Transduction of donor hematopoietic stem-progenitor cells with Fas ligand enhanced short-term engraftment in a murine model of allogeneic bone marrow transplant

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Short title for running head: Overexpression of FasL in bone marrow transplant

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Abstract

Fas-mediated apoptosis is a major physiologic mechanism by which activated T cells are eliminated after antigen (Ag)-stimulated clonal expansion generates a specific cellular immune response. Since activated T cells are the major effectors of allograft rejection, we hypothesized that genetically modifying allogeneic bone marrow (BM) cells prior to transplant could provide some protection from host T cell attack, thus enhancing donor cell engraftment in bone marrow transplantation (BMT). We undertook studies to determine the outcome of lentiviral vector-mediated transduction of Fas ligand (FasL) into lineage Ag-negative (lin−) mouse BM cells (lin− BMs), in an allogeneic BMT model. FasL modified lin− BMs killed Fas-expressing T cells in vitro. Mice transplanted with allogeneic FasL+ lin− BMs had enhanced short-term engraftment, after non-myeloablative conditioning, as compared to controls. We observed no major hepatic toxicity, or hematopoietic or immune impairment, of recipient mice at these time points. These results suggest potential therapeutic approaches by manipulating lympho-hematopoietic stem-progenitor cells to express FasL or other immune modulating genes in the context of BMT.
Introduction

General radio-pharmacologic immunosuppression is the primary method used to decrease the immune rejection response of the host against allogeneic donor hematopoietic and organ transplant grafts. Development of more specific, cellular therapies designed to induce antigen (Ag)-specific tolerance would be widely applicable in many transplant settings. Studies have begun to investigate potential avenues for novel cellular-based therapies by producing tolerance using immature dendritic cells (DCs) alone \(^1,2\), as well as DCs genetically modified to express a number of immunoregulatory genes, including IL-10 \(^3\), TGF-\(\beta\) \(^4\), CTLA4-Ig \(^4\) and Fas ligand (FasL) \(^5,6\). These approaches have been shown to confer tolerance in certain defined experimental settings. The Fas pathway offers an intriguing opportunity to manipulate the anti-donor immune response in the BMT setting, since allograft rejection is mediated primarily by activated host anti-donor T cells, and since activated T cells generally express high levels of the Fas receptor and are susceptible to Fas-mediated apoptosis, at least during the later stages of activation (“activation-induced cell death”) \(^7-9\).

Conflicting results have been obtained on the effects of expressing FasL in experimental solid organ allografts. For pancreatic islet cell allografts, the initial report found increased graft acceptance \(^10\), but later studies indicated that FasL\(^+\) pancreatic allografts became infiltrated with neutrophils and suffered enhanced rejection \(^11\). Tolerance to FasL\(^+\) allografts was shown in thyroid \(^12\) and lung \(^13\) models. FasL expression was also shown to inhibit allogeneic recognition of tumor cells \(^14\). The circumstances leading to these conflicting results are complex, and there are multiple differences among these model systems, including the strains of mice and the immunosuppressive regimens. In addition, the local environment may influence the nature of the response to FasL expression. For example, TGF-\(\beta\) inhibited the proinflammatory effects of FasL in a tumor rejection model \(^15\).

In this initial investigation of this approach, we sought to determine whether allogeneic hematopoietic grafts might be protected from acute rejection, early after non-
myeloablative transplant, by genetically expressing FasL in donor lineage Ag− bone marrow (BM) cell preparations (lin− BMs) enriched in lympho-hematopoietic stem-progenitor cells. Allogeneic BMT is an important treatment option for many cases of hematologic malignancies and blood diseases, as well as selected non-hematologic cancers and inherited disorders, but is limited by complications, including graft versus host disease (GVHD). Rigorous isolation of transplanted lympho-hematopoietic stem-progenitor cells removes mature T cells and thereby prevents or reduces GVHD; but in the absence of donor T cells, host versus graft (HVG) rejection becomes a major problem. The effects of FasL expression have not been previously reported in a BMT model; “armed” FasL+ donor lin− BMs might engraft, and then they and their multilineage FasL+ progeny could kill T cells that attack them. This would tend to down-regulate the anti-donor immune response and protect the donor graft. FasL+ lympho-hematopoietic stem-progenitor cells for BMT might be more effective than FasL+ DCs have been in organ transplant models, because transduced lympho-hematopoietic stem-progenitor cells could generate large numbers of donor FasL+ progeny cells. On this basis, we undertook studies to determine whether lentiviral transduction of donor lin− BMs with FasL protected the donor graft from acute rejection, in an allogeneic mouse nonmyeloablative BMT model. Results indicate that FasL+ lin− BMs killed activated anti-donor T cells and enhanced short-term donor cell engraftment, without producing major acute hepatotoxicity or generalized acute myelosuppression or immunosuppression.

Methods

Viral vector construction and production.

