

## BLOCKING OF THE B7-CD28 PATHWAY INCREASES THE CAPACITY OF FasL<sup>+</sup> (CD95L<sup>+</sup>) DENDRITIC CELLS TO KILL ALLOACTIVATED T CELLS

Lina Lu,<sup>1,2</sup> Shiguang Qian,<sup>1,2</sup> Thomas E. Starzl,<sup>1,2</sup> David H. Lynch,<sup>4</sup> and  
Angus W. Thomson<sup>1,2,3\*</sup>

<sup>1</sup>Thomas E. Starzl Transplantation Institute

<sup>2</sup>Department of Surgery and

<sup>3</sup>Department of Molecular Genetics and Biochemistry  
University of Pittsburgh

Pittsburgh, Pennsylvania 15213

<sup>4</sup>Immunex Research and Development Corporation  
Seattle, Washington 98101

### 1. INTRODUCTION

Dendritic cells (DC) are antigen presenting cells of hemopoietic origin, uniquely well-equipped to activate naive T cells.<sup>1</sup> Evidence also exists however, for DC tolerogenicity.<sup>2</sup> During primary activation, mature T cells change from an activation-induced cell death (AICD) -resistant to an AICD-sensitive phenotype.<sup>3</sup> The complete molecular basis for this transition remains to be determined, but CD95 (Fas/Apo-1) and CD95L (Fas ligand; FasL) appear to play an important role in the homeostatic regulation of T cell responses.<sup>4</sup> It seems that CD95L can mediate opposite effects (T cell activation or apoptosis) depending on the state of activation of the responding T cells. Previously, we have shown that mouse bone marrow (BM)-derived DC progenitors deficient in expression of cell surface costimulatory molecules B7-1 (CD80) and B7-2 (CD86) are capable of inducing alloantigen-specific T cell hyporesponsiveness<sup>5</sup> and prolonging allograft survival.<sup>6</sup> Others have shown recently that a major subpopulation of freshly-isolated mouse lymphoid tissue DC is FasL<sup>+</sup>, and can induce apoptosis in allogeneic T cells.<sup>7</sup> In the course of studies on DC propagated *in vitro* from mouse BM, we observed that these cells expressed Fas L. Our aim was to determine whether these cells could induce T cell apoptosis and whether costimulatory molecule expression could affect this activity.

\* Address correspondence to: Angus W. Thomson, D.Sc., Thomas E. Starzl Transplantation Institute, 15th Floor, Biomedical Science Tower, University of Pittsburgh Medical Center, 200 Lothrop Street, Pittsburgh, PA 15213.

## 2. MATERIALS AND METHODS

### 2.1. Animals

10–12-week-old male B10.BR/SnJ (BR; H-2<sup>k</sup>), C57BL/10J (B10, H-2<sup>b</sup>), C57BL/6J (B6; H-2<sup>b</sup>) and B6.Smn.C3H-*gld* (B6.*gld*, H-2<sup>b</sup>; FasL-deficient) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

### 2.2. Propagation and Purification of BM-Derived DC

The DC were propagated from normal B10.BR mouse BM in GM-CSF+IL-4 as described.<sup>5,8</sup> They were purified over metrizamide (to approximately 90% purity; in some experiments, DEC-205<sup>+</sup>, B7-2<sup>+</sup> DC (purity >95%) sorted using a FACStar® plus cell sorter (Becton Dickinson & Co, Mountain View, CA) were used.<sup>9</sup>

### 2.3. Flow Cytometric Analysis of FasL Expression Using Fas-Fc Fusion Protein

FasL expression on DC was analyzed by FACS. Metrizamide-purified BM DC were first incubated with 0.1 mg/ml mouse Ig (PharMingen, San Diego, CA), 1% v/v normal goat serum (NGS; Vector Laboratories Inc., Burlingame, CA) and 50 µg/ml anti-FcR mAb 2.4 G2 (PharMingen) for 10 min at 4°C to block Fc receptor binding. Mouse Fas-Fc fusion protein (Immunex Corporation, Seattle, WA) or purified human IgG was then added at 50 µg/ml and the cells incubated for 30 min. They were then washed and stained with anti-human IgG-FITC (Caltag Laboratories, South San Francisco, CA) at the same concentration.

