## Early Th1 immunity promotes immune tolerance and may impair graft-*versus*-leukemia effect after allogeneic hematopoietic cell transplantation

Acute graft-*versus*-host disease (aGvHD) is an unpredictable immunological complication that affects the skin, gut, or liver following allogeneic hematopoietic cell transplantation (HCT). Donor Th1 cells can initiate aGvHD after recognition of recipient alloantigens and migration to target tissues *via* homing receptors.[1] Paradoxically, experiments in animal models of transplantation suggest that deficiency of the inflammatory Th1 cytokine IFN-γ can exacerbate aGvHD severity and mortality.[2,3] Investigators have interpreted these results to mean that other cell types are capable of generating aGvHD or that loss of a protective function related to IFN-γ was responsible for the intensified alloreactivity observed in these animal models.

Indeed, IL-17 secreting Th17 cells can initiate aGvHD with skin as the principal target under certain experimental conditions.[4] IFN-γ also up-regulates the inhibitory molecule, programmed cell death 1 ligand [PD-L1,

**Table 1.** Characteristics of total cohort, 42 patients undergoing HLA identical sibling donor transplant using mobilized peripheral blood stem cells.

| | N. of patients with characteristic, for indicated grade of aGvHD (percentage) | | | | |
|---|---|---|---|---|---|
| Characteristic | GvHD Grade 0-1 N= 11 | Grade 2-4* N= 31 | Univariate analysis *P* | Multivariate HR (95% CI) | *P* |
| Age in years | | | | | |
| Median | 54 | 52 | 0.529 | | |
| Range | 42-65 | 24-70 | | | |
| Sex | | | | | |
| Male | 9 (82) | 16 (52) | 0.080 | | |
| Female | 2 (18) | 15 (48) | | | |
| Diagnosis | | | | | |
| AML + MDS | 3 (27) | 13 (42) | – | | |
| CML + MPD | 1 (9) | 1 (3) | | | |
| Lymphoid malignancy | 7 (64) | 17 (55) | | | |
| Disease risk** | | | | | |
| Standard | 4 (36) | 16 (52) | 0.384 | | |
| High risk | 7 (64) | 15 (48) | | | |
| Conditioning regimen | | | | | |
| Myeloablative | 4 (36) | 13 (42) | 0.746 | 1 | |
| Reduced intensity | 7 (64) | 18 (58) | | 0.94 (0.40-2.21) | 0.887 |
| Donor / recipient sex | | | | | |
| Female to male | 1 (9) | 4 (13) | 1.00 | | |
| Other | 10 (91) | 27 (87) | | | |
| aGvHD prophylaxis | | | | | |
| CSA + methotrexate | 4 (36) | 13 (42) | 0.746 | | |
| CSA + MMF | 7 (64) | 18 (58) | | | |
| CD34+, x $10^6$/kg | | | | | |
| Median | 7.79 | 6.93 | 0.910 | | |
| Range | 4.63-10.4 | 3.92-10.1 | | | |
| $CD3^+$, x $10^7$/kg§ | | | | | |
| Median | 32.1 | 34.3 | 0.864 | | |
| Range | 16.8-87.9 | 16.0-162 | | | |
| Neutrophil engraftment | | | | | |
| Median day | 18 | 19 | 0.897 | | |
| Range | 10-31 | 14-27 | | | |
| aGvHD target organ | | | | | |
| None | 8 (73) | – | – | | |
| Skin only | 3 (27) | 4 (13) | | | |
| Gut only | – | 18 (58) | | | |
| Multi-organ | – | 9 (29) | | | |
| Day 100 survival† | | | | | |
| Alive | 10 (91) | 30 (97) | – | | |
| Dead | 1 (9) | 1 (3) | | | |
| Engrafted sST2 (ng/mL) | | | | | |
| Median | 16.0 | 22.0 | 0.081 | | |
| Range | 0.1-215 | 6-435 | | | |
| Day+30 sST2 (ng/mL) | | | | | |
| Median | 14.0 | 26.0 | 0.043 | 1.02 (1.01-1.02) | <0.001 |
| Range | 0.1-232 | 6.0-521 | | | |

