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Selective Reduction of Graft-versus-Host Disease-Mediating Human T Cells by Ex Vivo Treatment with Soluble Fas Ligand

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Previous work done in our laboratory, using mouse models, showed that soluble Fas ligand (sFasL) can efficiently delete donor anti-host T cells during their activation against irradiated host cells in MLCs. In the mouse models, this ex vivo sFasL treatment abrogated graft-vs-host disease (GVHD) while sparing donor T cells with antitumor reactivity. The present work was performed with human cells, to extend our work toward reduction of clinical GVHD. PBMC responders from a given individual (first party) were separated, to a therapeutically useful extent (5–7). In addition, sFasL/MLR-treated cell populations contained influenza-specific T cells, CD4⁺FOXP3⁺ T cells, and CD4⁺CD25⁺ T cells. These data indicate that this ex vivo sFasL/MLR depletion of alloreacting human donor anti-host T cells was efficient and selective. The Journal of Immunology, 2009, 183: 696–705.

Allogeneic (Allo) hematopoietic stem cell transplantation is the only curative treatment for several malignant and nonmalignant hematopoietic disorders. The efficacy of this treatment is limited by graft-vs-host disease (GVHD), the major cause of morbidity and mortality in this setting. GVHD is a complex inflammatory disease mediated mainly by allo donor graft T cells that are stimulated by APCs to attack genetically disparate host tissues, especially skin, mucosa, and liver (1–3). Although pan-T cell depletion of the donor allograft can reduce GVHD, this approach has not translated into improved overall survival because of the following: 1) increased rate of graft failure, 2) increased frequency of leukemia relapse, 3) increased risk of posttransplantation EBV-associated lymphoproliferative disorders, and 4) delayed immune reconstitution with increased incidence of opportunistic infections (4). Although these observations support the overlapping role of donor alloreactive T cells in mediating both GVHD and graft vs leukemia (GVL), cases with complete remission of leukemia, but no clinical signs of GVHD after hematopoietic stem cell transplantation or donor leukocyte infusion support the presence of allo T cells with antileukemia activity that do not mediate (clinically detectable) GVHD, and suggest that GVHD and GVL activities can be separated, to a therapeutically useful extent (5–7). In addition, the existence of allo T cells with antileukemia activity, recognizing leukemia-specific Ags or minor histocompatibility Ags that are preferentially expressed on leukemia cells, has been reported by several groups (6, 8–11). These findings indicate that the GVL potential of the donor allograft and support the search for a simple, clinically applicable method that would allow the transfer of allo donor T cells with reduced potential to mediate GVHD against the transplant host.

Several methods for depletion of donor anti-host GVHD-mediating T cells have been developed. These methods target ex vivo alloantigen-activated T cells (from a MLR culture with irradiated allo host cells) based on the expression of various T cell activation markers (such as CD25, CD69, CD71, HLA-DR, CD137, and CD134 (12–21)), the dilution of CFSE (22), or the uptake of the dye 4,5-dibromorhodamine 123 (TH9402) by activated cells, making them sensitive to light (23).

We recently reported a new approach for depletion of allo donor anti-host T cells that is based on the sensitivity of activated T cells to Fas (CD95)-mediated apoptosis (24). Using mouse models, we showed that recombinant human soluble Fas ligand (sFasL) can efficiently and selectively deplete donor antihost T cells during their activation against irradiated host cells in a MLC. This ex vivo sFasL treatment reduced GVHD while sparing antitumor reactivity and hematopoietic engrafting capacity in mouse models (24). The feasibility of our method is supported by a similar study in which an agonistic Ab to Fas (CD95) was used to reduce donor antihost-alloreacting cells (25, 26). To move our work toward potential use for reduction of clinical GVHD, we extended these studies to a human model system.
Materials and Methods

Cell isolation for MLR cultures

Human PBMCs were isolated from heparinized normal human blood by density gradient centrifugation using Ficoll-Paque (Amersham/GE Healthcare Bio-Sciences), according to the manufacturer’s instructions. Signed informed consent was obtained for use of each human blood sample under an Institutional Review Board-approved protocol. Mouse splenocytes were isolated by mincing the spleens, and treating (30 min, room temperature) with liberase blendzymes and DNase I (Roche Applied Science; following manufacturer’s instructions), followed by EDTA (10 mM, 5 min, room temperature; Sigma-Aldrich). Light density cells were isolated after centrifugation in Nycodenz medium (Accurate Chemical & Scientific).

