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Apopotic Cells Induce Immunosuppression through Dendritic Cells: Critical Roles of IFN-γ and Nitric Oxide

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Apopotic cells induce immunosuppression through unknown mechanisms. To identify the underlying molecular mediators, we examined how apoptotic cells induce immunoregulation by dendritic cells (DC). We found that administration of DC exposed to apoptotic cells (DC\textsubscript{ap}) strongly inhibited the expansion of lymphocytes in draining lymph nodes in vivo and the subsequent Ag-specific activation of these lymphocytes ev x vivo. Unexpectedly, DC\textsubscript{ap} supported T cell activation to a similar extent as normal DC in vitro, leading to proliferation and IL-2 production, except that DC\textsubscript{ap} did not support T cell production of IFN-γ. Surprisingly, when DC\textsubscript{ap} were cocultured with normal DC, they completely lost their ability to support T cell activation, an effect reversed by anti-IFN-γ or inhibitors of inducible NO synthase (iNOS). As expected, exposure to apoptotic cells rendered DC\textsubscript{ap} capable of producing much more NO in response to exogenous IFN-γ than normal DC. Furthermore, DC\textsubscript{ap} from iNOS\textsuperscript{-/-} or IFN-γR1\textsuperscript{-/-} mice were not inhibitory in vitro or in vivo. Therefore, the IFN-γ-induced production of NO by apoptotic cell sensitized DC plays a key role in apoptotic cell-mediated immunosuppression. The Journal of Immunology, 2008, 181: 3277–3284.

One of the most significant characteristics of cell death by apoptosis is the quick and quiet removal of the cell corpse by surrounding cells (1–4). Unlike the eradication of necrotic cells, which is often associated with local inflammation, the clearance of apoptotic cells has been shown to suppress immune responses (2, 3, 5, 6). Therefore, it is highly likely that the interaction of phagocytes with apoptotic cells results in different consequences compared with their interaction with necrotic cells (3). Phagocytosis conducted by unicellular organisms is believed to be involved primarily in food up-take (7). Phagocytosis exists throughout the metazoan subkingdom. In the lower nematodes, such as Caenorhabditis elegans, one of the main functions of phagocytosis is the removal of apoptotic cells by neighboring cells (8). In higher animals, phagocytosis of apoptotic cells is largely accomplished by cells of the innate immune system such as macrophages and dendritic cells (DC)\textsuperscript{5} (9). Therefore, it is possible that during evolution, phagocytosis arose first for nutritional purposes in unicellular organisms, and then evolved for the removal of apoptotic cells. The phagocytic functions of innate immune cells may therefore have evolved from cells with the capability of apoptotic cell removal.

Although a century has passed since Élie Metchnikoff first discovered that the clearance of regressing tissue during amphibian morphogenesis occurs without inflammation (10), the link between immune tolerance and phagocytosis of apoptotic cells was made only in recent years. Several groups have studied how apoptotic cells signal for their clearance without eliciting inflammatory responses by the engulfing phagocytes (5, 9). The hallmarks of apoptotic cell death are plasma membrane blebbing, cell shrinkage, and the loss of membrane phosphatidylserine (PS) asymmetry. The exposure of PS on the outer leaflet of the cytoplasmic membrane is recognized by a phagocyte PS receptor, milk fat globule epidermal growth factor factor 8 (11). In addition, a number of PS-binding proteins, such as plasma-protein β\textsubscript{2}-glycoprotein I and protein S, can act as a bridge between apoptotic cells and phagocytes. In previous studies, we showed that blocking the interaction between PS and phagocytes with annexin V prevents apoptotic cell-induced tolerance for allogeneic heart transplants (6). In addition, recent studies have shown that carbohydrate changes on the surface of apoptotic cells may be important in triggering recognition by lectin receptors (12). C1q has also been found to bind to the blebs of apoptotic cells and interact with collectins during apoptotic cell phagocytosis (13). Furthermore, apoptotic cells display a carbohydrate group that binds to CD14 (14). Thus, phagocytosis is mediated by a number of different receptors and ligands.

