A Subclass of Dendritic Cells Kills CD4 T Cells Via Fas/Fas-Ligand-induced Apoptosis

By Gabriele Süss and Ken Shortman

Summary
Dendritic cells (DC), the most efficient antigen-presenting cells, are well equipped for activation of naive CD4+ T cells by their expression of high levels of major histocompatibility complex and costimulator molecules. We now demonstrate that some DC are equally well equipped for killing these same T cells. Murine splenic DC consist of both conventional CD8α− DC and a major population of CD8α+ DC. Whereas CD8α− DC induce a vigorous proliferative response in CD4 T cells, CD8α+ DC induce a lesser response that is associated with marked T cell apoptosis. By using various mixtures of T cells and DC from Fas-mutant lpr/lpr mice and Fas-ligand (FasL) mutant gld/gld mice, we show this death is due to interaction of Fas on activated T cells with FasL on CD8α+ DC. Furthermore, we show by direct surface staining that CD8α+ DC, but not CD8α− DC, express FasL at high levels. These findings indicate that FasL+ CD8α+ DC are a specialized subgroup of DC with a role in the regulation of the response of primary peripheral T cells.

Materials and Methods

Animals. The normal mouse strains used were C57BL/6J, Wehi (B6) (H-2d), CBA/J (H-2k), BALB/c (H-2d), and C3H/HeJ (H-2k). The mutant mouse strains used were C3H/HeJ lpr (C3H.lpr) (H-2k), C3H/HeJ gld (C3H.gld) (H-2k), and B6Smn.C3H-Fasl(gld) (B6.gld) (H-2k). The hemagglutinin-specific class II MHC I-E-restricted TCR-transgenic mice, on a BALB/c (H-2k) background and heterozygous for the transgene, were originally obtained from H. von Boehmer (Basel Institute for Immunology, Basel, Switzerland) and are described elsewhere (18). All mice were bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute, except the B6.gld mice, which were purchased from The Jackson Laboratory (Bar Harbor, ME). For all experiments, 5–6 wk-old female mice were used.

Isolation of DC. The procedure has been described previously (5). Briefly, mild collagenase digestion for 25 min at room temperature and EDTA treatment were applied to release DC from murine spleen fragments, after which all procedures were at 0–4°C. Light density cells were selected by centrifugation in Nycodenz
medium (Nycomed Pharma, Oslo, Norway; 1.078 g/cm³, pH 7.2, 0.308 osmolar). These cells were coated with mAb against CD3, CD4, Thy 1, IL-2Rα, Gr-1, Mac-1, F4/80 antigen, FcRII, B220, and erythrocyte antigen TER 119; non-DC were then removed using anti-Ig-coated magnetic beads (Dynabeads, Dynal, Oslo). The enriched cells (~70% DC) were stained with anti-CD11c (N418 [4]) and with anti-CD8 (PharMingen, San Diego, CA), together with propidium iodide (PI) to allow rejection of dead cells. CD11c⁺ DC, either the total population or separated into CD8⁺ and CD8⁻ fractions, were then obtained by sorting on a FACStar Plus® instrument (Becton Dickinson & Co., San Jose, CA). Reanalysis of the sorted cell populations confirmed purity ≥98%.

**Purification of CD4 T Cells.** CD4 T cells were isolated from the lymph nodes of the appropriate mice by coating lymphocyte suspensions with saturating levels of mAb recognizing CD8 (53-6.7), MHC class II (M5/114), and red blood cells (TER 119). In some experiments, mAb recognizing CD44 (IM7.81) was added to deplete any preactivated T cells and so select for naive CD4 T cells. The cells were washed and coated as well as B cells were then removed using a 1:1 mixture of anti-α and anti-β mouse IgG-coupled magnetic beads (Dynabeads, Dynal) at a 8:1 bead-to-cell ratio. The recovered cells were 98% pure, as determined by FACSort® analysis. For the isolation of CD4 T cells from C3H, C3H lpr and B6.129 mice, anti-B220 (RA3-6B2) was included in the depletions to exclude any B220⁺ T cells (19).

