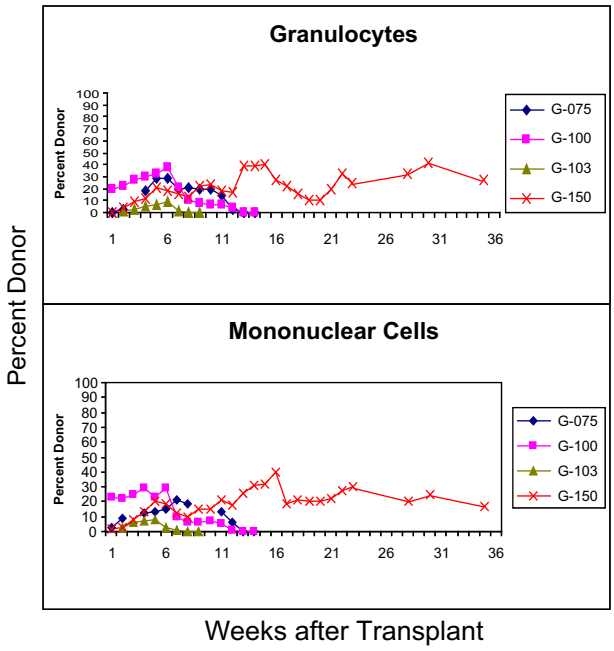


**Figure 1.** Mixed hematopoietic chimerism in dogs (n = 4) prepared with 50-cGy total-body irradiation (TBI) and given DLA-identical marrow and G-PBMC grafts, followed by cyclosporine (CSP) and mycofenolate mofetil (MMF) for immunosuppression after transplant. CSP, 15 mg/kg twice daily orally on days 1 to 35; MMF, 10 mg/kg twice daily subcutaneously on days 0 to 27. Donor chimerism was assessed as described in Methods in the granulocyte fraction (upper panel) and mononuclear cell fraction (lower panel). Identification numbers of recipient dogs are listed in the box.

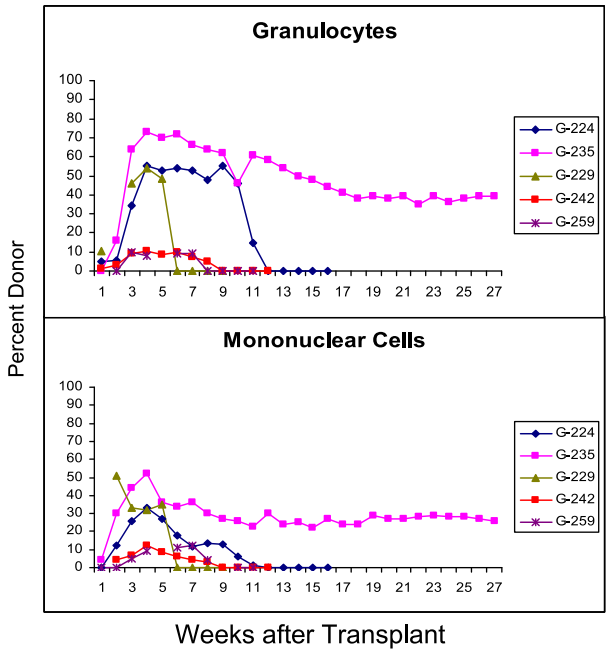


**Figure 2.** Mixed hematopoietic chimerism in dogs (n = 5) prepared with donor PBMC infusions and intravenous methotrexate (MTX) before 50-cGy total-body irradiation (TBI) and DLA-identical marrow/G-PBMC transplantation, followed by cyclosporine (CSP) and mycofenolate mofetil (MMF) immunosuppression after transplant. Donor PBMC ($5 \times 10^6$/kg) infusions were given on days −5 and −3. MTX (0.4 mg/kg i.v.) was given on days −4 and −2. CSP, 15 mg/kg twice daily orally on days 1 to 35; MMF, 10 mg/kg twice daily subcutaneously on days 0 to 27. Donor chimerism was assessed as described in Methods in the granulocyte fraction (upper panel) and mononuclear cell fraction (lower panel). Identification numbers of recipient dogs are listed in the box.

model, subsequent experiments were aimed at overcoming the engraftment barrier encountered after 50-cGy TBI by tolerizing or killing rejection-mediating host T cells before transplant. For this purpose, host T cells were activated by repetitive donor-specific PBMC infusions that were followed by administration of methotrexate (MTX), CTLA4-Ig, or Ontak (Experiments 3-5). Using these 3 approaches, rates of sustained engraftment ranged between 0% and 25% and, hence, were not significantly different from the 25% observed with controls given no immunomodulatory treatment before transplant (exemplified in Figure 2). Even though peak chimerism levels within the first 12 weeks after HCT appeared higher in recipients given pretransplant donor-specific PBMC and MTX compared to controls (median, 55% versus 25%), this difference was not statistically significant ($P = .56$) (Figure 2). Furthermore, combined modalities of pretransplant conditioning of recipients with donor-specific PBMC followed by CTLA4-Ig and MTX, or 5c8 antibody and MTX proved as ineffective as the singular strategies tested in Experiments 3-5.