The FasL gene expression vector was created by fusion of the mouse FasL coding region to the 5’ end of the enhanced green fluorescent protein (GFP) gene, and subsequent insertion into a lentivirus vector under the control of the CMV-β–actin fusion promoter. The control vector expresses only GFP. The lentivirus packaging system 16 and the self-inactivating lentiviral parent vector pRLL.hPGK.GFP SIn-18 17 have been described previously. The PGK promoter was removed from the parent vector, and additional
restriction sites were added (5’XhoI-EcoRV-BamHI-AgeI-NheI-KpnI-MluI-Spel-HpaI-EcoRI-XBamHI) by insertion of a T4 kinase treated oligonucleotide pair: (a) CGAGATATCGGATCCACCGGTGCTAGCGGTACCACGCGTACTAGTGTTAACGAATTC (b) GATCGAATTCGTTAACACTAGTACGCGTGGTACCGCTAGCACCAGGTGGATCCGATATC. Next, the 1.75 kb CMV β-actin fusion promoter (CAG) derived from the pCAGGS vector from Dr. J. Miyazaki was inserted into the modified parent vector preceding the GFP reporter sequence, and the GFP was removed. A plasmid containing the mouse full-length FasL cDNA coding sequence, as an 880 bp insert in the p43 plasmid, was obtained from Dr. T. August (Johns Hopkins Medical Institutions [JHMI]). The FasL cDNA was digested from the p43 plasmid using Bgl II and Sal I, then cloned into those sites of the pEGFP-C2 plasmid (Clontech, Palo Alto, CA). The resulting FasL-EGFP fusion sequence was digested from the plasmid with NheI and MluI, then using these sites, inserted into the lentiviral parent vector under the control of the CMV-β-actin fusion promoter. The insert sequence was verified by DNA sequencing.

For generation of producer lines, 293T cells cultured in DMEM containing 10% fetal calf serum (FCS; Life Technologies, Carlsbad, CA) were transfected using Effectene (Qiagen, Valencia, CA). Viral supernatants were collected for 3 days and filtered (0.45µM Millipore filter, Fisher Scientific, Pittsburgh, PA). Supernatants were titered on 293T cells by adding 100 µl supernatant to 2 x 10^5 cells/well in 1 ml DMEM containing 10% FCS in a 6 well dish (Costar, Bedford, MA), incubating for 2 days, then measuring the percent GFP-positive cells by FACS analysis of 488 nm-excited fluorescence in the FL1 channel using a FACScan flow cytometer and Cellquest software (Becton Dickinson (BD), San Jose, CA). Supernatant from 293T cells was either used fresh for transduction or stored at −80°C until use. Prior to use, supernatants were concentrated using Centricon filters (100 kD cutoff; Millipore Corporation, Bedford, MA). Sufficient supernatant to achieve an MOI of 3–5 was concentrated to a volume of 50-100 µl, then added to lin− BMs in the presence of 8 µg/ml polybrene (Sigma, St. Louis, Missouri).
BM harvest, DC cultures, lin- BM enrichment, and transduction.

All animal studies were conducted under approved animal protocols at JHMI. Mice were obtained from the National Cancer Institute at 6-8 weeks of age, except for the 2C transgenics, which were bred onto a C57BL/6 background and kindly provided to us by Dr. J. Schneck (JHMI). For BM harvest, femurs and tibias were removed from sacrificed mice, flushed with ice-cold isotonic phosphate-buffered saline (PBS; pH 7.4, 0.05M Phosphate), and the resulting BM cells were washed and counted. DCs were generated from BM cells as previously described. After transduction, DCs were evaluated on day 8 by FACS analysis for expression of both the transgene (indicated by GFP fluorescence in the FACScan FL1 channel) and DC markers (including PE-labeled MHC class II, CD80, CD86, and DEC-205, measured in the FL2 channel). PE-labeled antibodies were obtained from BD-Pharmingen (San Diego, CA), except DEC-205 was from Serotec (Raleigh, NC).

Lin- BMs were enriched from mouse BM by immunomagnetic depletion of cells expressing mature hematopoietic “lineage” Ags, following the manufacturer’s procedure (Stem Cell Technologies, Vancouver, British Columbia, Canada), then plated at 10⁶ cells/ml in RPMI 1640 with 5% serum (Life Technologies, Carlsbad, CA) containing recombinant flt-3 ligand (FL; 50 ng/ml; R & D Systems, Inc., Minneapolis, MN), kit ligand (KL; 100 ng/ml; Peprotech, Rocky Hill, NJ) and thrombopoietin (10 ng/ml; Peprotech, Rocky Hill, NJ). Lin- BMs were transduced three times, by addition of concentrated viral supernatant (MOI 3-5) as described above, on days 1, 2 and 3 of culture. On day 4 or 5, aliquots of the transduced cells were analyzed by FACS for GFP expression, plated for CFC, or transplanted intravenously (iv, via the dorsal tail vein) into recipient mice. In addition, transduced cells were analyzed for cytotoxic function by co-incubation with PKH26 (PKH; Sigma, St. Louis, MO)-labeled Jurkat target cells (labeled according to manufacturer’s instructions), known to be sensitive to Fas-mediated killing. Transduced lin- BMs were sorted for GFP expression, on a FACSVantage flow cytometer (BD) for the dose titration studies. Then, using multi-color flow cytometry, labeled
Jurkat target cells (PKH+) were distinguished from (unlabeled) effector lin- BMs by PKH fluorescence (FACScan FL2 channel), and live versus dead PKH+ Jurkat target cells were quantified using 7-aminoactinomycin (7-AAD) (BD-Pharmingen) incorporation (measured in FACScan FL3 channel), following the manufacturer’s procedure.

