The Journal of Immunology

# Inhibitory Effects of Apoptotic Cell Ingestion upon Endotoxin-Driven Myeloid Dendritic Cell Maturation

Lynda M. Stuart, Mark Lucas, Cathy Simpson, Jonathan Lamb, John Savill and Adam Lacy-Hulbert

*J Immunol* 2002; 168:1627-1635; ;
doi: 10.4049/jimmunol.168.4.1627
http://www.jimmunol.org/content/168/4/1627





*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Inhibitory Effects of Apoptotic Cell Ingestion upon Endotoxin-Driven Myeloid Dendritic Cell Maturation[1]

**Lynda M. Stuart,[2] Mark Lucas, Cathy Simpson, Jonathan Lamb, John Savill, and Adam Lacy-Hulbert**

**Dendritic cells (DCs) are the sentinels of the immune system, able to interact with both naive and memory T cells. The recent observation that DCs can ingest cells dying by apoptosis has raised the possibility that DCs may, in fact, present self-derived Ags, initiating both autoimmunity and tumor-specific responses, especially if associated with appropriate danger signals. Although the process of ingestion of apoptotic cells has not been shown to induce DC maturation, the exact fate of these phagocytosing DCs remains unclear. In this paper we demonstrate that DCs that ingest apoptotic cells are able to produce TNF-$\alpha$ but have a diminished ability to produce IL-12 in response to external stimuli, a property that corresponds to a failure to up-regulate CD86. By single-cell analysis we demonstrate that these inhibitory effects are restricted to those DCs that have engulfed apoptotic cells, with bystander DCs remaining unaffected. These changes were independent of the production of anti-inflammatory cytokines TGF-$\beta$1 and IL-10 and corresponded with a diminished capacity to stimulate naive T cells. Thus, the ingestion of apoptotic cells is not an immunologically null event but is capable of modulating DC maturation. These results have important implications for our understanding of the role of clearance of dying cells by DCs not only in the normal resolution of inflammation but also in control of subsequent immune responses to apoptotic cell-derived Ags.** ***The Journal of Immunology,*** **2002, 168: 1627–1635.**

Dendritic cells (DCs)[3] are the sentinels of the adaptive immune system and have an important role not only in induction of immunity but also in maintenance of tolerance (1). Immature DCs exist in the periphery, where they capture and process exogenous Ag. Upon receipt of maturation stimuli they migrate to draining lymph nodes, a process associated with phenotypic changes, including down-regulation of their Ag-capturing machinery, up-regulation of MHC and costimulatory molecules, and production of IL-12, becoming fully functional APC (2). This maturation process is affected by a variety of endogenous or exogenous factors and can be modeled in vitro by LPS and other bacterial products (3).

Immature DCs acquire Ag by many pathways including uptake of soluble Ag or protein complexes by endocytosis and macropinocytosis and ingestion of entire cells by phagocytosis. Ingestion of certain necrotic cells is capable of inducing DC maturation, while ingesting apoptotic cells fails to activate DCs, appearing to be an immunologically null event (4, 5). However, such DCs are capable of responding to strong external stimuli, such as monocyte-conditioned medium or IFN-$\gamma$, to mature and present Ag derived from the ingested apoptotic cells to T cells (6–9). Apoptotic cells are a preferential source of many autoantigens (10), often found localized to apoptotic blebs, and the ability of DCs to present such Ags unchecked might initiate autoimmunity. In support of this, perturbations in apoptotic cell death and clearance of these cells have been shown to contribute to the induction of autoimmunity (11, 12).

However, a growing body of evidence implicates DCs that ingest dying cells in maintaining self-tolerance, by constantly sampling peripheral self Ags and presenting them in a tolerogenic way to the adaptive immune system. Thus, a dichotomy exists in responses of DCs that may be either "friend or foe" (13). The ability of a DC to deliver "signal 2," either as costimulation or IL-12, singly or in combination, appears key in determining subsequent immune responses and is likely to be tightly controlled. Interestingly, the ingestion of apoptotic cells by macrophages generates an active anti-inflammatory response through the production of TGF-$\beta$1 and other anti-inflammatory molecules and down-regulates subsequent release of proinflammatory cytokines (14–16). Because of the close lineage relationship of macrophages and myeloid DCs, we postulated that apoptotic cell ingestion by DCs might similarly modulate their effector functions.

In this paper we confirm that immature murine bone marrow-derived DCs ingest apoptotic cells and, after phagocytosis, become functionally distinct. We demonstrate that endotoxin-induced production of IL-12, but not TNF-$\alpha$, is selectively diminished in DCs that have ingested apoptotic cells. In addition, endotoxin-driven up-regulation of the costimulatory molecule CD86 is inhibited in those DCs that had phagocytosed apoptotic cells, but not in neighboring DCs. We show the functional consequences of these changes by demonstrating that these DCs have a reduced capacity to stimulate T cell proliferation. Thus, phagocytosis of apoptotic cells affects subsequent maturation of DCs in a manner analogous to the anti-inflammatory effects in macrophages, generating mature, $CD86^{low}$ cells that produce less IL-12. These data confirm that the apoptotic cell is not immunologically null, but, by inhibiting DC activation, may contribute to down-regulation of the response to apoptotic cell-derived self Ag and maintenance of self tolerance.

