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Aldehyde Dehydrogenase Expression Drives Human Regulatory T Cell Resistance to Posttransplantation Cyclophosphamide

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Abstract

High-dose, posttransplantation cyclophosphamide (PTCy) is an effective strategy for preventing graft-versus-host disease (GVHD) after allogeneic blood or marrow transplantation (alloBMT). However, the mechanisms by which PTCy modulates alloimmune responses are not well understood. We studied early T cell reconstitution in patients undergoing alloBMT with PTCy and the effects of mafosfamide, a cyclophosphamide (Cy) analog, on CD4⁺ T cells in allogeneic mixed lymphocyte reactions (MLRs) in vitro. Patients exhibited reductions in naïve, potentially alloreactive conventional CD4⁺ T cells with relative preservation of memory CD4⁺Foxp3⁺ T cells. In particular, CD4⁺CD45RA[−]Foxp3^{hi} effector regulatory T cells (T_{regs}) recovered rapidly after alloBMT and, unexpectedly, were present at higher levels in patients with GVHD. CD4⁺Foxp3⁺ T cells from patients and from allogeneic MLRs expressed relatively high levels of aldehyde dehydrogenase (ALDH), the major in vivo mechanism of Cy resistance. Treatment of MLR cultures with the ALDH inhibitor diethylaminobenzaldehyde reduced the activation and proliferation of CD4⁺ T cells and sensitized T_{regs} to mafosfamide. Finally, removing T_{regs} from peripheral blood lymphocyte grafts obviated PTCy's GVHD-protective effect in a xenogeneic transplant model. Together, these findings suggest that T_{reg} resistance to Cy through expression of ALDH may contribute to the clinical activity of PTCy in preventing GVHD.

Introduction

Allogeneic blood or marrow transplantation (alloBMT) is a potentially curative modality for a variety of otherwise incurable hematologic diseases. However, the effectiveness of alloBMT is limited by graft-versus-host disease (GVHD), which is a significant source of

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Competing interests: R.J.J. holds the patent for Aldefluor and, under a licensing agreement between Aldagen and Johns Hopkins University, is entitled to a share of royalties received by the University. The terms of this arrangement are managed by Johns Hopkins University in accordance with its conflict of interest policies. No other authors have competing interests.

morbidity and mortality from alloBMT. Although standard pharmacologic GVHD prophylactic regimens are effective in preventing acute GVHD (aGVHD), most have been less effective in limiting chronic GVHD (cGVHD). Moreover, calcineurin inhibitor (CNI)–based standard regimens may impair immune reconstitution, prevent tolerance induction (1), and potentially increase the risk of relapse (2).

An emerging body of work suggests that high-dose, posttransplantation cyclophosphamide (PTCy) is an effective strategy for GVHD prevention (3). PTCy facilitates both partially human leukocyte antigen (HLA)–mismatched alloBMT (4) and combined kidney/bone marrow (BM) transplantation (5) without severe GVHD. Furthermore, PTCy as a single agent is sufficient to prevent both aGVHD and cGVHD after HLA-matched alloBMT (6).

Although the exact mechanisms by which PTCy prevents GVHD and induces immunologic tolerance in humans are not well understood, it has been assumed that the selective killing of proliferating, alloreactive T cells is the dominant mechanism (7). This hypothesis has been based on murine data showing loss of particular V β T cell receptor–expressing T cells after PTCy treatment to facilitate skin allografting (8–10). However, two murine studies suggested that maintenance of tolerance to skin allografts after PTCy may depend on the generation of suppressor T cells (10, 11). The potential role of regulatory T cells (T_{regs}) in the mechanism of cyclophosphamide (Cy)–induced tolerance contrasts with other studies showing a negative impact of Cy on murine T_{reg} survival, leading to augmented antitumor immunity (12).

T_{regs} are increasingly recognized as key mediators in promoting tolerance and preventing and ameliorating GVHD in mouse models (13, 14), with mounting evidence implying a conserved critical role in human allografting. Several correlative studies suggest that higher T_{reg} peripheral blood (PB) levels in patients after alloBMT are associated with a lower incidence of aGVHD using standard GVHD prophylactic regimens (15–17). Recently, early-phase clinical studies have shown that administration of T_{regs} can modulate GVHD in patients after alloBMT (18) and that T_{regs} may even be effective as sole GVHD prophylaxis (19).

Here, we sought to better understand the role of T_{regs} in PTCy-mediated GVHD prophylaxis. Using peripheral blood mononuclear cells (PBMCs) collected from patients treated on a clinical protocol using PTCy as sole GVHD prophylaxis (6), we first characterized T_{reg} reconstitution and its relationship to the development of aGVHD. Next, we performed in vitro studies to examine the effects of Cy on CD4⁺ T cell subsets, particularly T_{regs}, in allogeneic reactions. Our results show that human T_{regs} are resistant to Cy in allogeneic reactions at least in part through increased expression of aldehyde dehydrogenase (ALDH) and that this sparing of T_{regs} contributes to PTCy's activity in preventing GVHD.

Results

Effects of PTCy on T cell subsets after alloBMT

PBMCs were collected from patients treated on the clinical study that established the effectiveness of high-dose PTCy as single-agent, short-course GVHD prophylaxis (6). In that study using HLA-matched donors and myeloablative conditioning (6), the overall incidences of grade II to IV and III to IV aGVHD were 43 and 10%, respectively. For the current analysis, all available specimens for patients with grade III to IV aGVHD were analyzed along with similar numbers of randomly selected patients with grade II aGVHD or without aGVHD, respectively. The clinical characteristics of the patients whose samples were analyzed are shown in Table 1. The onset of aGVHD was at a median of 42 (range, 20 to 94) days after alloBMT.