**Mixed lymphocyte reactions and T cell proliferation assays.**

Spleen responder cells were incubated with irradiated (3000 cGy) DCs or spleen cell stimulators, depending on the experiment. 10^5 DC stimulators were incubated with 10^6 responders. 2 x 10^6 spleen cell stimulators were added to 2 x 10^6 responders. Cultures were incubated in 96 well U bottom plates (Costar) for 4 days, then 1 µCi/ml ^3^H-thymidine (Amersham, Piscataway, NJ) was added for 16 hours, at which time the plates were harvested and counted.

**Allogeneic BMT and engraftment analysis.**

For non-myeloablative allogeneic BMT in a multiple minor MHC mismatch setting, B6.SJL (CD45.1+) donor lin- BMs were infused into 400 cGy irradiated recipient C3H.SW (CD45.2+) mice. These mice are MHC matched (H-2^b_), but differ at multiple minor histocompatibility loci, many of which are still undefined. Mice were sacrificed at 3–24 weeks after transplant, then single cell suspensions of organs were prepared for FACS analysis (BM and spleen), colony-forming cell (CFC) assays (BM), and MLR assays including responsiveness to third party stimulators (spleen). In these FACS analyses, BM and spleen were evaluated for the numbers of donor cells (CD45.1+) and transduced donor cells (GFP+/CD45.1+). PE-CD45.1 monoclonal antibody was obtained from Pharmingen.

**CFC assays.**

CFC analysis was conducted on BM cells prior to transplant by plating 3 x 10^3 transduced lin- BMs (in triplicate) in 1 ml Marrow-Gro methylcellulose medium
(generously provided by Quality Biologicals, Gaithersburg MD) supplemented with recombinant KL (50 ng/ml), IL-3 (10 ng/ml), granulocyte-monocyte-colony-stimulating factor (GM-CSF, 10 ng/ml) and erythropoietin [Epo] (5 U/ml). Unless otherwise specified, growth factors were obtained from Peprotech. After 7 days incubation, CFC-Mix, CFC-granulocyte-macrophage (CFC-GM) and erythroid burst-forming unit (BFU-E) colonies were counted. Upon sacrifice of the transplanted mice, 3 x 10^5 whole BM cells were assayed for CFC as above.

For the studies with soluble FasL (sFasL), BM cells were plated in QBSF-58 (Quality Biologicals) containing KL, GM-CSF, and erythropoietin, with a range of concentrations of sFasL (Alexis Pharmaceuticals, San Diego, CA) for 48 hours prior to plating in CFC assays.

**Listeria monocytogenes** **challenge.**

BALB/c mice, known to be susceptible to *Listeria* from preliminary studies, were lethally irradiated (850 cGy) and transplanted with syngeneic BALB/c lin^−^ BMs that had been transduced with either the control GFP, or the FasL-GFP lentiviral vector. Three weeks later, transplanted mice were tail bled to quantify GFP^+^ cells, then injected intraperitoneally (ip) with 10^6 colony-forming units (cfu) attenuated *Listeria monocytogenes* bacteria. Four days after challenge, mice were sacrificed. Livers and spleens were removed, and portions were fixed in paraformaldehyde and analyzed histologically. The remainders of these organs were crushed to obtain single cell suspensions that were stained with CD8 Cy-chrome and either CD3-PE or CD4-PE monoclonal antibodies (BD-Pharmingen), then analyzed by FACS.

**Results**

Transduced lin^−^ BMs were analyzed for expression of the transgene by (1) determination of GFP fluorescence (which should be present in both control vector- and experimental vector-transduced groups, since the experimental lentivirus encodes FasL as a GFP-fusion) and (2) determination of function by analyzing killing of FasL-sensitive Jurkat T
cells by transduced cells. 10-20% of the transduced lin− BMs used for were GFP+, consistently in control and experimental groups throughout the experiments described herein; specific percentages are shown for representative experiments.

**Transduced FasL+ lin− BMs killed activated T cells.**

For the functional assay, lin− BMs were incubated with PKH-labeled Jurkat cells, then the co-cultured cells were analyzed by FACS for the presence of 7-AAD (indicating cell death) in PKH+ (Jurkat) cells (Figure 1a). To determine whether there was a dose-response effect for the killing of the Jurkat cells by FasL+ cells, transduced lin− BMs were FACS sorted for GFP expression, then mixtures of FasL+ (i.e., based on GFP fluorescence) with FasL− (i.e., GFP−) cells were prepared and incubated for 24 hours with PKH-labeled Jurkat target cells. Mixtures containing 1 or 5% FasL+ cells mediated only a slight increase in killing, compared to negative control cultures. The mixture containing 20% FasL+ lin− BMs was markedly more effective at killing Jurkat cells, and the mixture containing 80% FasL+ lin− BMs was slightly more potent than the 20% mixture (Fig 1a shows 1 of 2 similar experiments).