MLR cultures and proliferation assays

One-way allo MLRs were performed by culturing either of the following: 1) 2/10^5 responder PBMCs from one normal human donor (first party) with 4 × 10^5 irradiated (1,500 cGy, Gammacell 137Cs source; Atomic Energy) stimulator PBMCs of an unrelated normal human donor (second party); or 2) 10^5 responder PBMCs from a donor with 0.2/10^5 irradiated (10,000 cGy) allogeneic stimulator cells from a human B-lymphoblastoid cell line (LCL; established in the Levitsky laboratory by standard EBV transformation of peripheral blood B lymphocytes). One-way xenogeneic (xeno) MLRs were performed by culturing 2/10^5 responder PBMCs from a donor with 2/10^5 irradiated (5,000 cGy) mouse splenocytes as stimulators. Responder PBMCs cultured without stimulators were the background proliferation controls in all experiments. Cells were cultured in RPMI 1640 medium (Life Technologies) containing 10% human AB serum (GemCell; Gemini Bio-Products), 2 mM L-glutamine (Life Technologies), 10 mM HEPES (Life Technologies), and 100 g/ml primocin (Amaxa Biosystems) in 96-well plates for 1–7 days in a humidified incubator (37°C, 5% CO2) with (0.5 g/ml sFasL plus cross-linker; or without (filled; cross-linker only) sFasL treatment, were labeled with CD3-allophycocyanin and the fluorescent inhibitor of caspases (FLICA) 3/7 (which binds active caspases 3 and 7; Molecular Probes, Vybrant FAM caspase 3/7 assay kit), as per manufacturer’s instructions. A representative experiment is shown; in three repeat experiments, following sFasL/MLR treatment, 14% (mean ± 4% SD) of gated CD3+ cells expressed active caspase 3/7. A total of 5.4% (mean ± 1.1% SD) of the unstimulated CD3+ cells expressed active caspase 3/7 following the sFasL treatment.

In [3H]thymidine assays, MLCs (in 96-well round-bottom tissue culture plates) were pulsed for their last 18–24 h of cell culture with 1 μCi/well of [3H]thymidine incorporation or CFSE dilution.
[3H]thymidine (Amersham/GE Healthcare Bio-Sciences). Then cells were harvested onto glass fiber filters (Printed Filtermat A; Wallac), and incorporated [3H]thymidine was measured (Wallac 1450 MicroBeta liquid scintillation counter; PerkinElmer Wallac). All samples were tested in triplicate, and values for individual experiments are reported as means ± SD. Combinations of results of 3 independent experiments are presented as mean ± SEM. Values of p were measured by Student’s t test.

In CFSE assays, responder PBMCs (10⁷) were labeled with 1 μM CFSE (Molecular Probes) for 10 min at 37°C in 5 ml of staining buffer (PBS containing 0.1% (w/v) BSA; Sigma-Aldrich). CFSE was removed by three washes with RPMI 1640 medium containing 10% heat-inactivated FBS (BenchMark; Gemini Bio-Products). Combinations of results of 3 independent experiments are presented as mean ± SD.

In the experiments of supplemental Fig. 2, 4 CD4⁺CD25⁻ cells were isolated from fresh PBMCs using a MACS isolation kit (Miltenyi Biotec; kit 130-091-301), then labeled with CFSE and mixed with unlabeled PBMCs from the same donor.

