Dendritic Cells Genetically Engineered to Express Fas Ligand Induce Donor-Specific Hyporesponsiveness and Prolong Allograft Survival[1]

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Polarization of an immune response toward tolerance or immunity is dictated by the interactions between T cells and dendritic cells (DC), which in turn are modulated by the expression of distinct cell surface molecules, and the cytokine milieu in which these interactions are taking place. Genetic modification of DC with genes coding for specific immunoregulatory cell surface molecules and cytokines offers the potential of inhibiting immune responses by selectively targeting Ag-specific T cells. In this study, the immunomodulatory effects of transfecting murine bone marrow-derived DC with Fas ligand (FasL) were investigated. In this study, we show that FasL transfection of DC markedly augmented their capacity to induce apoptosis of Fas$^+$ cells. FasL-transfected DC inhibited allogeneic MLR in vitro, and induced hyporesponsiveness to alloantigen in vivo. The induction of hyporesponsiveness was Ag specific and was dependent on the interaction between FasL on DC and Fas on T cells. Finally, we show that transfusion of FasL-DC significantly prolonged the survival of fully MHC-mismatched vascularized cardiac allografts. Our findings suggest that DC transduced with FasL may facilitate the development of Ag-specific unresponsiveness for the prevention of organ rejection. Moreover, they highlight the potential of genetically engineering DC to express other genes that affect immune responses. *The Journal of Immunology,* **2000, 164: 161–167.**

Dendritic cells (DC)[3] are APC that play a critical role in the initiation of immune responses (1). Although the potent immunostimulatory capacity of DC is well recognized, recent evidence suggests that DC are also capable of inducing donor-specific hyporesponsiveness (2). It is not yet clear whether these apparently opposing functions of DC reflect distinct subpopulations of DC (3, 4), or alternatively, distinct functions expressed at unique stages in the developmental cycle of the same cell (5). Understanding the nature of the heterogeneity of DC function would promote the development of DC-based immunotherapy for the treatment of many diseases, including the induction of tolerance in transplantation and in autoimmune disorders.

Genetic modification of DC with genes encoding immunoregulatory molecules is an alternative approach for artificial generation of tolerogenic DC. Indeed, recent reports suggest that transfection of DC with IL-10 and TGF-β can increase their tolerogenic potential (6, 7). Several attributes make DC ideal vehicles for the delivery of such molecules. They are potent activators of naive T cells, a function related to their Ag-processing capacity and to high levels of expression of MHC and costimulatory molecules. In addition, they have unique migratory capability, enabling them to move from peripheral tissues to secondary lymphoid organs, where they interact with T and B cells (8, 9). The cognate recognition of DC and T cells provides the theoretic opportunity of these immunomodulatory molecules to influence the immune response in an Ag-specific manner.

One molecule that may enhance the tolerance-inducing capacity of DC is Fas ligand (FasL), a type II integral membrane protein that belongs to the TNF superfamily (10). Engagement of Fas by FasL initiates a signaling cascade that leads to apoptotic cell death of Fas-bearing cells. Apoptosis induced by Fas/FasL interactions is thought to play a pivotal role in the immune system, regulating both peripheral T cell homeostasis and lymphocyte-mediated cytotoxicity. FasL is expressed in immunoprivileged organs, including the eye and testis, where it has been proposed to contribute to their tolerogenic milieu and paucity of infiltrating inflammatory cells (11–13). There is evidence that FasL constitutively expressed on splenic DC and bone marrow-derived DC may be involved in the killing of activated CD4$^+$ T cells (14, 15). More recently, tolerance induced by infusion of donor bone marrow cells was shown to be dependent on the expression of FasL on the infused cells (16). In the studies described below, we investigate the immunomodulatory effect of DC transduced to express high levels of FasL in vitro and in vivo.

Materials and Methods

Mice

Male C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H (H-2^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*lpr/lpr* mice were purchased originally from The Jackson Laboratory and bred in our animal facility. All mice were used at 8–12 wk of age.

Generation of bone marrow-derived DC

DC were generated from bone marrow progenitor cells, as described by Inaba et al. (17) and modified by Suri et al.[4] Briefly, bone marrow cells

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[3] Abbreviations used in this paper: DC, dendritic cell; DNFB, dinitrofluorobenzene; FasL, Fas ligand.

[4] R. M. Suri, M. B. Lutz, A. L. J. Ogilvie, S. Robner, M. Nimi, N. Kukutsch, G. Schuler, and J. M. Austyn. 1999. Stably immature dendritic cells induce T cell unresponsiveness in vitro and prolong allograft survival in vivo. *Submitted for publication.*

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were flushed from femurs and tibias of C57BL/6 mice, washed, and cultured in 6-well plates (2×10^6/ml) in 4 ml RPMI 1640 containing rGM-CSF (10 ng/ml; Peprotech, Rocky Hill, NJ) and mouse rIL-4 (10 ng/ml; Peprotech). All media and additives were documented to be free of LPS contamination (18). Nonadherent granulocytes were removed after 48 h of culture, and fresh media added every 48 h. By day 4 to 6 of culture, proliferating clusters of cells with typical dendritic morphology were seen, and by day 7 to 9 more than 90% of the cells expressed the DC cell surface marker DEC-205. The proportion of cells staining for T (CD3) and B (B220) lymphocytes was consistently <3%.

Gene transfection

pBK-CMV phagemid vector (2 μg) containing full-length human FasL cDNA or empty control vector was incubated with 8 μl of Lipofectin (Life Technologies, Gaithersburg, MD) in a volume of 100 μl of PBS at room temperature for 45 min. This mixture was added to 7-day cultured DC in a final volume of 1 ml of serum-free medium. After 4-h incubation at 37°C with 5% CO_2, the cells were washed and cultured in RPMI 1640 with 10% FCS for 48 h.