**Fig 1a**

<table>
<thead>
<tr>
<th>% FasL cells</th>
<th>% Jurkats killed</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
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<tr>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>80</td>
<td>42</td>
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FasL+ DCs inhibited allogeneic T cell proliferation.

Untransduced DCs or DCs transduced with the FasL(-GFP fusion) or the control GFP vector were irradiated and incubated with responder spleen cells. In the allogeneic mixture (B6 DCs and BALB/c splenocyte responders), the control GFP+ DCs stimulated a robust proliferative response, while the FasL+ DCs failed to stimulate a response above background (Fig 1b). 2C mice are transgenic for a CD8+ T cell receptor that recognizes H2 Ld (displayed on BALB/c cells) [18]. Proliferation of 2C cells in response to BALB/c stimulators was essentially eliminated with the FasL+ DCs.

![Fig 1b](image)

To begin to address the question of the specificity of the effects of FasL+ DCs, we tested whether proliferation of responder T cells would be “non-specifically” inhibited by FasL+ cells syngeneic to the responders. The results of Fig 1c indicate that the proliferative response of BALB/c T responder cells was potently inhibited by FasL+ allogeneic B6 DCs. A mixed population of B6 DC and FasL+ syngeneic BALB/c DC stimulators resulted in some inhibition of the response of BALB/c T cells to untransduced B6 DCs, but the inhibition was less. Thus, while some non-specific inhibition was observed with syngeneic FasL+ cells, allogeneic FasL+ DCs mediated nearly complete inhibition of the proliferative immune response.
Constitutive FasL expression by lin- BMs did not impair generation of CFCs.

Lin- BMs were enriched from mouse BM as described, transduced with either the GFP or FasL vector (resulting in 10-20% GFP+ cells pre BMT, determined by FACS analysis prior to plating) and plated in CFC assays. Colonies were counted 7 days later. No significant difference was observed in numbers or types of CFCs from the two groups (Figure 2a).
sFasL pre-treatment of untransduced lin\(^-\) BMs did not affect CFCs.

Since only 10-20% of the transduced BM cells in the experiments above expressed FasL, we undertook experiments to determine the effect of exposing 100% of the BM cells to agonistic sFasL. A range of concentrations of sFasL was added to cultures of untransduced lin\(^-\) BMs for 48 hours prior to plating in CFC assays as shown. Pretreatment of lin\(^-\) BMs with sFasL did not inhibit CFC numbers or alter the distribution of CFC types (Fig 2b).

![Graph showing the effect of sFasL on CFC numbers and types](https://example.com/graph.png)

**Fig 2b**

Constitutive FasL expression by lin\(^-\) BMs did not impair syngeneic engraftment, assessed early post-BMT.

To investigate the effect of FasL expression on the capacity of transduced FasL\(^+\) lin\(^-\) BMs to engraft, syngeneic transplants were performed. BALB/c lin\(^-\) BMs were transduced with either the GFP control or FasL vector, then \(10^5\) cells were transplanted into 850 cGy irradiated syngeneic mice (BALB/c). Fig 2c shows GFP fluorescence of the lin\(^-\) BMs prior to transplantation. The entire population of cells (transduced and untransduced) was injected for transplant.
GFP transduced (10% GFP⁺)  
FasL transduced (14% GFP⁺)

Fig 2c

Three weeks after BMT, mice were tail-bleed to determine the percentage of circulating cells that expressed the transgene (Fig 2d). Both groups had similar percentages of GFP⁺ cells, which were also similar to the percent GFP⁺ input cells (Fig 2c).

Fig 2d

FasL⁺ lin⁻ BMs generated enhanced allogeneic engraftment early after BMT.
A multiple minor mismatch (B6.SJL→C3H.SW) was selected as an MHC-matched non-myeloablative BMT model. Lin⁻ BMs from B6.SJL mice (CD45.1⁺) were transduced with either the control (GFP) or experimental FasL(-GFP) vector. Fig 3a shows GFP fluorescence of the B6.SJL lin⁻ BMs prior to transplantation into sub-lethally irradiated recipient C3H.SW mice.
Approximately three weeks later, mice were sacrificed and analyzed for donor cell hematopoietic engraftment. Donor cells and transduced cells were analyzed by correlated CD45.1 and GFP fluorescence. Table 1 shows the percentages of transplanted mouse BM cells derived from donor (CD45.1⁺) cells or transduced donor (CD45.1⁺/GFP⁺) cells. Mice transplanted with FasL⁺ lin⁻ BMs had significantly higher levels of donor chimerism than those that received the control lin⁻ BMs, in BMs (p = 0.01) (Table 1 and Fig3b).
Table 1. FasL+ lin BMs generated enhanced allogeneic engraftment early after BMT. BM from mice was FACS analyzed at 3 weeks post-BMT to determine the presence of both donor cells (CD45.1+) and donor cells that were transduced (GFP+ & CD45.1+). The graph (Fig 3b) summarizes the averages and SEM of all the mice shown in table 1. In the table, each value is representative of a single mouse, with results combined from four separate experiments. The first three columns are the total percent CD45.1+ cells in mice receiving unmodified, GFP-, and FasL- modified transplants as indicated; the last row is the percentage of CD45.1+ cells that are GFP positive also, in the FasL transplants. (CD45.1+/GFP+)

Representative histogram analyses for CD45.1 from BM from a mouse transplanted with BM expressing GFP (top) or FasL (bottom).
Fig 3c

Representative histogram analyses for GFP from BM from transplanted mice, as labeled.