**Mixed Leukocyte and Antigen-specific Cultures.** The MLR assays were carried out in V-bottomed 96-well culture plates to ensure efficient DC–T cell contact. CD4 T cells (20,000) were mixed with various numbers (500-4,000) of DC in 0.2 ml modified RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% FCS (Commonwealth Serum Laboratories, Melbourne, Australia) and 10⁻⁴ M 2-mercaptoethanol; they were then incubated at 37°C in humidified 10% CO₂-in-air for 1-6 d. In some experiments, exogenous murine (mu) rIL-2 (Immunex, Seattle, WA) at 100 U/ml was added. For the antigen-specific assays, CD4 T cells from heterozygous hemagglutinin-specific, TCR-transgenic mice (18) were prepared as described above; 85% expressed the transgenic TCR. These CD4 T cells (20,000) were cultured with titrating numbers of splenic DC from BALB/c mice, together with the specific peptide (FERFEIFPK) at 1 μg/ml. For thymidine incorporation, proliferation assay cultures were incubated at the end of the incubation period with 0.5 μCi [³H]thymidine (ICN, Irvine, CA) for 12 h; the cells were then harvested on glass fiber sheets for counting. A gas-phase [³H] counter was used that had an ~20-fold lower sensitivity than liquid scintillation counting. Thymidine pulses of 6-16 h gave similar results. For cell-counting experiments, 10 replicate cultures were pooled after 1-6 of incubation and centrifuged at 500 g for 7 min. The cells were resuspended in 200 μl EDTA-containing medium to disrupt clumps, then diluted in eosin-containing medium; viable/eosin-excluding cells were counted in duplicate under phase contrast microscopy.

**Assays for Apoptosis.** DC (2,000) were cultured with 20,000 CD4 T cells for 3 d as described above; pools of 12 cultures were then harvested. For analysis of DNA content, the cells were resuspended in 200 μl saline, containing 0.1% sodium acetate, 0.2% Triton X-100, and 0.005% PI, then analyzed on the FACSort®. For the terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end labeling (TUNEL) assay, the procedure was similar to that of Sgonc et al. (20), involving incubation of fixed, permeabilized cells with FITC-12-dUTP (Boehringer Mannheim, Mannheim, Germany), dATP, and TdT (Promega, Madison, WI) to label apoptotic cells. Cells were then analyzed by FACSort® for FITC-fluorescence and forward light scatter. Negative controls omitting TdT gave no FITC labeling.

**Staining for FasL Expression.** Purified but not FACSort®-separated DC (as described above) were first incubated at 10⁶ cells/sample with 0.1 mg/ml mouse Ig, 10% goat serum, and saturating levels of anti-α-FcR mAb 2.4G2 for 10 min at 0-4°C to block Fc receptor binding and minimize unspecific binding. The μFas-Fc fusion protein (provided by D. Lynch, Immunex) was added at 50 μg/ml and incubated 30 min. Cells were washed and stained with anti-human IgG-FITC (Caltag Laboratories, South San Francisco, CA), and blocked again with 0.3 mg/ml rat Ig. The cells were then stained with CD8-PE and N418-biotin followed by Texas red-avidin (Caltag) and PI to allow identification of the DC subpopulations. The background controls were samples incubated with 50 μg/ml purified human IgG instead of fusion protein. The cells were analyzed for Fasl FITC-fluorescence, gating on viable CD8⁺ or CD8⁻ DC as in Fig. 1.