Flow cytometry

Phenotypic analysis of DC was performed at day 9 (2 days after gene transfection) of culture using an EPICS XL-MCL Cell Analysis System (Coulter, Miami, FL). The following mAbs were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada), unless otherwise indicated, and used for staining cells as primary mAbs: anti-DEC205 (clone NLDC-145), anti-mouse H-2^b, anti mouse I-E^b, and anti-mouse CD40 (PharMingen, San Diego, CA). The secondary mAbs used were FITC-conjugated anti-rat IgG2a (Caltag Laboratories) or FITC-conjugated anti-mouse IgG2a (PharMingen). The following mAbs, purchased from PharMingen, were used directly as FITC-conjugated mAb: anti-mouse CD80 (B7-1), anti-mouse CD86 (B7-2), anti-mouse CD3ϵ, anti-mouse CD4, anti-mouse CD8, anti-mouse Mac1, and anti-mouse B220. FasL-transfected and control DC were stained with anti-human FasL mAb (MBL, Nagoya, Japan) or isotype control, followed by secondary PE-conjugated anti-hamster IgG (Cedarlane Laboratories)

RT-PCR

Total RNA was extracted from DC (1×10^7) 48 h after transfection with FasL or the empty control vector, with TRIzol reagent (Life Technologies), as per the manufacturer's instructions. First strand cDNA was synthesized using an RNA PCR kit (Life Technologies) with the supplied oligo$(dT)_{16}$ primer.

One microliter of the reverse-transcription reaction product was used for the subsequent PCR reaction. The sequence of the human FasL primers, which generated a 293-bp fragment of human FasL, was: sense, 5′-AAT AGGCCACCCCAGTCCA-3′; antisense, 5′-CCCCTCCATCATCACCA GA-3. The sequence of the mouse β-actin primers was: sense, 5′-AGGCA TCCTGACCCTGAAGTAC-3′; antisense, 5′-TCTTCATGAGGTAGTCT GTCAG-3′. The samples were denaturated for 1 min at 94°C, annealed for 1 min at 53°C, and extended for 1 min at 72°C, for a total of 35 cycles. The PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. β-actin was used as an internal control for RNA integrity.

DNA fragmentation (JAM assay)

Jurkat cells (1×10^4) were labeled with 5 μCi/ml [^{3}H]thymidine for 4 h at 37°C (19) and seeded in triplicate in U-bottom 96-well tissue culture plates as target cells. They were incubated with the indicated ratio of FasL- or control-transfected DC in a total volume of 200 μl/well for 18 h. Unfragmented high m.w. DNA was harvested onto glass fiber filters and counted in a Beckman scintillation counter. Data are expressed as percentage of DNA fragmentation: $100 \times [(1 - \text{cpm in experimental group})/(\text{cpm of unstimulated targets alone})]$.

Mixed leukocyte reactions

Two days after gene transfection, FasL- or control-transfected DC (1×10^4) were treated with 50 μg/ml of mitomycin C at 37°C for 20 min, washed twice with RPMI, and seeded in triplicate in flat-bottom 96-well culture plates (Corning Glass, Corning, NY) for use as stimulator cells. Responder spleen cells (2×10^5/well) from BALB/c mice were added to the DC in a total volume of 200 μl of RPMI 1640 containing 10% FCS, 50 μM 2-ME, 1 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) at the indicated time points, cultured an additional 16 h, and collected onto glass fiber filters; [^{3}H]thymidine incorporation was quantified using a Beckman scintillation counter. Results were expressed as the mean cpm of triplicate cultures.

For MLR using C57BL/6-*lpr/lpr* responder splenocytes, the stimulating DC were generated from BALB/c mice and transfected as described above.

Blockade of FasL

Soluble Fas:Fc fusion protein (Alexis, San Diego, CA), or control human IgG (Calbiochem, San Diego, CA) was added at the beginning of MLR and DNA fragmentation assays at the concentrations indicated, and as described by Desbarats et al. (20).

Flow-cytometric analysis for T cell apoptosis

Quantitative determination of T cell apoptosis was analyzed by flow cytometry, as described by Nicoletti et al. (21). Purified T cells were activated with Con A (5 μg/ml) for 72 h and collected over Ficoll-Hypaque. Viable blasts (5×10^6) were incubated with 5×10^5 FasL-transfected or control DC for 24 h. Cell suspensions were centrifuged at $200 \times g$ for 10 min, gently resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide, 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, 0.1% Triton X-100), and stored in the dark for 3–4 h before being analyzed by flow cytometry. The apoptotic cells were quantified as the percentage of cells with subdiploid DNA.

Donor-specific hyporesponsiveness induced by allogeneic DC injection

FasL- or empty control vector-transfected BALB/c DC (2×10^6) were injected i.p. into groups of nine C57BL/6 and four C57BL/6-*lpr/lpr* mice at 3-day intervals for a total of six injections, as per Zhang et al. (22). Mice were sacrificed 3 days after the last injection, and MLR cultures were initiated with fresh mitomycin C-treated BALB/c spleen stimulator cells, as described above. Paraffin sections of Formalin-fixed liver and spleen biopsies from these animals were stained with hematoxylin and eosin for histologic evaluation.

Heterotopic heart transplantation with DC pretreatment

FasL- or empty control vector-transfected BALB/c DC (2×10^6) were injected i.p. into groups of six C57BL/6 at 3-day intervals for a total of six injections. Within 3 days of the last injection, vascualized heterotopic heart transplants from BALB/c mice were performed and monitored daily, as described (23). Rejection was defined by the cessation of heartbeat.

Statistical analysis

Continuous variables were compared with Student *t* tests. Cardiac graft survival curves were calculated by the Kaplan-Meier method, with differences between groups compared by the log-rank test. A p value <0.05 was considered significant.

Results

Transfection and expression of functional FasL in DC

DC were propagated from bone marrow cells cultured with GM-CSF and IL-4, as described in *Materials and Methods*. By the seventh day of culture, cells with characteristic DC morphology and immunophenotype (DEC-205^+, MHC class II^+, $CD40^+$, $CD80^+$, $CD86^{2+}$, $Mac1^{low}$, $B220^-$, $CD3^-$, $CD4^-$, $CD8^-$) were observed.