### 2.4. Immunoperoxidase Staining for FasL

Fas-Fc fusion protein was also used for staining of cytopins and was added (10 µg/ml) to unfixed preparations of DC after incubation with 0.1 mg/ml mouse Ig, NGS (1%) and 50 mg/ml anti-FcR mAb 2.4G2 (PharMingen) for 10 min at 4°C. Peroxidase-conjugated mouse anti-human IgG was then added, and the Fas-Fc fusion protein localized by the addition of chromogenic AEC substrate for 5 min.

### 2.5. Reverse Transcriptase PCR and DNA Sequencing

Total cellular RNA was extracted with TRIzol (Life Technologies, Gaithersburg, MD) and 2 µg was used for cDNA synthesis using SuperScript II RT (Life Technologies) and both Fas L and β-actin primers. The FasL amplified product from the purified DC cDNA was cloned into Bluescript II SK (Stratagene, La Jolla, CA) and sequenced with an automated sequencer (Applied Biosystems Inc., Foster City, CA).

### 2.6. Mixed Leukocyte Reactions (MLR)

These were performed using nylon wool purified splenic T cells as responders, as described previously in detail.<sup>10</sup>

## 2.7. Costimulatory Molecule Blockade

In some experiments, mouse CTLA4-Ig fusion protein (a gift from Dr. P.S. Linsley, Bristol-Myers-Squibb, Seattle, WA) or control human Ig was added at the start of the cell proliferation or DNA fragmentation assays.

## 2.8. Radiometric Assay for DNA Fragmentation

Splenic T cells activated either with Con A (3 µg/ml; 48 h) or irradiated allogeneic spleen cells (1:1 ratio; 72 h) were labeled for 18 h with 5 µCi/ml [<sup>3</sup>H] thymidine at 37°C in complete medium. The target cells (10<sup>5</sup>) were then combined with various numbers of DC or spleen cells (as effectors) in a total volume of 200 µl per well in complete medium in 96 well plates and incubated at 37°C for 4 - 18 h. Unfragmented labeled high molecular weight DNA was collected by filtration through glass fiber filters (Pharmacia) and counted in a liquid scintillation counter. Data are expressed as % DNA fragmentation = 100 x (1-cpm in the presence of effectors/cpm in the absence of effectors).

## 2.9. Spectrofluorometric Assay for DNA Fragmentation

The protocol was as described previously in detail by McCarthy *et al.* (11).

## 2.10. In Situ Nick-End Labeling

DNA strand breaks in fixed cytospin preparations were identified by *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated (dUTP) nick end labeling (TUNEL) (12).

## 3. RESULTS

### 3.1. Culture, Purification, and Allostimulatory Activity of Bone Marrow (BM)-Derived DC

The DC propagated in GM-CSF+IL-4 and purified as described in the Materials and Methods (DEC 205<sup>+</sup>; MHC class II<sup>hi</sup>, B7-2<sup>+</sup>[CD86<sup>+</sup>], CD40<sup>+</sup>, CD11c<sup>+</sup>) were highly efficient inducers of primary allogeneic T cell responses. Blockade of the B7-CD28 costimulatory pathway however, by addition of CTLA4-Ig at the start of cultures, significantly inhibited the MLR.

### 3.2. FasL Expression on BM-Derived DC

FasL expression was detected on DC by immunoperoxidase labeling, and by flow cytometric analysis using the mouse Fas-Fc fusion protein (Fig. 1).