Medical Research Council Center for Inflammation Research, University of Edinburgh, Edinburgh, Scotland, United Kingdom

Received for publication September 6, 2001. Accepted for publication December 12, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] L.M.S. was supported by Wellcome Trust Clinical Training Fellowship 34842, and J.S. was supported by Wellcome Program Grant 34561.

[2] Address correspondence and reprint requests to Dr. Lynda M. Stuart, Medical Research Council Center for Inflammation Research, University of Edinburgh, Teviot Place, Edinburgh, Scotland, U.K. EH8 9AG. E-mail address: lynda.stuart@ed.ac.uk

[3] Abbreviations used in this paper: DC, dendritic cell; ac, apoptotic cell; SAC, *Staphylococcus aureus* (Cowan strain).



0022-1767/02/$02.00

## Materials and Methods

### *Mice*

BALB/c mice were purchased from B & K Universal (Hull, U.K.) and were used at 8 wk for bone marrow-derived DC and macrophage preparation. T cells were isolated from the DO11.10 transgenic mice expressing TCR specific for the chicken OVA peptide, $OVA_{323–339}$, in association with $I\text{-}A^d$. The mice were typed for the presence of the transgene using Abs against CD4 (BD PharMingen, San Diego, CA) and biotin peak 2 (KJ1-26; Scottish Antibody Production Unit, Lanarkshire, U.K.) and streptavidin-PE (BD PharMingen) on PBL.

### *DC and macrophage culture*

Murine cells were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, $5 \times 10^{-5}$ M 2-ME, and 15% heat-inactivated FBS unless otherwise stated. DC complete medium also contained 10–15% conditioned supernatant from a hybridoma (gift from Prof. D. Gray, Edinburgh, U.K.) expressing rGM-CSF, resulting in a final GM-CSF concentration of 20–30 ng/ml. This hybridoma also produces IL-10, at a final concentration of ~2 ng/ml, but no TNF-$\alpha$ or IL-12. All culture reagents were obtained from Life Technologies (Grand Island, NY) unless otherwise stated.

DCs were cultured as described previously (17). Briefly, femurs from BALB/c mice were removed, dipped in 70% ethanol for 10 s, and then placed in DC complete medium. Bone marrow was flushed from femurs, and 10 ml of a single-cell suspension of bone marrow cells at $2 \times 10^5$/ml was plated in non-tissue culture grade petri dishes. On day 3 an additional 10 ml of fresh medium was added to the cultures. On day 6 half the medium was removed, and the cells were pelleted, resuspended in fresh medium, and added back to the petri dishes. On day 7 nonadherent cells were removed, leaving strongly adherent macrophages on the plate. These cells were pelleted, resuspended at $2 \times 10^5$ cells/ml, and replated before use. On day 7 these were a heterogeneous population, 65–80% of the cells having surface phenotype and morphology of immature DCs (Fig. 1*A*), with granulocytes being the main contaminant. Maturation was initiated on day 7 with 0.1–1 $\mu$g/ml LPS (*Escherichia coli* serotype 026:B6; Sigma-Aldrich, St. Louis, MO) or 0.02% (w/v) *Staphylococcus aureus* (Cowan strain) (SAC; Calbiochem, La Jolla, CA) (3), and cells were assessed on day 8.

Bone marrow-derived macrophages were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 10% heat-inactivated FBS, and 10% conditioned supernatant from L929 cells, which was changed on day 2 of culture. Macrophages were used for experiments on day 7 of culture.

### *Generation of apoptotic cells*

Neutrophils were extracted from peripheral blood of healthy volunteers as described previously (18). Briefly, blood was separated using dextran sedimentation and a Percoll gradient. This yielded highly pure human neutrophils (>90%), which were allowed to undergo constitutive apoptosis by aging overnight in Iscove's medium supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% autologous serum. After this period the cells were 40–80% apoptotic by cytospin morphology. This method of generating apoptotic bodies was preferred because there was no significant necrosis (<1%) by trypan blue exclusion, as confirmed by annexin-propidium iodide staining and flow cytometry. Apoptotic murine thymocytes were also generated for use in some experiments by treating single-cell suspensions of thymocytes with dexamethasone for 4–6 h. This method yielded apoptotic cells, but these preparations often contained contaminating postapoptotic cells and other nonapoptotic thymic cells. For most experiments cells were stained using a green cell tracker dye (Molecular Probes, Eugene, OR) before overnight culture.

### *DC-apoptotic cell coculture*

Fluorescently labeled apoptotic cells were cocultured with day 7 DCs at a ratio of 2–5:1, apoptotic cells:DC. Interaction of DCs with apoptotic cells was assessed by removing cells after 2 h and staining with allophycocyanin-CD11c for FACS analysis. All FACS analyses were conducted on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Maximum interaction was seen at ratios of 5:1 apoptotic cells/DCs, but the large numbers of uningested apoptotic cells reduced the efficiency of cell sorting, so ratios of 2:1 were used in most experiments. For fluorescent microscopy DCs were grown on chamber well slides, allowed to interact with red fluorescent apoptotic cells, then fixed with 4% paraformaldehyde. Slides were stained in PBS with 0.5% BSA and 0.2% sodium azide with $I\text{-}A^d$-FITC (BD PharMingen) in the presence of 10% normal mouse serum. Slides were mounted and examined under ×63 oil lens using an inverted microscope (Zeiss, New York, NY), and images were captured using Open Lab software (Improvision, Coventry, U.K.) and CoolSnap digital camera (Media Cybernetics, Silver Spring, MD).