The differences in percentages and absolute numbers of defined T cell subsets between donors ($n = 16$) and patients after alloBMT ($n = 47$), as well as between patients with ($n = 31$) or without aGVHD ($n = 16$), were analyzed using generalized estimating equations (GEEs) (20) (Table 2). The mean absolute CD4⁺ count on day 30 after alloBMT was 98 cells/ μ l and on day 60 was 124 cells/ μ l. At days 30 and 60 after alloBMT, the mean absolute CD8⁺ T cell counts were already within the donor range (Fig. 1A). This CD4⁺ and especially CD8⁺ T cell recovery compared favorably with that previously reported for T cell-replete BM allografting using standard GVHD prophylactic regimens (21).

CD4⁺Foxp3⁻ conventional T cells (T_{cons}) can be divided into naïve (CD45RA⁺) and memory-like (CD45RA⁻) subsets. The naïve T_{cons} percentages of CD4⁺ T cells were greatly reduced in patients after alloBMT compared with donors (GEE, $P = 0.002$). On the other hand, the percentages of memory-like T_{cons} were slightly increased in patients after alloBMT (Table 2).

CD4⁺CD25⁺Foxp3⁺ T_{regs} have been suggested to have diagnostic and prognostic utility as a biomarker for aGVHD (15). We found that the percentages of CD4⁺CD25⁺Foxp3⁺ T cells were increased in patients after alloBMT when compared with donors (GEE, $P = 0.002$) (Fig. 1B). Unexpectedly, the percentages of CD4⁺CD25⁺Foxp3⁺ T cells were higher in patients with aGVHD than in those without aGVHD (GEE, $P < 0.0001$) (Fig. 1B and Table 2).

Persistence of memory CD4⁺Foxp3⁺ T cell subsets in patients treated with PTCy

Foxp3 is not a specific marker for human T_{regs} because activated human T_{cons} transiently also express Foxp3 (22). Given the limited amounts of prospectively collected PBMCs on the clinical study for conducting functional studies, it was important to overcome the potential short-coming of using only Foxp3 expression (with or without CD25 and CD127) to assess the in vivo effects of PTCy on T_{regs} . Therefore, to explore the unexpected finding of increased CD4⁺CD25⁺Foxp3⁺ T cells in patients with aGVHD, we used the strategy of Miyara and colleagues (22), which phenotypically and functionally delineated three fractions of human CD4⁺Foxp3⁺ T cells on the basis of expression of Foxp3 and CD45RA: fraction I (Fr I) (CD45RA⁺Foxp3^{hi}) representing naïve, resting, natural T_{regs} (nT_{regs}); Fr II (CD45RA⁻Foxp3^{hi}) representing activated, effector T_{regs} (eT_{regs}); and Fr III

(CD45RA⁻Foxp3⁺¹⁰) representing cytokine-secreting, nonsuppressive T cells (22). We were able to reproducibly identify these fractions (Fig. 2A). We confirmed that the two T_{reg} fractions (Fr I and II) were suppressive, were largely demethylated within the Foxp3 T_{reg}-specific demethylation region (TSDR), and showed differential cytokine production (figs. S1 to S3), verifying that Fr I and II were indeed true T_{reg} fractions.

The results of analyses using this approach are shown in Fig. 2 and summarized in Table 2. The percentages of both memory CD4⁺CD45RA⁻Foxp3⁺ fractions (Fr II and III) were increased in patients after alloBMT when compared with donors. Despite marked posttransplant CD4⁺ lymphopenia, the total numbers of Fr II (eT_{regs}), but not that of Fr III or total CD4⁺CD25⁺Foxp3⁺ T cells, recovered to donor levels by 30 days after alloBMT. The percentages of Fr III cells were increased in patients with aGVHD compared with those without aGVHD (GEE, $P = 0.0003$). Surprisingly, patients with aGVHD also had higher percentages of eT_{regs} (GEE, $P = 0.01$). At day 30 after alloBMT, patients had low percentages of recent thymic emigrants and relatively high Ki-67 expression in both memory CD4⁺Foxp3⁺ fractions, consistent with rapid peripheral expansion of CD4⁺Foxp3⁺ T cells surviving PTCy (fig. S4). In contrast to eT_{regs}, the percentages and counts of CD4⁺CD45RA⁺Foxp3⁺ nT_{regs} (Fr I) were lower in patients after alloBMT compared with donor levels, most likely reflecting their known ability to convert to eT_{regs} (22). Thus, overall, in patients treated with PTCy, there was a relative preservation of memory CD4⁺Foxp3⁺ fractions, particularly eT_{regs}, with loss of naïve T_{cons}. Both of these effects seemed to be accentuated by the presence of aGVHD. Although a potential confounder for the effects of GVHD on particular T cell fractions, corticosteroid use seemed to be associated with reductions of CD4⁺ counts while not appearing to affect the relative skewing of particular T cell fractions induced by aGVHD (fig. S5).

Resistance of CD4⁺Foxp3⁺ fractions to Cy in mixed lymphocyte reactions in vitro

Because of the unexpected finding of increased CD4⁺Foxp3⁺ T cells, particularly eT_{regs}, in patients with aGVHD, we further explored the effects of PTCy using the in vitro model of the mixed lymphocyte reaction (MLR). Since Cy is a prodrug that requires hepatic activation, the effects of the in vitro active Cy analog mafosfamide on MLRs were studied and compared with MLRs treated with cyclosporine A (CsA) or rapamycin. Mafosfamide was administered as a 1-hour incubation on day 3 of MLR to mimic the in vivo short half-life of Cy (23). Two different doses of each treatment drug were tested. For all in vitro studies, cells were first gated on viable cells using the LIVE/DEAD viability dye. The viability dye alone was effective in identifying >90 to 95% of non-viable cells when assessed by a combination of staining with the viability dye and annexin V (fig. S6). The percentages or total numbers reported are of viable cells unless otherwise specified.