### 3.3. RT-PCR Analysis and cDNA Cloning

The expression of message for Fas L in purified DC was confirmed by RT-PCR analysis. Levels of FasL mRNA in DC were similar to those expressed by Con A-activated T cells. The FasL amplified product from the DC cDNA was cloned into Bluescript II SK, sequenced and found to be identical to the mouse FasL sequence deposited in GenBank, Accession No. U06948.

### Flow Analysis of FasL Expression Using Fas-Fc Fusion Protein

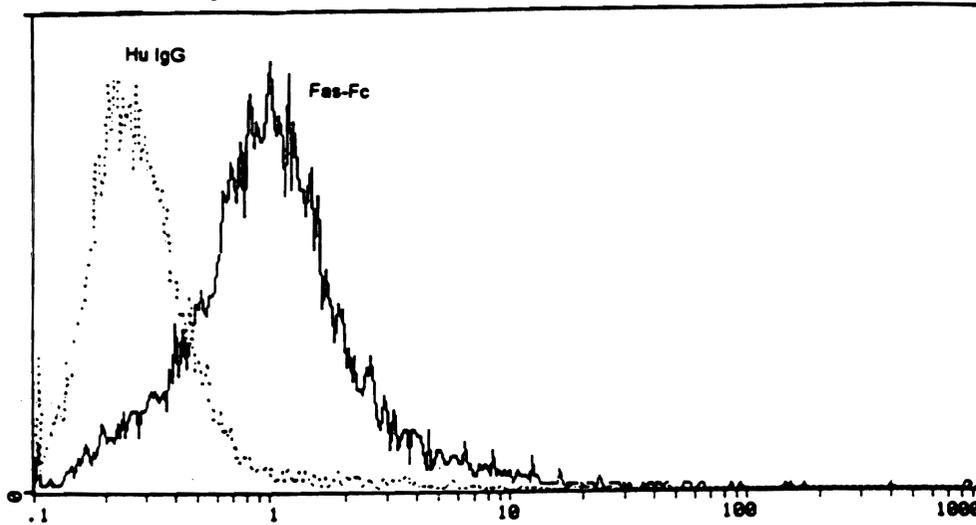


Figure 1. Immunofluorescence staining for FasL on in vitro generated DC using Fas-Fc fusion protein and detected by flow cytometric analysis.

#### 3.4. Induction of DNA Fragmentation in Fas<sup>+</sup> Jurkat T Cells and Its Inhibition by Fas-Fc Fusion Protein

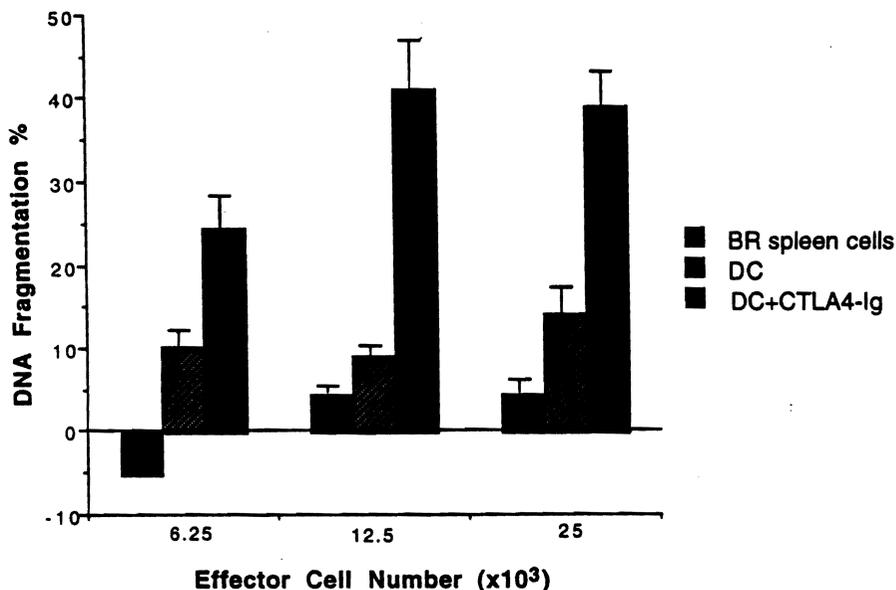
DC propagated from the BM of "wild-type" B6 mice but not those from *gld* (FasL-deficient) mice induced dose-related levels of DNA fragmentation. Similar data were obtained using DC propagated from B10.BR BM. Killing was partially blocked however, by the addition of Fas-Fc fusion protein at the start of the assay (data not shown).