**Results**

**Isolation of DC Subpopulations.** The FACSort® separation of B6 and BALB/c mouse splenic DC using mAb against CD11c (N418 [4]) and CD8α (53-6.7) is illustrated in Fig. 1. Since we found neither mAb caused inhibition nor stimulation when added to MLR cultures of unseparated DC and CD4 T cells, sorting on the basis of these two markers was unlikely to affect DC or T cell function. Distinct CD8⁻ and CD8⁺ populations of the CD11c⁺ DC were seen in the spleen of all mouse strains studied. Although the relative proportions varied in a mouse strain–dependent manner, CD8⁺ DC were always more frequent in the spleen. The CD8 molecule has been shown to be in the form of a CD8α homodimer and to be synthesized by the DC themselves (5). Both DC populations were found to express high levels of class II MHC, as well as B7-1 and B7-2 (data not shown).

**T Cell–proliferative Responses to CD8⁺ and CD8⁻ DC.** CD4 T cells from CBA mice proliferated in a dose–dependent manner in response to CD11c⁺ DC from B6 mouse spleen (Fig. 2 A). However, there was a marked difference in the extent of T cell proliferation when CD8⁻ and CD8⁺ DC were compared, with the CD8⁻ DC consistently pro...
Figure 2. The interaction of sorted DC populations with allogeneic, syngeneic, or antigen-specific CD4 T cells. Representative experiments are shown, with the values representing the mean ± SD of triplicate cultures. Combined data from 17 MLR experiments show that the difference between CD8+ and CD8- DC-stimulated MLR cultures is consistent and statistically significant (P < 0.0001). No exogenous cytokines were added to the cultures. Proliferation of CD4 T cells was assessed by [3H]thymidine incorporation after a 12-h pulse. The background with either CD4 T cells or DC cultured alone was <50 cpm. (A) DC dose-response of an allogeneic MLR proliferation assay. CD4 T cells from CBA mice were stimulated with CD8- DC (○), CD8+ DC (●) and unseparated splenic DC (■) from B6 mice. Culture time was 3 d. (B) Kinetics of an allogeneic MLR proliferation assay. 2,000 B6 CD8- DC (○) or CD8+ DC (●) were combined with 20,000 CD4 T cells from CBA mice. CD4 T cells were also cultured alone (▲). Cultures were pulsed with [3H]thymidine after 1–5 d. (C) A comparison of the DC dose-response of an allogeneic and a syngeneic MLR proliferation assay. CD4 T cells from CBA mice were stimulated with CD8- or CD8+ DC from B6 mice (allogeneic response, ▲ and □, respectively) or CBA mice (syngeneic response, ○ and ●, respectively) and cultured for 3 d. (D) DC dose-response of a proliferation assay, stimulating hemagglutinin-specific TCR-transgenic CD4 T cells with BALB/c splenic CD8+ DC (●) and CD8- DC (○) presenting the specific peptide. Culture time was 3 d.

producing a 2–10-fold greater CD4 T cell response. The CD4 T cell response to the unseparated CD11c+ DC (containing CD8- and CD8+ DC at a ratio of 1:3, as found in the B6 spleen; Fig. 1) was intermediate and in accordance with the proportions of its CD8- and CD8+ DC components. This suggested that by stimulation with unseparated DC, both types of responses occur side by side without influencing each other. The kinetics of the MLR. (Fig. 2 B) showed that the difference in the CD4 T cell response to CD8- and CD8+ DC persisted over the entire duration of the MLR response and was not due to a shift in the peak of proliferation. The CD8- DC gave T cell stimulation indices 200–250-fold above the T cell background, indicating they were potent stimulators of CD4 T cells. Other allogeneic combinations showed the same difference between CD8- and CD8+ DC (data not shown).

When DC are used as stimulators, it is known that a small but definite syngeneic MLR can be observed (21). This phenomenon is believed to mimic a response to self-antigens. As demonstrated in Fig. 2 C, this syngeneic response was also greater with CD8- than with CD8+ DC.

To determine if the allogeneic MLR was a valid model of responses to a specific antigen, a defined peptide antigen from influenza hemagglutinin was added to cultures of CD4 T cells from hemagglutinin-specific, class II MHC-restricted TCR transgenic mice together with syngeneic CD8+ or CD8-DC. Results equivalent to those from the MLR were obtained (Fig. 2 D), CD8- DC producing much greater specific T cell proliferation than CD8+ DC.