Day 7 MLR cultures treated with the higher dose of mafosfamide (7.5 µg/ml) yielded CD4⁺ T cell counts that were significantly lower than those of both untreated cells and cells treated with either CsA or rapamycin [repeated-measures analysis of variance (RM-ANOVA), $P < 0.001$; $n = 10$] (Fig. 3A). Much of the drop in CD4⁺ cells in the mafosfamide-treated MLR cultures was a result of loss of naïve T_{cons} (Fr VI) (RM-ANOVA, $P < 0.001$; $n = 10$) (Fig. 3B). Memory/effector CD4⁺ T_{con} (Fr IV/V) counts declined as well (RM-ANOVA, $P <$

0.001; $n = 10$). However, because the decline of memory/effector T_{con} counts was less marked than the losses of naïve T_{cons} , the percentages of memory/effector T_{cons} actually increased (RM-ANOVA, $P < 0.001$; $n = 10$) (Fig. 3B).

The percentages of the three $CD4^+Foxp3^+$ fractions were significantly elevated in MLR cultures treated with the higher dose of mafosfamide compared with untreated cultures or those treated with CsA or rapamycin (RM-ANOVAs, $P < 0.001$ for all three fractions; $n = 10$) (Fig. 3C). Despite the loss of the majority of $CD4^+$ T cells after mafosfamide treatment, the total numbers of these three $Foxp3^+$ fractions were similar at day 7 of MLR between untreated and mafosfamide-treated cultures (Fig. 3C), suggesting that all three $CD4^+Foxp3^+$ fractions were resistant to mafosfamide-induced cytotoxicity.

Treatment with mafosfamide did not affect the suppressive activity of T_{regs} (Fr I and II), the methylation patterns within the $Foxp3$ TSDR, or the cytokine expression profiles (figs. S1 to S3), thus confirming the preservation of bona fide T_{regs} after mafosfamide treatment. Furthermore, Fr III cells, which in MLR comprised a heterogeneous population with partial suppressive activity, contained a higher content of true T_{regs} after mafosfamide treatment on the basis of heightened suppressive capability, increased demethylation within the $Foxp3$ TSDR, and near depletion of $CD127^{+hi}$ cells contained within this fraction (figs. S1 and S2). High expression of CD95 was present in memory $Foxp3^+$ fractions at MLR day 7 and did not differ substantially between mafosfamide-treated and untreated cells (fig. S7).

CsA treatment led to lower numbers of both Fr II and III cells at day 7 of MLR (Fig. 3C) and significantly reduced cytokine production (fig. S3) compared with other treatment groups. Of note, CsA administered the day after mafosfamide treatment did not prevent similar increases in percentages of $Foxp3^+$ fractions as seen with mafosfamide treatment alone and differed from CsA treatment alone from days 0 to 7 (fig. 8).

Expression of ALDH by T_{regs} after allogeneic stimulation both in vitro and in vivo

The major mechanism of Cy inactivation in vivo is through ALDH1 (24). Therefore, we hypothesized that $CD4^+Foxp3^+$ T cells might express ALDH1 as a potential mechanism of resistance to Cy. Flow cytometry with Aldefluor was used to assess ALDH1 activity in viable cells (25, 26). Because intracellular $Foxp3$ staining could not be tested simultaneously with Aldefluor, we relied on the cell surface markers CD25, CD45RA, and CD127 to distinguish six $CD4^+$ T cell fractions as previously described (22, 27) and in the same way we utilized fluorescent-activated cell sorting (FACS) for the suppression and methylation studies (figs. S1 and S2). Diethylamino-benzaldehyde (DEAB), a specific, competitive, pharmacologic inhibitor of ALDH1, was coincubated with half of each sample during staining for use as a negative control (Fig. 4A) (25, 26).

There was minimal ALDH activity in circulating $CD4^+$ T cells from healthy donors (Fig. 4B). However, at day 3 after allogeneic stimulation in MLR, 5 to 13% of T_{regs} (Fr I and II) expressed ALDH. By day 7 of MLR, 15 to 30% of the eT_{reg} and other memory $CD25^+$ fractions were ALDH⁺ (Fig. 4B). Although the Aldefluor staining was indistinct even with optimization, it was clearly positive based on the DEAB negative control (Fig. 4A). Overall, the staining was lower in expression than that seen in normal hematopoietic stem cells but

similar to that seen in normal or malignant stem cells of other organs (28). Mafosfamide treatment on day 3 did not eliminate the ALDH⁺ populations at day 7; CsA- or rapamycin-treated cells had minimal ALDH activity at either day 3 or day 7 (Fig. 4C).

Given the indistinct nature of the staining, we validated ALDH expression through quantitative reverse transcription polymerase chain reaction (RT-PCR), which confirmed up-regulation of ALDH1A1 with allogeneic stimulation in MLR compared with no expression in unstimulated cells (Fig. 4D). ALDH1A1 up-regulation was seen in all fractions, but ALDH1A1 expression was 10-fold higher in the T_{reg} fractions than in T_{cons} at day 3 of MLR. Up-regulation of Foxp3 expression also occurred with allogeneic stimulation and mirrored the changes in ALDH1A1 expression (fig. S9). However, the expression of ALDH3A1, which is thought also to be involved in both drug resistance to Cy and cell proliferation (29), did not consistently change and was generally undetectable (fig. S10).