To optimize gene delivery into DC by lipofection, we first used the *Escherichia coli* β-galactosidase gene under the control of the CMV immediate promoter as a reporter system. The optimal DNA (μg):Lipofectin (μl) ratio was found to be 1:4 using an incubation time of 4 h. With these conditions, the transfection efficacy was 50–70%, and cell viability was more than 90% (data not shown). Transfection of DC with the FasL vector construct using the same conditions resulted in high levels of FasL gene expression, but not with the empty control vector, as determined by RT-PCR and flow-cytometric analysis (Fig. 1). Forty-eight hours after transfection, ~50% of the DC stained positive for FasL (range, 29% to 68% in 20 independent experiments). Transfection with either FasL or the control vector did not adversely affect cell viability or the expression levels of cell surface molecules (Fig. 2).

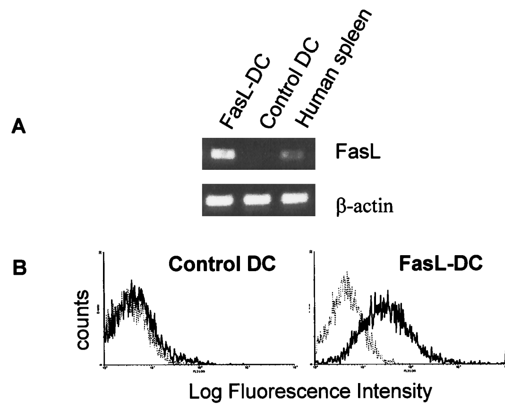

FIGURE 1. *A*, Expression of human FasL in murine DC. *Top,* Human FasL mRNA expression in FasL-DC and human spleen by RT-PCR; *bottom*, β-actin mRNA expression by RT-PCR. Control, empty vector-transfected DC. *B*, FasL expression in DC by flow-cytometric analysis. Two days after transfection with FasL or empty control vector, DC were collected and stained with anti-human FasL mAb and PE-conjugated anti-hamster IgG, as described in *Materials and Methods*. Thin dotted lines denote isotype control.

The biological activity of FasL in transfected DC was confirmed in a DNA fragmentation assay that used Fas^{+} Jurkat cells (Fig. 3*A*). DC transfected with FasL induced high levels of DNA fragmentation as compared with those transfected with the control vector. Moreover, we found that the addition of blocking Fas-Fc effectively inhibited DNA fragmentation. This inhibition was dose dependent and specific, as it was unaffected by the addition of control Ig (Fig. 3*B*).

FasL-transfected DC down-regulate T cell responses in vitro

To assess the functional activity of transfected DC in stimulating allogeneic T cell responses, MLR reactions were performed using FasL- or control-transfected DC. Allogeneic T cell proliferation was significantly decreased when incubated with FasL-DC, but not with the control DC (Fig. 4*A*). The stimulatory capacity of FasL-DC could be restored in the presence of soluble Fas-Fc, but not control Ig, indicating that inhibition of allogeneic MLR by FasL-DC was specific to FasL (Fig. 4*B*). Furthermore, these results confirmed that DC transfected with FasL were viable and capable of presenting alloantigen.

FasL-transfected DC do not inhibit T cell responses in Fas-deficient mice

To further establish the functional relevance of Fas:FasL interactions in the inhibition of the MLR response by FasL-DC, the MLR studies were repeated with lymphocytes from C57BL/6-*lpr/lpr* mice, which do not express Fas. In these experiments, DC were propagated from BALB/c bone marrow cells and transfected as described above. FasL-DC inhibited the proliferative response of wild-type lymphocytes, whereas there was no inhibition of C57BL/6-*lpr/lpr* lymphocytes (Fig. 5).

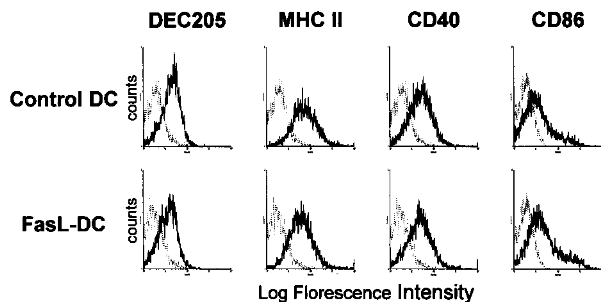

FIGURE 2. Phenotypic analysis of DC after transfection with FasL. DC were transfected after 7 days of culture in GM-CSF and IL-4 with empty control vector (*top*) or FasL (*bottom*). They were stained for DEC-205, MHC class II, CD40, and CD86, and analyzed by flow-cytometric analysis 48 h after transfection. Thin dotted lines denote isotype controls.

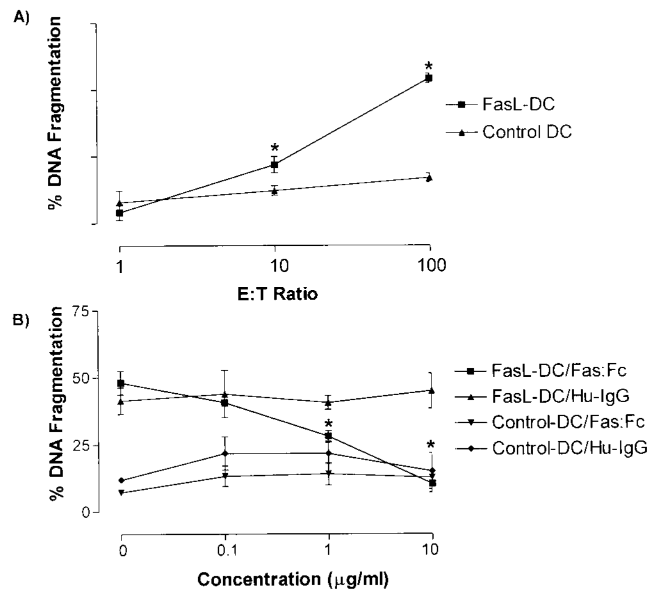

FIGURE 3. DNA fragmentation assay. *A*, Jurkat cells were labeled with [^{3}H]thymidine and incubated with graded numbers of FasL- or empty vector-transfected DC, for 18 h. The percentage DNA fragmentation was calculated as described in *Materials and Methods*. *B*, Inhibition of DNA fragmentation by soluble Fas:Fc. Radiolabeled Jurkat cells were mixed with FasL- or control-transfected DC (E:T ratio 50:1), and human Fas:Fc protein or control human IgG was added at the beginning of the cultures. Results are expressed as mean ± SD, and are representative of three independent experiments; *, $p < 0.02$.

