Prevention of Murine Graft-Versus-Host Disease and Bone Marrow Alloengraftment Across the Major Histocompatibility Barrier After Donor Graft Preincubation With Anti-LFA1 Immunotoxin

By Bruce R. Blazar, Stephen F. Carroll, and Daniel A. Vallera

We have investigated the effects of the in vitro depletion of LFA1 positive cytolytic T lymphocytes, natural killer (NK) cells, and monocytes on the afferent phase of graft-versus-host disease (GVHD). Lethal GVHD was induced across the murine major histocompatibility complex by injecting C57BL/6 (H-2b) bone marrow (BM) cells (a source of stem cells) and splenocytes (S) (a source of T cells) into lethally irradiated B10.BR (H-2d) recipients. Because anti-LFA1 does not bind complement (C1), we conjugated anti-LFA1 \( \alpha \) chain monoclonal antibody (MoAb) to ricin toxin A chain (RTA) as a means of facilitating target cell elimination. A 2-hour preincubation of C57BL/6 bone marrow/spleen (BMS) with anti-LFA1-RTA in the presence of ammonium chloride (a potentiator of immunotoxin toxicity), but not a control immunotoxin (IT), reduced CTL activity by greater than 2 logs, significantly reduced NK cell activity, and prevented B10.BR mice from developing GVHD. Depletion of target cells by toxin-labeled MoAb and not the blockade of the LFA1 molecule by the anti-LFA1 MoAb accounted for our results, because incubating cells with IT in the absence of a potentiator had no effect on GVHD prevention. In contrast, C57BL/6 recipients of C3H BMS grafts only partially benefited from anti-LFA1-RTA preincubation, demonstrating that in this system, different cells not expressing LFA1 were involved in GVHD generation. The same findings observed with anti-LFA1-RTA preincubation were observed with preincubation with L-leucyl-L-leucine methyl ester, a chemical compound eliminating cytolytic cells, providing further support that GVHD induction in the C3H/HeJ into C57BL/6 system is not entirely mediated by classical cytolytic T cells. We next tested anti-LFA1-RTA in a model devised to measure its effect on alloengraftment (B10.BR recipients given lower doses of irradiation). Anti-LFA1-RTA BM preincubation selectively reduced alloengraftment in the model. This observation, combined with experiments showing that LFA1-RTA preincubation, but not anti-Thy1.2 + C' or control IT preincubation, reduced colony-forming unit-spleen formation, indicates that anti-LFA1 \( \alpha \) chain IT may remove accessory cells or stem cells critical to engraftment. Still, anti-LFA1-RTA may be useful for clinical GVHD prevention when combined with positive selection techniques designed to enrich for stem cells.

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tion of donor grafts on GVHD prevention and allograftment.

MATERIALS AND METHODS

**Preparation of anti-T-cell ITs.** Anti-LFA1 α chain MoAb (FD441.8) (Dr Frank Fitch, University of Chicago, Chicago, IL)\(^{20}\) and anti-Thy1.2-RTA (obtained from the American Type Culture Collection [ATCC], Rockville, MD) are rat IgG2b MoAb. Anti-Thy1.2-RTA and anti-LFA1-RTA were synthesized by linking purified MoAb to RTA or RTA\(_s\) (a purified fraction of RTA with reduced carbohydrate content). Both had similar in vitro and in vivo activities against mouse cells. The conjugation technique has been previously described in detail.\(^{26}\) H55 is a control mouse IgG2a anti-human CD5 MoAb\(^{37}\) that was conjugated to RTA. Mel-RTA is a control IT made by linking mel, a mouse IgG2a MoAb recognizing human melanoma antigen,\(^{27}\) to RTA. These reagents are referred to as control IT. We have noted no differences in rat, mouse, or isotype control MoAb used as control IT.

**Gel analysis.** To assess the purity of IT, analysis was performed on a 10% polyacrylamide gel electrophoresis (PAGE) plate (Daiichi Pure Chemicals, Tokyo, Japan) using a Mini Protean II slab cell (BioRad, Richmond, CA) as previously described.\(^{27}\) Quantitation of the bands was performed by densitometric analysis using a Gel Scan Soft Pack Module Software Package (Beckman Instruments, Fullerton, CA).