For secondary challenges, day 2 sFasL/MLR-treated (and control) cells were washed twice with RPMI 1640 medium containing 10% human AB serum, and then cultured overnight in sFasL-free medium (to allow apoptosis to take place). On day 3, equal numbers of control- and sFasL-treated cells from primary MLCs were cultured, with or without the sFasL treatment. A. On day 0, the general caspase inhibitor Z-VAD-fmk (50 μM final concentration; R&D Systems) or the vehicle control (0.25% DMSO final concentration) was added to control or sFasL/MLRs. On day 3, [3H]thymidine uptake was assessed; background control [3H]thymidine incorporation was subtracted. Background controls were <20% of experiments in all cases. A representative experiment is shown. In three repeat experiments, proliferation was reduced by 85% (mean ± 4% SEM) in sFasL/MLRs, as compared with controls; 100% blockade of the sFasL effect was achieved by Z-VAD in all the experimental repeats (p = 0.0058; Student’s t test). B-D, Cells from 7-day allo MLCs done with CFSE-labeled PBMC responders, and control unstimulated responders, cultured for the first 2 days with or without sFasL treatment, were labeled with CD3-allophycocyanin and CD25-PE-Cy7. sFasL/MLR treatment reduced the numbers of gated large CD3⁺CFSE⁻ cells by 97% (mean ± 2% SD; n = 5, a representative experiment is shown in B) and the numbers of the CD3⁺CFSE⁺CD25⁻, CD3⁺CFSE⁺CD25⁺, and CD3⁺CFSE⁻CD25⁻ cell subsets by 60 ± 27%, 98 ± 1%, and 93 ± 4% (mean ± SD; n = 5), respectively, as compared with control cells (a representative experiment is shown in C; combined data from five independent experimental repeats are in D).

Flow cytometry

FACS analyses were performed using the following directly fluorochrome-labeled mAbs: CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD25 (M-A251), CD38 (HIT2/HB7), HLA-DR (L243), CD45 (H30 and 30-F11), CD95 (DX2), CD107a (H4A3) (BD Biosciences), FOXP3 (PCH101), and IFN-γ (4S.B3) (eBioscience). mAbs or isotype controls (mouse IgG1, IgG2a; BD Biosciences) were added to cells suspended in 100 μl of cold PBS containing 2.5% heat-inactivated FBS (PBS-FBS) for 30 min at 4°C. Cell suspensions were washed once, resuspended in 200 μl of PBS-FBS, and then analyzed using a FACScalibur cytometer (BD Biosciences).

Influenza (Flu)-specific CD8⁺ cells were detected by incubating 10⁶ PBMCs from a HLA-A*0201 Flu-positive donor (characterized by the manufacturer in an ELISPOT assay for ability to respond to the influenza A viral peptide GILGFVFTL; SeraCare catalog numbers 72000-020805).
and 72000-031705-B) with 10 μl of PE-labeled HLA-A*0201/GILG FVFTL MHC class I Flu pentamer (ProImmune) for 10 min at room temperature, washing once with PBS-FBS, and then labeling with CD8-allophycocyanin and either CD25-FITC, CD38-FITC, CD95-FITC, or HLA-DR-FITC. The sFasL/MLR treatment reduced the numbers of CD4hiCD38+ and CD4hiCD95+ cells, which was subtracted (bar graph). The number of cells taken from each sorted population matched its actual frequency in the day 7 MLC, as assessed by FACS. B, Cells from a day 7 allo MLR, and control unstimulated responders, that were cultured for the first 2 days with or without sFasL treatment, were labeled with CD4-allophycocyanin and either CD25-FITC, CD38-FITC, CD95-FITC, or HLA-DR-FITC. The sFasL/MLR treatment reduced the numbers of CD4hiCD25+, CD4hiCD38+, and 4D4HLA-DR+ cells by 96 ± 1.4%, 96 ± 3%, 96 ± 0.9%, and 98.5 ± 0.5% (mean ± SD; n = 3), respectively, as compared with control cells. In the representative experiment shown, the dot plots of PBMC responders stimulated with allo LCLs (red) were overlaid onto dot plots of unstimulated PBMC responders (black). C, Cells from a day 7 allo MLR using CFSE-labeled PBMC responders and allo LCL stimulators were labeled with CD4-allophycocyanin and either CD25-PE-Cy7, CD38-PE-Cy7, or HLA-DR-PE-Cy7 only; the CD38-defined subsets from a representative experiment are shown (left panel). The CD4 ‘CD38’ and CD4‘CD38’ cell subsets were gated and analyzed for CFSE (right panel); similar results were obtained for the CD4‘CD25’ and CD4‘HLA-DR’ T cell subpopulations (data not shown). The experiment shown is representative of three repeats. D, Cells from a 7-day allo MLR that were cultured for the first 2 days with or without sFasL treatment were restimulated for 5 h with the same second party allo LCLs, PMA, and ionomycin, and then FACS analyzed after immunostaining with CD4-PE and IFN-γ-allophycocyanin. Day 7 unstimulated PBMC responders treated with PMA and ionomycin were controls.