Investigations in the last few years have indicated a strong correlation between defects in apoptotic cell phagocytosis and the development of autoimmunity in both humans and genetically modified mice (12–17). Apoptotic cells are important modulators of immune responses and are believed to be a primary source of tolerogen (3, 18). There are several examples showing the induction of tolerance after injection of apoptotic cells and the role of APCs, such as macrophages and DCs, in the...
induction and maintenance of tolerance after phagocytosis of dead cells (6, 19–22). Because DC are the most potent APCs capable of activating naïve T lymphocytes, the processing of apoptotic cells by DC is likely to be important for apoptotic cell-mediated immune regulation (19, 21). Indeed, several studies have demonstrated that phagocytosis of apoptotic cells by DC leads to the generation of regulatory T cells (23) and the induction of IL-10 production (24). Additionally, TGFβ (19, 25) and PGE₂ (26) are also believed to play a role.

We have reported that, in an allogeneic rat heart transplant model, a single transfusion of a few million apoptotic splenocytes from the donor strain before transplant resulted in dramatically prolonged survival of heart allografts. This effect was prevented by administration of gadolinium chloride, which disrupts phagocyte function, and by annexin V, which blocks the binding of exposed phosphatidylserine to its receptor on phagocytes (6). In this study, we investigated the molecular mechanism controlling this apoptotic cell-mediated immunosuppression. We found no role for IL-10, TGF-β, PGE₂, CTLA-4, BTLA-4, or regulatory T cells. Instead, DC exposed to apoptotic cells (DC apoptotic) are affected in at least two ways: reduced ability to prime T cells to produce IFN-γ and increased propensity for IFN-γ-induced production of NO. In the presence of both DC apoptotic and normal DC, T cells are activated by normal DC to produce IFN-γ, which in turn induces DC apoptotic to release NO and thus suppress immune responses. Therefore, our studies reveal a novel mechanism through which apoptotic cells induce immunosuppression.

Materials and Methods

Mice

C57BL/6 mice (6–8 wk) were obtained from National Cancer Institute. IFN-γR1−/− and iNOS−/− mice (6–8 wk), both in a C57BL6 background, were purchased from The Jackson Laboratory. All mice were maintained in the Robert Wood Johnson Medical School Vivarium, with food and water provided ad libitum. Animals were used 1 wk after receipt and were matched for gender and age in each experiment, all approved by the Institutional Animal Care and Use Committee.

Reagents

Recombinant mouse IFN-γ, TNF-α, IL-1α, anti-mouse CTLA-4, anti-mouse BTLA-4, and PE-conjugated anti-mouse CD11c, CD40, CD80, CD86, and MHC class II were from eBioscience. Abs against IL-10 and TGF-β were from R&D Systems. Ab against IFN-γR1 was produced by Harlan Sprague Dawley. Biotin-conjugated anti-mouse IFN-γR1 was from Biolegend. Indomethacin and NG-nomethyl-l-arginine were purchased from Sigma-Aldrich.

Generation of bone marrow-derived DC

Mice were euthanized and the femurs were isolated. Bone marrow was flushed out with RPMI 1640 medium using a 1 ml syringe (27G). The cells were washed twice with PBS and then cultured in complete RPMI 1640 medium supplemented with GM-CSF (20 ng/ml) and IL-4 (10 ng/ml). On day 3, 75% of the medium was changed. On day 5, the suspended cells were removed and the loosely adherent cells were collected as immature DC (CD11c+ cells were >80%). For mature DC, the immature DC were stimulated with LPS (1 μg/ml) for 24 h.

Generation of apoptotic cells and DC apoptotic

Apoptotic cells were generated by treating splenocytes with dexamethasone (1.0 μM) for 12 h. At least 80% of the resultant cells were apoptotic, as determined by 2-color staining with FITC-conjugated annexin V and propidium iodide, as well as DNA content analysis (27). Cells were washed twice with PBS before use. Immature DC apoptotic were generated by culturing apoptotic splenocytes with immature DC at a 10:1 ratio for 24 h followed by removal of buoyant splenocytes. Mature DC apoptotic were obtained from immature DC apoptotic by LPS (1 μg/ml) stimulation for 24 h.

Proliferation assay

CD4+ T cells were isolated by negative selection using a mouse CD4+ T cell isolation kit (Miltenyi Biotec). Cells were cultured in 0.1 ml medium in 96-well plates for the indicated times. To assay de novo cell proliferation, 0.5 μCi of [3H]thymidine (Tdr, GE Healthcare BioSciences) was added to each well 6 to 8 h before termination of the cultures by freezing. Plates were then thawed, harvested, and incorporated [3H]Tdr was counted using a Wallac Microbeta scintillation counter (PerkinElmer).