**Indirect immunofluorescence.** Because IT does not immediately destroy target cells, the binding of anti-Thy 1.2-RTA or anti-LFA1-RTA was determined by standard indirect immunofluorescence techniques. For in vitro analysis, 10\(^5\) Thy 1.2- and LFA1-EL4 cells were incubated with 10 \(\mu\)g/mL IT or a previously determined saturating amount of MoAb for 30 minutes at 4°C. The cells were then washed and incubated for 30 minutes at 4°C with 50 \(\mu\)L of a 1\(\times\) dilution of fluoresceinisothiocyanate (FITC)-conjugated goat anti-rat IgG (GARG). Cells were washed three times, diluted in buffer, and analyzed. Flow cytometry was performed on the FACSscan (Becton Dickinson, Mountain View, CA) as previously described.\(^{2}\) Background binding using a labeled control antihuman T-cell MoAb, 3A1E,\(^{26}\) was subtracted from the results.

**Mice.** C57BL/6 (H-2\(^d\)) and C3H/HeJ (H-2\(^j\)) donor mice, and B10.BR/SpSnJ (H-2\(^d\)) recipient mice were purchased from Jackson Laboratory (Bar Harbor, ME). Donors and recipients were female mice that were 4 to 6 weeks old at the time of BMT.

**BMT.** Our GVHD transplant procedure was described.\(^{24,25}\) When C57BL/6 donors were used, B10.BR recipients were conditioned with 9.0 Gy TBI. Donor BM was collected from the shafts of femurs and tibias. Single cell suspensions of splenocytes were obtained by debranched bone marrow. BM cells plus 1 to 25 \(\times\) 10\(^6\) lymph node (LN) T cells were substituted for donor BM, and TCD with 0.05 to 1 \(\times\) 10\(^6\) LN cells, 0.05 to 1 \(\times\) 10\(^6\), were added to the BM inoculum. Recipients were monitored for the onset of GVHD by weight loss, diarrhea, and alopecia.\(^{24,29}\)

For engraftment experiments, 18 \(\times\) 10\(^6\) donor marrow cells (without supplemental S or LN cells) were incubated with the appropriate reagent and then transferred to irradiated (6.0 to 6.5 Gy TBI) fully allogeneic recipients as described previously.\(^{20}\)

** Colony-forming unit spleen (CFU-S) assay.** BALB/c BM cells were treated with LLME or IT and then washed twice.\(^{1}\) Thirty thousand cells were infused into irradiated (7.5 Gy TBI by \(\gamma\) emulsion) syngeneic recipients as previously described.\(^{29}\) Seven mice per group were transplanted. Twelve days post-BMT, the number of CFU-S cells was determined by placing the spleens into Bouin’s solution and enumerating the number of macroscopic colonies.

In vitro depletion of donor allografts with MoAb, IT, or LLME. C57BL/6 BM with or without splenocytes (1:1 ratio) were suspended at 20 \(\times\) 10\(^6\)/mL. The donor inoculum was TCD with anti-Thy 1.2 plus low background rabbit C’ (Pel-Freez, Rogers, AK; diluted 1:6) as described.\(^{24}\) Marrow treated with LLME or anti-Thy 1.2 + C’ was treated similarly except for the omission of anti-Thy 1.2. For LLME treatment, aliquots were incubated 15 minutes with LLME at room temperature. LLME was added to a final concentration of 250 \(\mu\)mol/L to 10 \(\times\) 10\(^6\) cells/mL.\(^{21}\) At this concentration, all cytolytic and monocytic functions were eliminated (and Results below). For IT treatment, anti-LFA1-RTA, anti-Thy1.2-RTA, or irrelevant RTA (H65-RTA or mel-RTA) was incubated for 2 hours at 37°C, 5% CO\(_2\), in the presence of pH adjusted (pH 7.4) 20 mmol/L NH\(_4\)Cl at concentrations ranging from 1 to 30 \(\mu\)g/mL. All BM groups were then washed once in cold media. Cells were injected via caudal vein in 0.5 mL volume.