Aldefluor activity on day 3 after alloBMT before PTCy administration was examined in six consecutive patients undergoing myeloablative alloBMT (five HLA-matched-related, one HLA-haploidentical-related). Consistent with our previous data in unstimulated cells, there was minimal Aldefluor positivity in donor PB and BM CD4⁺Foxp3⁺ T cells (Fig. 4E). However, by day 3 after alloBMT, Foxp3⁺ subsets, particularly eT_{regs}, had significantly increased Aldefluor activity (Fig. 4E). Chimerism studies performed by our clinical laboratory for five of these six patients on day 3 showed donor chimerism of ~20% each in CD3⁺ and CD3⁻ fractions. Thus, increases in CD4⁺Foxp3⁺ T cells expressing ALDH occur after allogeneic stimulation in vivo and likely contribute to bidirectional tolerance induction with PTCy.

Reduced activation and proliferation of CD4⁺ T cells in MLR after ALDH inhibition

We next examined the relationship between ALDH activity and alloantigen-induced activation and proliferation by culturing cells with or without the ALDH inhibitor DEAB. By MLR day 7, the vast majority of eT_{regs} and other memory CD25⁺ fractions were robustly proliferating (Fig. 5A). DEAB treatment from days 0 to 3 inhibited the proliferation of CD4⁺ T cells at day 7 when compared with controls (paired *t* test, *P* < 0.001; *n* = 6) but was insufficient to fully block the proliferation of eT_{regs} (Fig. 5A). These results support a role for ALDH in CD4⁺ T cell proliferation after allogeneic stimulation and are consistent with the impact of abrogated ALDH activity on proliferation in previously published studies (29).

Similar to its effects in blocking proliferation, DEAB treatment prevented the activation of alloreactive CD4⁺ T cells. By MLR day 3, the vast majority of Aldefluor⁺CD4⁺CD25⁺ T cells expressed CD69 (Fig. 5B), consistent with known expression of CD69 upon alloantigen-induced T_{reg} activation in vitro (30). Treatment with DEAB from days 0 to 3 significantly reduced the expression of CD69 on CD4⁺ T cells at days 3 and 7 of MLR (paired *t* tests, *P* < 0.001 for each comparison; *n* = 6) (Fig. 5C). Given the increased expression of CD25 and Foxp3 by alloantigen-stimulated T_{cons}, we also examined the effects of DEAB treatment on CD4⁺CD127^{hi} T_{cons} and found that DEAB treatment from days 0 to 3 significantly reduced CD25 and Foxp3 expression by T_{cons} at MLR day 7 in a dose-dependent manner (RM-ANOVAs, *P* = 0.005 for each comparison; *n* = 4) (fig. S11).

Sensitization of T_{regs} to Cy through ALDH inhibition during allogeneic reactions

Given the effects of ALDH on CD4⁺ T cell activation and proliferation, we next examined the impact of ALDH expression on the relative mafosfamide sensitivity of CD4⁺ T cells. Aldefluor⁺CD4⁺ cells were significantly more resistant to mafosfamide than Aldefluor⁻CD4⁺ cells (unpaired *t* test, *P* = 0.022; *n* = 4) (fig. S12), despite the former being almost entirely activated, proliferating cells and the latter being predominantly small, resting lymphocytes, which would be expected to be less susceptible to an alkylating agent.

To confirm that ALDH was a driving mechanism by which CD4⁺Foxp3⁺ T cells were resistant to Cy, we assessed the effects of DEAB on MLR cultures treated with mafosfamide by examining changes in cell viability after DEAB and/or mafosfamide treatment. Treatment with DEAB alone from days 0 to 3 or with mafosfamide alone on day 3 did not substantially affect the viability of T_{regs} (Fig. 5D). However, pretreatment with DEAB before mafosfamide treatment sensitized T_{regs} to mafosfamide, leading to a dose-dependent marked increase in cell death (RM-ANOVAs, *n* = 6; nT_{regs}, *P* < 0.001; eT_{regs}, *P* < 0.001) (Fig. 5D). Overall, these data suggest that ALDH is necessary for T_{regs} to survive Cy treatment in allogeneic reactions.

Role of T_{regs} in the GVHD-preventive effects of PTCy in vivo

Although we have shown that T_{regs} are resistant to Cy through ALDH expression, it was unknown whether survival of these T_{regs} played any role in PTCy's clinical activity in preventing GVHD. Therefore, we used a xenogeneic GVHD model to assess the effects that removing T_{regs} from a PBMC graft might have on mice treated with PTCy. In four separate experiments using a single human donor for all mice in a given experiment, NOD/Lt-*scid*/IL-2γ^{null} (NSG) mice treated with PTCy (100 mg/kg) on day 3 after receiving T_{reg}-depleted PBMC grafts (*n* = 14) (Fr I and II removed by FACS) had lower weights, had higher GVHD scores, and died more quickly than mice treated with PTCy after receiving whole PBMC grafts (*n* = 13) (survival, Gehan-Breslow, *P* < 0.001; Holm-Sidak post-test for these two groups, *P* = 0.016) (Fig. 6). Thus, removal of T_{regs} abrogated the protective effects of PTCy in this xenogeneic model. Furthermore, the fact that NSG mice receiving PTCy did eventually die suggests that alloreactive T cells were not completely eliminated by PTCy and eventually escaped suppression and caused fatal GVHD. These findings support an essential role for T_{regs} in the mechanism of GVHD prevention by PTCy.