Fig 3d

BM cells from the allo-transplanted mice sacrificed at 3 weeks post-BMT (Table 1) were assessed for CFCs. No significant differences were observed in numbers or types of CFCs from the FasL- versus control GFP-transduced groups of mice (Figure 4).
Additional mice were transplanted as above in separate experiments; the transduction efficiency pre-BMT averaged 17%. In 2 experiments (combined results), BM cells from mice were FACS analyzed at 20-24 weeks post-BMT for correlated expression of CD45.1 (representing donor) and GFP (representing transduced) cells. Each value shows the result for an individual mouse. The first column is the percent of donor cells in BM from mice that received GFP-modified transplants. The second column is the percent of donor cells in BM from mice that received FasL modified transplants. The third column correlates with the second and shows the percent of donor cells that also are GFP⁺. None of 7 mice transplanted with control GFP transduced lin⁻ BMs, but 4/7 mice transplanted with FasL transduced lin⁻ BMs, had >2% donor cells in BM. None of the mice in either group had detectable GFP⁺ BM cells.
Table 2.

<table>
<thead>
<tr>
<th>GFP</th>
<th>FasL</th>
<th>% FasL(GFP+)</th>
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<tbody>
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<td>&lt;0.5</td>
<td>9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0.52</td>
<td>11</td>
<td>&lt;1</td>
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<td>&lt;1</td>
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<td>&lt;0.5</td>
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<td>&lt;1</td>
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<tr>
<td>&lt;0.5</td>
<td>11</td>
<td>&lt;1</td>
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<tr>
<td>&lt;0.5</td>
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Mice transplanted with FasL⁺ lin⁻ BMs did not have significant hepatic toxicity or immune impairment.

A significant concern with expressing FasL in lin⁻ BMs is the potential for in vivo toxicity due to FasL. The mice transplanted with syngeneic or allogeneic FasL⁺ lin⁻ BMs were not different from control groups in overall health, or on gross pathology at autopsy. Since hepatic cells express high levels of Fas, and since hepatotoxicity was reported after administration of one, but not another, anti-Fas antibody, we evaluated whether transplant with FasL⁺ lin⁻ BMs produced histologic hepatotoxicity. Histological analysis of hematoxylin/eosin stained slides by a pathologist (FKR) revealed no detectable injury to hepatic cells of mice that had received a transplant of FasL⁺ versus control GFP⁺ lin⁻ BMs (Fig 5a, b). Mild hepatic inflammation was noted in both groups but there was no difference in the levels of hepatic inflammation between the two groups. In the groups followed for 4-6 months, inflammation persisted to varying degrees in the transplanted mice; one of four FasL mice that were analyzed had detectably worse inflammation.
To assess the immune responsiveness of allo-transplanted mice, splenocytes were taken at the time of sacrifice and used as responders in an MLR to a third party antigen stimulator (BALB/c, H-2^d). No significant difference was observed between the two groups in the level of proliferation. (Fig 5c).

![Fig 5c](image)

To further evaluate hepatotoxicity and to test the immune responsiveness of the transplanted mice, we challenged transplanted mice with a sublethal dose of *Listeria monocytogenes* as a model infectious agent. *Listeria* was selected since it is known to produce hepatic inflammation, and thus any inflammatory or hepatic in vivo toxicity of FasL^+^ lin^-^ BMs or their progeny might be highlighted by this challenge. In addition, since T cells have been reported to die in the liver based on Fas-FasL interactions^{24}, significant Fas-mediated toxicity should prevent accumulation of T cells recruited in response to this challenge. BALB/c lin^-^ BMs were transduced with FasL or GFP as above, and transplanted into lethally irradiated syngeneic BALB/c recipients. All mice were then injected ip with a sublethal dose of *Listeria*, observed for 4 days, and sacrificed. All mice in both groups exhibited decreased activity, starting one day after
Listeria injection. On pathology, all mice had mild inflammation in the liver, with no gross differences between the two groups (Fig 6a). All mice had high numbers of T cells in the livers in response to this challenge (Fig 6b).

**Fig 6a**
Results of this study suggest that HVG rejection was inhibited at time points early after transplant when FasL was genetically expressed in a fraction of the transplanted donor marrow cells. Increased levels of donor cells were detected in the host early after non-myeloablative BMT, and no significant toxicity to the recipient mice was detected. These results may serve as a paradigm for developing systems in which immune modulatory genes may be inserted into donor lin⁻ BMs prior to BMT in order to engineer the recipient’s immune response after BMT. Insertion of immunomodulatory genes into BM prior to transplant has the potential to be applied to diseases for which BMT is already utilized, to enhance the transplant effects, or to develop new therapies in which transplant could be employed for the purpose of introducing such genes. Thus, the capacity to modify immune responses through BMT would provide a significant potential for improvement of therapies for a number of diseases.
In the present experiments, transduced FasL+ lin− BMs killed Fas-sensitive T cells in vitro. Expression of FasL in mouse lin− BMs did not appear to be toxic to BM function, since (a) there was no difference in the CFC numbers or types from FasL+ lin− BMs compared to controls, and (b) FasL+ donor lin− BMs engrafted to as high (in syngeneic transplants) or higher (in allogeneic transplants) levels than did control lin− BMs. Although syngeneic engraftment was assessed at only the early time point of 3 weeks post-BMT, this finding is consistent with other studies in which human CD34+ cells were shown to not be susceptible to Fas-mediated apoptosis, possibly due to high level expression of the caspase pathway inhibitor, FLICE inhibitory protein (FLIP)25.

