Quantitative Assessment of Posttransplant Host-Specific Interleukin-2-Secreting T-Helper Cell Precursors in Patients With and Without Acute Graft-Versus-Host Disease After Allogeneic HLA-Identical Sibling Bone Marrow Transplantation

By Thomas Nierle, Donald Bunjes, Renate Arnold, Hermann Heimpel, and Matthias Theobald

Recent studies in mice and humans have emphasized an important contribution of host-reactive minor histocompatibility antigen (mH)-specific lymphokine-secreting donor T-helper cells (Th) for the induction of acute graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT). By using limiting dilution (LD) and clonal specificity analyses, we investigated in 14 patients with and without acute GVHD after non–T-depleted HLA-identical sibling BMT whether posttransplant host-reactive mH-specific interleukin-2 (IL-2)-secreting Th are involved in the development of clinically significant acute GVHD and the establishment of tolerance. At different time intervals posttransplant (I, days 0 through 45; II, days 45 through 90; III, days 90 through 180), host-specific IL-2-secreting Th-precursors (Th-p) were quantitatively assessed in six patients during clinically apparent grade II-III acute GVHD. Frequencies of responding Th-p ranged from 1/13,000 to 1/174,000. The presence of host-specific Th-p was significantly correlated with the development of grade II-III acute GVHD (P = .0003 by Fisher’s exact test). The detectability of host-specific Th-p preceded the clinical onset of grade II-III acute GVHD. Host-specific Th-p were no longer detectable after the clinical resolution of grade II-III acute GVHD. No subsequent chronic GVHD was observed in these patients. However, prolonged occurrence of host-specific Th-p was accompanied by clinically persisting acute GVHD and the onset of secondary chronic GVHD. In patients with no acute GVHD (grade 0) (n = 7) and grade I (n = 1) acute GVHD, host-specific Th-p were not detectable at all. We conclude that host-reactive Th are critically involved in the development and maintenance of acute GVHD and may contribute to the establishment of tolerance after genotypically HLA-identical sibling BMT.

MATERIALS AND METHODS

Patients. Fourteen mixed lymphocyte culture (MLC)-negative and lymphocyte crossmatch-negative, genotypically HLA-identical sibling donor-patient pairs were investigated posttransplant for the presence of circulating host-specific Th-p. The relevant clinical details of the study group are listed in Table 1. All patients received non-T-depleted marrow grafts from their genotypically HLA-identical sibling donor-patient pairs, posttransplant for the presence of circulating host-specific Th-p. The relevant clinical details of the study group are listed in Table 1. All patients received non-T-depleted marrow grafts from their genotypically HLA-identical sibling donors in the BMT-Unit of the Ulm University Hospital (Ulm, Germany). Successful engraftment was achieved in all patients. This was proved by assessment of erythrocyte antigens of donor-type after BMT, by posttransplant detection of donor sex chromosomes in the case of sex mismatch between donor and recipient, and by the amplification of variable number of tandem repeats (VNTR) in cell sorter-separated T-lymphocyte and monocyte cell populations using the polymerase chain reaction (PCR). Even in three cases of early leukemic relapse (patients no. 11, 12, and 14), T lymphopoiesis appeared to be of complete donor origin, as determined by the latter approach (data not shown). In all patients, cyclosporine (CsA) and methotrexate (Mtx) were administered as posttransplant immunosuppressive agents for the prevention of GVHD, as previously reported. The diagnosis of acute and chronic GVHD was established by clinical and histopathologic criteria. Chronic GVHD was classified according to Sullivan et al. Six patients developed grade II-III acute GVHD. Grade I acute GVHD was diagnosed in one patient.

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Seven patients never showed any clinical signs of acute GVHD. Two patients developed secondary chronic GVHD and one patient developed primary chronic GVHD. GVHD was treated with high-dose inactivated pooled human AB serum, L-glutamine, 5\( \times \)10\(^{-2}\) mol/L 2-mercaptoethanol, 100 \( \mu \)g/mL streptomycin, and 0.5 mmol/L HEPES, and continued at 100 mg/mL penicillin. The culture medium used for the establishment of pretransplant donor and host as well as completely HLA-mismatched unrelated EBV-LCL was found free of mycoplasma contamination on repeated testing and served as responder and stimulator cells.