** In vitro effect of LLME or IT treatment on splenocyte lytic function.** LDA analysis of CTL precursors in the spleens of C57BL/6 (donor strain) mice were determined as previously described.\(^{31}\) Minimal estimates of the CTL precursor frequencies were obtained from the Poisson distribution relationship between the responding cell number culture and the logarithm of the percentage of nonresponding (negative) cultures. When plotted according to the zero-order Poisson equation, the data define a straight line with a slope equal to the frequency of CTL precursors.

** NK cell activity was measured as previously described.** Reponder cells were plated with Na\(_2\)CrO\(_4\)-labeled YAC-1 target cells. Four hours after adding targets, 100 \(\mu\)L of supernatant was removed and counted in a gamma counter. The percent specific cytotoxicity was calculated.

**Chimerism analysis post-BMT.** Chimerism of peripheral blood mononuclear cells was analyzed between days 53 and 70 post-BMT in experiment 1. Engraftment was measured by analyzing the cell surface binding of anti–H-2 MoAb linked to fluorochromes on a FACSscan. Anti–H-2\(^d\) (clone 11-4-1, mouse IgG2a; ATCC) or anti–H-2\(^d\) (clone E1144, mouse IgG2b; provided by Dr T.V. Rajan, Albert Einstein University, New York, NY) was directly conjugated to FITC or phycoerythrin (PE), respectively, as previously described.\(^{2}\) 3A1E was conjugated to FITC or PE to determine background binding.

**Hematologic evaluation of recipients post-BMT.** Fifty microliters of peripheral blood was obtained by retro-orbital venupuncture on days 7, 14, and 28 post-BMT. Leukocyte number and morphology were determined by examination of Wright-Giemsa stained slides.\(^{34}\) Hematocrit values were determined by capillary tube red blood cell to plasma volume ratios after centrifugation.

**Skin grafting.** To measure immunocompetence, donor and
third party skin grafting was performed. Rejection of donor and third-party (BALB/c-H-2\(^d\)) skin was tested (n = 3 mice/group) as previously described.\(^2\)

Statistical analysis. Groupwise comparisons of continuous data were made using the Student's t-test. The computer program for compiling life table and statistical analysis by the log-rank test was provided by Dr Bruce Bostrom (Department of Pediatrics, University of Minnesota, Minneapolis).

RESULTS

Gel analysis. Sodium dodecyl sulfate (SDS) PAGE under nonreduced conditions was performed. Gel profiles from scanning anti-LFA1-RTA showed three bands of IT exceeding 150 Kd, the molecular weight of anti-LFA1 MoAb alone (not shown). The IT bands from the smallest to the largest represented 31%, 27%, and 13% of the total protein applied to the gradient. Nineteen percent free antibody and less than 5% free RTA were present. Gel profiles for this lot of anti-Thyl.2-RTA have been reported.\(^2\)

Binding of IT to the EL4 cell line as measured by indirect immunofluorescence. Eighty-nine percent of the LFA1 positive EL4 cell line bound anti-LFA1-RTA with a similar binding intensity (mean fluorescent channel) to anti-LFA1 MoAb, which bound 90% of EL4 cells (not shown). These data suggest that the conjugation procedure did not substantially alter MoAb binding.