Detection of NO

NO was detected using a modified Griess reagent (Sigma-Aldrich). In this method, all NO is converted into NO₂ by nitrate reductase, and the total amount of NO₂ is detected as a colored azo dye product of the Griess reaction.

Flow cytometry

For cell surface Ag staining, DC were blocked with anti-CD16/CD32 and stained for CD40 (IC10), CD80 (16-10A1), CD86 (GL1), and MHC class II (M5/14.15.2) by direct immunofluorescence. Cells were evaluated by flow cytometry, using a FACScan instrument and CellQuest software for acquisition and analysis (BD Immunocytometry Systems). For the detection of IFN-γR1 expression, DC were first stained with biotin-conjugated anti-mouse IFN-γR1, which was in turn detected by PE-conjugated streptavidin. CD4+ Foxp3+ regulatory T cells were tested by a mouse regulatory T cell staining kit (eBioscience).

Detection of cytokines

Cytokines in the culture supernatant were assayed by a Luminex microbead-based multiplexed assay using commercially available kits according to the manufacturer’s protocol (BioPlex, Bio-Rad).

Treatment of mice with Ag-loaded DC

Mature DC or DC apoptotic (1 × 10⁶ in 50 μl PBS) preincubated with 50 μg/ml OVA for 6 h were injected into the right footpad of mice. After 7 days, draining popliteal lymph nodes were isolated and single-cell suspensions were prepared. Cell activation was determined by cultivating the cells (1 × 10⁶ cells/ml) with 10 μg/ml OVA, and measuring proliferation after 48 h by [3H]thymidine incorporation.

Real-time PCR

RNA was isolated from cell pellets using an RNasy mini kit. First-strand cDNA synthesis was performed using the Superscript RT Kit with random hexamer primers (all kits from Qiagen). mRNA were quantitated by real-time PCR (MX-4000 from Stratagene) using SYBR Green Master Mix (Applied Biosystems). The amount of mRNA was normalized to endogenous β-actin mRNA. Primer sequences for STAT1 gene were: forward, 5’-CTCCCTTCGCTGCTGGATTG-3’; reverse, 5’-GCCCATATACTGACCCATCATC-3’.

Statistical analysis

Differences in treatment groups were assessed by two-tailed Student’s t test or by ANOVA for repeated measures, as specified. Statistical significance was determined as p values: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Results

The immunosuppressive effect of DC apoptotic in vivo and ex vivo

Previous studies in various systems have shown that apoptotic cells can induce immune tolerance (3, 5, 9). We have reported that in vivo administration of apoptotic cells prolongs engraftment of allogeneic heart transplants (6). To investigate the molecular mechanisms controlling this immunosuppression, we examined the effect of apoptotic cells on DC, APCs that are critical for the activation of resting T cells. DC were derived from mouse bone marrow and exposed to syngeneic apoptotic splenocytes (DC apoptotic) in vitro. Both normal DC and DC apoptotic were matured by LPS stimulation and then used for the following experiments.

The DC apoptotic, normal mature DC, or a mixture of both DC types, were incubated with OVA in vitro to load OVA peptide and then injected into mouse hind footpads. In this in vivo model, the DC would migrate from the site of injection to the draining lymph nodes (dLNs) and cause swelling (28). Seven days after injection, lymphocytes in the popliteal dLNs were isolated and restimulated with OVA ex vivo. As shown in Fig. 1A, mice immunized with OVA-loaded normal DC showed a dramatic increase in cellularity in the
They also produced less of the Th1 cytokines, IFN-γ, while normal DC showed much less Ag-specific proliferation (Fig. 1). When normal DC and DCap were injected together, the increment in dLN cellularity was significantly less than with normal DC alone, indicating that DCap inhibited stimulation by normal DC. To directly assess the effect of apoptotic cells on T cell stimulation by DC, we examined the response of dLN lymphocytes to OVA stimulation in vitro. Equal numbers of dLN cells were stimulated with OVA, and proliferation and cytokine production determined 48 h later. As expected, dLN cells stimulated with OVA-exposed normal DC (DCap) were prepared as described in Materials and Methods. Normal DC, DCap, or a 1:1 mixture of both (same total cell number) were cultured in vitro with 50 μg/ml OVA at 37°C for 6 h, to load with OVA peptide. Cells were then washed extensively with PBS and injected s.c. into mouse hind footpads (1 × 10⁶ cells in PBS per footpad), with PBS alone as control. Seven days later, lymphocytes in the draining popliteal lymph nodes were collected and enumerated (A). These cells were stimulated with OVA (10 μg/ml) in vitro and cell proliferation was assessed by [³H]thy- midine incorporation after 48 h (B). Culture supernatants were collected at 48 h and assayed for cytokines (IFN-γ, IL-2, and IL-4) using the Luminex microbead-based multiplexed assay system (C). Data are representative of four independent experiments.