Depletion of alloreacting CD4hiCD38+ cells

Cells from a day 7 allo-MLR were harvested, dead cells were removed using the MACS dead cell removal kit (Miltenyi Biotec; kit 130-090-101), and viable cells were labeled with CD4-allophycocyanin and CD38-FITC (BD Biosciences). A high-speed MoFlow sorter (DakoCytomation) was used to FACS sort the CD4hiCD38+ cells and the complementary population depleted of these cells. Secondary responses of the FACS-sorted cell populations to the same second party stimulators, to unrelated third party cells, and to CMV Ags were assessed by [3H]thymidine incorporation.

Xeno-GVHD assay

Highly immunodeficient NOD.Cg-Pkdcre+/−Il2rg−/−SzJ (NOD-scid Il2rg−/−) mice (27, 28), purchased from The Jackson Laboratory, were bred and maintained in an immune-compromised mouse facility, under protocols approved by the Johns Hopkins Medical Institution Animal Use and Care Committee. Xeno GVHD was induced in NOD-scid Il2rg−/− mice following the protocol previously described by van Rijn et al. (29), with slight modifications. Briefly, 8-wk NOD-scid Il2rg−/− females...
received a single (sublethal) dose of 250 cGy of total body irradiation before injection (tail vein) of human cells on the same day. Radiation control mice did not receive human cells. Weight loss was monitored every 7 days. When mice in an experimental group developed clinical signs of severe GVHD (severe weight loss, hunched posture, ruffled fur, reduced mobility, tachypnea), one representative ill mouse from that group was sacrificed, and multiple organs/tissues (spleen, bone marrow, liver, lung, blood) were harvested for FACS analysis of human cell engraftment (using directly fluorochrome-labeled mAbs recognizing mouse CD45 and human CD45 (BD Biosciences)) and T cell activation. Blood was obtained via the retro-orbital sinus from the mice under brief anesthesia (Isoflurane USP; Hospira), then FACS analyzed, after RBC lysis (RBC lysis buffer; eBioscience). Histopathologic analysis of organs (skin, lungs, liver, gut, spleen) harvested postmortem from each mouse was done by an expert veterinary pathologist (C.B.).

Results

sFasL/MLR treatment reduced activated donor T cells

Alloreacting CD3+ T cells began to up-regulate Fas (CD95; Fig. 1A) as early as day 1–2 of the allo MLR. Alloreacting CD4+ and CD8+ T cell subsets exhibited similar kinetics of Fas up-regulation (Fig. 1, B and C), and T cells became sensitive to Fas-mediated apoptosis by day 2 (Fig. 1D). In preliminary experiments, we varied sFasL concentrations (with or without cross-linker), cell concentrations, and responder to stimulator ratios to determine the optimal conditions for efficient reduction of donor antihost T cell alloreactivity. In 7-day MLCs using PBMC responders taken from one person (first party) and irradiated PBMC or LCL stimulators from a second person (second party), at a responder to stimulator ratio of 1:2 or 5:1, respectively, treatment with 0.5 μg/ml sFasL plus 2 μg/ml cross-linker efficiently depleted anti-second party alloreactivity (data not shown). To shorten the ex vivo sFasL/MLR treatment to a minimum (potentially to reduce toxicity for future clinical application), we compared the efficacy of 2, 3, 4, and 5 days sFasL treatment. We found that a 2-day sFasL/MLR treatment was sufficient to eliminate most of the reactivity against the second party allostimulators (data not shown). These conditions were used in the experiments presented in this study, unless otherwise indicated.

sFasL/MLR-treated cells had reduced [3H]thymidine incorporation and CFSE dilution, compared with controls (Fig. 2, A and B); the pan-caspase inhibitor Z-VAD blocked the effect of sFasL treatment (Fig. 2A). CFSE-labeled responder cells were FACS analyzed for activated T cell subsets on each day of the 7-day allo
MLR culture. Consistent with other reports (22), we found that, by day 5 of the allo MLC, three subpopulations of alloreacting CD3\textsuperscript{+} cells were evident: CFSE\textsuperscript{hi}CD25\textsuperscript{+}, CFSE\textsuperscript{lo}CD25\textsuperscript{+}, and CFSE\textsuperscript{lo}CD25\textsuperscript{hi} (where “hi” represents “high” and “lo” represents “low”) (Fig. 2C). The sFasL/MLR treatment reduced the numbers of CFSE\textsuperscript{hi}CD25\textsuperscript{+}, CFSE\textsuperscript{lo}CD25\textsuperscript{+}, and CFSE\textsuperscript{lo}CD25\textsuperscript{hi}CD3\textsuperscript{+} cells by 60–98% in five experiments, as compared with control cells cultured without sFasL (Fig. 2D).