| | | | | | |
|---|---|---|---|---|---|
| T-cell subsets‡ | | | | | |
| Total CD4+ (%) | | | | | |
| Median | 61.3 | 59.1 | 0.553 | | |
| Range | 16.4-77.0 | 35.3-78.9 | | | |
| Total Th1 (%) | | | | | |
| Median | 18.7 | 6.29 | 0.027 | 0.90 (0.93-0.97) | 0.004 |
| Range | 3.16-28.8 | 1.18-16.9 | | | |
| CLA+ Th1 (%) | | | | | |
| Median | 0.78 | 2.78 | 0.001 | | |
| Range | 0.18-3.54 | 0.39-11.2 | | | |
| α4β7+ Th1 (%) | | | | | |
| Median | 21.2 | 31.5 | 0.021 | | |
| Range | 5.72-59.0 | 12.7-41.2 | | | |
| CLA-α4β7- Th1 (%) (Conventional Th1) | | | | | |
| Median | 77.4 | 66.0 | 0.016 | | |
| Range | 39.5-94.1 | 47.3-82.9 | | | |
| Total Th17 (%) | | | | | |
| Median | 0.41 | 0.72 | 0.219 | | |
| Range | 0.05-2.13 | 0.06-2.46 | | | |
| CLA+ Th17 (%) | | | | | |
| Median | 12.0 | 9.16 | 0.567 | | |
| Range | 1.08-51.6 | 2.34-35.9 | | | |
| α4β7+ Th17 (%) | | | | | |
| Median | 14.4 | 13.6 | 0.699 | | |
| Range | 1.92-41.9 | 3.84-41.7 | | | |
| CLA-α4β7- Th17 (%) (Conventional Th17) | | | | | |
| Median | 70.2 | 74.9 | 0.122 | | |
| Range | 46.0-84.7 | 41.7-92.5 | | | |

*aGvHD: acute graft-versus-host disease; HR: hazard ratio; CI: confidence interval; AML: acute myelogenous leukemia; MDS: myelodysplastic syndrome; CML: chronic myeloid leukemia; MPD: myeloproliferative disorder; CSA: cyclosporine; MMF: mycophenolate mofetil; sST2: soluble suppression of tumorigenicity 2; CLA: cutaneous lymphocyte antigen. *Maximum grade of aGvHD during first 100 days. aGvHD occurred at a median of 37 days (range 13-93 days) after allogeneic hematopoietic cell transplantation. **Standard risk disease is defined by acute leukemia in CR1 or 2, CML in chronic phase 1, MDS without excess blasts. All others were considered high-risk disease. §Data were available for 38 of the 42 patients. †Causes of death included: relapse of malignancy (n=1) and infection (n=1). ‡CD4+ cells and Th subsets were analyzed at time of neutrophil engraftment. Total CD4+ T cells were expressed as the percentage of CD3+ T cells, while total Th1 and Th17 cells were expressed as the percentage of CD4+ T cells. Skin-homing (CLA+), gut-homing (α4β7+), and conventional (CLA-α4β7-) Th1 or Th17 subsets were expressed as a percentage of the total Th1 or Th17 population.*

(CD274)].[5,6] Binding of PD-L1 to its receptor programmed cell death 1 [PD1, (CD279)] on T cells leads to apoptosis and promotes immune tolerance.[7] Thus, IFN-γ seems to have opposing functions during HCT. IFN-γ expression is increased at sites of active aGvHD while complete Th1 deficiency intensifies aGvHD severity. Although such findings present an apparent contradiction, it is possible that differing migration patterns by the various cell types or expression of effector molecules in the systemic *versus* local compartments might explain the contrasting outcomes of such studies. We tested the hypothesis that effector T cells can be inflammatory [(mediating pro-GvHD and graft-*versus*-leukemia effects (GvL)] or inhibitory (mediating anti-GvHD/GvL effects) depending on their expression of tissue-homing molecules on T cells, such as cutaneous lymphocyte antigen (CLA) or the gut-homing marker α4β7.