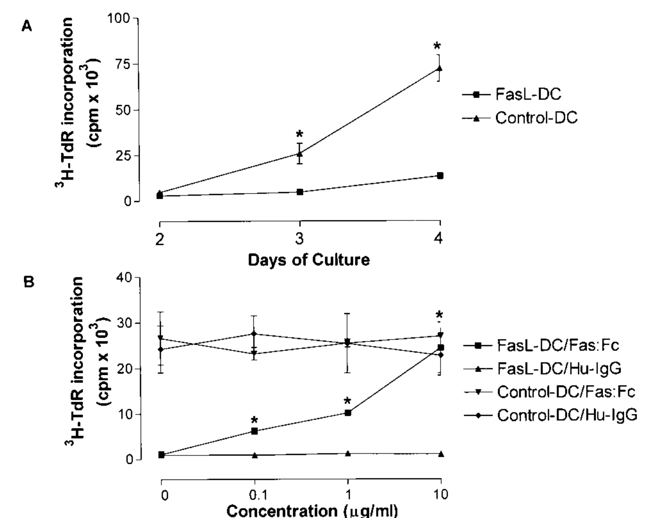

FIGURE 4. FasL-DC inhibit allogeneic MLR. *A*, Cultures were set up in triplicate with 2×10^{5} BALB/c responder spleen cells and 1×10^{4} mitomycin-treated DC that were transfected with FasL or control vectors. Proliferation at the indicated time points was determined by [^{3}H]thymidine incorporation. *B*, The stimulatory capacity of FasL-DC was restored with the addition of soluble Fas:Fc, but not control human IgG, at the beginning of a 3-day MLR. Results are expressed as mean cpm $\times 10^{3}$ ± SD, and are representative of three different experiments; *, $p < 0.01$.

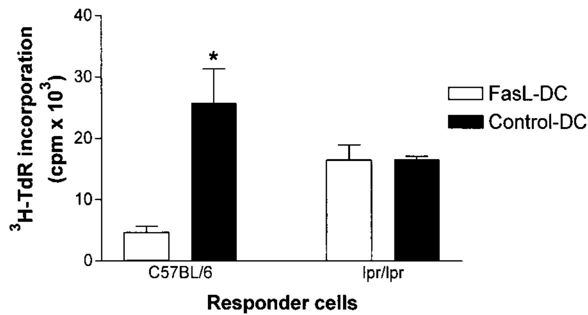

FIGURE 5. FasL-DC do not inhibit proliferation of splenocytes from *lpr* mice. DC (1×10^4) propagated from BALB/c BM cells were transfected with FasL or control vector, treated with mitomycin C, and cocultured with 5×10^5 responder splenocytes from C57BL/6-*lpr/lpr* or C57BL/6 wild-type mice for 3 days. Proliferation was determined by [^{3}H]thymidine incorporation. Results are expressed as mean cpm $\times 10^3 \pm$ SD, and are representative of three different experiments; *, $p < 0.02$.

FasL-DC induce T cell apoptosis

To explore the mechanism by which FasL-DC inhibit MLR, we determined their ability to induce apoptosis of activated T cells using a quantitative fluorometric assay for hypodiploid DNA. Con A blasts were incubated with FasL-transfected or control-transfected DC for 24 h. As shown in Fig. 6, DC transfected with FasL induced significantly higher levels of apoptosis than the control-transfected cells.

FasL-DC induce unresponsiveness after injection into mice

To determine whether Ag-specific inhibition of alloreactivity would be seen after pretreatment of naive mice with FasL-DC, C57BL/6 or C57BL/6-*lpr/lpr* mice received i.p. injections of 2×10^6 BALB/c-derived DC (FasL or control vector transfected) at 3-day intervals for a total of six injections. Three days after the last injection, all mice were sacrificed and MLR cultures were initiated using spleen cells from all individuals stimulated with BALB/c (allogeneic) or C3H (third-party) mitomycin-treated spleen stimulator cells. Control cultures used cells from nonimmunized (no DC treatment) mice.

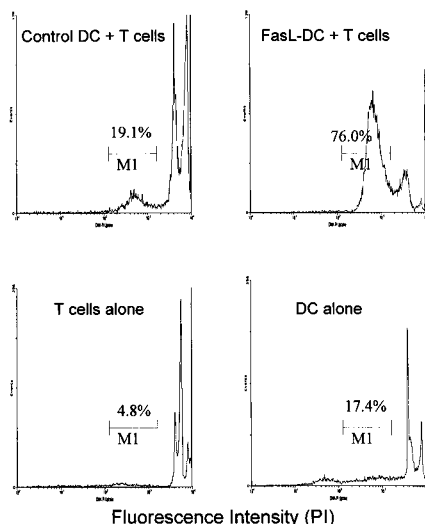

FIGURE 6. FasL-DC induce apoptosis of Con A blasts. DC (1×10^5), propagated from C57BL/6 mice and transfected with FasL or control vector, were cocultured with BALB/c Con A blasts (1×10^6) for 24 h. Flowcytometric analysis for subdiploid DNA is indicated in the region marked M1. The results are representative of three independent experiments.