### *T cell proliferation assays*

CD4 T cells were isolated from spleens of DO11.10 transgenic mice. In brief, spleens were made into a single-cell suspension by passing through a 53-$\mu$m pore size filter in PBS, and debris and red cells were removed by density gradient sedimentation through Lympholyte-M (Cedarlane Laboratories, Ontario, Canada) according to the manufacturer's instructions. Cells were then isolated using L3T4 (CD4) microbeads and the MACS purification system (Miltenyi Biotec, Auburn, CA). Day 7 DCs were cocultured with apoptotic cells for 4 h, stimulated with LPS overnight, and then pulsed with 5 $\mu$g/ml OVA peptide ($OVA_{323–339}$; Albachem Laboratories, Edinburgh University, Edinburgh, U.K.) for 2 h. They were then washed thoroughly, and cells were sorted into DCs containing apoptotic cells and those not, using a FACSVantage cell sorter (BD Biosciences). Contaminating apoptotic cells could be excluded from the sort by their smaller size and bright fluorescence. OVA-TCR-transgenic T cells ($3 \times 10^5$) were cocultured in 24-well tissue culture dishes with varying doses of DCs for 5 days in a final volume of 2 ml. Proliferation was assessed by removing triplicate 100-$\mu$l samples pulsed with 1 $\mu$Ci/well [$^3$H]thymidine (Sigma-Aldrich) for 16 h. Cells were harvested, and thymidine incorporation was measured using a scintillation counter. Interactions were performed in duplicate.

### *FACS analysis of costimulatory molecules*

DCs were cocultured with apoptotic cells for 4 h before overnight stimulation with LPS. Nonadherent cells were then removed from plates and resuspended in PBS with 0.5% BSA and 0.2% sodium azide. Blocking was performed using 10% mouse serum for 15 min, then cells were stained with relevant Abs at 4°C in the dark for 30 min. Cells were then washed and resuspended in 200 $\mu$l of FACS wash and analyzed using FACSCalibur and FlowJo software. The following Abs were used (all from BD PharMingen unless otherwise stated): $FITC\text{-}I\text{-}A^d/I\text{-}E^d$, PE-CD40, PE-CD86, PE-CD54, PE-CD11c, allophycocyanin-CD11c, and FITC-F4/80 (Serotec, Oxford, U.K.). All samples were compared with appropriate isotype controls. The geometric mean fluorescence of cells positive to isotype control was used in analysis unless stated otherwise.

### *Cytokine detection*

For intracellular cytokine staining, cells were cocultured with apoptotic cells for 4 h and stimulated with LPS for 5 h in the presence of GolgiPlug (BD PharMingen) according to the manufacturer's instructions. Cells were harvested and stained for cell surface markers as described above. Cells were then fixed using 4% paraformaldehyde and permeabilized with 2% saponin in PBS with 0.5% BSA, 0.2% sodium azide, and 10% mouse serum while staining with allophycocyanin-TNF-$\alpha$, IL-10, and IL-12p40/p70 (BD PharMingen). IL-10 could not be reliably detected above background by intracellular staining and so was also measured in the supernatant after 24–48 h of interaction with apoptotic cells and LPS using a Quantakine ELISA kit (R&D Systems, Minneapolis, MN). Interactions were performed in duplicate wells, and triplicate readings of each supernatant were made. Soluble forms of IL-10 and TGF-$\beta$R were obtained from R&D Systems and used at the recommended concentrations (1.25 and 0.5 $\mu$g/ml, respectively).

## Results

### *Immature murine myeloid DCs ingest apoptotic cells*

Day 7 murine myeloid DCs cultured as described above were immature by cell surface phenotype ($CD11c^+$MHC class $II^+CD40^-CD80^{low}CD86^{low}$) and could be matured (as evidenced by up-regulation of costimulatory molecules and MHC class II) over 24 h by the addition of LPS in a dose-dependent manner (Fig. 1*A*). Similar results were seen by stimulation with SAC (data not shown). Apoptotic cells stained with a fluorescent cell-tracker dye were cultured with DCs at a ratio of 5:1, and interactions with DCs were quantified by flow cytometry. In a typical experiment, 2 h after coincubation ~50% of $CD11c^+$ cells had interacted with apoptotic cells. Such interaction was exhibited by <5% of DCs at 4°C, demonstrating that the interaction assay used predominantly detected phagocytosis rather than binding (Fig. 1*B*). Ingestion was further confirmed by fluorescence microscopy (Fig. 1*C*). Similar