FasL+ lin− BMs directly killed Fas-sensitistive Jurkat target cells, and FasL+ DCs inhibited allogeneic T cell proliferation. Transplanted FasL+ lin− BMs have the potential to differentiate in vivo into DCs, which could potentiate the tolerizing effect mediated by the FasL+ lin− BMs, per se. Our observation that FasL+ DCs decreased an immune response is consistent with findings in multiple other in vitro and model organ transplant systems. Mice transplanted with FasL+ donor lin− BMs had significantly higher levels of donor hematopoietic cell chimerism than did those transplanted with control donor lin− BMs. This was likely the result of inhibition of HVG attack by the FasL+ lin− BMs and progeny, since in the syngeneic transplants conducted for the Listeria challenge, no significant differences in levels of total or GFP+ cells were observed between the FasL+ and control groups. Presumably, FasL expression would confer no selective advantage in a syngeneic transplant. In the non-myeloablative allogeneic BMT recipient hematopoietic chimeras, not all of the donor cells were transduced or expressed high levels of FasL, as assessed by GFP fluorescence from the fusion protein. Thus, HVG rejection was significantly down-regulated, even though only a fraction of the donor cells were FasL+. It is possible that FasL+ cells generated donor tolerance, and once tolerance to donor cells was achieved, the FasL+ cells no longer had a selective advantage over the untransduced donor cells. Over time, the percent FasL+ cells appeared to decrease, consistent with this hypothesis. Since the percent donor cells decreased somewhat over time, ongoing studies will address relative contribution of the graft in longer term experiments using this approach, as compared to (or combined with) other non-
myeloablative approaches. The goal of the present studies was to determine effects on acute allograft rejection. In addition, these studies do not address the potential for long term non-specific toxicity, e.g., late GVHD or late non-specific immunosuppression. However, it appears that the FasL expression provides bystander protection and that not all the cells need to express FasL. In these experiments, clonal expansion of FasL+ cells did not occur. Thus, it may be possible to translate the effects of FasL+ cells to clinical use in the future using only a low percent FasL+ cells that express FasL for a relatively short time. However, more extensive analysis of long term transplants will be necessary to determine the full extent of the beneficial and potential toxic effects.

These results are consistent with other model systems in which organs modified to express FasL have been protected from rejection, as discussed above. One still unresolved issue in the use of FasL is the results from studies in which FasL generated enhanced rejection of organs and inflammatory responses. The conditioning regimen may affect the level of engraftment or rejection, and the degree of non-specific immunosuppression. For example, BMT preparative radiation may non-specifically sensitize cells that may upregulate Fas, potentially leading to non-specific killing by FasL+ cells. It is likely that the microenvironment surrounding the FasL+ cells may contribute to differences in published results, since FasL has been shown to have different effects depending on the cytokines present in the host. A greater understanding of these phenomena would increase the utility of FasL in vivo.

One significant potential for a limitation in this approach of using FasL+ BM cells is that constitutive hematopoietic cellular expression of FasL might be toxic to the host. For example, many subsets of immune cells express Fas and so might be non-specifically killed by FasL+ BM cells and their progeny. Long term effects of constitutively expressed FasL by donor cells could lead to chronic GVHD or potentially generate cells that would be inappropriately resistant to killing. Inducible vector systems would be one potential method to address these limitations. In addition, Fas is not the sole determinant of sensitivity to FasL-mediated apoptosis. As examples, DCs may express Fas but are protected by high levels of FLIP, and T cells are only highly sensitive to FasL upon
activation \(^{31}\). In addition, we have recently found that CD34\(^+\) cells are resistant to Fas-mediated cytotoxicity, and express low levels of Fas and high levels of FLIP. \(^{25}\) However, since the detailed long-term effects of \textit{in vivo} administered transduced FasL\(^+\) BM cells are not fully known, this significant concern must be investigated. In these studies, the mice transplanted with FasL\(^+\) lin\(^-\) BMs appeared as healthy as the controls. No significant difference was observed in BM cellularity or CFC of mice transplanted with FasL\(^+\) versus control lin\(^-\) BMs. Since FasL has been shown to produce acute hepatotoxicity in some systems, we analyzed livers from transplanted mice histologically. Although there was minimal inflammation in both experimental and control mice (mild inflammation might be expected after a BMT), no difference was noted between the groups.