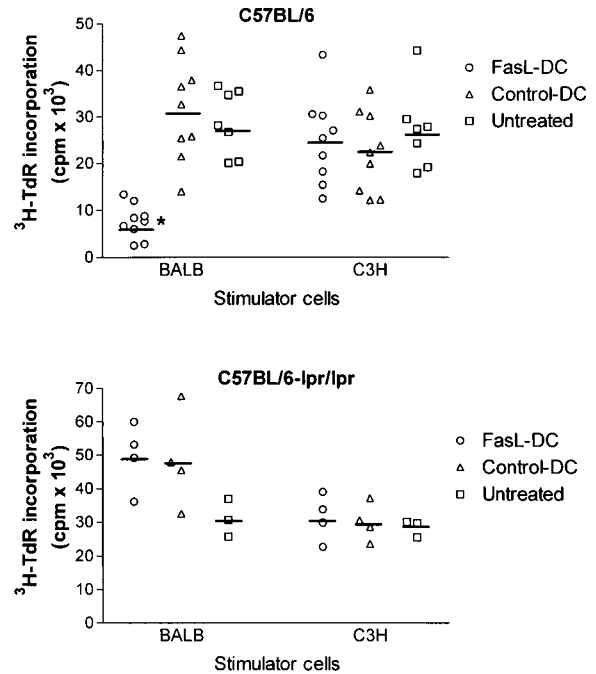

FIGURE 7. FasL-DC induce donor-specific hyporesponsiveness in vivo. Nine C57BL/6 mice per group (*upper panel*) or four C57BL/6-*lpr/lpr* (*lower panel*) mice per group received six injections of 2×10^6 BALB/c DC transfected with FasL or control vector, or were untreated (see *Materials and Methods* and text for details). Spleen cells were harvested 3 days after the last injection and stimulated in vitro with mitomycin-treated BALB/c or C3H (third party) spleen cells for 3 days. The proliferative response, as determined by [^{3}H]thymidine incorporation, is shown for each mouse. The horizontal bars represent the mean proliferative response of each group; *, $p < 0.001$ compared with mice treated with control DC. Data are representative of two independent experiments

FasL-DC treatment induced allospecific hyporesponsiveness during restimulation with fresh stimulators in vitro, while splenocytes from mice treated with control DC showed normal secondary responsiveness (*upper panel* of Fig. 7). This suppression of secondary proliferative responses by FasL-DC was abrogated in *lpr* mice (*lower panel* of Fig. 7).

FasL-DC prolong cardiac allograft survival

The capacity of FasL-DC to induce alloantigen-specific hyporesponsiveness in vivo suggested that these cells might prolong allograft survival. We tested this possibility with a vascularized heterotopic cardiac transplant model. Groups of five to six C57BL/6 mice were pretreated with i.p. injections of 2×10^6 BALB/c control- and FasL-DC at 3-day intervals for a total of six injections. As shown in Fig. 8, mean graft survival was significantly longer in mice pretreated with FasL-DC as compared with both untreated controls and those pretreated with control-DC (20 ± 4 vs 10 ± 2 and 9 ± 3 days, respectively; $p = 0.01$ by log-rank test).

Discussion

In this study, we show that primary murine bone marrow-derived DC can be successfully transduced to express high levels of FasL. FasL-DC were capable of killing Fas$^+$ cells through apoptosis, down-regulating allogeneic MLR in vitro, and inducing donor-specific hyporesponsiveness to alloantigen in vivo. Finally, we

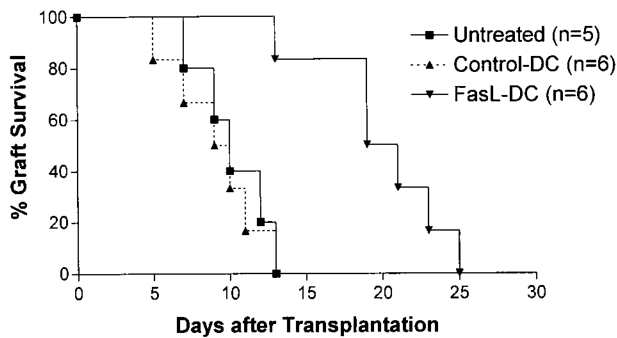

FIGURE 8. FasL-DC prolong cardiac allograft survival. C57BL/6 recipients were pretreated i.p. with six injections of 2×10^6 BALB/c DC transfected with FasL or control vectors, or were untreated. Within 3 days of the final injection, BALB/c cardiac allografts were transplanted, and monitored daily. $p = 0.01$ by log-rank test. Data are representative of two independent experiments.

show that these cells can prolong organ allograft survival when administered before transplantation.

A promising strategy for inducing tolerance to alloantigens is infusion of tolerogenic DC. Previous studies have shown that immature DC, which characteristically express low levels of costimulatory molecules such as CD80 and CD86, can promote the development of donor-specific tolerance and prolong cardiac and islet allograft survival (24, 25). In vitro growth conditions can be manipulated to enhance the generation of immature DC from bone marrow cells (26, 27); however, subsequent maturation in vivo may limit their tolerogenic potential (25). Genetic engineering of DC with genes encoding immunoregulatory molecules provides an alternative method of generating tolerogenic DC that might be more effective. The feasibility of this approach is supported by recent studies showing that DC can be genetically modified using retroviral and adenoviral vectors to express model tumor Ags that promote both protective and antitumor immunity (28–30), and cytokines that augment (IFN-γ, IL-12) and inhibit (IL-10, TGF-β) immune responses (6, 7, 31). Other molecules that could potentially enhance the capacity of DC to promote tolerance include CTLA4-Ig, which would block CD80 and CD86 costimulatory pathways, and OX-2, which we and others have recently shown down-regulates T cell responses through a unique costimulatory pathway (32, 33).

Apoptosis induced by Fas/FasL interactions is one mechanism implicated in peripheral T cell tolerance. Enhanced or elevated expression of FasL on specific tissues or cells by a transgene technique is being extensively applied for gene therapy of tumors (34) (35), rheumatoid arthritis (36), autoimmune diseases (37, 38), and regulation of rejection in transplantation (39–41). Thus, significant prolongation of allogeneic grafts has been achieved by directly transducing FasL gene into donor tissue or organs before transplantation (39, 40), or by cotransplanting FasL-transfected carrier cells (41). The studies we report suggest that DC transfected with FasL are capable of down-regulating T cell responses. This modulating effect appears to function by inducing T cell apoptosis via a Fas/FasL pathway, because inhibition was blocked by a Fas:Fc fusion protein, and failed to occur in lymphocytes from C57B6-*lpr/lpr* Fas mutant mice. Furthermore, we show that systemic administration of these cells not only inhibits donor-specific alloresponsiveness, but also prolongs cardiac allograft survival.