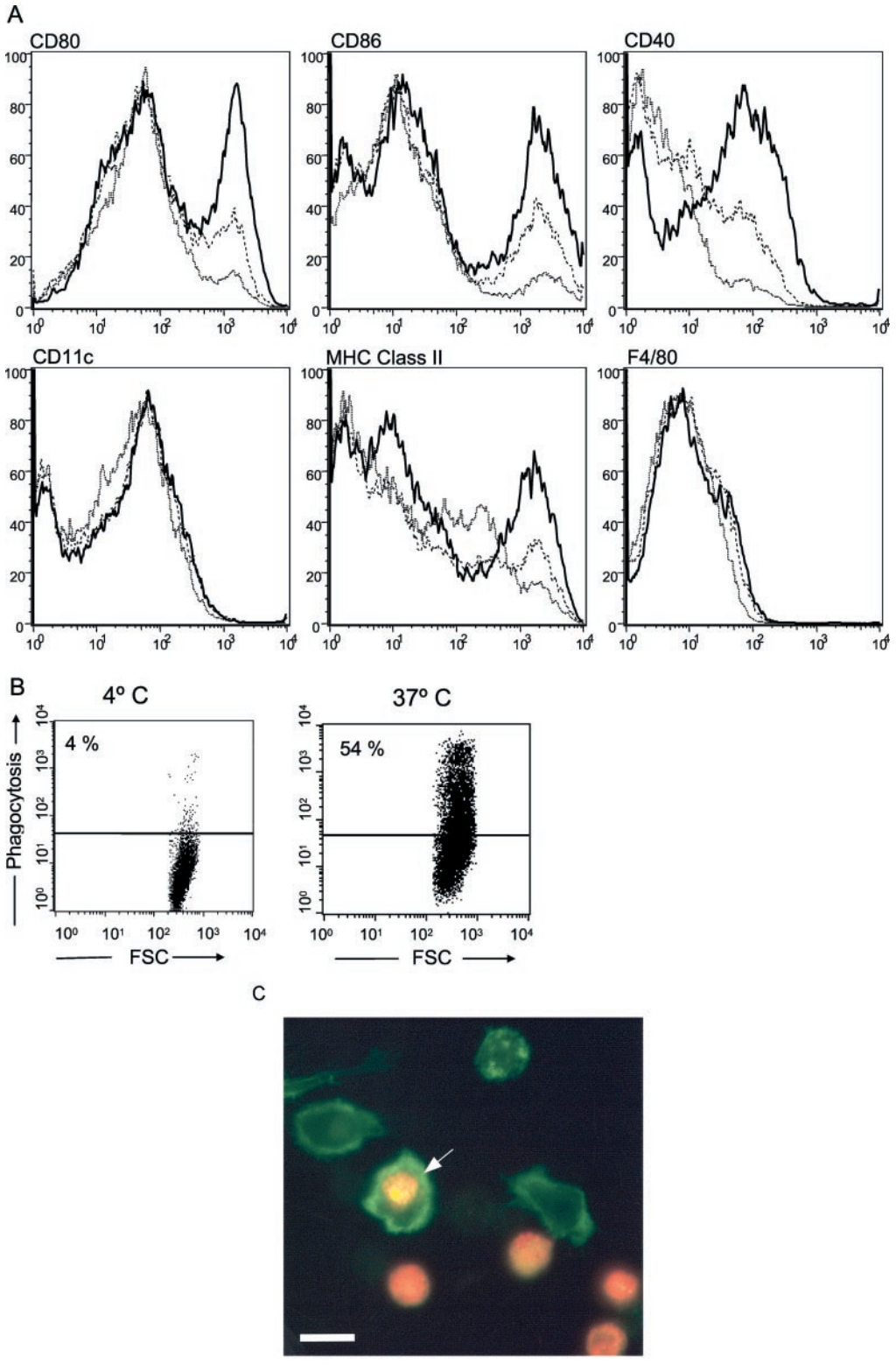


**FIGURE 1.** Immature murine myeloid DCs ingest apoptotic cells. *A*, DCs cultured as described are immature on day 7, as assessed by levels of expression of costimulatory molecules and MHC class II. Stimulation by LPS for 24 h induced a mature phenotype in a dose-dependent manner. DCs were prepared as described in *Materials and Methods* and stimulated with 0 (thin lines), 0.1 (dashed lines), or 0.5 $\mu$g/ml (thick lines) LPS for 24 h and assessed by flow cytometry. Isotype controls are omitted for clarity but sit in the first log order of fluorescence. Data shown are from one experiment, representative of at least five similar experiments. *B*, Immature DCs were incubated with fluorescently labeled apoptotic neutrophils at a ratio of 1:5. Flow cytometric assay of interaction between DCs and apoptotic cells demonstrating at 37°C green fluorescence of phagocytic DC, which was almost completely inhibited at 4°C. Events were gated for CD11c-positive cells and were representative of at least three experiments. *C*, DC were incubated with fluorescent apoptotic cells and counterstained with FITC-conjugated I-$A^d$/I-$E^d$ (MHC II). By fluorescence microscopy, red fluorescent apoptotic bodies interact with MHC II-positive DCs (green) and, after internalization by a DC, change their fluorescent properties (arrow). Original magnification, ×63 oil. Scale bar represents 10 $\mu$m.

rates of phagocytosis were seen when dexamethasone-treated murine thymocytes were used as an alternative source of apoptotic cells (data not shown). Thus, immature murine DCs exhibited a capacity for phagocytosis of apoptotic cells similar in magnitude to that reported in studies of immature human DC (7, 8).