Alloreacting CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were reduced by sFasL.

Immunostaining of responder cells from day 4–7 MLCs revealed CD4\textsuperscript{hi} (Fig. 3) and CD8\textsuperscript{hi} (supplemental Fig. 1) subpopulations. In
three independent experiments, depletion of the CD4^hi^CD38^hi^ subpopulation by FACS sorting reduced the secondary proliferative response against second party cells to 12% (mean ± 4% SEM) of the total cell control; however, the responses to third party and CMV Ags were 82% (mean ± 12% SEM) and 134% (mean ± 24% SEM) of the total cell control, respectively (Fig. 3A). The CD4^hi^ and CD8^hi^ cells from these MLCs coexpressed multiple T cell activation markers (Fig. 3B and supplemental Fig. 1A) and were actively dividing (CFSElo) (Fig. 3C and supplemental Fig. 1B). These CD4^hi^ and CD8^hi^ T cell subpopulations were >94% eliminated by the sFasL/MLR treatment (Fig. 3B and supplemental Fig. 1A). In preliminary analyses, we found that the anti-second party cytotoxic CD8^hi^ T cells resided within the CD8^hi^CD38^hi^ T cell subpopulation (O. Bohana-Kashtan et al., manuscript in preparation).

In addition, sFasL/MLR treatment reduced the numbers of IFN-γ-secreting CD4^+^ and CD8^+^ cells by 99% (mean ± 1% SD; n = 3) and 89% (mean ± 5% SD; n = 3), respectively, compared with untreated controls (Fig. 3D and supplemental Fig. 1C). Moreover, the numbers of anti-second party allo-activated CTLs (expressing the degranulation marker, lysosome-associated membrane protein-1 (CD107a; a sensitive marker of CTL activity that is briefly exposed on the surface of CD8^+^ T cells during the act of killing) (30–32%)) were markedly reduced by the sFasL/MLR treatment (91–94% reduction, compared with untreated controls; n = 2; supplemental Fig. 1D).

Retention of overall immune competence following sFasL/MLR treatment

We tested the reactivity of equal numbers of cells from primary allo MLCs, with or without sFasL, in secondary MLCs. In three independent experiments, 87% (mean ± 4% SD) fewer CD3^+^ CFSE^lo^ cells were generated in response to second party cells, in the sFasL/MLR-treated group. However, there was no detectable decrease in the CD3^+^CFSE^lo^ cells generated in response to CMV.
Ags (as calculated after subtraction of the background control proliferation that represents residual anti-second party alloreactivity); the response to PHA was reduced by only 4% (mean ± 2% SD); and the response to third party cells (calculated after subtraction of the background control) was reduced by only 33% (mean ± 34% SD) (Fig. 4, A and B). Similar results were obtained in secondary proliferative responses assessed by [3H]thymidine incorporation (Fig. 4C).

There were no significant differences between sFasL/MLR and control-treated MLCs in the frequencies of Flu-specific CD3+CD8+ cells (Fig. 5). In day 7 allo MLRs, there were no Flu-positive cells detected in the gated CD8+CD25+ cell subpopulation (data not shown).

CD4+FOXP3+ and CD4+CD25+ T cells in sFasL-treated MLCs

We assessed the effect of sFasL on CD4+FOXP3+ cells in the populations of stimulated responders. We used LCLs (rather than PBMCs) as stimulators to avoid the presence of CD4+FOXP3+ cells in the stimulator cell population. Consistent with previous reports (33–35), all or most of the human alloreacting T cells (CD4+hCD38+ and CD4+CD25+) had up-regulated FOXP3 expression (Fig. 6A). Indeed, in MLR cultures at day 3, a 41% (mean ± 10.5% SD; n = 3) increase in cells expressing FOXP3 was observed (Fig. 6B). In sFasL-treated MLRs, the frequency of FOXP3+ cells was similar to that seen in unstimulated controls (4.08 vs. 5.14%; n = 3). All of the surviving CD4+FOXP3+ cells coexpressed CD25 (data not shown). Unstimulated sFasL-treated responders contained 56% (mean ± 9% SD; n = 3) of the numbers of CD4+FOXP3+ cells in the untreated (unstimulated) controls (Fig. 6B).