**FIGURE 1.** DC exposed to apoptotic cells are immunosuppressive in vivo and ex vivo. Mature bone marrow-derived DC and apoptotic cell-exposed DC (DCap) were prepared as described in Materials and Methods. DC and DCap were stimulated with LPS (1 μg/ml) for 24 h. Surface expression levels of costimulatory molecules CD40, CD80, CD86, and MHC class II were measured by immunoflourescence and flow cytometry (heavy line) and compared with isotype control Ab staining (shaded area). Values indicate mean fluorescence intensity of specific staining (A). Culture supernatants were assayed for cytokines (IL-12, IL-10, TNF-α, and IL-1α) (B) as in Fig. 1. Data are representative of three independent experiments for A and of four independent experiments for B.

DLNAs, as compared to a PBS control, while those receiving DCap-exposed OVA exhibited much smaller increases. Importantly, when normal DC and DCap were injected together, the increment in dLN cellularity was significantly less than with normal DC alone, indicating that DCap inhibited stimulation by normal DC. To directly assess the effect of apoptotic cells on T cell stimulation by DC, we examined the response of dLN lymphocytes to OVA stimulation in vitro. Equal numbers of dLN cells were stimulated with OVA, and proliferation and cytokine production determined 48 h later. As expected, dLN cells from mice stimulated with OVA-loaded normal DC exhibited vigorous proliferation and high levels of several cytokines. In contrast, dLN cells from mice primed with DCap or with mixed DCap and normal DC showed much less Ag-specific proliferation (Fig. 1B). They also produced less of the Th1 cytokines, IFN-γ and IL-2, as well as the Th2 cytokine, IL-4 (Fig. 1C). Cytokines could not be detected from the lymphocytes isolated from PBS treated mice (data not shown). Thus, DC exposed to apoptotic splenocytes exhibited an immunosuppressive function both in vivo and ex vivo.

**Apoptotic cells induce functional and phenotypic changes in DC**

Our in vivo data clearly demonstrate that apoptotic cells dramatically affect a specific Ag-induced immune response through their interaction with DC. Therefore, we explored the changes that occur in DC after exposure to apoptotic lymphocytes. We first examined the expression of several costimulatory molecules. As shown in Fig. 2A, after stimulation with LPS, DCap showed a significantly less mature phenotype compared with normal DC: surface expression levels of CD40, CD80, CD86, and MHC-II were all down-regulated. We also examined the function of DCap by stimulating them with LPS and measuring proinflammatory cytokine production. We found that DCap produced less proinflammatory cytokines, such as TNF-α, IL-1α, and IL-12, but more IL-10, than do normal DC (Fig. 2B). Taken together, these results show that coculture with apoptotic cells, causes mature DC to exhibit significant functional and phenotypic changes.