### *Ingestion of apoptotic cells specifically inhibits the ability of DCs to up-regulate CD86*

To ascertain whether ingestion of apoptotic cells alters DC phenotype, cell surface expression of activation markers was studied by flow cytometry after phagocytosis. No significant difference in cell surface expression of the costimulatory molecules CD40, CD80, and CD86 was seen between immature DCs that had ingested apoptotic cells ($ac^+$) and those that had not ($ac^-$) either immediately (2 h, data not shown) or 24 h (Fig. 2) after phagocytosis, confirming that ingestion of apoptotic cells did not activate DCs. However, on DC maturation with LPS a marked difference in surface expression of CD86 was detected between $ac^+$ DCs and $ac^-$ DCs. Immature DCs were predominantly $CD86^{low}$, with a small population of $CD86^{high}$ cells. Upon maturation driven by LPS the proportion of $CD86^{high}$ cells increased in a dose-dependent manner (Fig. 1*A*). However, fewer $ac^+$ DCs became $CD86^{high}$ compared with $ac^-$ DCs; in a typical experiment at 0.1 $\mu$g/ml LPS only 13.2% of $ac^+$ DCs became $CD86^{high}$ vs 42% $ac^-$ DCs, and this difference was maintained at the highest LPS dose of 0.5 $\mu$g/ml, with only 24% of $ac^+$ DCs becoming $CD86^{high}$ compared with 46% of $ac^-$ DCs (Fig. 2). Similar results were seen when DCs were matured with SAC (data not shown). Mature DCs were heterogeneous for CD54 with distinct populations of $CD54^{low}$ and $CD54^{high}$ cells, becoming most apparent at the highest dose of LPS (0.5 $\mu$g/ml). Despite the general increase in fluorescent intensity of the $ac^+$ DCs after phagocytosis, it is apparent that the percentage of $CD54^{high}$ cells was lower in $ac^+$ than $ac^-$ populations (Fig. 2). CD40 expression was unaffected by apoptotic cells, with maturation in response to 0.5 $\mu$g/ml generating a single $CD40^+$ population (Fig. 2), and no statistically significant difference was seen in MHC class II or CD80 expression (data not shown). Hence, the failure to up-regulate CD86 in the $ac^+$ DCs was not due to general unresponsiveness to LPS but appeared to affect a subset of costimulatory molecules.

Failure to up-regulate CD86 expression in $ac^+$ DCs could reflect preferential ingestion of apoptotic cells by a subpopulation of DCs destined not to become $CD86^{high}$ in response to maturation stimuli. However, when the mean CD86 fluorescence for the whole DC population was compared between DCs matured in the presence or the absence of apoptotic cells (Fig. 3, *A* and *B*), the presence of apoptotic cells resulted in significantly lower CD86 fluorescence for the whole DC population compared with the control. No such difference would have been detectable had the capacity to ingest apoptotic cells merely marked a subpopulation of DCs destined not to up-regulate CD86 in response to LPS. This and the observation that no significant difference in costimulatory molecule expression was detected between the $ac^+$ DCs and $ac^-$ DC population immediately after ingestion (data not shown) support the fact that phagocytosis had not preferentially occurred in a subpopulation destined to be $CD86^{low}$. Also, this inhibitory effect was not merely a result of particle ingestion, as immature DCs, when cocultured with latex beads and oxidized lipid, showed remarkably high levels (>85%) of phagocytosis and endocytosis, respectively, and exhibited no defect in LPS-driven up-regulation of CD86 expression, exhibiting, instead, apparently enhanced expression (Fig. 3*C*). Taken together these data support the hypothesis that apoptotic cell