Discussion

Here, we investigated the immunologic effects of PTCy on CD4⁺ T cells. Our results show that T_{regs} are resistant to Cy during alloimmune responses through expression of ALDH. Patients treated with PTCy have increased percentages of eT_{regs} compared with donors, and eT_{reg} counts recover quickly after alloBMT despite continued posttransplant CD4⁺ lymphopenia. In vitro MLRs confirmed that Cy relatively spares CD4⁺Foxp3⁺ fractions, which maintain suppressive capability and low demethylation within the Foxp3 TSDR. Although unstimulated cells express little ALDH, CD4⁺Foxp3⁺ T cells, particularly eT_{regs}, have significantly increased expression of ALDH after allogeneic stimulation both in vitro and in vivo. Furthermore, blocking ALDH activity with DEAB reduces the activation and

proliferation of CD4⁺ T cells in MLR and abolishes T_{reg} resistance to Cy. Finally, we demonstrated the critical importance of T_{regs} for mediating the clinical GVHD-protective effects of PTCy in a xenogeneic GVHD model.

Most published studies using CNI-based, standard GVHD prophylaxis have shown that lower circulating T_{reg} levels in patients after alloBMT (15–17) or in the donor graft (17) are associated with higher incidences of aGVHD. Our data using PTCy differ from these studies but are consistent with natural physiologic increases of T_{regs} in patients with active autoimmunity not treated with CNI-based pharmacologic immunosuppression (22, 31). Furthermore, our data are consistent with reports of increased organ-infiltrating T_{regs} in skin (32, 33) and gastrointestinal aGVHD (34). Even so, whether increases in circulating T_{regs} correlate with increased tissue infiltration by T_{regs} in patients treated with PTCy who develop aGVHD is an active area of investigation.

Some murine studies have suggested that Cy can reduce T_{regs} and thereby promote antitumor immunity (35, 36) or autoimmunity (37). However, these studies showed that the effects of Cy on T_{regs} are transient and are dependent on tissue type with a quick rebound in lymph node and splenic T_{regs} (37, 38). Moreover, the immunomodulatory effects of Cy on T_{regs} seen in these murine studies may not be specific for Cy since similar effects were seen with other chemotherapeutic drugs (39), and overall, the effects were fundamentally dependent on the timing of administration (39) and differed by dosing schema (12).

The context of Cy administration, particularly as it relates to relative ALDH expression, likely accounts for the differences seen between our work and these murine studies; ALDH is minimally expressed by human T_{regs} at equilibrium, but, as we have shown, ALDH is markedly up-regulated after allogeneic stimulation. Additionally, there are important differences in the dosing schedules and the source of T_{regs} evaluated (tissue versus PB). Moreover, there are intrinsic differences between human and murine T_{regs}; whereas nearly all murine CD4⁺CD25⁺ T cells are regulatory, there is considerable heterogeneity in humans with at least four different fractions of CD4⁺CD25⁺ T cells, only two of which are consistently regulatory (22).

T_{regs} are a resilient CD4⁺ subset, resistant to a variety of different cytotoxic treatments, including ionizing radiation (40), etoposide chemotherapy (40), and immunosuppressive drugs such as rapamycin (41). Moreover, human T_{regs}, particularly nT_{regs}, are resistant to oxidative stress (42). Our data support that T_{reg} resistance to PTCy appears to be related to induction of ALDH expression upon allogeneic stimulation. Although the degree of ALDH positivity at day 3 as measured by Aldefluor would not seem to fully explain the percentage of Foxp3⁺ cells that survive mafosfamide, the PCR and DEAB studies demonstrate a more dramatic effect. Part of this disparity may be due to conservative gating on the small lymphocyte population to avoid any size bias in Aldefluor expression. Moreover, it may be that Aldefluor is not sensitive enough to detect the extent of ALDH expression given the more marked up-regulation of ALDH RNA by PCR; because the Aldefluor reagent is a substrate for ALDH, Aldefluor testing may underestimate the amount of ALDH present due to competition by other substrates.

Further studies are needed to decipher the mechanism of increased ALDH expression after allogeneic stimulation. Potential mechanisms include regulation by Foxp3 or signaling through CD25. Both Foxp3 and CD25 have increased expression after allogeneic stimulation, and both are coexpressed with ALDH. In fact, the cells most highly expressing CD25 and Foxp3 are also the most ALDH-positive (Fig. 4), and the degree of Foxp3 expression on day 3 correlates with ALDH1A1 expression (fig. S9). Furthermore, the addition of CsA and rapamycin both inhibit ALDH expression (Fig. 4C), which is consistent with inhibition of ALDH by rapamycin in cell lines (43), suggesting that interleukin-2 (IL-2) and mammalian target of rapamycin (mTOR) signaling may be involved in ALDH expression.

The role of ALDH in cell activation and proliferation may explain its up-regulation in allogeneic reactions (29). Although this role is incompletely understood, it may be mediated through retinoic acid (RA). The ALDH1 family (retinaldehyde dehydrogenases) is the rate-limiting step in RA biosynthesis, and RA has numerous effects on T cell activation, proliferation, trafficking, differentiation, and function (44). Furthermore, RA has been shown to stabilize natural T_{regs} , induce T_{regs} in the periphery in the presence of transforming growth factor β (TGF β), and tilt the $T_{\text{reg}}/T_{\text{H}}17$ (T helper 17) balance against inflammation (44). Given the expression of ALDH1 after allogeneic stimulation, pharmacologic manipulation of the ALDH1/RA pathway could open a new avenue for modulating alloreactivity after alloBMT and is currently being explored.