**DCap act synergistically with normal DC to induce T cell hyporesponsiveness**

We next investigated the capability of DCap to support anti-CD3-stimulated proliferation of splenic CD4⁺ T cells in vitro. To our surprise, DCap were almost equally as efficient as normal DC in promoting T cell proliferation at a 1:5 ratio of DC to T cells (Fig. 3A), a big discrepancy from our ex vivo results (Fig. 1). Examination of the induction of T cell expression of cytokines by DCap revealed that, while IL-2 was still produced at the same level as that induced by normal DC, IFN-γ production was not induced by DCap (Fig. 3B). Surprisingly, in the presence of both DCap and normal DC (DCap: DC = 1:1), T cell proliferation was almost completely inhibited (Fig. 3A). This strongly suggests that an interaction between normal DC and DCap leads to the suppression of T cell responses induced by either type of DC alone. It is important to point out that the inhibition is very strong. Significant inhibition could be observed at a total DC: T cell ratio of 1:10 and 80% inhibition was achieved at 1:5 (Fig. 3C). We also found that maximal inhibition was at a DCap:DC ratio of 1:1 (Fig. 3D). Therefore, cells were cocultured at the ratios of 1:5 total DC:
To dissect the suppressive mechanism in the following experiments, we treated DCap with the supernatant from a culture of CD4+ H11001 T cells stimulated with normal DC, and vice-versa. The T cell-stimulatory capacity of these DC was then assayed by adding them to cultures of fresh CD4+ H11001 T cells and determining T cell proliferation in response to soluble anti-CD3. We found that DCap exposed to supernatant from the coculture of CD4+ T cells with normal DC largely lost their ability to subsequently activate T cells. In contrast, supernatant from DCap-activated CD4+ T cells had little effect on the stimulatory ability of normal DC (Fig. 4A). These results suggest that CD4+ T cells activated by normal DC secrete some factor(s) responsible for the induction of the immunosuppressive function of DCap.

**FIGURE 3.** DCap act synergistically with normal DC to induce T cell hyporesponsiveness. Naive CD4+ T cells were cocultured with the same total cell number of mature DC, mature DCap, or a mixture of both (DC:DCap = 1:1) at a 1:5 ratio of DC to T cells in the presence of soluble anti-CD3 (1 µg/ml). Cell proliferation (A) and cytokine production (IFN-γ and IL-2) (B) were assayed at 48 h, as in Fig. 1. Data are representative of five independent experiments. To titrate this effect, these cocultures were done at graded ratios of DC to T cells (C). Furthermore, to find the best proportion of DC to DCap, these cells were mixed at different ratios and cocultured with naive CD4+ T cells (1:5 ratio of total DC (DC+DCap) to T cells) in the presence of anti-CD3 and cell proliferation was assayed at 48 h (D). Data are representative of three independent experiments.

**FIGURE 4.** NO plays a critical role in immunosuppression induced by apoptotic cells. Naive CD4+ T cells were cocultured with mature DC, mature DCap, or mixture of both at a 1:5 ratio of DC to T cells in the presence of soluble anti-CD3 (1 µg/ml), as in Fig. 3, and proliferation assessed after 48 h. To some cultures, was added the following: supernatant (50% by volume) from cultures of CD4+ T cells activated in the presence of either DCap (DCap-sup) or normal DC (DC-sup) (A); or blocking Abs against CTLA-4, BTLA-4, IL-10, or TGFβ (25 µg/ml each); or the inhibitors indomethacin (10 µM) or NG-monomethyl-L-arginine (L-NMMA, 1 mM), to reveal molecules involved in T cell hyporesponsiveness (B). In the coculture of CD4+ T cells with DC, DCap, or DC plus DCap (1:5 ratio of total DCs to T cells), the presence of CD4+ Foxp3+ regulatory T cells was analyzed by flow cytometry at 48 h after stimulation with soluble anti-CD3 (1 µg/ml) (C). Data are representative of three independent experiments.
NO plays a critical role in immunosuppression induced by apoptotic cells

To probe the responsible factor(s) involved in immunosuppression by the combination of normal DC and DCap, we added various blocking Abs and inhibitors during the stimulation of CD4+ T cells and observed the effect on proliferation. First, the involvement of several costimulatory molecules was excluded by adding neutralizing Abs against CD80, CD86, CD40L (data not shown), CTLA-4, and BTLA-4 (Fig. 4B); none of these had an effect. We then tested the effects of anti-IL-10, anti-TGF-β, and indomethacin (a COX-2 inhibitor that blocks PGE2 production) but again found no effect (Fig. 4B). CD4+Foxp3+ regulatory T cells were also found to have no role in this process (Fig. 4C). Inhibition of inducible NO synthase (iNOS) by the arginine analog, NG-monomethyl-L-arginine, however, largely reversed the inhibition of T cell proliferation by the combination of DCap and normal DC (Fig. 4B). Other iNOS inhibitors, 1400W and l-NAME, also showed the same effect (data not shown), indicating that iNOS may be involved in the immunosuppression by DCap.

**IFN-γ is critical for the immunosuppression through induction of NO by DCap**

NO has been shown to suppress immune responses at high concentrations. In cells of the hematopoietic system, NO is generated found to have no role in this process (Fig. 4C). Inhibition of inducible NO synthase (iNOS) by the arginine analog, NG-monomethyl-L-arginine, however, largely reversed the inhibition of T cell proliferation by the combination of DCap and normal DC (Fig. 4B). Other iNOS inhibitors, 1400W and l-NAME, also showed the same effect (data not shown), indicating that iNOS may be involved in the immunosuppression by DCap.