In vitro studies with IT. Anti-Thyl.2-RTA potency and specificity was measured by PHA mitogenesis using splenocytes from a Thyl.2\(^+\) strain (C57BL/6) and a Thyl.2\(^-\) strain (AKR) as previously reported.\(^2\) In brief, Thyl.2-RTA completely eliminated PHA responsiveness in C57BL/6 splenocytes at a concentration of 10 \(\mu g/mL\), while reducing AKR PHA responsiveness by 19.8%. As shown in Table 1, anti-LFA1 was highly effective in reducing the CTL precursor frequency by greater than 2 logs and eliminating NK activity. In contrast, a control IT had negligible effects. In addition, anti-LFA1-RTA pretreatment inhibited NK function, as did anti-NK1.1-RTA in previous studies.\(^3\)

The efficacy of anti-LFA1-RTA incubation of donor grafts with IT in preventing GVHD induced in B10.BR recipients of C57BL/6 allografts. GVHD was induced by the injection of C57BL/6 BM and spleen cells or LN T cells (Fig 1). As few as 5 \(\times 10^6\) splenocytes or 10\(^6\) lymph node T cells resulted in the mortality of \(\geq 70\%\) of irradiated recipients, in contrast to the recipients of BM supplemented with 25 \(\times 10^6\) TCD splenocytes (n = 10/group). Because the mortality rate of mice given 15 to 25 \(\times 10^6\) nondeniplated supplemental splenocytes was 100% before 5 weeks post-BMT, we chose the highest splenocyte cell dose tested to study the efficacy of anti-LFA1-RTA pretreatment of donor grafts.

Cumulative actuarial survival data from two identical experiments in which bone marrow/spleen (BMS) grafts were preincubated with anti-LFA1-RTA (30 \(\mu g/mL\)), anti-Thyl.2-RTA (10 \(\mu g/mL\)), control IT, or control PBS are depicted in Fig 2. Overall, 90% of anti-LFA1-RTA treated BMS grafts survived the entire observation period. None of the mice had evidence of GVHD at any time period post-BMT. These results are similar to the 100% survival of anti-Thyl.2-RTA treated grafts. Recipients of control-RTA IT treated grafts had a median survival time of 40.5 days, with 20% of recipients surviving the observation period. Mice that received PBS-treated grafts had a median survival time of 33.9 days, with 11% surviving the observation period. There was no significant difference in survival in comparing these two different control groups, while each was significantly \((P < 3 \times 10^{-5})\) lower than recipients of anti-LFA1-RTA or anti-Thyl.1.2-RTA. At all periods post-BMT, recipients of anti-LFA1-RTA or anti-Thyl.1.2-RTA treated grafts did not experience the progressive weight loss (especially prominent 12 to 13 days post-BMT) characteristic of murine GVHD (data not shown).

Long-term chimerism studies on recipients shown in Fig 2 were performed at least 6 weeks post-BMT (Table 2). They demonstrated that the IT treatment did not interfere with alloengraftment. Four of six mice in each group surviving the observation period were skin grafted. All mice rejected third party (BALB/c) skin grafts within 13 days of graft placement. Donor skin grafts were accepted in five of six mice that received anti-LFA1-RTA treated BMS grafts and in five of six mice that received anti-Thyl.1.2-RTA treated grafts. It is possible that the single mouse rejection in each group was due to a technical failure.

In a third experiment (not shown), recipients of anti-LFA1-RTA at a dose of 30 \(\mu g/mL\) had a similar weight curve and similar survival curve as 10 \(\mu g/mL\) recipients

| Table 1. In Vitro Characterization of Anti-LFA1-RTA |
|-------------|-----------|-----------|-----------|-----------|
| Treatment   | Dose (\(\mu g\)) | CTLp Frequency | % NK-Mediated Cytolysis |
|             |           |            | 100:1 | 50:1 | 25:1 | 12.5:1 |
| Non-treated | NA        | 1/16,362   | 13    | 4    | 0    | 0     |
| LFA1-RTA    | 30        | 1/341,848  | 1     | 3    | 1    | 0     |
| Control-RTA | 30        | 1/18,865   | 13    | 3    | 1    | 0     |
| Non-treated | NA        | 1/16,362   | 12    | 7    | 2    | 0     |
| LFA1-RTA    | 0.1       | 1/50,000   | 1     | 0    | 0    | 0     |
| Control-RTA | 1         | 1/10,000   | 13    | 3    | 1    | 0     |

C57BL/6 S were incubated for 2 hours at 37°C with IT at the indicated concentrations. CTLp frequency and NK activity were determined as described in Materials and Methods.
C57BL/6 into B10.BR

Fig 1. Titration of C57BL/6 purified LN T cells or S on lethal GVHD induced in B10.BR recipients. Irradiated (8 Gy) B10.BR recipients were administered C57BL/6 BM (25 × 10^6) and spleen cells (5 to 25 × 10^6) or purified LN T cells (0.05 to 1 × 10^6). Control mice received anti-Thy1.2 + C' treated BM without additional cells. Ten mice per group were transplanted. Survival is plotted in an actuarial manner.