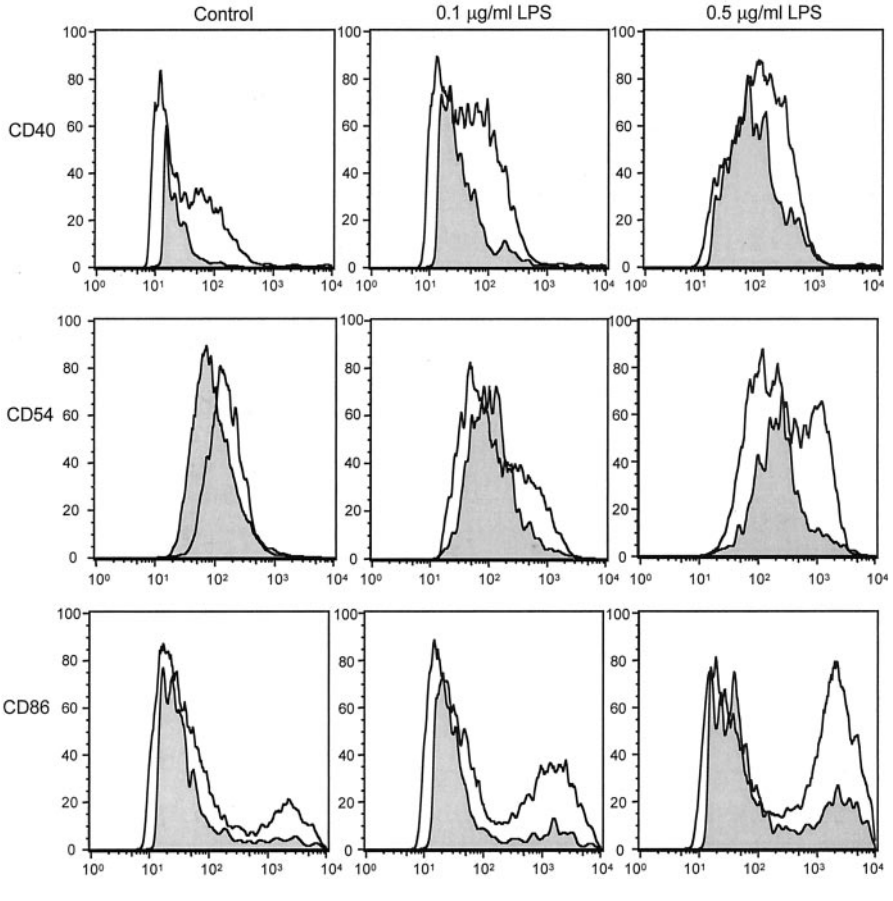


**FIGURE 2.** DCs that have internalized apoptotic cells, but not control particles, fail to up-regulate CD86 normally in response to LPS or SAC, but up-regulate other markers, such as CD40 and CD54. DCs were incubated with green fluorescent apoptotic cells, matured with LPS, and stained for surface costimulatory molecules. DCs were gated into $ac^+$ (filled histogram) and $ac^-$ (open histogram) by incorporation of green fluorescence. Isotype control Abs are not shown for clarity but sat in the first log order of fluorescence intensity. Data are representative of three experiments.

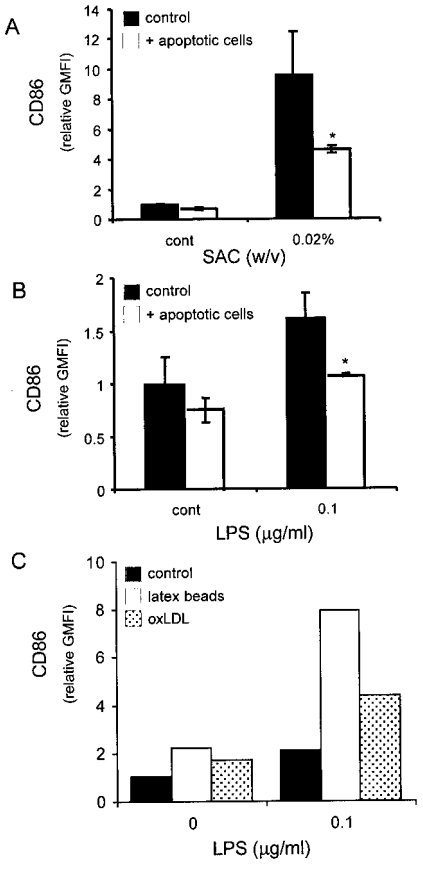


**FIGURE 3.** Surface CD86 expression of bulk DC cultures containing both $ac^+$ DCs and $ac^-$ DCs was inhibited by apoptotic cells. DCs were incubated with apoptotic cells and subsequently matured with SAC (0.02%, w/v; *A*) or LPS (0.1 $\mu$g/ml; *B*). Surface CD86 expression was measured by FACS and is expressed as mean fluorescence relative to control unstimulated DCs. Relative fluorescence of DCs cocultured with apoptotic cells (□) or medium alone (■) is shown, demonstrating that apoptotic cells can suppress both LPS- and SAC-driven DC maturation. Apoptotic cells were excluded from FACS analysis on the basis of small size and bright green fluorescence. The mean fluorescence ± SD for triplicate measurements from one culture is shown. Similar results were seen in three (SAC) or five (LPS) independent experiments. *, $p < 0.05$ (by ANOVA). *C*, DCs were cultured with latex beads (□) or oxidized LDL (▨); compared with incubation with no particles (■), these control particles augmented, rather than decreased, CD86 expression. The mean fluorescence of duplicate cultures is shown, and similar results were seen in two independent experiments.

ingestion alters the subsequent response of DCs to maturation stimuli.

### *Ingestion of apoptotic cells modulates proinflammatory cytokine expression by DCs*

Cytokines produced by DCs are especially important in determining subsequent T cell responses. We therefore examined the effect of ingestion of apoptotic cells on cytokine production by DCs by combining the fluorescent phagocytosis assay and intracellular cytokine staining of cells, allowing us to study the production of cytokines by individual DCs (Fig. 4). The autocrine response to TNF-$\alpha$ produced after LPS stimulation is an important factor in terminal maturation and activation of DCs as well as recruitment and activation of neighboring effector cells. Interestingly, virtually all the DCs containing apoptotic cells expressed TNF-$\alpha$ after stimulation with LPS for 5 h, demonstrating their functional viability and continuing responsiveness to LPS stimulation. A small population of the $ac^-$ DCs failed to produce TNF-$\alpha$ and probably represented a population of fully matured or "exhausted" DCs (19) (Fig. 4). IL-12 is produced predominately by DCs and orchestrates both the innate and adaptive immune responses. DCs express a functional IL-12R, ligation of which by bioactive IL-12p70 augments LPS maturation. In contrast to TNF-$\alpha$, $ac^+$ DCs failed to express IL-12 even when stimulated with 0.5 $\mu$g/ml LPS (Fig. 4). Similar results were seen when DCs were stimulated with SAC (data not shown).