In vitro Epstein-Barr virus-transformed B-lymphoblastoid cell lines (EBV-LCL). For the establishment of pretransplant donor and host as well as completely HLA-mismatched unrelated EBV-LCL, E-rosette-purified T and non-T cells were prepared from PBMC by incubation with neuraminidase-treated sheep red blood cells (SRBC), as previously described. Non-T cells (5 \( \times \)10\(^{6}\)/mL) were infected for 4 hours at 37°C with mycoplasma-free supernatant (SN) of the EBV-producing B95.6 cell line. After washing, EBV-infected cells were maintained in RPMI-1640 medium supplemented with 10% (vol/vol) heat-inactivated pooled human AB serum, 25 mmol/L HEPES, 2 mmol/L L-1-glutamine, 5 \( \times \)10\(^{-2}\) mol/L 2-mercaptoethanol, 100 \( \mu \)g/mL streptomycin, and 100 U/mL penicillin. The culture medium used for restimulation was supplemented with 200 \( \mu \)g/mL netilmicyn.

Cell suspensions. Heparinized peripheral blood was collected pretransplant from host and donor, from the host posttransplant, and from completely HLA-mismatched unrelated healthy volunteers after informed consent. The times of posttransplant peripheral blood harvesting ranged from day +20 to day +180 (time interval I, days 0 through 45 posttransplant; time interval II, days 45 through 90 posttransplant; time interval III, days 90 through 180 posttransplant). Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of the diluted blood over Ficoll-Hypaque (\( \rho = 1.077 \) g/mL; Biochrom). Cells were washed twice in phosphate-buffered saline (PBS; GHBCo Ltd, Paisley, UK) and cryopreserved in the vapour phase of liquid nitrogen. Before use, cells were immediately thawed, washed twice in PBS, and resuspended in complete medium, PBMC served as responder and stimulator cells.
tient pairs and unrelated healthy volunteers were performed by conventional serotyping of HLA-A, -B, -C, -DR, and -DQ alleles and by oligonucleotide typing of HLA-DPB1 alleles. All sibling donor-patient pairs were negative in the standard MLC and the lymphocyte crossmatch, and were genotypically matched for HLA-A, -B, -C, -DR, -DQ, and -DPB1 alleles (data not shown).

Limiting dilution (LD) cultures. The methodologic details of LD analysis of human Th-p have been recently reported. In brief, replicate microcultures (n = 16) were set up in 96-well V-shaped microtiter plates (Nunc, Wiesbaden, Germany) with graded numbers of posttransplant patient PBMC responder cells in the presence of 1 × 10^4 irradiated stimulator cells and 10 U human recombinant IL-2 (rIL-2)/mL (Eurocetus, Amsterdam, The Netherlands) in a final volume of 200 μL/well. Pretransplant host PBMC, pretransplant donor PBMC, or completely HLA-mismatched unrelated PBMC served as irradiated stimulator cells. LD cultures for posttransplant frequency analysis of host-reactive, donor-reactive (autoimmune), and allo-MHC-reactive Th-p were set up simultaneously for a given patient. A culture period of 14 days has been proved optimal for frequency calculation of Mh-specific Th-p (data not shown). Thus, restimulation of LD cultures was performed after 14 days. Cultures were washed twice with PBS and split into two aliquots, the first of which was restimulated with the original primary antigen and the second of which received pretransplant cells from the donor (if originally primed to host) or pretransplant host cells (donor and HLA-mismatched primed cultures) (1 × 10^5 irradiated EBV-LCL restimulator cells/well). After 20 to 24 hours, 75 μL of SN was removed and transferred to flat-bottom microtiter plates (Nunc) for IL-2 analysis.

Clonal specificity analysis. For clonal specificity analysis of posttransplant anti-host-induced Th-p from LD cultures, individual IL-2-producing colonies were only evaluated if they displayed a chance for clonality of greater than 70%, as calculated from the respective frequency values.