Our results are consistent with those of Zhang et al., who recently showed that a FasL-transfected macrophage cell line was capable of inducing allogeneic T cell hyporesponsiveness in vivo (22). They provide evidence with a CD8$^+$ transgenic model that FasL-transfected macrophages are capable of inducing rapid and profound clonal deletion of Ag-specific T cells (22). It is interesting to consider the possibility that the peritoneal macrophage cell line used these studies, which express MAC1, F4/80, MHC class I and II, as well as significant amounts of B7 contained population(s) of DC (42). This would explain their finding of fluorochrome-labeled cells in splenic T cell areas after i.v. injection, which is a property characteristic of DC (43). Furthermore, it would be valuable to investigate whether the clonal deletion suggested in their study might be due to the migration of Ag-specific clones out of the spleen (44)or down-regulation of CD8 itself (45). Another potential mechanism that might contribute to the hyporesponsiveness induced by FasL-DC is polarization of Th cells toward a Th2 phenotype, as Th1 cells are reported to be more sensitive to FasL-mediated apoptosis than Th2 cells (46). These issues are currently under investigation in our laboratory using highly purified subsets of FasL-DC in an allospecific transgenic model.

Similarly, Matsue et al. have recently reported the generation of killer DC from an immortalized DC line (47). The authors show that peptide-pulsed FasL-expressing DC are capable of inducing Ag-specific T cell hyporesponsiveness in delayed-type hypersensitivity and contact hypersensitivity responses both prophylactically and therapeutically. The homing capacity and mechanism of action of these cells in vivo were not addressed, however. Interestingly, these killer DC were unable to block the induction of immune responses to alloantigen or Ab responses to nominal Ag, suggesting that there may be important differences between these cells and those used in our and Zhang's (22) study.

Although our study indicates that FasL-DC can prolong survival of vascularized cardiac allografts, all grafts ultimately failed from rejection, suggesting that complete (or lasting) depletion of alloreactive cells did not occur. These results are in agreement with those of Matsue et al., who showed that hyporesponsiveness to dinitrofluorobenzene (DNFB) following treatment with a DNFB-pulsed FasL-DC clone was temporary and could be reversed with subsequent DNFB resensitization (47). Ongoing studies in our laboratory are being performed to determine whether the duration of allograft survival can be extended.

Earlier reports have shown that systemic administration of anti-Fas Abs and FasL-expressing viruses to mice causes massive hepatocyte apoptosis and liver failure, which has been attributed to high levels of expression of Fas on hepatocytes (48, 49). FasL also has proinflammatory properties mediated by recruitment and activation of neutrophils (50, 51). In our studies, however, treatment of mice with FasL-DC was remarkably well tolerated. Furthermore, histologic examination of livers from both the wild-type and *lpr/lpr*-treated mice showed no evidence of hepatitis or hepatocyte apoptosis (data no shown). Specific homing patterns of DC to secondary lymphoid organs may account for the lack of toxicity, and are currently being studied.

Recently, Matsue et al. reported that ligation of Fas on DC by FasL on T cells is capable of inducing DC apoptosis, and suggested that this may be one mechanism by which immune responses are normally terminated (52). Thus, one caveat of transfecting DC with FasL is that it might directly trigger DC apoptosis. However, the viability of DC after transfection in our studies was consistently greater than 90%, as determined by trypan blue exclusion and annexin V staining (data not shown). Whether FasL/Fas interactions induce DC apoptosis is most likely dependent on a variety of factors, including DC origin (e.g., spleen vs bone marrow), maturation stage, and expression levels of antiapoptotic proteins such as Bcl-2 and Bcl-x_L (53, 54). The use of phenotypically mature DC (MHC II^{high}, DEC205$^+$, CD40$^+$, CD86^{2+}) in the

present study, which express higher levels of Bcl-2 than immature DC, may account for their tolerability to FasL transfection. In addition, several ligand/receptor interactions have been shown to affect the outcome of FasL/Fas interaction. For example, DC survival signals expressed on T cells, such as TRANCE and CD40L, can prevent Fas-induced apoptosis (55–57).

Another potential limitation of using FasL-DC to inhibit immune responses is that the level of expression of functional Fas on naive T cells may be insufficient to trigger apoptosis. In fact, Nishimura et al. (58) have shown that murine T cells are resistant to anti-Fas mAb treatments. However, recent studies by Suda et al. (59) indicate that the membrane-bound form of FasL is capable of killing both fresh and in vitro activated peripheral blood T cells, whereas soluble FasL only kills the latter. The ability of FasL-DC to induce systemic T cell hyporesponsiveness in our study may be due, at least partly, to the use of the entire (membrane form) FasL molecule, which would be expected to provide a potent apoptotic signal.

A variety of gene delivery methods has been reported for transfecting DC, including viral vectors (7, 60–62), electroporation (22, 63), and gene guns (64). We have also used a replication-deficient adenoviral vector to transfect DC with FasL, but found that despite providing a high transfection efficiency, cell viability was significantly less than with the Lipofectin method used in the present study (unpublished observations). Whether this was due to direct viral injury or to the level of FasL expression is unclear. Other important advantages of liposomal gene transfer are the avoidance of potential biological hazards and antigenicity, which are associated with viral vectors.

In summary, the results of the present study suggest that transfection of DC with FasL may be a practical way to suppress allospecific immune responses in transplant recipients, and possibly for the treatment of autoimmune diseases. Furthermore, our results also highlight the potential of using DC genetically engineered to express other immunoregulatory genes. Currently, our efforts are directed toward optimizing gene delivery and expression in DC, and defining the conditions that maximize their ability to modulate in vivo immune responses.