The activity of T_{regs} in mediating the GVHD-preventive effects of PTCy is an important step forward in our understanding of this effective GVHD prophylactic strategy. Encouragingly, graft-versus-tumor activity does not appear to be compromised by high levels of circulating T_{regs} in high-risk patients treated with PTCy (6) or in patients treated with infused T_{regs} (18, 19). We are currently investigating why aGVHD occurs in almost half of PTCy-treated patients despite high T_{reg} levels. Nevertheless, we suspect that T_{reg} preservation by PTCy accounts for the unexpectedly low 10% incidences of severe aGVHD and cGVHD seen in these patients (6). Furthermore, deleterious effects of CsA on T_{reg} survival also may help explain the much higher rates of cGVHD seen with CNi-based GVHD prophylaxis. Importantly, the addition of a CNi to the in vitro allogeneic reactions after Cy treatment did not ablate Cy's protective effects on T_{regs} . This maintained effect may explain the low incidences of severe aGVHD and cGVHD that are also seen when CNIs are added to PTCy in the setting of related donor HLA-haploidentical alloBMT (4, 45).

Materials and Methods

Study design

This study was designed to characterize the immunophenotypic recovery of patients after alloBMT compared with donors and between patients with or without aGVHD. The sample size was based on the number of specimens available for patients with grade III to IV aGVHD. About equal numbers of patients with grade II aGVHD or without aGVHD were randomly selected from available specimens for comparisons. Samples were deidentified and then flow cytometrically analyzed and gated in a blinded fashion. Ki-67, CD31, and CTLA-4 expression was assessed secondarily on available randomly selected PB specimens

from BM donors and from patients at day 30 after alloBMT. On the basis of the finding of rapid recovery of eT_{reg} s after alloBMT, we confirmed T_{reg} resistance to Cy in in vitro MLRs, which led directly to the exploration of ALDH expression as a potential mechanism of resistance. The importance of T_{reg} s for the clinical activity of PTCy was assessed using a xenogeneic GVHD model. All data including outliers were included in all analyses.

Human subjects

As part of the Institutional Review Board (IRB)–approved clinical protocol (6) and after informed consent, PBMCs were prospectively collected from patients at predetermined time points and from their healthy BM donors and were cryopreserved. Informed consent on our IRB-approved cell banking protocol was obtained before specimen collection for an additional 6 patients undergoing myeloablative alloBMT, 2 donors of BM for these patients, and 12 healthy volunteers. PBMCs obtained from these donors were all used for studies immediately without cryopreservation.

Animals

NSG mice (The Jackson Laboratory) were bred and kept under sterile conditions in our animal facility. Studies were performed in compliance with a protocol approved by the Institutional Animal Care and Use Committee using littermate-controlled mice of the same age (12 to 14 weeks) and sex. A FACSaria II [Becton Dickinson (BD)] was used both to purify human PBMCs after Ficoll-Hypaque density centrifugation and to sort out $CD3^+CD4^+CD45RA^+CD25^{int/hi}CD127^{lo/-}$ and $CD3^+CD4^+CD45RA^-CD25^{hi}CD127^{lo/-}$ T_{reg} s [Fr I and II, respectively, as per Miyara *et al.*'s methodology (22)]. The FACS purity was >99%. Flow-sorted whole PBMCs or T_{reg} -depleted PBMCs were injected intravenously into irradiated (2.5 Gy) NSG mice at 5×10^6 cells per mouse, whereas control mice received RPMI alone after irradiation. Cy (100 mg/kg) (Baxter Healthcare) was administered intraperitoneally 72 hours after adoptive cell transfer.

Flow cytometry

Flow cytometry was performed on a FACSCanto (BD) and LSR II (BD) for patient and in vitro studies, respectively, and data were analyzed with FACSDiva (BD). The LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used for all in vitro studies. Fixation and permeabilization were performed with the Foxp3 Buffer Kit (eBioscience).

For the Aldefluor analyses, fresh human PBMCs were separated by $CD3^+$ negative selection with the Pan T Cell Isolation Kit (Miltenyi Biotec) and were either stained immediately for Aldefluor (STEMCELL Technologies) or placed in MLR culture as below. Because Aldefluor testing depends on a viable cell membrane (25) and thus does not allow Foxp3 costaining, the breakpoint between Fr II and III was chosen as the point where $CD25^{int/hi}$ cells transitioned from being heterogeneous in CD127 expression to strictly low/negative CD127. For assessment of proliferation in Aldefluor-stained cells, the CellTrace Violet Cell Proliferation marker (Life Technologies) was used at 5 μ M as per the manufacturer's instructions.

In vitro MLRs

After isolation from freshly drawn PB, responder T cells were cocultured with irradiated (30 Gy), HLA-mismatched, allogeneic CD3⁺ PBMCs at 1:1 with 1×10^5 cells of each population per well in 96-well round-bottomed plates. Mafosfamide (Baxter Oncology) was freshly reconstituted in phosphate-buffered saline and administered at 2.5 or 7.5 $\mu\text{g/ml}$ as a single, 1-hour incubation on MLR day 3. These doses were based on a dose-response curve and consistent with previous studies (46). Treatment with CsA (Sigma) was at 200 or 600 ng/ml from MLR days 0 to 7. Treatment with rapamycin (Sigma) was at 5 or 15 ng/ml from MLR days 0 to 7. These doses for CsA and rapamycin were chosen to be consistent with published studies (47–49) and clinically relevant doses. DEAB (STEMCELL Technologies) treatment (25 or 50 μM) was from the start of MLR through mafosfamide treatment on day 3. Cells treated with mafosfamide and/or DEAB underwent two washings after treatment to remove residual drug.