### *Inhibitory effects of apoptotic cells on DCs are not mediated by autocrine/paracrine effects of IL-10 or TGF-$\beta$1*

IL-10 is an important anti-inflammatory cytokine associated with induction of tolerance, resolution of inflammation, inhibition of production of proinflammatory cytokines, and DC maturation (20). IL-10 has been shown to inhibit DC maturation, acting in both a paracrine and an autocrine manner (21). Interaction of apoptotic cells with monocytes (22), but not macrophages (14), has been shown to induce the production of IL-10. To investigate whether changes in IL-10 expression by DCs ingesting apoptotic cells might contribute to the different phenotype, intracellular IL-10 production and release into the supernatant were studied. IL-10 was detectable in DC culture supernatant but was unaffected by interaction with apoptotic cells or the addition of LPS (Fig. 5*A*). Intracellular IL-10 was difficult to detect reliably over background staining, and no differences in levels of IL-10 staining between $ac^+$ and $ac^-$ DCs were detectable (data not shown). Furthermore, blockade of functional IL-10 by soluble IL-10R did not differentially affect costimulatory molecule expression in the two DC subpopulations (data not shown). Finally, LPS activation of DCs derived from bone marrow of IL-10-deficient mice was also inhibited by the ingestion of apoptotic cells. Interestingly, these DCs demonstrated a heightened responsiveness to LPS, confirming an autocrine feedback role for IL-10 in DC maturation (Fig. 5*B*).

TGF$\beta$-1 is another important inhibitory cytokine implicated in anti-inflammatory effects of apoptotic cells. TGF-$\beta$ is found in apoptotic cells, preferentially localized to the mitochondria (23), as well as being secreted by macrophages ingesting apoptotic cells (14–16). Although TGF-$\beta$1 could be found in our culture supernatants, levels of serum contamination made determining its origin difficult (data not shown). However, when a soluble TGF-$\beta$R was used to neutralize active TGF-$\beta$1 released by DCs they were still inhibited after ingesting apoptotic cells (Fig. 5*C*). In contrast, soluble TGF-$\beta$R was capable of blocking inhibition of TNF-$\alpha$ production by macrophages that had ingested apoptotic cells in a parallel system (Fig. 5*D*). Taken together these data mitigate against a role for autocrine IL-10 or TGF-$\beta$1 in inhibiting the DC response to LPS while confirming the previously reported role for TGF-$\beta$ in the inhibition of macrophages that have ingested apoptotic cells.

### *Ingestion of apoptotic cells generates DCs with diminished capacity to sustain Ag-dependent unprimed T cell proliferation despite LPS maturation*

To determine whether these cytokine and surface CD86 differences reflected a distinct functional phenotype of DCs, we chose to examine the capacity of $ac^-$ DCs and $ac^+$ DCs to sustain Ag-dependent naive T cell proliferation, a process critically dependent on IL-12 production and expression of costimulatory molecules. The use of unprimed T cells from DO11.10 TCR-transgenic mice allowed us directly to compare T cell proliferation in response to mature $ac^+$ DCs vs $ac^-$ DCs, pulsed in both cases with $OVA_{323–339}$ peptide after LPS maturation. Interestingly, $ac^+$ DCs retained the ability to sustain naive T cell proliferation but were only ~30% as effective as stimulators compared with $ac^-$ DCs or