Assay for IL-2 activity. IL-2 activity was determined using a cortisol bioassay, as recently described. Briefly, this approach uses the fact that only viable cells are capable of cleaving the dye MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; No. M2128; Sigma Chemical Co, St Louis, MO]. CTL-16 cells served as indicators. Growth of the CTL-16 cells is IL-2 dependent and not promoted by human IL-4. Each microwell was incubated with [5 × 10^4] CTL-16 cells for 20 to 24 hours in a final volume of 100 μL. Thereafter, 10 μL of MTT solution (3 mg/mL) was added. Three hours later, 100 μL of a solution of 10% sodium dodecyl sulfate (SDS)-0.01 N HCl was added to each microwell to dissolve the blue crystals. After overnight incubation, the optical density (OD) of individual microwells was read at a wavelength set to 570 nm using a Nunc enzyme-linked immunosorbent assay (ELISA) reader (Model NJ-2000). The maximum MTT cleavage was assessed in two wells containing 20 U rIL-2/mL. All microwell cultures with an OD that exceeded the mean OD plus 3 times the standard deviation (SD) of control cultures containing stimulator cells only were considered positive.

Statistical analysis. Calculation of frequencies from LD analysis was based on the Poisson distribution. The values for the frequency, 95% confidence limits for the frequency, and the probability for single-hit kinetics were calculated by likelihood maximization and χ^2 minimization as described by Taswell. Frequencies of posttransplant Th-p are only shown if the P value (probability for single-hit kinetics) and the χ^2 value were >0.05 and less than 10, respectively.

RESULTS

Frequency and specificity of posttransplant host-reactive Th-p. Fourteen patients with grade II-II (n = 6) and grade 0-I (n = 8) acute GVHD (Table 1) were investigated for the presence of circulating host-specific Th-p at different time intervals posttransplant. All patients received non-T-depleted marrow grafts from their MLC-negative and lymphocyte crossmatch-negative, genotypically HLA-identical sibling donors. As shown in Table 2, posttransplant host-reactive

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Frequencies of posttransplant host-reactive IL-2-secreting Th-p were calculated according to Poisson distribution. Abbreviations: 95%-CL, 95% confidence limit for each frequency value; P, probability of single-hit kinetics; χ^2, value for χ^2; ND, not detectable (frequency < 1/400,000); NA, not applicable.
Fig 1. Simultaneous frequency analysis of posttransplant host-reactive, donor-reactive (autoreactive), and allo-MHC-reactive IL-2-secreting Th-p in patients with grade II-III (○) and grade 0-1 (●) acute GVHD. Frequencies of posttransplant IL-2-secreting Th-p were calculated according to Poisson distribution and plotted. A logarithmic scale is used to indicate the frequencies of Th-p (not detectable, frequency < 1/400,000). (A) Time interval I, days 0 through 45 after BMT; (B) time interval II, days 45 through 90 after BMT; (C) time interval III, days 90 through 180 after BMT.
Th-п were detectable in six patients with grade II-III acute GVHD. Frequencies of responding Th-п ranged from 1/13,000 to 1/174,000. The presence of posttransplant host-reactive Th-п was highly correlated with the development of grade II-III acute GVHD, as determined by Fisher's exact test (P = .0003). Host-reactive Th-п occurred as early as 20 days posttransplant and preceded the clinical onset of grade II-III acute GVHD (patients no. 9, 10, and 13). After resolution of the clinical symptoms of grade II-III acute GVHD, host-reactive Th-п were no longer observed (patients no. 5, 8, and 9). These patients did not develop subsequent chronic GVHD. However, prolonged detectability of host-reactive Th-п was associated with clinically persisting acute GVHD and onset of secondary chronic GVHD (patients no. 10 and 13). In contrast, among eight patients with no (grade 0) (n = 7) or grade I (n = 1) acute GVHD, host-reactive Th-п have not been found at all (Fig 1A through C). Although one of these patients (patient no. 7) developed primary chronic GVHD at day 108, we did not detect host-reactive Th-п at this time (Fig 1C). The antigen specificity of host-reactive Th-п in patients with grade II-III acute GVHD was indicated by the finding that frequencies of anti-host-induced Th-п were beyond the threshold of detectability (<1/400,000) after restimulation of split-well cultures with pretransplant donor cells (Fig 1A through C). No autologous background reactivity occurred after primary induction of post-transplant Th-п with pretransplant donor stimulator cells (Fig 1A through C). In all 14 patients, frequencies of posttransplant allo-MHC–specific Th-п were individually and on average higher in magnitude as compared with host-reactive Th-п (Fig 1A through C), but were below those obtained in pretransplant donor and unrelated normal control individuals (data not shown). However, frequencies of allo-MHC–reactive Th-п were not influenced by acute or chronic GVHD, application of steroids, and infectious complications.