Reverse transcription polymerase chain reaction

RNA was isolated and purified from flow-sorted populations of lymphocytes with TRIzol (Life Technologies) as per the manufacturer's protocol. Synthesis of complementary DNA (cDNA) was performed with random hexamers (Life Technologies) and You-Prime First-Strand Beads (GE Healthcare). Relative expression of the target gene was determined by multiplexed real-time PCR on an ABI 7500 (Life Technologies). Samples were run in triplicate using primer/probe sets for the FAM-labeled target gene and VIC-labeled endogenous control GAPDH. The primer/probe sets used [Hs01085834_m1 (Foxp3), Hs00946916_m1 (ALDH1A1), Hs00964880_m1 (ALDH3A1), and 4326317E (GAPDH)] and other ABI reagents were obtained from Life Technologies.

Statistical analysis

For the patient analyses, the primary statistical endpoints were differences in T cell fractions between donors and patients after transplant and between patients with or without aGVHD. To account for the correlation between T cell values obtained from the same patient at different time points or between donor and patient pairs, we used GEEs (20) (assuming a compound symmetry correlation structure) for estimation and hypothesis testing of mean differences based on a χ^2 statistic. A natural logarithmic transformation was applied before analysis. Ninety five percent confidence intervals for fold changes on the natural scale were obtained by exponentiating confidence intervals obtained from regression models on the logarithmic scale. These analyses were exploratory, and no adjustment was made for multiple comparisons. Comparisons were visualized using box-and-whisker plots of the untransformed data, which were generated with R (R Foundation for Statistical Computing). All other statistical computations were performed with SAS version 9.2 (SAS Institute). The SAS procedure GENMOD was used for GEE regression analyses.

Laboratory data were analyzed with RM-ANOVA or paired *t* test when comparing the same fraction between different treatment groups or with standard ANOVA or unpaired *t* test when comparing across different fractions. A natural logarithmic transformation was applied before analysis. ANOVA or *t* test results are reported in the text, and post hoc test results are shown in the figures. SigmaPlot (version 12.3, Systat Software Inc.) was used for the

laboratory analyses, and GraphPad Prism 6 (GraphPad Software) was used for data presentation. For all comparisons, two-sided *P* values were used, and *P* < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CD4⁺, CD8⁺, and CD4⁺CD25⁺Foxp3⁺ T cell reconstitution is favorable after alloBMT using PTCy as single-agent GVHD prophylaxis

Freshly frozen PBMCs prospectively collected at predetermined time points from donors ($n = 16$) and patients ($n = 47$) before and after alloBMT were immunophenotyped. (A) T cell counts from donors and patients. (B) Percentages and counts of CD4⁺CD25⁺Foxp3⁺ T cells. Data are shown in box-and-whisker plots representing the median, quartiles, and 1.5 times the interquartile range. Open circles represent outliers beyond this range. Statistical comparisons were performed using GEEs, and detailed results are presented in Table 2.

Fig. 2. eT_{regs} are preserved after alloBMT using PTcy and are relatively increased in patients with aGVHD

(A) Representative flow cytometric plots of healthy donors ($n = 16$), patients without aGVHD ($n = 16$), and patients with aGVHD ($n = 31$) using a gating strategy that distinguishes five subsets among $CD4^+$ T cells. (B and C) Percentages and counts of eT_{regs} (B) and $CD4^+CD45RA^-Foxp3^{+lo}$ T cells (C). (D) Pie charts showing the relative distribution of $CD4^+$ T cell fractions for donors, patients before transplant, and patients after transplant. Median values of each fraction were used and normalized to 100% between all fractions for each group. Statistical comparisons were performed using GEEs, and detailed results are presented in Table 2.

Fig. 3. CD4⁺Foxp3⁺ T cell fractions, but not naïve T_{con}s, are resistant to mafosfamide in MLR
 Human CD3⁺CD4⁺ T cells were flow cytometrically separated and cocultured at a 1:1 ratio in triplicate with irradiated allogeneic CD3⁺ PBMCs. Cells were either untreated, pulse-treated with mafosfamide (Maf) on day 3, or treated with CsA or rapamycin (Rap) from days 0 to 7. **(A)** Total numbers of viable CD4⁺ T cell counts at day 7 of MLR. **(B and C)** Percentages (top) and total numbers (bottom) of various CD4⁺ T_{con} (B) and Foxp3⁺ (C) fractions. *n* = 10 for all groups except rapamycin (*n* = 4). Data are shown in box-and-whisker plots representing the median, quartiles, and range. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with control groups by RM-ANOVAs followed by Holm-Sidak post hoc tests.

Fig. 4. CD4⁺Foxp3⁺ T cell fractions express ALDH1 after allogeneic stimulation

(A to D) Human MLR cultures were set up using immuno-magnetically bead-separated CD3⁺ and irradiated allogeneic CD3⁻ PBMCs at a 1:1 ratio in sextuplicate. (A) Representative histograms of Aldefluor flow cytometric analysis from MLR day 7 using a gating strategy that distinguishes six CD4⁺ T cell subsets on the basis of the expression of CD25, CD45RA, and CD127. The threshold of Aldefluor positivity is based on the DEAB negative control. (B) Aldefluor positivity within various CD4⁺ fractions from unstimulated cells ($n = 8$) and cells stimulated in MLR for 3 or 7 days ($n = 6$). (C) Aldefluor positivity in MLR cultures that were either untreated or treated with mafosfamide, CsA, or rapamycin. The example shown is representative of six independent experiments. (D) ALDH1A1 expression by CD4⁺ T cell fractions for unstimulated cells or cells stimulated in MLR. The expression is shown relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Error bars show SEMs. Lines depict data from the same individual over time. (E) ALDH activity in PB CD4⁺ T cells retrieved from patients on day 3 after alloBMT ($n = 6$) and the PB or BM of their healthy BM donors ($n = 2$).