Patients with hematologic malignancies undergoing T-cell-replete, matched-related donor (MRD) HCT were accrued to an aGvHD biomarker study (n=42). All patients received HLA-identical peripheral blood stem cell grafts followed by prophylaxis with cyclosporine and either methotrexate or mycophenolate mofetil. By day+100, grade 2-4 or grade 3-4 aGvHD was diagnosed clinically in 31 or 4 patients, respectively (Table 1).[8] aGvHD was confirmed by biopsy in the majority (n=23).

Flow cytometry, soluble suppression of tumorigenicity 2 (sST2), and statistical analysis are presented in the Online Supplementary Methods.

Tissue-specific T cells were identified in blood from healthy volunteers (n=21) or from recipients of HLA-identical sibling donor transplants at 2 time points: neutrophil engraftment (n=42, median 19 days post HCT) and at day+30 (n=37) (Online Supplementary Figure S1). Among HCT recipients, circulating Th17 cells at engraftment preferentially expressed a skin-homing (CLA+) phenotype (10.5% vs. 2.14%; P<0.001), while Th1 cells demonstrated an increased frequency of gut-homing (α4β7+) phenotype (27.1% vs. 14.0%; P<0.001). Similar homing patterns were seen at day+30 and among healthy volunteers, supporting the importance of Th17 and Th1 subsets for cutaneous or gastrointestinal immunity, respectively (data not shown).

Among patients with grade 2-4 aGvHD (n=31), T-cell analysis could be performed before clinical alloreactivity in all but 3 individuals when sampling occurred during hyperacute symptoms. Both percentage and absolute number of circulating Th1 cells at engraftment were significantly lower in patients developing grade 2-4 aGvHD when compared to recipients with grade 0-1 disease, while Th1 cell percentages were similar between healthy volunteers and HCT recipients without alloreactivity

(Table 1 and Figure 1). After adjusting for conditioning intensity and day+30 sST2, decreased percentages of total Th1 cells at engraftment continued to be a significant predictor of moderate to severe aGvHD (Table 1).[9] We were unable to find an association between total Th1 cells at day+30 and Th17 cells with the development of aGvHD (Table 1 and Figure 1). The remainder of analyses focused on Th1 subsets at engraftment.

We hypothesized that the observed decrease in Th1 cells in patients with clinical alloreactivity could represent the migration of effector cells from the systemic circulation to an aGvHD target tissue. Consistent with this idea, patients with grade 2-4 aGvHD had increased frequencies of circulating skin-homing ($CLA^+$) Th1 cells and