We tested three doses of anti-LFA1-RTA for preincubation. In addition to the 10 or 30 µg/mL doses, we also studied 1 µg/mL, because this lower dose of anti-LFA1-RTA was sufficient to eliminate NK function (see Table 1). Survival data are depicted in Fig 5. In contrast to the highly efficacious effect of anti-LFA1-RTA in preventing GVHD induced by C57BL/6 splenocytes in irradiated B10.BR recipients, anti-LFA1-RTA incubation at concentrations of 10 or 30 µg/mL was only partially effective in preventing GVHD induced by C3H/HeJ grafts in irradiated C57BL/6 recipients. Recipients of anti-LFA1-RTA at the lowest concentration (1 µg/mL), control IT treated, or C' treated grafts died before 10 weeks post-BMT, while a 100% survival rate was noted for recipients of control anti-Thy1.2 + C' treated grafts. Weight curves showed that anti-LFA1-RTA pretreatment at the higher concentrations was only marginally protective against GVHD.

The effect of LLME incubation of donor grafts in preventing GVHD induced in C57BL/6 recipients of C3H/HeJ allografts. We next determined if the cells involved in the afferent limb of GVHD in this C3H into C57BL/6 model were distinct from those in the C57BL/6 into B10.BR system. Therefore, we tested BMS treatment with LLME, which like anti-LFA1-RTA will eliminate CTLs, NK cells, and monocytes. We have previously shown that 250 µmol/L LLME pretreat-
PREVENTION OF MURINE GVHD

Table 2. Chimerism of Recipients of IT-Treated C57BL/6 BMS Grafts in a GVHD Model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (µg)</th>
<th>No. BMT</th>
<th>No. H-2 Typed</th>
<th>% Donor*</th>
<th>% Host*</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA1-RTA</td>
<td>30</td>
<td>20</td>
<td>19</td>
<td>92 ± 1</td>
<td>0 ± 0</td>
<td>95</td>
</tr>
<tr>
<td>Thy1.2-RTA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>93 ± 1</td>
<td>4 ± 0</td>
<td>100</td>
</tr>
<tr>
<td>Control-IT</td>
<td>30</td>
<td>20</td>
<td>6</td>
<td>89 ± 2</td>
<td>0 ± 0</td>
<td>20</td>
</tr>
</tbody>
</table>

*Chimerism typing was performed 6 to 8 weeks post-BMT using directly conjugated anti-H-2 and anti-H-2 antibodies.

The effect of anti-LFA1-RTA preincubation of donor grafts on alloengraftment. Because anti-LFA1-RTA was efficacious in preventing GVHD in B10.BR recipients of C57BL/6 allografts, we sought to determine if anti-LFA1-RTA pretreatment of donor grafts had a detrimental effect on alloengraftment. We modified our BMT regimen according to our previous published results,1-4 by reducing the TBI dose from 8.0 to 6.0 Gy, deleting the additional splenocytes, and reducing the BM cell number to 18 x 10⁶. Recipients received BM treated with anti-LFA1-RTA at a concentration of 30 µg/mL, control IT at 30 µg/mL, or anti-Thy1.2 + C' (Table 3). Survival was ≥60% in all groups, and recipients did not have evidence of GVHD by clinical examination or weight curves (data not shown). Chimerism was assessed 5 weeks post-BMT, at a time of stability under conditions of high BM cells dose.35 Recipients of anti-Thy1.2 + C' had a significantly (P ≤ .01) reduced alloengraftment as compared with recipients of control IT that completely engrafted. An almost identical level of alloengraftment was noted in recipients of anti-LFA1-RTA treated grafts when compared with anti-Thy1.2 + C' treated grafts.