Clonal specificity of posttransplant anti-host-induced Th-п. After restimulation of anti-host-induced posttransplant Th-п with pretransplant donor cells, a small proportion of Th colonies produced IL-2. However, frequencies of anti-host-induced donor-reactive Th-п could not be determined because the few colonies showing this reactivity were both detectable independent of the responder cell dose seeded and not sufficient for frequency calculation. Therefore, the antigen specificity of anti-host-induced Th-п among patients with grade II-III acute GVHD was quantitatively confirmed at the clonal level. The relative distribution of cumulative posttransplant host-specific IL-2–producing Th colonies was evaluated from LD split-well cultures, displaying a chance of clonality of greater than 70%, as estimated from the respective frequency values. This selection was performed not only to sustain a high enough chance for clonality but also
to obtain a sufficient number of IL-2-producing colonies for specificity analysis. Results are summarized in Table 3. After restimulation of split-well cultures with posttransplant host primary stimulating antigens and pretransplant donor antigens among patients with grade II-III acute GVHD, the vast majority (63% to 83%) of posttransplant anti–host-induced IL-2-producing Th colonies appeared to be exclusively specific for host antigens. Because only few colonies of antihost-induced donor-reactive Th were found among cultures with even higher responder cell numbers, a similar high proportion of host-specific IL-2-producing colonies was detectable among cultures with a less than 70% chance for clonality (data not shown).

**DISCUSSION**

Our study demonstrates a significant correlation between the detection of posttransplant host-specific Th and the presence of grade II-III acute GVHD in HLA-identical sibling BMT. The persistence of host-specific Th was associated with the development of secondary chronic GVHD. The in vitro detectability of posttransplant host-specific Th preceded the clinical onset of grade II-III acute GVHD, as recently demonstrated for host-reactive CTL-p. Other factors, such as patient age, sex mismatch, and previous donor pregnancy, correlated with neither the Th functional status posttransplant nor with the development of acute GVHD.

Between genotypically HLA-identical sibling donor-host pairs, host-reactive donor T cells are, by definition, directed against mH antigens, presented in the context of molecules encoded by the MHC. Recently, we have demonstrated that between genotypically HLA-identical sibling individuals, mH-specific naive Th-p as well as previously in vivo-primed memory Th-p are detectable in substantial frequencies within the class I MHC-restricted CD8 and the class II MHC-restricted CD4 T-cell subsets. The relative contribution of murine CD8 and CD4 T cells to GVHD directed against mH antigens varies according to the particular strain combination examined. In humans, it has been suggested that Th, defined as noncytolytic proliferating T cells, are involved in posttransplant anti-host mH-specific T-cell responses. An important role for mH-reactive Th in the induction of acute GVHD after HLA-identical sibling BMT has been further indicated by the recent finding that the posttransplant detectability of host-specific noncytolytic proliferating T cells rather than CTL correlates with the incidence of severe acute GVHD. More recently, we have demonstrated that high frequencies of pretransplant host-reactive mH-specific donor Th-p significantly correlate with the development of grade II-III acute GVHD in HLA-identical sibling BMT. Accordingly, in four of six patients with grade II-III acute GVHD and detectable host-specific Th-p posttransplant (patients no. 5, 9, 13, and 14), high frequencies of host-specific donor Th-p have been determined pretransplant. Among six of eight patients with grade I acute GVHD and undetectable host-reactive Th-p posttransplant (patients no. 1, 3, 4, 7, 11, and 12), only low frequencies of anti–host-induced donor Th-p, which had to be attributed to an autologous background response, were observed pretransplant. The remaining four patients of the present study (patients no. 2, 6, 8, and 10) have not yet been analyzed pretransplant.