As a second approach to address the relative sensitivity of T regulatory cells (Tregs) to sFasL, we isolated CD4+CD25+ cells from unstimulated PBMCs, labeled them with CFSE, and mixed them with unlabeled PBMCs from the same person (first party). These first party PBMCs containing CD4+CD25+ cell-enriched CFSE-labeled cells were used as responders, and irradiated allo PBMCs as second party stimulators. CFSE-labeled CD4+CD25+ cells persisted in the sFasL/MLR-treated cultures, actually at an increased frequency (121%, mean ± 18% SD; n = 3) relative to untreated (stimulated) controls, in part due to the elimination of activated unlabeled cells in the sFasL-treated group (supplemental Fig. 2).4 Unstimulated sFasL-treated responders contained 87% (mean ± 13% SD; n = 3) of the numbers of CFSE−CD4+CD25+ cells in the untreated (unstimulated) controls (supplemental Fig. 2).

Mice transplanted with sFasL/MLR-treated cells had prolonged times to severe GVHD

We compared the xeno-GVHD-mediating capacity of human PBMC responders that had been stimulated in vitro with irradiated NOD-scid IL2rg−/− splenocytes in the presence or absence of 2 μg/ml super FasL. In the first of two independent experiments, mice that received 7 × 106 sFasL/MLR-treated cells had a median survival time (MST) of 83 days, compared with 36 days for mice that received control cells from a MLR without super FasL (p = 0.0018; data not shown). In the second experiment, done with a different PBMC donor, freshly isolated PBMCs provided an additional control group. Mice that received super FasL/MLR-treated cells had a significantly prolonged MST compared with both control groups, at each of the three human cell doses tested (Fig. 7).

Discussion

We developed a new method to selectively eliminate ex vivo activated first party (model donor) anti-second party (model host) alloreacting T cells based on their sensitivity to Fas-mediated apoptosis. We designed our strategy to be technically simple and robust, to facilitate potential translation to clinical use. In a haploidentical mouse model system, we previously demonstrated the ability of sFasL to efficiently and selectively reduce donor antihost T cells during their activation against irradiated host cells in a MLR culture. GVHD was potently reduced, whereas a graft-vs-tumor effect and the hematopoietic engrafting capacity of sFasL-treated bone marrow cells were retained (24).

The present study extends our work to a human model system and supports its potential clinical utility. The actively dividing CFSE−CD25+ and CFSE−CD25− subsets of alloreacting T cells were efficiently (>93%) reduced by the sFasL treatment during the allo MLR (Fig. 2). The identification of an activated proliferating CFSE−CD25− T cell subpopulation that no longer expressed the T cell activation marker CD25 demonstrates the importance of combined use of proliferation (CFSE) and activation markers to identify alloreacting T cells. The CFSE−CD25+ T cell subpopulation that was partially, but not completely (mean 60%), eliminated by the sFasL/MLR treatment may be composed of early activated T cells (that are about to divide) and Tregs (22) (see text below; Fig. 6 and supplemental Fig. 2).

Prior reports showed, in a mouse model system, that the Ag-specific CD4+ T cells responding to an Ag challenge in vitro and in vivo were CD4+h (36, 37). As demonstrated by our data (Fig. 3A; graph) and as recently reported (38), sorting out the CD4+hCD38− and FOXP3−CD4hiCD38+ cell subsets of alloreacting T cells in sFasL-treated MLRs had a MST of 50 days, whereas mice that received super FasL/MLR-treated cells had a significantly prolonged MST compared with both control groups, at each of the three human cell doses tested (Fig. 7).

The efficacy of the sFasL/MLR treatment was further tested using a human anti-NOD-scid IL2rg−/− mouse xeno-GVHD model (27, 28). This model was at least as sensitive as the similar RAG2−/−γc−/− xeno-GVHD model (29), because as few as 1–5 × 106 freshly isolated human PBMCs induced lethal GVHD, as was assessed by the development of clinical signs (severe weight loss, hunched posture, ruffled fur, reduced mobility, tachypnea) and histopathological (pulmonary and hepatic vascular-perivascular mononuclear infiltrates) findings of acute GVHD (Fig. 7 and data not shown). Mice that received 7 × 106 sFasL/MLR-treated cells had a MST of 50 days, whereas mice that received 2.5 × 106 and 1 × 106 of the (two types of) control cells had a MST of 28–45 days and 37–58 days, respectively (Fig. 7).