**IFN-γ is critical for the immunosuppression through induction of NO by DCap**

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**FIGURE 5.** IFN-γ induces immunosuppression by DCap through the induction of NO production. Cocultures of CD3-stimulated naive CD4+ T cells with mature DC or mature DCap were supplemented with neutralizing Ab against IFN-γ (25 μg/ml) and the effect on proliferation assessed after 48 h (A). Mature DC and DCap (1 x 10^6 cells/ml) were treated with IFN-γ, TNF-α, or IL-1α, alone or in various combinations (each at 10 ng/ml), as indicated. Culture supernatants were collected after 24 h, and total nitrate production was assessed using modified Griess reagent (B). DC and DCap derived from IFN-γ-R1^-/- or iNOS^-/- mice were cultured with or without addition of recombinant IFN-γ (10 ng/ml) and nitrate production determined, as above (C). Data are representative of three independent experiments.

**FIGURE 6.** DCap deficient in IFNγR1 or iNOS are incapable of immunosuppression in vitro. Mature DC and DCap derived from wild-type, IFN-γ-R1^-/-, or iNOS^-/- mice were cultured in the indicated combinations with naive CD4+ T cells at a 1:5 ratio (DC to T cells) in the presence of soluble anti-CD3 (1 μg/ml), and proliferation was determined after 48 h (A and B). Naive CD4+ T cells were cocultured with DC, DCap, or an equal number of both, as above, and IFN-γ production, NO release, and cell proliferation were assayed at 24, 48, and 72 h (C). Data are representative of three independent experiments.
exclusively by iNOS. Because IFN-γ has been shown to be one of the best inducers of iNOS expression, we next determined the role of IFN-γ in the induction of NO production by DCap. Although we have shown that DCap are inefficient in inducing IFN-γ production by T cells (Fig. 3B), normal DC are quite effective. Accordingly, we added neutralizing Ab against IFN-γ during the activation of CD4+ T cells with anti-CD3 by normal DC and DCap, and found complete reversal of the inhibition of T proliferation (Fig. 5A). Therefore, it is possible that IFN-γ-induced NO production is responsible for the immunosuppression induced by the combination of normal DC and DCap.

As shown in Fig. 3, exposure to apoptotic cells down-regulated the ability of DCap to stimulate T cells to produce IFN-γ. However, our data also show that IFN-γ is required for the immunosuppression induced by the combination of normal DC and DCap. To test this possibility, we directly compared the differential responses of normal DC and DCap to exogenous IFN-γ. Because IL-1 and TNF-α have been reported to be NO inducers, their effects were also determined with and without IFN-γ. Normal DC or DCap were incubated with IFN-γ with or without IL-1 and TNF-α for 24 h and NO production was assayed. Surprisingly, IFN-γ alone induced dramatic NO production by DCap, but only a low level in normal DC (Fig. 5B). The inclusion of IL-1 or TNF-α had no significant effect. It should be pointed out that high levels of NO are required to suppress T cell proliferation, while DC normally produce only low levels.

To further examine the role of IFN-γ-induced NO, normal DC and DCap were generated from mice deficient in iNOS (iNOS−/−) or IFN-γ receptor 1 (IFN-γR1−/−). Consistent with our prediction, after stimulation with IFN-γ, DCap derived from either iNOS−/− or IFN-γR1−/− mice produced little NO (Fig. 5C).

**FIGURE 7.** DCap deficient in IFN-γR1 or iNOS do not suppress immune responses in vivo and ex vivo.

Using the same procedure described in Fig. 1, mature DC and DCap derived from wild-type, IFN-γR1−/−, and iNOS−/− mice were loaded with OVA peptide, and then injected s.c. into mouse hind footpads (1 × 10⁶ cells in PBS per footpad), with PBS alone as a control. After 7 days, lymphocytes in the draining popliteal lymph nodes were collected and enumerated (A). These cells were then stimulated with OVA (10 μg/ml) in vitro and cell proliferation was assessed after 48 h (B). Culture supernatants were collected at 48 h and assayed for cytokines IFN-γ, IL-2, and IL-4 (C). Data are representative of two independent experiments.