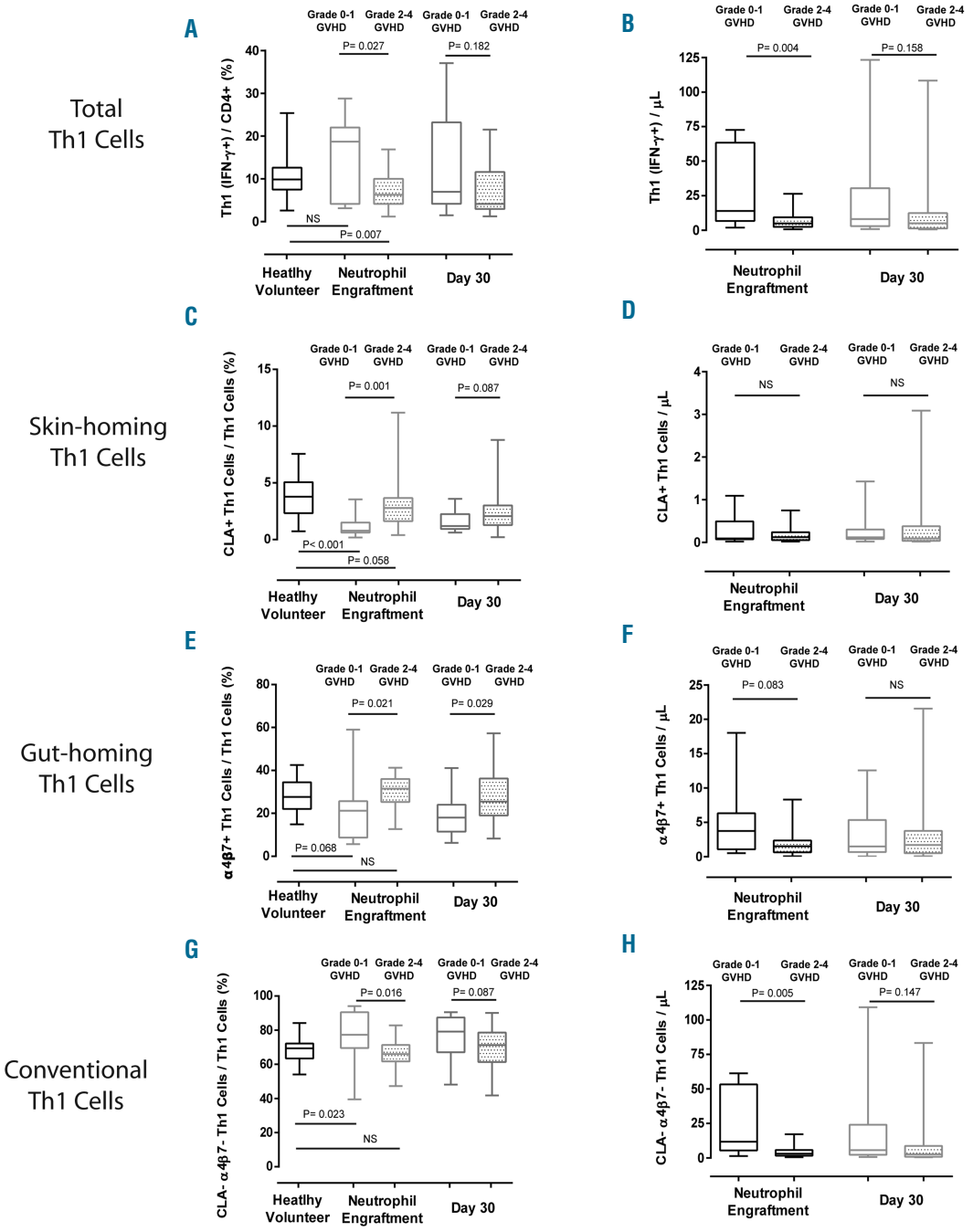


**Figure 1. The development of acute graft-*versus*-host disease (GvHD) is associated with changes in Th1 subsets early after allogeneic hematopoietic stem cell transplantation.** The percentages or absolute numbers of total (A and B), skin-homing (CLA+) (C and D), gut-homing (α4β7+) (E and F), or conventional (CLA-α4β7-) Th1 cells (G and H) are compared between healthy volunteers and patients with grade 0-1 or grade 2-4 acute GvHD. Box plots define the values for median, range, and 25th and 75th percentiles. Engraftment and day+30 Th1 subset frequencies are presented in graphs A, C, E, and G and absolute numbers of the respective populations are presented in graphs B, D, F, and H. Two-tailed *P* values were calculated using the Mann-Whitney *U* test.