By which mechanisms do host-reactive mH-specific donor Th contribute to the induction of acute GVHD? According to the classical concept of T-T cell cooperation, mH-specific Th are essential for the IL-2-mediated proliferation and differentiation of activated mH-specific CTL-p. Host-reactive mH-specific CTL have been observed during GVHD and histologic GVHD lesions may result from direct CTL-mediated target cell lysis. However, anti–host-directed mH-specific CTL can be generally isolated posttransplant, regardless of whether GVHD occurs. As a consequence, the induction of acute GVHD may depend on the posttransplant presence of host-reactive Th, capable of providing help via IL-2 release for the proliferation and differentiation of host-reactive CTL-p, which may trigger acute GVHD after maturation into cytolytic effector T cells. Apart from the cooperation with CTL-p, Th per se are capable of directly inducing a lethal GVHD-like syndrome via IL-2. Histologic lesions in acute GVHD may also result from IL-2. Recent experimental data emphasize the central role of various other cytokines such as TNF-α, IFN-γ, and IL-1 in the induction of acute GVHD. Inhibition of TNF-α and IL-1 by anti–TNF-α antibody and IL-1 receptor antagonist, respectively, prevents GVHD in animal models to some extent. TNF-α and IFN are produced by IL-2-secreting Th. More distal from Th, IFN may activate monocytes to release TNF and IL-1 and thereby further augment the lymphokine-driven inflammatory process underlying acute GVHD. Both IFN and TNF-α increase the expression of class I and class II MHC molecules as well as bound mH peptides on GVHD target tissues and thus participate in T-cell–dependent

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<td>5/6</td>
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For clonal specificity analysis, individual IL-2–producing colonies were only evaluated if they displayed a chance of clonality of greater than 70%, as estimated from the respective frequency values. The relative distribution of cumulative monoreactive (with either primary stimulating host antigen or donor antigen) and bireactive (with primary stimulating host antigen and donor antigen) IL-2–producing colonies is shown (time interval I, days 0 through 45 after BMT; II, days 45 through 90 after BMT; III, days 90 through 180 after BMT).
events during acute GVHD. In view of these various Th-dependent mechanisms and the results reported in the present study, Th may be involved as critical key effector cells in the pathophysiologic process underlying the development of acute GVHD.

In one patient with grade I acute GVHD, in six patients with no (grade 0) acute GVHD, and in one patient with primary chronic GVHD, posttransplant host-specific Th-p were not detectable at all. The same became evident after resolution of the clinical symptoms of grade II-III acute GVHD in three patients who did not develop subsequent chronic GVHD. The lack of host-specific Th-p in these patients was neither due to an overall absence of posttransplant Th-p nor to an effector cell compartmentalization as a result of steroid application. Thus, the pool size of circulating allo-MHC-reactive Th-p, although decreased to some extent posttransplant, as already established, remained unaffected by steroid therapy, the presence or absence of GVHD, and infectious complications. We conclude that the lack of posttransplant host-reactive Th-p is associated with the absence of severe GVHD, because intermediary cytokines would not be produced and host-reactive CTL-p would not be able to undergo IL-2-driven proliferation and differentiation to function as cytolytic effector T cells on GVHD target tissues. Whether mH compatibility between sibling donor-host pairs may lead to nonresponsiveness at the level of Th-p still remains unclear.

In patients with primary detection of host-specific Th-p during clinically apparent grade II-III acute GVHD and subsequent disappearance of these cells accompanied by the clinical resolution of acute GVHD without onset of chronic GVHD, secondary Th nonresponsiveness cannot be due to mH compatibility between donor and recipient. Does secondary nonresponsiveness of host-reactive mH-specific Th-p represent the establishment of tolerance at the level of IL-2-producing T lymphocytes? Tolerance is observed in a large proportion of mH-mismatched marrow graft recipients. The mechanisms responsible for the establishment of tolerance after genotypically HLA-identical sibling BMT are complex and not completely defined. It is well established that CsA acts to inhibit the synthesis of various lymphokines such as IL-1, IL-2, TNF-α, and IFN. Apart from the immunosuppressive therapy including steroid application, various complex mechanisms of central thymic and peripheral postthymic tolerance induction may contribute to tolerance after BMT. Central tolerance induced by clonal thymic deletion of potentially host-reactive immature T cells is unlikely to be involved in the patients reported here, as mature postthymic donor T cells were grafted with the non–T-depleted marrow inoculum. Whether different nonexclusive mechanisms of peripheral tolerance, including clonal deletion of mature postthymic T cells by the veto phenomenon and induction of clonal anergy of mature postthymic T cells by inadequate or absent costimulatory signals may contribute to tolerance at the level of posttransplant host-reactive Th-p is an open question at present.

In conclusion, our data support the view that host-reactive Th are critically involved in the development and maintenance of acute GVHD. IL-2-secreting Th may also serve as target cells for yet poorly defined mechanisms of peripheral tolerance. These results may further our understanding of acute GVHD and tolerance induction after allogeneic HLA-identical sibling BMT.

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