#### 3.5. Induction of DNA Fragmentation in Activated T Cells and Its Enhancement by CTLA4-Ig

Con A-stimulated syngeneic (B10.BR) or allogeneic (B10) T cell blasts or allo-activated T cells were labeled with [<sup>3</sup>H] thymidine and used as targets. Cultured DC or fresh bulk spleen cells from B10.BR mice were also used as effectors. The DC induced only a low level of DNA fragmentation. DC from B10.BR.BM induced similar levels of killing in B10 (allogeneic) or B10.BR (syngeneic) Con A blasts, indicating that the induction of killing by DC was not MHC restricted. The extent of DNA fragmentation was equal to or higher than that achieved with fresh bulk splenic effector cells. When the DC were incubated with T cells in the presence of CTLA4-Ig, the effect was increased up to 4-fold. Similar results were obtained using alloactivated T cells as targets (Fig. 2).

#### 3.6. Death of Activated T Cells Induced by DC Is Mediated by the Fas/FasL Pathway

The allostimulatory activity of DC from FasL-deficient mice was significantly higher and associated with lower DNA fragmentation of activated T cell targets compared

**B-7CD28 Blockage Augments Apoptosis Induced by DC in Alloactivated T Cells**

**Figure 2.** DNA fragmentation induced in alloactivated (B10) T cells by normal (BR) mouse BM-derived DC in the absence or presence of CTLA4-Ig. Addition of CTLA4-Ig (200ng/ml) at the start of the cultures markedly increased the death-inducing activity of the DC. Results are means  $\pm$  1SD and are representative of 3 separate experiments.

with DC from wild type B6 mice. However, increased levels of killing were induced by *gld* DC when CTLA4-Ig was added to the cultures (Fig. 3). It appears therefore, that Fas/FasL is not the only pathway by which DC can induce apoptosis of activated T cells.

#### 4. DISCUSSION

This study shows for the first time, that co-stimulatory (B7) and death-signaling (FasL) molecules expressed on *in vitro* propagated DC play counter-regulatory roles in determining activated T cell survival and proliferation. That the death-inducing capacity of the *in vitro* generated DC is linked to cell surface expression of FasL was shown by immunocytochemical techniques and by the capacity of Fas-Fc fusion protein to block T cell killing by the DC. These observations are consistent with a recent report<sup>7</sup> that a major FasL<sup>+</sup> subpopulation of freshly-isolated mouse lymphoid tissue DC (CD8<sup>+</sup> FasL<sup>+</sup>) can induce apoptosis in activated T cells. In contrast to the DC studied by Süss and Shortman,<sup>7</sup> however, the FasL<sup>+</sup> DC that we generated *in vitro* were CD8<sup>-</sup>. Thus it appears that the apoptosis-inducing activity of DC and the expression of CD8 (also present on mouse "veto" cells [13]) are not strictly inter-related. Others have shown that monocytes/macrophages, to which DC are related, and with which they may share a common precursor, can promote apoptosis in activated T cells via expression of Fas L.<sup>14,15</sup> Thus, cultured M-CSF-derived macrophages induce selective depletion of allospecific T cells (15). An alternative/additional mechanism by which macrophages have been shown to inhibit T cell