An evaluation of immune function of transplanted mice was conducted in two separate ways. First, spleen cells from the transplanted mice were used as responders in an MLR at the time of sacrifice, as a general determinant of intact immune responsiveness to an alloantigen. Mice that were transplanted with FasL\(^+\) lin\(^-\) BMs had no decrease in the ability to respond to allogeneic third party stimulation. Second, mice were evaluated for their ability to respond to an infectious agent (\textit{Listeria monocytogenes}) at a dose that was determined empirically to produce severe but sublethal toxicity (A. Jain, R. Schulick, D. Pardoll, unpublished observations). Mice that were significantly immunocompromised would be unable to mount an immune response to the agent and succumb. At 1-4 days after injection of the \textit{Listeria}, all mice were alive but lethargic. At this time, they were sacrificed, and livers were analyzed by FACS for T cell infiltration and by histology. Since each liver had a significant T cell infiltrate, the ability to mobilize T cells in response to an infectious antigen appeared to be intact in all mice.

These studies provide a novel approach to down-regulate graft rejection in BMT. Since FasL has the potential to kill multiple cell types and to produce organ toxicity, comprehensive analysis of potential toxicity in long-term engrafted recipients of FasL\(^+\) BM cells needs to be conducted prior to clinical application, and results at longer timepoints are needed, e.g. for assessment of potential effects on long-term engrafting lymphohematopoietic stem cells or chronic hepatotoxicity. In addition, the percentages
of cells that express the FasL may need to be titrated to achieve effective killing of T
cells with the minimum toxicity. Our results in vitro showed that only marginal killing of
T cells was achieved if 1-5% of the effector cells expressed FasL. Therefore, values
below this would not likely result in an effective decrease in graft rejection. Additional
studies are currently underway, both to assess the long term stability of a
nonmyeloablative transplant and for the expression of FasL. The present studies tested
the acute effects mediated by FasL expression in lin- BMs. In potential future application
in BMT, permanent immunosuppression may not be necessary to achieve stable
hematopoietic engraftment and tolerance. If so, one might minimize exposure of the
recipient to FasL by co-engineering the FasL+ BM cells with a suicide gene so that the
FasL+ cells could be deleted as soon as engraftment and tolerance were observed.

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References:


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Figure Legends

Figure 1. FasL in the transduced cells was functional: FasL+ lin− BMs killed Jurkat T cells in a dose-dependent fashion, and FasL+ DCs decreased T cell proliferative responses.

Fig 1a: B6.SJL lin− BMs were transduced 3 times to express either the control GFP vector or the vector expressing the GFP-FasL fusion. On day 5 of *ex vivo* transduction culture, the cells were FACS sorted into (FasL+)GFP+ or (FasL−)GFP− subsets. The (FasL+)GFP+ subset expressed >10-fold higher fluorescence that the (FasL−)GFP− subset; the lin− BMs with intermediate levels of fluorescence were discarded. Sorted (FasL+)GFP+ cells were mixed in varying ratios with (FasL−)GFP− cells for a total of 10⁵ lin− BMs, as indicated, in duplicate wells of a 96 well plate containing 10⁵ PKH labeled Jurkat cells. 24 hours later, 7-AAD was added to the cultures, which were then analyzed by FACS for the percent PKH+ (Jurkat) cells that had incorporated 7-AAD. Shown are histograms of 7-AAD fluorescence in gated PKH+ (Jurkat) cells. Mixtures containing 1, 5, 20, or 80% (FasL+)GFP+ lin− BMs, indicated by the graph labels, resulted in 5, 9, 36, or 42% dead Jurkat cells, respectively.

Fig 1b: Function of modified DCs was tested in a standard T cell proliferation assay. GFP control- or FasL-transduced B6.SJL or BALB/c DCs were generated as described, then irradiated (3000 cGy) and incubated with responder spleen cells (B6, transgenic 2C (on a B6 background) or BALB/c, as indicated in the graph axis labels). Proliferation was determined by incorporation of ³H-thymidine. This plot shows the average (+/- SD) of three separate experiments.

Fig 1c: T cell proliferative responses are shown from experiments in which allogeneic DCs were mixed with syngeneic DCs as stimulators, with either the allogeneic DCs (B6) or the syngeneic DCs (BALB/c) modified to express FasL. In this set of experiments, 10⁶ BALB/c spleen cells were mixed with the following (for each column shown in order): (1) 10⁵ control B6 DCs; (2) 10⁵ FasL+ B6 DCs; (3) 5 x 10⁴ control B6 DCs plus 5 x 10⁴ control BALB/c DCs; (4) 5 x 10⁴ control B6 DCs plus 5 x 10⁴ FasL+ BALB/c DCs. After 3 days’ incubation, 1µCi ³H-thymidine was added for 24 hours, then cells were harvested and proliferation was determined by ³H-thymidine incorporation. The results are shown for 2 separate experiments, with triplicates for each condition.

Figure 2. FasL expression in lin− BMs did not inhibit generation of CFCs or syngeneic in vivo engraftment, assessed early post-BMT.

Fig 2a: B6.SJL lin− BMs were transduced with either the FasL(-GFP fusion) or control GFP vector as described in Methods, then 3 x 10³ cells were plated in CFC assay medium
as described in Methods. The results are the averages (and SEM) of 4 separate experiments.