The differences seen in the MST of mice that received 7 × 106 cells in the two independent xeno-GVHD experiments can be partially explained by the fact that these experiments were done with different unrelated PBMC donors that may have differences in their human anti-mouse xeno-GVHD-mediating capacity.

The selectivity of this sFasL/MLR method is indicated by the detection of no depletion of the immune response to CMV, 4% depletion of the response to PHA, 33% depletion of the response to third party alloantigens, and no depletion of Flu-specific T cells (Figs. 4 and 5). The somewhat reduced response to third party alloantigens may be due to our use of PBMCs isolated from a third
party blood donor who was unrelated, but not HLA typed, and thus might have HLA genes in common with the second party donor (as will often be the case in the clinical situation that we are modeling).

The ability of naturally occurring donor-derived Tregs to suppress allogeneic immune responses, in vitro and in vivo, has been reported by several groups (39–43). As shown by Edinger et al. (44) and others (45, 46), donor-derived CD4⁺CD25⁺ T cell-mediated reduction of GVHD did not reduce the ability of donor effector T cells to mediate antileukemia responses. The importance of donor-derived Tregs for control of GVHD is further suggested by clinical studies associating a high CD4⁺FOXP3⁺ T cell content in donor stem cell allogeneic transplant grafts with a low incidence of GVHD (47). In addition to their ability to inhibit GVHD, CD4⁺CD25⁺ cells have also been shown to support long-term allogeneic hematopoietic cell engraftment (48). A complicating factor is that, whereas FOXP3 expression is highly specific for Tregs in mice, our data confirmed previous reports (33–35) that most activated human T cells up-regulate FOXP3 (Fig. 6). We observed that 55% of the CD4⁺FOXP3⁺ T cells survived the sFasL/MLR treatment (Fig. 6), as compared with the untreated, stimulated cells; this represents ~75% of the numbers of CD4⁺FOXP3⁺ T cells in the untreated, unstimulated responder cell population. These surviving CD4⁺FOXP3⁺ T cells coexpressed CD25, and we speculate that Tregs are included in this subset.

In another approach to investigate the sensitivity of a T-reg-enriched subset to the sFasL/MLR treatment, we isolated CD4⁺ CD25⁺ cells from fresh unstimulated PBMCs and labeled them with CFSE. These CFSE-labeled CD4⁺CD25⁺ cells were mixed with unlabeled whole PBMCs from the same donor, and this cell mixture was used as the first party PBMCs responders. We observed that the CFSE-labeled CD4⁺CD25⁺ cells survived the sFasL/MLR treatment (supplemental Fig. 2). Taken together, the survival of substantial fractions of CD4⁺FOXP3⁺ and CD4⁺CD25⁺ cells suggests that some Tregs survived the sFasL treatment. These findings are consistent with other reports demonstrating lack of sensitivity of activated Tregs to Fas-mediated apoptosis. Murine Tregs were shown to be resistant to clonal deletion induced by viral superantigen in vivo and to Fas-mediated apoptosis following polyclonal activation in vitro (49, 50). Consistent with these observations, Fritzscheing et al. (51) showed that Tregs freshly isolated from human PBMCs expressed high levels of Fas, but upon stimulation with anti-CD3 and anti-CD28, they became resistant to activation-induced cell death. Similar to Fritzscheing et al. (51), we found that the CD4⁺ FOXP3⁺ and CD4⁺ CD25⁺ subsets of the unstimulated cells appeared to be more sensitive to Fas-mediated apoptosis (Fig. 6 and supplemental Fig. 2).

