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

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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 Ipr/Ipr 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.

Dendritic cells (DC) are APCs of hemopoietic origin, found in many tissues but most abundant in the T-dependent areas of lymphoid organs. Only DC have the capacity to activate naive T cells (1). DC from murine lymphoid organs can be identified by their expression of high levels of class II MHC proteins or of the integrin CD11c (2-4). A substantial proportion of murine DC express CD8α, although this is only evident if the DC are efficiently released from the tissue, as in our recent isolation procedure (5, 6). This direct procedure avoids culture and adhesion steps, thus maintaining DC in a physiologically native state. In the present study we have used mAbs against CD11c and CD8α to sort pure splenic CD8α+ and CD8α- DC from such DC preparations. To elucidate the functional differences between CD8α+ and CD8α- splenic DC, we first tested their capacity to stimulate purified allogeneic CD4 T cells in a primary MLR. The general relevance of the findings from the MLR system was then tested in a specific response to a defined peptide antigen.

Our initial results indicated that CD8α+ DC caused the apoptotic death of activated CD4 T cells. Recent reports (7, 8) indicate that a signal for the apoptosis of mature T cells is delivered through the Fas molecule (CD95/APO-1) expressed on the surface of activated T cells. The ligand for Fas (FasL/CD95L) has been demonstrated on the surface of T cells after activation (9, 10), and it has been shown that even a single T cell can kill itself by Fas–FasL interaction (11-13). This is considered to be a physiological control system preventing excess T cell proliferation. This control system is disregulated in mouse strains with functional mutations for Fas (lpr/lpr [14]) or FasL (gld/gld mice) (9, 16, 17). We therefore used these mutant mouse strains to analyze Fas/FasL involvement in DC–T cell interactions. The system we now describe involves FasL expression by a major population of DC, potentially regulating or eliminating CD4 T cell responses at the initial step of T cell activation by an APC.

Materials and Methods

Animals. The normal mouse strains used were C57BL/6J Wehi (B6) (H-2b), CBA/J (H-2k), BALB/c (H-2d), and C3H/HeJ (H-2k). The mutant mouse strains used were C3H/HeJlpr (C3H.lpr) (H-2k), C3H/HeJgld (C3H.gld) (H-2k), and B6Smn.C3H-Fasl(gld) (B6.gld) (H-2b). The hemagglutinin-specific class II MHC I-E restricted TCR-transgenic mice, on a BALB/c (H-2b) 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, FeRII, 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-CDllc (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 activated T cells and so select for naïve CD4 T cells. The cells were washed and coated as well as B cells were then removed using a 1:1 mixture of anti–rat and anti–mouse IgG-coupled magnetic beads (Dynabeads, Dynal) at a 8:1 bead-to-cell ratio. The recovered cells were 98% pure, as determined by FACS® analysis. For the isolation of CD4 T cells from C3H, C3H lpr and B6 gld mice, anti-B220 (RA3-6B2) was included in the depletion 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 routine (muFas-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 then analyzed for Fasl FITC-fluorescence, gating on viable CD8+ or CD8– DC as in Fig. 1.

**Results**

**Isolation of DC Subpopulations.** The FACS® 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 CD8αβ 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 CD11chigh 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 (O), 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 (O) or CD8⁺ DC (□) were combined with 20,000 CD4 T cells from CBA mice. CD4 T cells were also cultured alone (A). 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, ■-■, □-□, respectively) or CBA mice (syngeneic response, □-□, □-□, 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 (O) 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-high 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), reference of dead cells in the cultures to be monitored. CD8- DC gave a greater accumulation of viable cells in the cultures than did CD8+ DC, resulting in a twofold increase of viable cells at the peak of response (Fig. 3). Associated with the reduced response, the CD8+ DC-stimulated cultures contained about half the level of T cell blasts, but more dead cells. This reduced T cell response was not due to insufficient production of IL-2 in the CD8+ DC-stimulated cultures. Although addition of exogenous 100 U/ml murIL-2 enhanced the proliferative responses to both CD8+ and CD8- DC, it maintained the marked difference between them (Fig. 3). These results confirmed the differences between CD8+ or CD8- DC stimulation obtained with the [3H]thymidine uptake assays.

Cellular DNA Analysis on Cultures Stimulated with CD8+ or CD8- DC. In view of the fact that in relative numbers more dead cells could be detected by microscope counting in cultures stimulated by splenic CD8+ DC, we performed PI staining of cells from day 3 MLR cultures of DC-stimulated CD4 T cells to monitor their nuclear DNA content. This allowed us to identify apoptotic cells as having hypodiploid DNA as well as to distinguish them from dividing cells with ongoing DNA synthesis. Results, summarized in Table 1, demonstrate a significantly larger population of apoptotic cells in CD4 T cell cultures stimulated by CD8+ DC (29%) compared with those stimulated by CD8- DC (14%). In addition, more dividing cells were present in CD8- DC-stimulated cultures, supporting our earlier findings obtained with [3H]thymidine incorporation and direct cell counting.

Evidence for Fas-mediated Apoptosis of CD4 T Cells. To assess the role of Fas in the observed apoptotic T cell death, we used Fas-deficient C3H/HeJ/lpr CD4 T cells in our MLR cultures (14, 19), stimulating them with B6 CD8+ or CD8- DC in the presence of exogenous IL-2 to ensure this was not a limiting factor. Whereas control C3H/HeJ CD4 T cells gave the same result as that obtained with CBA T cells, namely a greater response to CD8- than to CD8+ DC (Fig. 4 A), Fas-deficient lpr CD4+ T cells responded at least as well to CD8+ as to CD8- DC (Fig. 4 B). Similar results were obtained in the absence of exogenous IL-2 (data not shown). This demonstrates that lack of inherent stimulatory ability by CD8+ DC was not the reason for the reduced response of normal T cells. The decreased proliferation appeared rather to be due to Fas-mediated killing of the CD4 T cells after their activation by CD8+ DC.

This T cell death was further analyzed by the TUNEL technique (20) which detects DNA strand breaks in cells undergoing apoptosis (Fig. 4 C). Normal CD4 T cells stimulated for 3 d by CD8+ DC showed ~20% apoptotic cells, a sixfold increase compared with stimulation by CD8- DC; associated with this, the CD8+ DC again gave only half the number of blast cells compared with stimulation by CD8- DC. However, when lpr CD4 T cells were used there were few apoptotic cells and an equivalent number of blasts.

| Stimulating DC population | DNA content of cultured CD4 T cells |
|---------------------------|-----------------------------------|
|                           | <2n (apoptotic cells) | 2n (G0 or G1 cells) | >2n (dividing cells) |
| CD8+                      | 29 ± 1                   | 61 ± 1              | 10 ± 1               |
| CD8-                      | 14 ± 1                   | 63 ± 1              | 23 ± 1               |

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.
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 \(Ipr\) 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 DC, indicating that the FasL was 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\(^+\) 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...
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 CD8\(\alpha\) as a marker for the separation of DC, there is no evidence to date that CD8\(\alpha\) 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 CD8\(\alpha\) 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
behave as expected for "conventional" DC, very efficiently stimulating CD4 T cell responses. The CD8+ DC, in contrast, constantly express high levels of FasL and thereby are able to kill interacting CD4 T cells.

The expression of FasL by testicular Sertoli cells (25), and by parenchymal cells of the anterior chamber of the eye (26), enables them to kill any activated, Fas-expressing T cells and makes these tissues immune-privileged sites. The presence of FasL+ CD8+ DC within a tissue should 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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