Fig. 5. ALDH inhibition with DEAB blocks proliferation and activation of CD4⁺ T cells in MLR and sensitizes T_{reg}s to mafosfamide

Human MLR cultures were set up as in Fig. 4. (A to C) ALDH activity and alloantigen-induced activation and proliferation in MLR cultures were assessed by combining Aldefluor testing and the CellTrace Violet proliferation marker. Cells were first gated for viability before other analyses. $n = 6$. (A) Proliferation patterns of six CD4⁺ T cell subsets based on the expression of CD25, CD45RA, and CD127 for untreated cells or cells treated with 50 μ M DEAB from days 0 to 3. The percentages of proliferating cells within each fraction are shown. (B) Expression of the early activation marker CD69 by Aldefluor⁺ cells of various CD4⁺ T cell fractions. (C) CD69 expression at days 3 and 7 of MLR for CD4⁺ cells that were either untreated or treated with DEAB from days 0 to 3. Lines connect paired samples. (D) DEAB was added to designated MLR cultures from days 0 to 3 followed by either no additional treatment or treatment with mafosfamide (7.5 μ g/ml) on day 3. Cells were analyzed with the gating strategy using Foxp3 and CD45RA expression before assessment for viability. $n = 6$ for all treatments except DEAB alone ($n = 4$). * $P < 0.05$, ** $P = 0.01$, *** $P = 0.001$ by paired t tests (C) or RM-ANOVAs (D).

Fig. 6. GVHD protection conferred by PTCy is dependent on the presence of T_{reg}
 NSG mice were irradiated (2.5 Gy) followed by infusion of 5×10^6 human PBMC grafts or RPMI. Human PBMC grafts were flow cytometrically sorted to include all PBMCs or PBMCs selectively depleted of nT_{reg} s and eT_{reg} s. Designated groups received PTCy (100 mg/kg) on day +3. All animals were followed daily for survival. Combined results of four separate experiments are shown.

Table 1
Clinical characteristics of patients included for analyses

	No aGVHD (<i>n</i> = 16)	Grade II aGVHD (<i>n</i> = 16)	Grade III–IV aGVHD (<i>n</i> = 15)
Age range (median), years	25–58 (48)	22–63 (50.5)	27–61 (52)
Male/female (%)	38/62	50/50	60/40
Disease type	AML 8, ALL 2, MDS 2, HL 2, CML 1, NHL 1	AML 9, ALL 3, MDS 2, HL 1, MM 1	AML 8, ALL 4, MDS 1, HL 1, CML 1
Matched-related/matched-unrelated donors (%)	62/38	69/31	47/53
cGVHD (%)	6	12	20
Corticosteroids used for aGVHD treatment (%)	N/A	88	100
Second systemic immunosuppressants used for aGVHD treatment (%)	N/A	69	93

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; HL, Hodgkin lymphoma; CML, chronic myelogenous leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; N/A, non-applicable.

Table 2

Statistical comparisons of various T cell fractions

As per statistical methods, calculations were performed on natural logarithmically transformed numbers but, for more intuitive comprehension by the reader, are presented as raw percentages or counts. Arrows in parentheses indicate the direction of trending differences. T_{eff}: effector T cell.

T cell fraction	Immunophenotype	Cell type	Percentages			Total numbers		
			Change	Fold change (95% CIs)	P	Change	Fold change (95% CIs)	P
Patients post-alloBMT compared with donors								
All CD8	CD3 ⁺ CD8 ⁺	CD8				↔	0.33–2.66	0.91
All CD4	CD3 ⁺ CD4 ⁺	CD4				↓	0.071–0.17	0.0001
CD25 ⁺ Foxp3	CD3 ⁺ CD4 ⁺ CD25 ⁺ Foxp3 ⁺	T _{reg} /T _{eff}	↑	1.53–3.08	0.002	↓	0.28–0.63	0.005
I	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{lo}	Naïve T _{reg}	↓	0.69–0.93	0.02	↓	0.15–0.29	0.001
II	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{hi}	eT _{reg}	↑	1.79–3.99	0.004	↔	0.51–1.44	0.58
III	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{lo}	Fr III	↑	1.49–2.46	0.004	↓	0.19–0.46	0.004
IV–V	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 [–]	Memory/effector T _{con}	↑	1.13–1.58	0.02	↓	0.12–0.25	0.001
VI	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 [–]	Naïve T _{con}	↓	0.14–0.28	0.002	↓	0.015–0.06	0.001
Patients with acute GVHD compared with patients without acute GVHD								
All CD8	CD3 ⁺ CD8 ⁺	CD8				↔	0.34–1.27	0.23
All CD4	CD3 ⁺ CD4 ⁺	CD4				↔	0.40–1.08	0.18
CD25 ⁺ Foxp3	CD3 ⁺ CD4 ⁺ CD25 ⁺ Foxp3 ⁺	T _{reg} /T _{eff}	↑	1.71–2.94	<0.0001	↑	1.10–2.56	0.04
I	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{lo}	Naïve T _{reg}	↑	1.05–1.52	0.02	↔ (↑)	1.02–1.25	0.07
II	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{hi}	eT _{reg}	↑	1.15–2.26	0.01	↔ (↑)	0.99–2.03	0.09
III	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 ^{lo}	Fr III	↑	1.54–2.75	0.0003	↔ (↑)	1.16–2.52	0.06
IV–V	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 [–]	Memory/effector T _{con}	↓	0.80–0.92	0.001	↔ (↓)	0.36–0.98	0.06
VI	CD3 ⁺ CD4 ⁺ CD45RA ⁺ Foxp3 [–]	Naïve T _{con}	↓	0.59–0.99	0.05	↔ (↓)	0.42–0.98	0.06