gut-homing (α4β7⁺) Th1 cells, but lower numbers of conventional (CLA- α4β7⁻) Th1 cells than patients with grade 0-1 aGvHD (Table 1 and Figure 1). When analyzing patients with isolated skin aGVHD, CLA⁺ Th1 cells were increased in patients with more severe skin involvement (stage 3-4) *versus* those with stage 0-2 (6.51% *vs.* 0.78%; *P*=0.013). After excluding individuals with skin aGvHD, α4β7+ Th1 cells tended to be higher in patients with any stage of gut-only aGvHD (32.8% *vs.* 21.8%; *P*=0.08). The absolute decrease in conventional (CLA- α4β7-) Th1 cells in the blood of patients developing aGvHD could result from upregulation of homing-markers within this lymphocyte population followed by migration from the circulation to a peripheral compartment, thus explaining why the relative frequency of tissue-specific T cells in blood changed but not the absolute numbers (Figure 1).

Since alloimmunity can lead to both detrimental aGvHD and beneficial GvL effects, exploratory analyses were performed to determine whether circulating total Th1 subsets at engraftment could impact the cumulative incidence of malignancy relapse in patients with MDS/AML (n=16; 7 relapses post HCT). The majority of individuals had standard risk disease (n=13), and all patients had less than 5% blasts prior to HCT. As suggested by the aGvHD data, decreased percentages of total Th1 cells at engraftment predicted a lower risk of MDS/AML relapse after adjustment for conditioning intensity and disease risk (HR 0.84; 95%CI: 0.71-0.99; *P*=0.047). To investigate the potential inhibitory mechanisms of IFN-γ on GvL in our study, we examined PD1 and PD-L1 expression on circulating CD4⁺ T cells and CD14⁺ monocytes, respectively, between engraftment and day +30 after HCT (*Online Supplementary Figure S2*).[5,10]

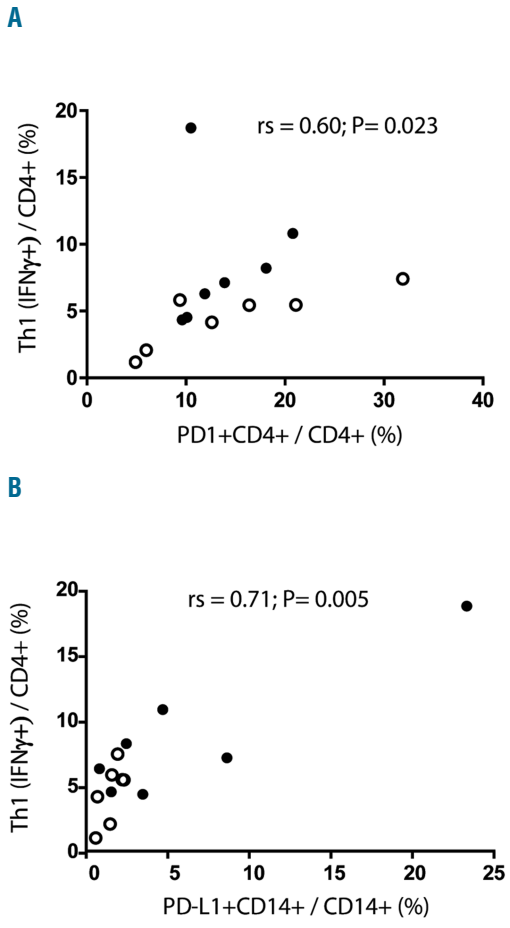


**Figure 2. Th1 subsets correlate positively with PD1 and PDL1 expression by CD4⁺ T helper cells** (A) **and CD14⁺ monocytes** (B), **respectively.** Data were derived from 14 patients with myelodysplastic syndrome/acute myelogenous leukemia undergoing HLA-identical sibling donor allogeneic hematopoietic cell transplantation. Filled circles represent patients with malignancy relapse after transplant. Two-tailed *P* values were calculated using the Spearman's rank correlation coefficient.