Several methods have been developed to ex vivo deplete donor antithost T cells during their activation in a MLC (12–20, 22, 23, 52). Allodepletion based on CD25 expression (14) has the disadvantage of deleting CD4⁺CD25⁺ Tregs. Compared with methods involving prelabeling (CFSE, TH9402) (22, 23), transduction (HSV-tk) (52), or FACs sorting (15–17), the sFasL/MLR approach is simple and rapid (2 days ex vivo incubation, which could be done in a closed system), and should be safe and nontoxic (e.g., cells can be washed to remove sFasL after the MLC). Pachnio and colleagues (53) recently reported that, similar to Godfrey et al. (22), nonproliferating CFSE⁺ T cells (FACS sorted following an allo MLC) have reduced ability to mediate GVHD. However, these nonproliferating CFSE⁺ T cells did not mediate GVH in vivo and did not support engraftment (53). The inability of CFSE⁺ T cells to mediate in vivo immune response might be explained, in part, by CFSE toxicity (54, 55).

Removal of CD25⁺ cells has been tested clinically (12, 13, 56). Patients who received allografts that had been treated with the CD25 immunotoxin had a low incidence of GVHD, but had high rates of leukemia relapse. The high rates of leukemia relapse seen in patients receiving CD25-depleted grafts (12, 13) raise the possibility that the use of host PBMCs as stimulator cells may not be optimal; this may lead to the elimination of donor T cells that recognize minor hematopoietic-expressed histocompatibility Ags important in GVL. As demonstrated by van Dijk et al. (19), host keratinocytes can be used as alternative allo stimulators. In support of this idea, Jones et al. (57) demonstrated the inability of donor CD4⁺ T cells to induce GVHD in chimeric mice hosts that express only hematopoietic-derived alloantigens. Approaches to be considered in the future should also include attempts to selectively enhance the antileukemia immune response (6, 58, 59).

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Disclosures

The authors have no financial conflict of interest.

References


**Supplemental Figure 1:** Alloreacting CD8⁺ T cells were eliminated by the sFasL/MLR treatment.

(A) Cells from a 7 day allo MLC, and control unstimulated responders, that were cultured for the first 2 days with or without sFasL treatment, were labeled with CD8-APC and either CD25-FITC, CD38-FITC, CD95-FITC or HLA-DR-FITC. The sFasL/MLR treatment reduced the numbers of CD8⁺CD25⁺, CD8⁺CD38⁺, CD8⁺CD95⁺, and CD8⁺HLA-DR⁺ cells by 97% ± 0.6%, 98% ± 1.6%, 94% ± 2%, and 97% ± 0.5% (mean ± SD; n=3), respectively, as compared to control cells. In the representative experiment shown, the dot plots of PBMC responders stimulated with allo LCLs (gray) were overlaid onto dot plots of unstimulated PBMC responders (black).

(B) Cells from a 7 day allo MLC using CFSE-labeled PBMC responders and allo LCL stimulators were labeled with CD8-APC and either CD25-PE-Cy7, CD38-PE-Cy7, or HLA-DR-PE-Cy7; only the CD38-defined subsets from a representative experiment are shown (left panel). The CD8⁺CD38⁻ and CD8⁺CD38⁺ cell subsets were gated and analyzed for CFSE (right panel); similar results were obtained for the CD8⁺CD25⁺ and CD8⁺HLA-DR⁺ T cell subpopulations (data not shown). The experiment shown is representative of 3 repeats.

(C, D) Cells from a 7 day allo MLC that were cultured for the first 2 days with or without sFasL treatment, were re-stimulated for 5 hrs with the same 2ⁿᵈ party allo LCLs, PMA and ionomycin (C), or only with the 2ⁿᵈ party allo LCLs (D), then FACS analyzed after immunostaining with CD8-FITC and IFN-γ-APC (C) or with CD8-APC and CD107a-PE (D). CD107a expression on gated CD8⁺ cells in unstimulated (control, filled histograms) and stimulated (solid lines) responder cells is shown in D.

**Supplemental Figure 2:** CD4⁺CD25⁺ cells were present after the sFasL/MLR treatment. Unlabeled responder PBMCs that had been mixed with CFSE-labeled
CD4⁺CD25⁺ cells isolated from the same person (1st party) were cultured with (stimulated) or without (unstimulated) allo PBMCs (2nd party), with or without sFasL treatment, for 2 days. At day 7, cells from these cultures were FACS-analyzed after immunostaining with CD4-APC and CD25 PE-Cy7.
Figure 1- Supplemental Data

A.

B.

C.

D.
Figure 2-Supplemental data

Unstimulated vs Stimulated

Control

dFasL

CFSE

CD4

CD25

Unstimulated: 1.76, 1.69, 1.76, 1.69
Stimulated: 0.59, 1.55, 0.98, 1.55