**FIGURE 8.** STAT1, but not IFN-γR1 expression was highly inducible in DCap cells by IFN-γ. IFN-γR1 expression by mature DC and DCap cells was compared by flow cytometry, with hamster IgG as an isotype control (A). To quantify the level of STAT1 gene expression induced by IFN-γ, mature normal DC and DCap were treated with or without addition of IFN-γ (10 ng/ml) for 12 h. STAT1 expression was then assayed by real-time PCR. The level of STAT1 in normal DC without IFN-γ was arbitrarily defined as 1 unit (B).


**Discussion**

Cellular apoptosis occurs throughout the lives of higher metazoan animals (32). Although the extent of apoptosis in different tissues varies depending on the tissue type, age, and physiological and ambient conditions, it has been shown in mice and humans that deficiency in the removal of apoptotic cells can lead to autoimmune disorders such as systemic lupus erythematosus (33). In addition, apoptotic cells by themselves can actually lead to immunosuppression (3, 18, 20, 34–38). It was reported that decreased phagocytosis of apoptotic cells contributes significantly to the development of systemic lupus erythematosus in mice and humans (39). Thus, removal of the remnants of apoptotic cells by phagocytes not only scavenges the degradation products of apoptotic cells, but also result in neutral responses from phagocytes or active suppression of inflammation through production of anti-inflammatory mediators. It has also been reported that apoptotic cells trigger tolerogenic responses in the adaptive immune system. Apoptotic cells loaded with exogenous Ag induce specific tolerance to the Ag when injected i.v. into mice (40, 41). We have reported that apoptotic cell administration also prevents the rejection of allogeneic heart transplants (6). Various immunosuppressive factors such as IL-10, TGF-β, and PGE₂ have been proposed to be responsible for these effects (42). In this study, we examined the effects of apoptotic cells on DC and found that, although DC apoptogen (DCap) showed little decrement in their ability to activate T cells, they were strongly immunosuppressive when combined with normal DC. Our further analysis revealed that DC exposed to apoptotic cells lost their ability to induce IFN-γ production by T cells, while enabling DC to be highly responsive to IFN-γ by producing large amounts of NO. It is this NO that is responsible for T cell suppression in this system. We also examined IL-10, TGFβ, and PGE₂ and found no role for these factors. Therefore, we report a novel mechanism through which apoptotic cells affect immune responses.

Several studies have found that apoptotic cells exhibit a dramatic immunosuppressive effect in vivo (6, 43, 44). Our studies with heart transplantation showed that a single injection of apoptotic cells could extend successful engraftment for >40 days on the average. When tested in vitro, however, we found that apoptotic cell-pulsed DC lost little of their capacity to activate T cells, in terms of T cell proliferation and IL-2 production, except for decreased secretion of IFN-γ. In fact, most studies of immunosuppression by apoptotic cells have been demonstrated using in vivo systems; few have been reported in vitro. Considering the dramatic immunosuppressive effects of apoptotic cells in vivo, we reasoned that it is highly probable that an interaction exists between apoptotic cell-pulsed phagocytes and other cells. By combining apoptotic cell-treated DC with normal DC, we found that the cooperation of both cell types significantly inhibits T cell activation. This novel finding has significant ramifications for our understanding of the critical role of apoptotic cells in the maintenance of immune homeostasis, autoimmunity and cancer immunology, especially in pathophysiological processes with significant apoptosis.

This study puts NO at the center of the immunosuppression mediated by apoptotic cells. This novel conclusion was based on studies with iNOS inhibitors and iNOS-deficient mice. In fact, it has been shown that iNOS−/− mice are more responsive to immunological challenge and are more prone to development of autoimmune diseases (45). Although NO is known to be very labile and can act only at short range, it is astonishing that such strong immunosuppressive effects can be achieved by this unstable gaseous molecule. Interestingly, we found that IFN-γ also dramatically upregulated the expression of several T cell chemokines, more so in DC than in DC. Blockade of the chemokine receptors, CXCR3 and CCR5, reduced the immunosuppressive effect of DAp (data not shown). Therefore, upon stimulation by IFN-γ produced by T cells that have been activated by normal DC, in turn DAp produce

\[ \text{DC}_{\text{ap}} \text{ deficient in IFNyR1 or iNOS lack the immunosuppressive effect} \]