Direct Cell Counts on Cultures Stimulated with CD8+ or CD8- DC. To obtain a more direct measure of cell expansion than that obtained by [3H]thymidine uptake assays, direct cell counts were performed on the MLR cultures. This approach allowed blast cell formation as well as occur-
Figure 3. Direct cell counts on cultures of CD4 T cells stimulated with allogeneic CD8− DC (○) and CD8+ DC (■). MLR cultures were set up as in Fig. 1 B. After 1–6 d, pools of replicate cultures were counted under the microscope. The numbers of viable cells per culture are shown. Exogenous IL-2 at 100 U/ml was added to the cultures (bottom).

Table 1. Apoptosis Analysis by PI labeling

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<th>Stimulating DC population</th>
<th>DNA content of cultured CD4 T cells</th>
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<td>&lt;2n (apoptotic cells)</td>
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<td>2n (G0 or G1 cells)</td>
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<td>&gt;2n (dividing cells)</td>
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<td>CD8+</td>
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2,000 DC isolated from B6 spleens were cultured with 20,000 CD4 T cells from CBA mice. After 3 d, cells of pooled cultures were permeabilized, stained with PI, and analyzed for DNA content by flow cytometry. 2n designates the DNA content of a normal diploid cell.
Figure 4. The response of normal and Fas-deficient mice to allogeneic CD8− and CD8+ DC. All experiments have been repeated twice with similar results. Recombinant mouse IL-2 at 100 U/ml was added to the cultures; these results obtained in the presence of exogenous IL-2 were confirmed in three experiments where IL-2 was not added to the cultures. Proliferation was assessed as described in Fig. 2. The background with either CD4 T cells or DC cultured alone was <100 cpm. (A) Proliferative response of control C3H CD4 T cells to B6 CD8− DC (●) or CD8+ DC (○) after 3 d of culture. (B) Proliferative response of mutant C3H.lpr CD4 T cells to B6 CD8− DC (●) or CD8+ DC (○) after 3 d of culture. (C) FACS® profiles of a combined TUNEL assay for apoptosis and forward light scatter analysis for cell size. Samples consisted of C3H and C3H.lpr CD4 T cells stimulated by B6 CD8− or CD8+ DC for 3 d. Cells that incorporated FITC-dUTP were classified as apoptotic.

whether stimulated by CD8− or CD8+ DC. The apoptotic cells detected were T cells rather than DC, because at this time point T cells were in vast excess in the cultures; the reduction of apoptotic cells in lpr T cell cultures further validates this conclusion. These results demonstrate that Fas is involved in an early, postactivation T cell death that limits the proliferative expansion of normal CD4 T cells stimulated by CD8+ DC.

Evidence for FasL on CD8+ DC. The possibility that APC could express FasL and so deliver a death signal has been suggested (22), but not yet demonstrated. To investigate the role and cellular location of FasL in death induced by CD8+ DC, FasL mutant gld mouse strains were utilized (10, 15, 23). When CD4 T cells from C3H/HeJ gld were stimulated with B6 CD8− or CD8+ DC in the presence of exogenous IL-2, the difference between the proliferative responses was maintained and was equal to that obtained using normal T cells, indicating that the FasL was being provided by the CD8+ DC (Fig. 5 A). When normal C3H CD4 T cells were stimulated by FasL-deficient CD8+ or CD8− DC from B6.gld mice, there was still some difference between the proliferative responses obtained, although it was much less than with normal DC (Fig. 5 B). This suggested a small part of the killing was inter-T cell (suicide or fratricide). However, when both the CD4 T cells and the DC were isolated from gld mutant mice (C3H.gld and B6.gld, respectively) there were equally strong CD4 T cell–proliferative responses to CD8+ DC or to CD8− DC (Fig. 5 C), showing that CD8+ DC directly signaled most of the T cell death in the normal cultures. TUNEL analyses on the gld cultures revealed no differences in the level of apoptotic cells between CD8+ and CD8− DC, confirming that in the absence of FasL on both DC and T cells, apoptosis was abrogated (not shown).