Because anti-LFA1-RTA pretreatment of donor grafts was detrimental to alloengraftment in the C57BL/6 into B10.BR model, we tested its effect on the C3H/HeJ into C57BL/6 model (Table 3). For alloengraftment studies, we used 6.0 Gy TBI and a BM dose of 18 x 10⁶ without splenocytes. Recipients received BM treated with 30 µg/mL anti-LFA1-RTA, control IT, or anti-Thy1.2 + C'. Survival was 73% to 86% in all groups, and recipients showed no evidence of GVHD by clinical examination or weight curves (data not shown). Chimerism was assessed 7 weeks post-BMT. Recipients of anti-Thy1.2 + C' had a significantly (P < .001) reduced alloengraftment as compared with recipients of control IT. In recipients of anti-LFA1-RTA, all 12 mice that were H-2 typed had 0% donor cells.

Sequential blood studies were performed in the mice due to our concerns that anti-LFA1 was indeed interfering with engraftment (not shown). Early post-BMT (day 7), recipients of control IT had higher numbers of circulating leukocytes than either of the other two groups. In fact, none of the recipients of anti-LFA1-RTA treated BM achieved a leukocyte count greater than 200/µL, while 14 of 15 recipients of control IT and 7 of 15 recipients of anti-Thy1.2 + C' treated grafts had leukocyte counts ≥400/µL. By day 14, 12 of 15 mice that received control IT treated grafts had leukocyte numbers of greater than 1,000/µL, in contrast to 3 of 15 anti-Thy1.2 + C' treated and none of 15 anti-LFA1-RTA treated grafts. By day 28, 11 of 15 control IT, 9 of 15 anti-LFA1-RTA treated grafts, and 12 of 15 anti-Thy1.2 + C' treated grafts had leukocyte counts ≥1,000/µL. The absolute numbers of circulating neutro-
phils in recipients of anti-LFA1-RTA treated grafts were also decreased at this time post-BMT. They were less than 40% of values observed in recipients of either anti-Thy1.2 + C' or control IT treated grafts. Hematocrit values were equivalently and significantly ($P < .05$) decreased in recipients of anti-LFA1-RTA or anti-Thy1.2 + C' treated grafts 14 days post-BMT as compared with recipients of control IT treated grafts. Therefore, these data indicate that in the C3H/HeJ into C57BL/6 model, anti-LFA1-RTA pretreatment interferes with donor cell engraftment.

**The effect of IT pretreatment or anti-Thy1.2 + C' depletion on bone BM derived day 12 CFU-S formation.** Because data in both engraftment systems were consistent with an adverse effect of anti-LFA1-RTA pretreatment on alloengraftment, we examined the effect of IT on day 12 CFU-S formation, an indicator of early progenitor cells contained within the BM graft. Anti-LFA1-RTA (30 µg/mL) significantly ($P < .01$) reduced the number of day 12 CFU-S, as compared with recipients of anti-Thy1.2 + C' treated, control IT treated (30 µg/mL), or LLME (250 µmol/L) treated syngeneic BM cells (Table 4). These data show that in addition to its effect on immune cells, anti-LFA1-RTA pretreatment also reduces the number of early BM progenitor cells.

**DISCUSSION**

Our understanding of the afferent cells involved in GVHD is limited in part by our inability to adequately remove certain types of BM cells in vitro. The use of antibodies that bind to a cell surface epitope provides an important strategy for the selective removal of cell types which may be involved in GVHD generation. In vitro, antibody-mediated cellular elimination is most often accomplished by incubating the target cell populations with antibody followed by C', which provides a means of lysing cells. However, not all antibodies efficiently bind C'. This is the case for FD441.8, a rat antimus HLA-A chain MoAb, which inefficiently depletes in vivo. Panning with antibody-coated plates is an alternative strategy, but two or more panning steps are usually required, and a large amount of MoAb is needed for large-scale depletions. Therefore, we conjugated FD441.8 to RTA. Once internalized, RTA catalytically inhibits ribosomal protein synthesis with high efficiency such that a molecule of RTA will inactivate 1,500 ribosomes/minute. With the hybrid molecule, specificity of the IT is conferred by the antibody moiety which is necessary for efficient internalization.