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References

1. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
2. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
3. Pulendran, B., J. L. Smith, G. Caspary, K. Brasel, D. Pettit, E. Maraskovsky, and C. R. Maliszewski. 1999. Distinct dendritic cell subset differentially regulates the class of immune responses in vivo. *Proc. Natl. Acad. Sci. USA 96:1036.*
4. Rissoan, M. C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt, and Y.-J. Liu. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science 283:1183.*
5. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med. 185:317.*
6. Takayama, T., Y. Nishioka, L. Lu, M. T. Lotze, H. Tahara, and A. W. Thomson. 1998. Retroviral delivery of viral interleukin-10 into myeloid dendritic cells markedly inhibits their allostimulatory activity and promotes the induction of T-cell hyporesponsiveness. *Transplantation 66:1567.*
7. Lee, W. C., C. Zhong, S. Qian, Y. Wan, J. Gauldie, Z. Mi, P. D. Robbins, A. W. Thomson, and L. Lu. 1998. Phenotype, function, and in vivo migration and survival of allogeneic dendritic cell progenitors genetically engineered to express TGF-β. *Transplantation 66:1810.*
8. Austyn, J. M., J. W. Kupiec-Weglinski, D. F. Hankins, and P. J. Morris. 1988. Migration patterns of dendritic cells in the mouse: homing to T cell- dependent areas of spleen, and binding within marginal zone. *J. Exp. Med. 167:646.*
9. Austyn, J. M. 1998. Dendritic cells. *Curr. Opin. Hematol. 5:3.*
10. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science 267:1449.*
11. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science 270: 1189.*
12. Korbutt, G. S., J. F. Elliott, and R. V. Rajotte. 1997. Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. *Diabetes 46:317.*
13. Bellgrau, D., D. Gold, H. Selawry, J. Moore, A. Franzusoff, and R. C. Duke. 1995. A role for CD95 ligand in preventing graft rejection. *Nature 377:630.*
14. Suss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J. Exp. Med. 183:1789.*
15. Lu, L., S. Qian, P. A. Hershberger, W. A. Rudert, D. H. Lynch, and A. W. Thomson. 1997. Fas ligand (CD95L) and B7 expression on dendritic cells provide counter-regulatory signals for T cell survival and proliferation. *J. Immunol. 158:5676.*
16. George, J. F., S. D. Sweeney, J. K. Kirklin, E. M. Simpson, D. R. Goldstein, and J. M. Thomas. 1998. An essential role for Fas ligand in transplantation tolerance induced by donor bone marrow. *Nat. Med. 4:333.*
17. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 176:1693.*
18. Suri, R. M., and J. M. Austyn. 1998. Bacterial lipopolysaccharide contamination of commercial collagen preparations may mediate dendritic cell maturation in culture. *J. Immunol. Methods 214:149.*
19. Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods 145:185.*
20. Desbarats, J., R. C. Duke, and M. K. Newell. 1998. Newly discovered role for Fas ligand in the cell-cycle arrest of $CD4^+$ T cells. *Nat. Med. 4:1377.*
21. Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods 139:271.*
22. Zhang, H., X. Su, D. Liu, W. Liu, P. Yang, Z. Wang, C. K. Edwards, H. Bluethmann, J. D. Mountz, and T. Zhou. 1999. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. *J. Immunol. 162: 1423.*
23. Corry, R. J., H. J. Winn, and P. S. Russel. 1973. Primarily vascularized allografts in mice: the role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation 16:343.*
24. Rastellini, C., L. Lu, C. Ricordi, T. E. Starzl, A. S. Rao, and A. W. Thomson. 1995. Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. *Transplantation 60:1366.*
25. Fu, F., Y. Li, S. Qian, L. Lu, F. Chambers, T. E. Starzl, J. J. Fung, and A. W. Thomson. 1996. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II^+, $CD80^{dim}$, $CD86^-$) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation 62:659.*
26. Lu, L., W. Li, F. Fu, F. G. Chambers, S. Qian, J. J. Fung, and A. W. Thomson. 1997. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation 64:1808.*
27. Yamaguchi, Y., H. Tsumura, M. Miwa, and K. Inaba. 1997. Contrasting effects of TGF-β1 and TNF-α on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells 15:144.*
28. Song, W., H. L. Kong, H. Carpenter, H. Torii, R. Granstein, S. Rafii, M. A. Moore, and R. G. Crystal. 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med. 186:1247.*
29. Wan, Y., J. Bramson, R. Carter, F. Graham, and J. Gauldie. 1997. Dendritic cells transduced with an adenoviral vector encoding a model tumor-associated antigen for tumor vaccination. *Hum. Gene Ther. 8:1355.*
30. McArthur, J. G., and R. C. Mulligan. 1998. Induction of protective anti-tumor immunity by gene-modified dendritic cells. *J. Immunother. 21:41.*
31. Ahuja, S. S., S. Mummidi, H. L. Malech, and S. K. Ahuja. 1998. Human dendritic cell (DC)-based anti-infective therapy: engineering DCs to secrete functional IFN-γ and IL-12. *J. Immunol. 161:868.*
32. Borriello, F., J. Lederer, S. Scott, and A. H. Sharpe. 1997. MRC OX-2 defines a novel T cell costimulatory pathway. *J. Immunol. 158:4548.*
33. Gorczynski, R. M., M. S. Cattral, Z. Chen, J. Hu, J. Lei, W. P. Min, G. Yu, and J. Ni. 1999. An immunoadhesin incorporating the molecule OX-2 is a potent immunosuppressant that prolongs allo- and xenograft survival. *J. Immunol. 163: 1654.*
34. Shimizu, M., Y. Takeda, H. Yagita, T. Yoshimoto, and A. Matsuzawa. 1998. Antitumor activity exhibited by Fas ligand (CD95L) overexpressed on lymphoid cells against Fas^+ tumor cells. *Cancer Immunol. Immunother. 47:143.*
35. Arai, H., D. Gordon, E. G. Nabel, and G. J. Nabel. 1997. Gene transfer of Fas ligand induces tumor regression in vivo. *Proc. Natl. Acad. Sci. USA 94:13862.*
36. Okamoto, K., H. Asahara, T. Kobayashi, H. Matsuno, T. Hasunuma, T. Kobata, T. Sumida, and K. Nishioka. 1998. Induction of apoptosis in the rheumatoid synovium by Fas ligand gene transfer. *Gene Ther. 5:331.*
37. Stassi, G., M. Todaro, F. Bucchieri, A. Stoppacciaro, F. Farina, G. Zummo, R. Testi, and R. De Maria. 1999. Fas/Fas ligand-driven T cell apoptosis as a consequence of ineffective thyroid immunoprivilege in Hashimoto's thyroiditis. *J. Immunol. 162:263.*
38. Saito, I., K. Haruta, M. Shimuta, H. Inoue, H. Sakurai, K. Yamada, N. Ishimaru, H. Higashiyama, T. Sumida, H. Ishida, et al. 1999. Fas ligand-mediated exocrinopathy resembling Sjogren's syndrome in mice transgenic for IL-10. *J. Immunol. 162:2488.*