To verify that NO is indeed produced by DC in response to IFN-γ in the mixed coculture of DC and DCap, we mixed normal DC and DCap derived from iNOS-deficient, IFN-γR1-deficient, or wild-type mice. We found that both types of DC derived from all mice were capable of inducing T cell activation. When DAp from iNOS−/− or IFN-γR1−/− mice were cocultured with wild-type normal DC, immunosuppression was not observed (Fig. 6A), indicating that IFN-γ receptor-mediated signaling and NO production in DCap are the key to immunosuppression. However, when the respective deficient normal DC were mixed with wild-type DCap, immunosuppression still occurred (Fig. 6B) because normal DC from iNOS−/− or IFN-γR1−/− mice did not lose the ability to support T cells to produce IFN-γ (data not shown). We also excluded the possibility that IFN-γ produced from T cells by DCap at early time points could lead to NO production at later time points. By analyzing IFN-γ production, NO release, and T cell proliferation on day 1, 2, and 3, we found that, DCap did not support the production of large amounts of IFN-γ by T cells (Fig. 6C). Thus, these results demonstrate that in the mixed culture of DC and DCap, it is the DCap that produce NO in response to IFN-γ that is provided mainly by normal DC.

To extend these in vitro results to an in vivo system, we tested the effect of DCap on an Ag-specific immune response. DC or DCap from wild-type, IFN-γR1−/− and iNOS−/− mice were injected into the footpads of wild-type mice, using the same protocol as in Fig. 1. Unlike DCap derived from wild-type mice, DCap derived from IFN-γR1−/− or iNOS−/− mice were capable of activating dLN lymphocytes to the same extent as normal DC, as shown by the effects on cell number (Fig. 7A), Ag-specific lymphocyte proliferation (Fig. 7B) and T cell cytokine production (Fig. 7C). These results indicate a critical role for IFN-γ and NO in immunosuppression by apoptotic cell-educated DC in vivo.

The greatest difference between DCs that have or have not been exposed to apoptotic cells lies in their distinct responses to IFN-γ. Therefore, we next investigated the molecular mechanism of these responses. Because it has been reported that some microbes can alter the IFN-γ response by modulation of IFN-γR1 expression (29), we examined whether DCap express greater levels of IFN-γR1. We found no difference in IFNγR1 expression between mature DCap and mature DC (Fig. 8A). We also found that addition of exogenous IFN-γ did not alter the expression pattern of IFN-γR1 (data not shown). We then compared the expression of STAT1 in IFN-γ-treated DC and DCap since STAT1 signaling is the most prevalent pathway of IFN-γ-induced NO production (30, 31). We found that STAT1 induction after treatment with IFN-γ was significantly higher in DCap than in normal DC, which may explain the greater IFN-γ response of DCap cells (Fig. 8B). Further molecular investigations should elucidate the mechanism by which distinct IFN-γ signaling occurs in DCap cells.

In conclusion, our study reveals a novel mechanism by which apoptotic cells mediate immunosuppression. Upon exposure to apoptotic cells, DC lose their capacity to induce IFN-γ production by T cells, while at the same time they become capable of producing large amounts of NO in response to IFN-γ. With IFN-γ provided by T cells stimulated by other normal DC, the high levels of NO produced by DCap can suppress T cell responsiveness, leading to immunosuppression.
NO and chemokines. It is presumed that the latter attracts T cells to the near vicinity of DC_{ap}, where the locally high concentrations of NO suppress T cell responsiveness.

The removal of apoptotic cells by phagocytes involves a complex array of still poorly defined molecules. Besides those on the cell surface, soluble factors that link apoptotic cells and phagocytes are also involved. Although the detailed molecular mechanisms are unknown, our data shows that apoptotic cells induce two major changes in DC cells: DC_{ap} lose their capability to stimulate T cells to produce IFN-γ and become highly responsive to IFN-γ to produce high levels of NO. This is a novel mechanism by which apoptotic cells induce immunosuppression. Because several molecules have been reported to be involved in the phagocytosis of apoptotic cells, more studies are needed to understand which are involved in these changes in DC_{ap}. In addition, further studies of recently identified molecules such as calreticulin, which has been shown to be critical in converting apoptotic cells from tolerogenic to immunogenic, may shed new light on our understanding of autoimmunity and lead to novel tactics for cancer immunotherapy.

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Disclosures

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