We were interested in the contribution of LFA1 α chain
to 20% in recipients of PBS-treated or control IT-treated grafts. Because as few as 10% to 30% survival rate, it is likely that 80% or more of the cells involved in the afferent GVHD response were eliminated. The action of the IT was selective because control IT did not protect against GVHD.

An interesting finding was that the complete anti-GVHD effect induced by anti-LFA1-RTA treatment in the C57BL/6 into B10.BR model was not observed in the C3H/HeJ into C57BL/6 model. It has been shown that different phenotypic cell populations are more prevalent in the induction of GVHD in recipients of class I only, class II only, minor antigen only, or class I + II + multiple antigen disparate donor grafts. CD4⁺ cells mediate GVHD in congenic models expressing only class II differences and CD8⁺ cells mediate GVHD in congenic models expressing only class I differences. However, responses to minor antigens are always mediated by CD8⁺ T cells, although in some cases, CD4⁺ T cells are also involved.

Thus, cell types involved in GVHD induction may depend on the donor/recipient strain combinations chosen. In the strain combination C57BL/6 into B10.BR, the donor and recipient differ in the H-2 region and the minor antigen H-9 (which can serve as a sufficient disparity for the induction of GVHD at high T-cell doses). In this combination, the type of target antigens that dominate the afferent limb of GVHD response in vivo appears to require the involvement of LFA1⁺ donor cells. In the C3H/HeJ into C57BL/6 system, a number of different minor antigenic disparities (not including H-9) exist (H-1, -3, -7, -8, -12, -13, Tla, Mls). Therefore, classical LFA1⁺ or LLME sensitive donor cytolytic cells may not be involved in these responses. For example, Parkman has described autoreactive clones isolated from mice with acute GVHD, which are CD4⁺ and noncytolytic, as well as a minority of CD8⁺ noncytolytic cells. Soluble mediators produced by CD4⁺ (or CD8⁺) cells, such as interferon γ, which have been implicated in minor antigen disparity induced chronic GVHD, may be involved in some acute GVHD

![Fig 6. The effect of LLME on lethal GVHD induced in C57BL/6 recipients of C3H/HeJ BMS grafts. Irradiated (9 Gy) recipients were administered C3H/HeJ BM (20 x 10⁶) and spleen cells (5 x 10⁶). The inoculum was incubated for 15 minutes with LLME at 250 μmol/L. Controls received anti-Thy1.2 + C' or nontreated BMS. Ten mice per group were transplanted. Survival is plotted in an actuarial manner.](image)

**Table 3. Chimerism After Pretreatment of Donor Grafts With Anti-LFA-1-RTA in C57BL/6 Into B10.BR or C3H/HeJ Into C57BL/6 Allotransplants**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg)</th>
<th>No. BMT</th>
<th>No. H-2 Typod*</th>
<th>% Donor</th>
<th>% Host</th>
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<tr>
<td>C57BL/6 into B10.BR</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LFA1-RTA</td>
<td>30</td>
<td>15</td>
<td>9</td>
<td>75 ± 12⁺</td>
<td>21 ± 10⁺</td>
</tr>
<tr>
<td>Control-IT</td>
<td>30</td>
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<td>14</td>
<td>99 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Thy + C'</td>
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<td>15</td>
<td>15</td>
<td>74 ± 6⁺</td>
<td>18 ± 6⁺</td>
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<tr>
<td>C3H/HeJ into C57BL/6</td>
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<tr>
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<td>10 ± 7⁺</td>
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<td>93 ± 14⁺</td>
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<td>15</td>
<td>15</td>
<td>67 ± 11</td>
<td>26 ± 9</td>
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*Chimerism studies were performed 6 to 8 weeks post-BMT using fluorochrome-labeled anti-H-2⁺ and anti-H-2⁻ MoAbs and flow cytometry.