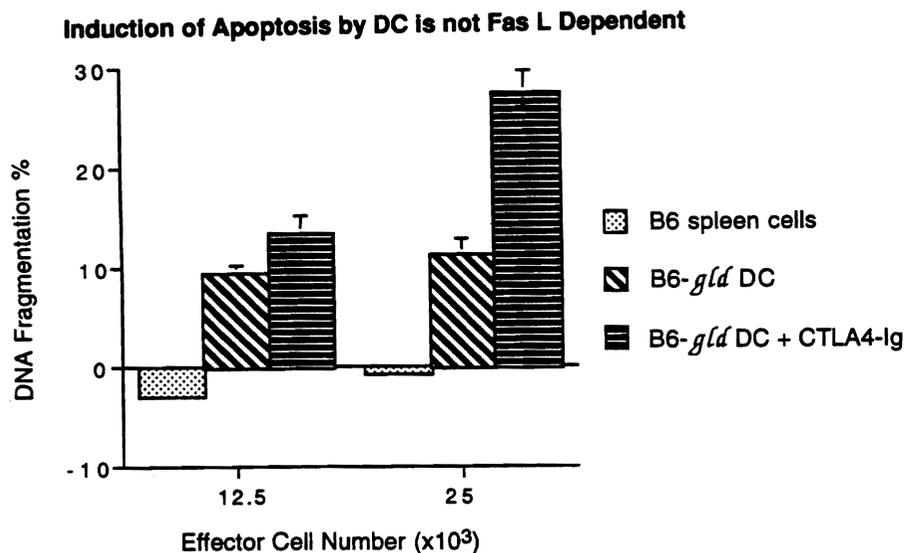


Figure 3. DNA fragmentation in Con A blasts following 4h incubation with FasL-deficient (B6.*gld*) DC in the absence or presence of CTLA4-Ig. Blockade of the B7-CD28 costimulatory pathway by CTLA4-Ig was associated with increased T cell death. Results are means  $\pm$  1SD and are representative of 2 separate experiments.

proliferation/induce apoptosis is by the secretion of NO.<sup>9,16,17</sup> NO may also be induced in DC by IFN- $\gamma$ , endotoxin or interaction with allogeneic T cells.<sup>9</sup>

The Fas (APO-1; CD95) FasL system has emerged as an important pathway regulating the induction of T cell apoptosis<sup>18-20</sup> and has been implicated in the deletion of graft-infiltrating cells in immunologically privileged sites.<sup>21</sup> Both Fas and FasL are induced on T cells upon activation, resulting in cell death by suicide or fratricide. In the present study, the expression of FasL on *in vitro* generated mouse DC was linked to the ability of these cells to induce low levels of killing in activated T cells. No T cell death significantly above background levels could be induced however, by DC propagated from FasL-deficient (B6.*gld*) mice. The capacity of CTLA4-Ig to markedly enhance the ability of potent, antigen-presenting DC to induce DNA fragmentation in T cells implicates a role for CD28 costimulation in preventing T cell death. Others studying both human and mouse systems, have shown recently that CD28 costimulation enhances T cell survival following their activation by TCR cross-linking. The resistance conferred by costimulation has been linked to increased expression of the survival gene *bcl-x<sub>L</sub>* within the T cells.<sup>22</sup>

Our finding that DC generated from BM of FasL-deficient mice can induce apoptosis in activated T cells in the presence of CTLA4-Ig suggests that Fas-independent pathways are also involved in DC-induced apoptosis. Indeed, it has also been shown that Fas is not necessary for activation-induced T cell death (23). We are investigating the expression of TNF family members<sup>23</sup> on DC and in addition, the influence of other costimulatory pathways, such as CD40/CD40L and IL-12/IL-12R on the resistance of activated T cells to apoptosis.

The *in vivo* relevance of FasL expression on DC remains to be elucidated and such studies are clearly warranted. The potential of DC to induce death of activated T cells in the absence of costimulation may have key implications for interpretation of the docu-

mented tolerogenic properties of DC. Since cells engineered to express FasL, have immunosuppressive properties,<sup>24</sup> the therapeutic potential of Fas L<sup>+</sup> APC, in particular appropriately "engineered" DC is worthy of evaluation.

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