We had previously shown that the TBI dose required for sustained engraftment after DLA-identical marrow transplantation (without G-PBMC coinfusion) could be reduced from 200 cGy to 100 cGy if recipients were tolerized against donor marrow with pretransplant donor PBMC/CTLA4-Ig infusions [17]. In addition, donor-specific PBMC infusions and CD154 blockade with the 5c8-antibody not only delayed marrow graft rejection but also improved rates of sustained engraftment [9]. Even though these concepts were directly translated to the current model, they proved unsuccessful. The difficulty to attain sustained engraftment with 50-cGy TBI in this model is likely related to the fact that the chosen immunosuppressive strategies did not sufficiently compromise host T cell function to shift the immunologic balance toward the donor. We have shown previously that TBI doses of 200 cGy cause substantially more effective T cell depletion and suppression of T cell function than TBI doses of 100 cGy, illustrating the steep dose-response relationship at low doses of ionizing radiation [18].

An alternative explanation for the difficulty to attain sustained engraftment with 50-cGy TBI may be related to the fact that, in preparative regimens that primarily rely on low-dose TBI for host immunosuppression, ionizing radiation may serve the additional purpose of providing a competitive advantage to donor hematopoietic stem cells [19]. In fact, competitive

repopulation studies using irradiated versus nonirradiated human CD34 cells in NOD/SCID mice showed that 100 cGy reduced engraftment by 24% [19]. Therefore, although the pharmacological pretransplant strategies tested might tolerize or kill host T cells, and thereby have immunosuppressive activity, one could argue that they might not sufficiently compromise the recipient's stem cell compartment. However, the validity of the latter hypothesis is challenged by the observation that sustained engraftment has been achieved in dogs given marrow space-sparing lymph node irradiation (450 cGy) before and pharmacological immunosuppression with CSP and MMF after DLA-identical marrow transplantation [8]. Furthermore, clinical observations suggest that long-term stable donor/host chimerism can be achieved in patients with primary immunodeficiency disorders without using a preparative regimen before HCT provided pharmacological immunosuppression is given after HCT [20,21].

Our second approach aimed at achieving sustained engraftment after 50 cGy involved enhancing engraftment-facilitating GVH reactions. For this purpose, recipients were treated with FL to expand the dendritic cell (DC) compartment and make T cells more "visible" to engraftment-facilitating GVH-reactions (Experiments 8 and 9). This approach has been shown to facilitate engraftment in the canine HCT model that uses DLA-identical bone marrow and an otherwise suboptimal TBI dose of 450 cGy without postgrafting immunosuppression [22]. We had shown previously that 10 days of FL treatment of dogs resulted in a doubling of the total white blood cell count, which was largely attributable to an approximately 10-fold increase in the number of $CD14^+$ monocytes [14]. Moreover, FL treatment led to the emergence of a distinct $CD11c^+/HLA\text{-}DR^+/CD14^-$ cell population, a surface marker profile consistent with that of myeloid DC in humans. As few as $5 \times 10^3$ irradiated $CD11c^+/HLA\text{-}DR^+/CD14^-/DM5^-$ cells elicited strong proliferative T cell responses in unidirectional, allogeneic MLC [14]. The identification of these highly potent antigen-presenting cells in the peripheral blood of dogs, which may represent the equivalent of myeloid DC in humans, prompted us to investigate whether expansion of these cells would enhance GVH reactions leading to sustained engraftment.

Our results showed that 10-day FL treatment of recipients before HCT (FL +/–), or 10 days before HCT and 10 days after HCT (FL +/+), did not have engraftment-facilitating effects in the 50-cGy TBI model. Indeed, levels of peak granulocyte chimerism in the FL +/+ group (Experiment 8) were significantly lower than those in controls (median, 7% versus 25%, $P = .03$) (Table 1), suggesting a counterproductive effect of FL administration *after* HCT. Only 1 of 9 dogs conditioned with FL had sustained engraftment. This dog did not experience clinically apparent graft-versus-host disease (GVHD), which could have been a concern after recipient-DC expansion [23].

In summary, only 5 of 37 (14%) recipients had sustained mixed chimerism when conditioned with 50-cGy TBI and given combined DLA-identical marrow/G-PBMC grafts followed by immunosuppression with CSP and MMF after transplant. None of the tested immunomodulatory regimens improved rates of engraftment or levels of donor chimerism above those of controls. Even though we did not provide direct evidence in the present study that the chosen immunomodulatory approaches inhibited host-antidonor activity or enhanced donor-antihost reactions, there is ample evidence from previously conducted and published studies that this is indeed the case [9,17,24]. The fact that substituting minimally toxic pharmacological immunomodulation for TBI did not overcome the engraftment barrier in this model is evidence for the potent immunosuppressive effects of even low doses of ionizing radiation [18]. Thus, in HCT preparative regimens without any or with very low doses of TBI, conventional immunosuppressive drug therapy may not suffice to achieve sustained engraftment. In order to more forcefully shift the immunologic balance in favor of the incoming donor cells without having to rely on TBI, more innovative pharmacotherapy needs to be developed.

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