Fig 2b: CFC were assayed from untransduced B6.SJL lin− BMs that were exposed to sFasL in vitro, at the concentrations indicated, for 24 hours prior to plating in methylcellulose. Colonies were counted 7 days later; shown are the averages of 2 separate experiments.

Fig 2c: BALB/c lin− BMs were transduced with either the GFP control or FasL vector, then 10^5 cells were transplanted into 850 cGy irradiated syngeneic mice (5 mice per group). Shown is the histogram of GFP expression in the starting population of transduced lin− BMs.

Fig 2d: Three weeks after transplant of the cells shown in Fig 2c, mice were tail bled to determine the percentage of circulating transduced cells. Whole blood was collected by tail bleeds and red cells removed by hypotonic lysis, then analyzed by FACS for GFP expression. The graph shows the percent GFP+ blood cells after transplant (each point represents one mouse).

Figure 3. Mice transplanted with FasL+ allogeneic lin− BMs had enhanced engraftment early after BMT.

Fig 3a: B6.SJL (CD45.1+) lin− BMs were transduced with either control GFP or FasL(-GFP) vector. Cells were analyzed by FACS to determine the level of GFP fluorescence of the transduced cells prior to transplant.

Fig 3b and Table 1: 10^5 transduced lin− BMs were transplanted iv into 400cGy irradiated C3H.SW (CD45.2+) recipients. Upon sacrifice at 3 weeks post-BMT, mouse organs were analyzed by FACS for correlated expression of CD45.1 and GFP. The graph shows the compilation of data for all mice shown in Table 1, with the average percentage of CD45.1+ cells in BM of each group. Table 1 shows the data for each mouse analyzed, with the first column being the percent donor cells for mice receiving unmodified lin-BM, the second column being the percent donor cells for mice receiving GFP modified BM, the third showing percent donor cells from the FasL modified BM, and the fourth being the percent donor cells in the FasL transplants that were also GFP+.

Fig 3c: The figure shows a representative plot for CD45.1 for a mouse that was transplanted with GFP modified cells (top) and a mouse transplanted with FasL modified cells (bottom).

Fig 3d: These histograms show the GFP fluorescence of gated CD45.1+ (donor) cells (from Fig 3b) of representative mice transplanted with lin− BMs transduced with either control GFP or FasL and analyzed at 3 weeks post-BMT.
Figure 4. Mice transplanted with FasL$^+$ lin$^-$$^-$ BMs did not have diminished numbers of BM CFCs early after BMT.

Upon sacrifice of the mice described in Figure 3, whole BM (3x10$^5$ cells) was assayed for CFC (triplicates). Seven days after plating, colonies were counted (averages +/- SEM are shown).

Table 2. Retention of graft at 4-6 months after transplant.
Table 2 shows the percent donor chimerism of mice that were transplanted with modified cells and analyzed 4-6 months later. Four of 7 transplanted FasL mice retained the graft at detectable levels, while 0/7 GFP transplants retained the graft. No GFP positive cells were detected in the FasL transplants at this later timepoint.

Figure 5. Mice transplanted with FasL$^+$ lin$^-$$^-$ BMs did not have hepatocellular injury or enhanced hepatic inflammation and retained immune responsiveness to a third party alloantigen.

Fig 5a and 5b: Livers from mice shown in Fig 3 were fixed in formaldehyde, cut into paraffin blocks, then stained with hematoxylin/eosin for evaluation of inflammation. Fig 5a is a representative section from a control GFP transplanted mouse, and Fig 5b is from the liver of a representative FasL$^+$ transplanted mouse.

Fig 5c: Splenocytes from transplanted mice were incubated as responders with irradiated (3000 cGy) allogeneic third party stimulator (BALB/c) spleen cells. Four days after adding stimulators, $^3$H-thymidine was added overnight. Cells were then harvested, and incorporation of $^3$H-thymidine was determined. Fig 5c shows the proliferative responses (group average +/-SEM) from 8 mice transplanted with FasL-transduced lin$^-$$^-$ BMs, 7 mice transplanted with control GFP-transduced lin$^-$$^-$ BMs, 4 mice transplanted with untransduced lin$^-$$^-$ BMs, and 3 untransplanted control C3H.SW mice, taken from three separate experiments.

Figure 6. Mice transplanted with syngeneic FasL$^+$ lin$^-$$^-$ BMs responded to an antigenic infectious challenge.

Fig 6a: Representative liver histologies of mice four days after Listeria monocytogenes injection. Some hepatic inflammation was seen in all mice in both control and experimental groups.

Fig 6b: Livers were analyzed for T cell infiltration by making single cell suspensions from a portion of the liver and staining for T cell markers. Shown are representative FACS plots of correlated CD4 and CD8 staining of the liver cells from a normal uninfected mouse liver, a liver from a mouse transplanted with control GFP$^+$ lin$^-$$^-$ BMs, and a liver from a mouse transplanted with FasL$^+$ lin$^-$$^-$ BMs.
Transduction of donor hematopoietic stem-progenitor cells with Fas ligand enhanced short-term engraftment in a murine model of allogeneic bone marrow transplant

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