Since cryopreserved specimens at the time of relapse were not available, PD-L1 expression by monocytes was used as a surrogate measure of IFN-γ effects on residual MDS/AML cells persisting after HCT. In the 14 MDS/AML patients with accessible samples, total Th1 cells at engraftment correlated positively with both PD1⁺CD4⁺ T cells and PD-L1⁺CD14⁺ monocytes (Figure 2). Increased PD-L1 expression by CD14⁺ monocytes also predicted a greater risk for MDS/AML relapse (HR 1.11; 95%CI: 1.01-1.24; *P*=0.048).

Early increases in Th1 cells were associated with decreased alloreactivity and greater PD-L1 expression following HCT . Furthermore, we expanded upon the findings from previous animal studies by identifying tissue-homing subsets within this Th1 population that are critically important for either generating or preventing aGvHD.

Clinical manifestations of aGvHD often occur early after engraftment and are restricted to particular tissues, suggesting tissue-specific expression of effector molecules or peripheral localization of lymphocytes are responsible for aGvHD. Similar to our previous Treg data, our current report supports lymphocyte tissue-trafficking as an important factor for alloreactivity, as patients developing grade 2-4 aGvHD had increased skin-homing (CLA⁺) and gut-homing (α4β7+) Th1 cells and tended to have more cutaneous or gastrointestinal symptoms, respectively.[11,12] As opposed to lymphocyte migration determining aGvHD, it is also plausible that conventional (CLA-α4β7-) Th1 cells possess immunoregulatory properties. When we analyzed Foxp3 expression, the Foxp3⁺ cells appeared separate and distinct from the Th1 subsets, indicating that these IFN-γ+ lymphocytes likely were not traditional Tregs (*Online Supplementary Figure S3*). IFN-γ induces expression of programmed death ligand 1 (PD-L1) on antigen presenting cells, which leads to apoptosis of PD1-expressing T cells and anergy.[7] Consistent with the idea of IFN-γ mediated immune regulation, we have shown a very strong, positive correlation between the percentage of total Th1 cells at engraftment with the expression of PD1 and PD-L1 by CD4⁺ T cells or CD14⁺ monocytes, respectively. This preliminary data indicate that early Th1 cell proliferation primarily within the CLA-α4β7- compartment could promote peripheral tolerance by up-regulating the inhibitory protein PD-L1. Although we showed a correlation between IFN-γ and PD-L1, it is unknown whether the expression of PD-L1 on monocytes is a valid surrogate for IFN-γ effects on AML cells or the cause for relapse, and further experimentation is required.

Some, but not all, research supports a role for Th17 cells in the generation of cutaneous aGvHD.[4,13] We found that CLA was expressed more commonly by Th17 cells than by Th1 cells; however, the frequency of cells in Th17 subsets did not correlate with aGvHD. These analyses were possibly limited by the low incidence of cutaneous alloreactivity. Strikingly, about 70% of our MRD cohort developed aGvHD with gastrointestinal

symptoms. Reassuringly, sST2, a validated biomarker for aGvHD, was elevated in patients developing alloreactivity.[9] The high incidence of gastrointestinal aGvHD likely was due to early endoscopy, as previously reported.[14]

Malignancy relapse and severe aGvHD are the primary causes for early HCT failure. Blockade of immune checkpoints including CTLA-4 with ipilimumab or PD1 with nivolumab and pembrolizumab could improve GvL, but also may increase aGvHD.[10,15] Concerns regarding aGvHD exacerbation may have dampened enthusiasm for PD1 blockade after allogeneic HCT.[15] Our data confirm preclinical research that early Th1 immunity is associated with immune tolerance and increased PD-L1 expression. It also potentially explains the paradoxical nature of IFN-γ during HCT in terms of lymphocyte compartmentalization (i.e. inflammatory tissue-homing Th1 cells *vs.* inhibitory conventional Th1 cells). If validated, these results could identify patients with low aGvHD risk who may benefit from therapies targeting immune checkpoints to promote GvL and prevent malignancy relapse.

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