* C3H/HeJ into C57BL/6 BM (18 x 10⁶) was pretreated and administered to irradiated (6.5 Gy TBI) C57BL/6.

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because fewer T cells (lo6) mediate 100% lethality in the graftment model because some other ex vivo TCD strategies in mice (eg, anti-Thyl.2)

...that is by the omission of NH4Cl. Without NH4Cl, under conditions in which binding but not lysis was optimized, that is by the omission of NH4Cl. Without NH4Cl, anti-LFA1-RTA was not effective in GVHD prevention, demonstrating that lysis was required for GVHD protection.

We tested the effects of anti-LFA1-RTA in an alloengraftment model because some other ex vivo TCD strategies in mice (eg, anti-Thyl.1.2 + C') that prevent GVHD have done so at the expense of engraftment. It was possible that anti-LFA1-RTA pretreatment would not adversely affect alloengraftment, because LLME pretreatment did not interfere with alloengraftment in this same model in previous studies. However, the clinical use of LLME may be somewhat problematic due to its documented toxicity to committed human progenitor cells. We do not know whether the α chain of LFA1 was expressed on pluripotent BM stem cells, but engraftment was not compromised in our GVHD model. In previous studies, doses of anti-LFA1 IT were selected that do not affect human progenitor cells in CFU-GEMM assays in vitro, but still inhibit T-cell function. Additionally, investigators have used in vivo injections of anti-LFA1 to successfully promote engraftment in mice and humans. In these situations, blockade of the host antidonor alloreactive cells is likely occurring and probably impairs T-cell expansion, inhibiting the host-versus-graft effect. Moreover, because in vivo the anti-LFA1 α chain antibody does not deplete efficiently, accessory cells that facilitate alloengraftment may be preserved, as presumably would be the capacity of progenitor cells to yield progeny. This situation does not exist with anti-LFA1-RTA pretreatment in vitro because the IT depletes rather than blocks LFA1 expressing cells. In addition, the cell surface expression of a determinant on stem cells does not necessarily preclude its use for targeting. For example, Thy-1 is expressed on murine stem cells. Despite its expression, BM pretreatment with anti-Thyl.1.2 IT combined with high-dose TBI conditioning regimens resulted in 100% donor engraftment in mismatched recipients.

In our studies, anti-LFA1-RTA pretreatment significantly decreased long-term alloengraftment compared to pan-T cell depletion with anti-Thyl.1.2 + C'. In addition, anti-LFA1-RTA pretreatment reduced hematopoietic recovery to a more significant degree for a longer time as compared with anti-Thyl.1.2 + C' pretreatment. We performed day 12 CFU-S assays and observed an impressive reduction in CFU-S formation in recipients of anti-LFA1-RTA treated, but not anti-Lyl-1-RTA treated, LLME treated, control IT treated, or anti-Thyl.1.2 + C' treated syngeneic BM grafts. These data suggest that anti-LFA1-RTA pretreatment removed critical accessory cells preserved by anti-Thyl.1.2 + C', or alternatively, reduced the number of early progenitor cells more efficiently than anti-Thyl.1.2 + C'. While day 12 CFU-S formation is not necessarily a direct reflection of stem cell repopulation capacity, BM cells giving rise to day 12 CFU-S cells are early progenitor cells. Therefore, a decrement in the number of early committed progenitor cells will likely slow early post-BMT hematopoietic recovery.

In conclusion, anti-LFA1-RTA pretreatment of donor grafts is an effective means of preventing murine GVHD against some, but not all, of the cells that are involved in the induction of murine GVHD. Its potential may be limited by the expression of the α chain of LFA1 on accessory cells or on stem cells responsible for alloengraftment. Yet, anti-LFA1-RTA may still be useful for clinical GVHD prevention when combined with positive selection techniques such as elutriation designed to enrich for stem cells.

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