39. Li, X. K., T. Okuyama, A. Tamura, S. Enosawa, Y. Kaneda, S. Takahara, N. Funashima, M. Yamada, H. Amemiya, and S. Suzuki. 1998. Prolonged survival of rat liver allografts transfected with Fas ligand-expressing plasmid. *Transplantation 66:1416.*
40. Gainer, A. L., W. L. Suarez-Pinzon, W. P. Min, J. R. Swiston, C. Hancock-Friesen, G. S. Korbutt, R. V. Rajotte, G. L. Warnock, and J. F. Elliott. 1998. Improved survival of biolistically transfected mouse islet allografts expressing CTLA4-Ig or soluble Fas ligand. *Transplantation 66:194.*
41. Lau, H. T., M. Yu, A. Fontana, and C. J. Stoeckert, Jr. 1996. Prevention of islet allograft rejection with engineered myoblasts expressing FasL in mice. *Science 273:109.*
42. Rosen, H., and S. Gordon. 1990. The role of the type-3 complement receptor in the induced recruitment of myelomonocytic cells to inflammatory sites in the mouse. *Am. J. Respir. Cell Mol. Biol. 3:3.*
43. Kupiec-Weglinski, J. W., J. M. Austyn, and P. J. Morris. 1988. Migration patterns of dendritic cells in the mouse: traffic from the blood, and T cell-dependent and -independent entry to lymphoid tissues. *J. Exp. Med. 167:632.*
44. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*
45. Hammerling, G. J., G. Schonrich, F. Momburg, N. Auphan, M. Malissen, A. M. Schmitt-Verhulst, and B. Arnold. 1991. Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of foreign MHC class I antigen. *Immunol. Rev. 122:47.*
46. Zhang, X., T. Brunner, L. Carter, R. W. Dutton, P. Rogers, L. Bradley, T. Sato, J. C. Reed, D. Green, and S. L. Swain. 1997. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2 effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Immunol. 185:1837.*
47. Matsue, H., K. Matsue, M. Walters, K. Okumura, H. Yagita, and A. Takashima. 1999. Induction of antigen-specific immunosuppression by CD95L cDNA- transfected "killer" dendritic cells. *Nat. Med. 5:930.*
48. Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature 364:806.*
49. Muruve, D. A., A. G. Nicolson, R. C. Manfro, T. B. Strom, V. P. Sukhatme, and T. A. Libermann. 1997. Adenovirus-mediated expression of Fas ligand induces hepatic apoptosis after systemic administration and apoptosis of ex vivo-infected pancreatic islet allografts and isografts. *Hum. Gene Ther. 8:955.*
50. Kang, S.-M., D. Schneider, Z. Lin, D. Hanahan, D. Dichek, P. Stock, and S. Baekkeskov. 1997. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat. Med. 3:738.*
51. Chen, J.-J., Y. Sun, and G. Nabel. 1998. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science 282:1714.*
52. Matsue, H., D. Edelbaum, A. Hartmann, A. Morita, P. Bergstresser, H. Yagita, K. Okumura, and A. Tadashima. 1999. Dendritic cells undergo rapid apoptosis in vitro during antigen-specific interaction with $CD4^+$ T cells. *J. Immunol. 162: 5287.*
53. Josien, R., B. Wong, H. Li, R. Steinman, and Y. Choi. 1999. TRANCE, a TNF family member, is differentially expressed on T cell subset and induces cytokine production in dendritic cells. *J. Immunol. 162:2562.*
54. Santiago-Schwarz, F., M. Borrero, J. Tucci, T. Palaia, and S. E. Carsons. 1997. In vitro expansion of $CD13^+$ $CD33^+$ dendritic cell precursors from multipotent progenitors is regulated by a discrete Fas-mediated apoptotic schedule. *J. Leukocyte Biol. 62:493.*
55. Wong, B. R., R. Josien, S. Y. Lee, B. Sauter, H. L. Li, R. M. Steinman, and Y. Choi. 1997. TRANCE (tumor necrosis factor (TNF)-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med. 186:2075.*
56. Bjorck, P., J. Banchereau, and L. Flores-Romo. 1997. CD40 ligation counteracts Fas-induced apoptosis of human dendritic cells. *Int. Immunol. 9:365.*
57. Ludewig, B., D. Graf, H. R. Gelderblom, Y. Becker, R. A. Kroczek, and G. Pauli. 1995. Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-α, but strongly enhanced by interleukin-10. *Eur. J. Immunol. 25:1943.*
58. Nishimura, Y., A. Ishii, Y. Kobayashi, Y. Yamasaki, and S. Yonehara. 1995. Expression and function of mouse Fas antigen on immature and mature T cells. *J. Immunol. 154:4395.*
59. Suda, T., H. Hashimoto, M. Tanaka, T. Ochi, and S. Nagata. 1997. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J. Exp. Med. 186:2045.*
60. Bregni, M., S. Shammah, F. Malaffo, M. Di Nicola, M. Milanesi, M. Magni, P. Matteucci, F. Ravagnani, C. T. Jordan, S. Siena, and A. M. Gianni. 1998. Adenovirus vectors for gene transduction into mobilized blood $CD34^+$ cells. *Gene Ther. 5:465.*
61. Reeves, M. E., R. E. Royal, J. S. Lam, S. A. Rosenberg, and P. Hwu. 1996. Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. *Cancer Res. 56:5672.*
62. Di Nicola, M., S. Siena, M. Bregni, P. Longoni, M. Magni, M. Milanesi, P. Matteucci, R. Mortarini, A. Anichini, G. Parmiani, et al. 1998. Gene transfer into human dendritic antigen-presenting cells by vaccinia virus and adenovirus vectors. *Cancer Gene Ther. 5:350.*
63. Van Tendeloo, V. F., H. W. Snoeck, F. Lardon, G. L. Vanham, G. Nijs, M. Lenjou, L. Hendriks, C. Van Broeckhoven, A. Moulijn, I. Rodrigus, et al. 1998. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Ther. 5:700.*
64. Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to $CD8^+$ T cells after gene gun immunization. *J. Exp. Med. 188:1075.*