To test directly for the presence of FasL, we stained CD8+ and CD8− DC with the muFas-Fc fusion protein recently engineered by Lynch et al. (10). The CD8+ DC all stained strongly with this reagent, whereas the CD8− DC included only a small proportion of positive cells (Fig. 6). Under these conditions, only a low level of staining could be detected on Con A–activated T cells. It should be emphasized that freshly isolated, uncultured DC were used.
Figure 5. The proliferative responses of normal and FasL mutant CD4 T cells to allogeneic normal and FasL mutant CD8+ and CD8- DC. Experiments were repeated two to four times and one representative example is shown. Exogenous IL-2 at 100 U/ml was added to the cultures. Proliferation was assessed as described in Fig. 2. The background with either CD4 T cells or DC cultured alone was <100 cpm. (A) C3H.gld CD4 T cells were cultured with B6 CD8- DC (●) or CD8+ DC (○) for 3 d. (B) C3H CD4 T cells were cultured with CD8- DC (●) or CD8+ DC (○) from mutant B6.gld mice for 3 d. (C) C3H.gld CD4 T cells were cultured with B6.gld CD8- DC (●) or CD8+ DC (○) for 3 d.

in these experiments, suggesting a high level of FasL expression is the normal state of CD8+ DC within the mouse spleen.

Discussion

Our results lead to the conclusion that CD8+ DC express FasL and so are functionally specialized for killing activated, Fas-expressing CD4 T cells. By using mouse strains with genetic null-mutations for Fas and FasL, lpr/lpr and gld/gld, respectively, we demonstrated that FasL-expressing CD8+ DC are functionally capable of eliminating interacting CD4 T cells. Although we have used CD8α as a marker for the separation of DC, there is no evidence to date that CD8α itself is functionally important. Since some CD8- DC express FasL (Fig. 6), the FasL itself is potentially a better marker for the complete segregation of stimulatory from regulatory DC.

This study establishes that murine splenic DC can be subdivided phenotypically and functionally into two distinct populations based on their expression of CD8α and of FasL. CD8+ DC are found in other lymphoid tissues (5, 6); their function and level of FasL expression are currently being assessed. Since the CD8+ DC of thymus represent a distinct lineage (6, 24), we suspect the CD8- and CD8+ DC of spleen differ in lineage origin. The present study demonstrates that they differ in function. The CD8- DC...
likewise eliminate or restrict T cell responses to antigens presented by these DC, either in order to regulate normal T cell responses to foreign antigens or to purge the tissue of self-reactive CD4 T cells. We envisage that the expression of FasL on the same DC that presents antigen and activates naive T cells is an effective way to block responses at an early stage, in contrast to the suicidal effect of FasL on activated T cells that would limit responses at a later stage when the number of activated T cells becomes too high. Whole animal approaches are now needed to assess the relative importance of different FasL-expressing cells in the control of immune responses.

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Address correspondence to Dr. Ken Shortman, The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Victoria 3050, Australia.

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References

gle positive cells with a class II major histocompatibility com-
20. Sgonc, R., G. Boeck, H. Dietrich, J. Gruber, H. Recheis,
and G. Wick. 1994. Simultaneous determination of cell sur-
face antigens and apoptosis. Trends Genet. 10:41–42.
Muramatsu, T. Masuda, and S. Ikehara. 1991. Distinct mech-
anisms of neonatal tolerance induced by dendritic cells and
lecular cloning and expression of the Fas ligand, a novel mem-
ic dendritic cells and T cells develop simultaneously in the
and R.C. Duke. 1995. A role for CD95 ligand in preventing
26. Griffith, T.S., T. Brunner, S.M. Fletscher, D.R. Green, and
T.A. Ferguson. 1995. Fas ligand–induced apoptosis as a mech-
anism of immune privilege. Science (Wash. DC). 270:1189–
1192.