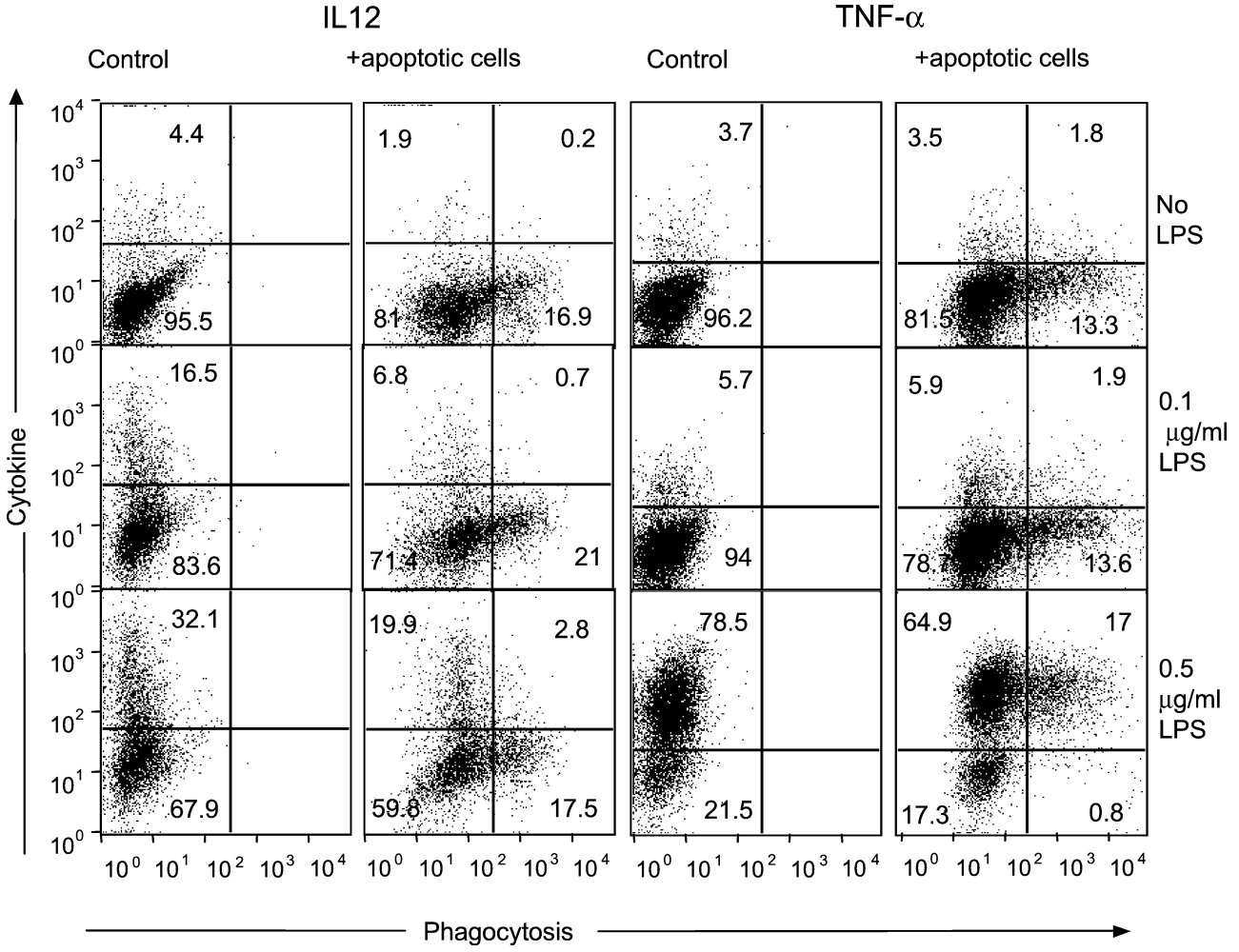


**FIGURE 4.** Apoptotic cells inhibit IL-12 production, but not TNF-$\alpha$ production, by LPS-stimulated DCs. DCs were incubated with green fluorescent apoptotic cells and stimulated with LPS. Intracellular cytokine production was measured after 4 h by FACS analysis gated on CD11c-positive cells (DCs). DCs that have ingested apoptotic cells can be distinguished by the incorporation of green fluorescence. The quadrant markers are set on isotype control Abs (cytokine) or DCs prepared without apoptotic cells (phagocytosis), and figures give the percentage of cells in each quadrant. The data are representative of three independent experiments.

DCs matured without apoptotic cells when cultured at a ratio of 10:1, T cells:DCs (Fig. 6).

## Discussion

The data presented demonstrate that DC ingestion of apoptotic cells, but not control particles, results in subsequent down-regulation of LPS-driven IL-12 production and CD86 expression. Furthermore, this correlated with impaired Ag-dependent T cell activation in vitro. Interestingly, these effects were restricted to those DCs that had ingested apoptotic cells and were not due to anti-inflammatory cytokine production, implicating ligation of specific phagocytic receptors in this process.

Bone marrow-derived DCs and macrophages arise from common myeloid precursors and share many characteristics while maintaining subtle differences in responses and effector functions. Ingestion of apoptotic cells stimulates macrophages to adopt an anti-inflammatory phenotype, inhibiting LPS-induced release of TNF-$\alpha$ and up-regulating release of TGF-$\beta$1 and other anti-inflammatory mediators (24, 25). Furthermore, previous reports and our unpublished data emphasize that this phenotypic change in macrophages ingesting apoptotic cells is spread to surrounding cells through the paracrine action of cytokine release triggered by the ingestion of apoptotic cells. This is in contrast to the response of DCs ingesting apoptotic cells, which, in this current study, did not affect the ability of neighboring DCs that had not ingested apoptotic cells to mature or stimulate T cells. This would implicate a direct and cell-specific effect of apoptotic cell ingestion on DC expression of CD86 and cytokine production, rather than a paracrine effect of secreted anti-inflammatory cytokines. Our data lend weight to the possibility that the different recognition mechanisms employed for ingestion of apoptotic cells by monocyte-derived phagocytes might determine the different responses seen between macrophages and DCs (7, 26), although we have not set out to define the receptors for apoptotic cells implicated in the inhibition of DC maturation in this study. The preferential inhibition of IL-12 has been demonstrated by ligation of a large number of phagocytic receptors used by macrophages, including some that are also expressed on DCs (27, 28). The CD36/integrin $\alpha_v\beta_5$/thrombospondin complex has been implicated as the major receptor for apoptotic cells in DC phagocytosis, and recently binding of both malaria-infected erythrocytes (29) or apoptotic cells (30) to this complex has been shown to inhibit human DC maturation. Although these studies did not address whether apoptotic cell internalization must

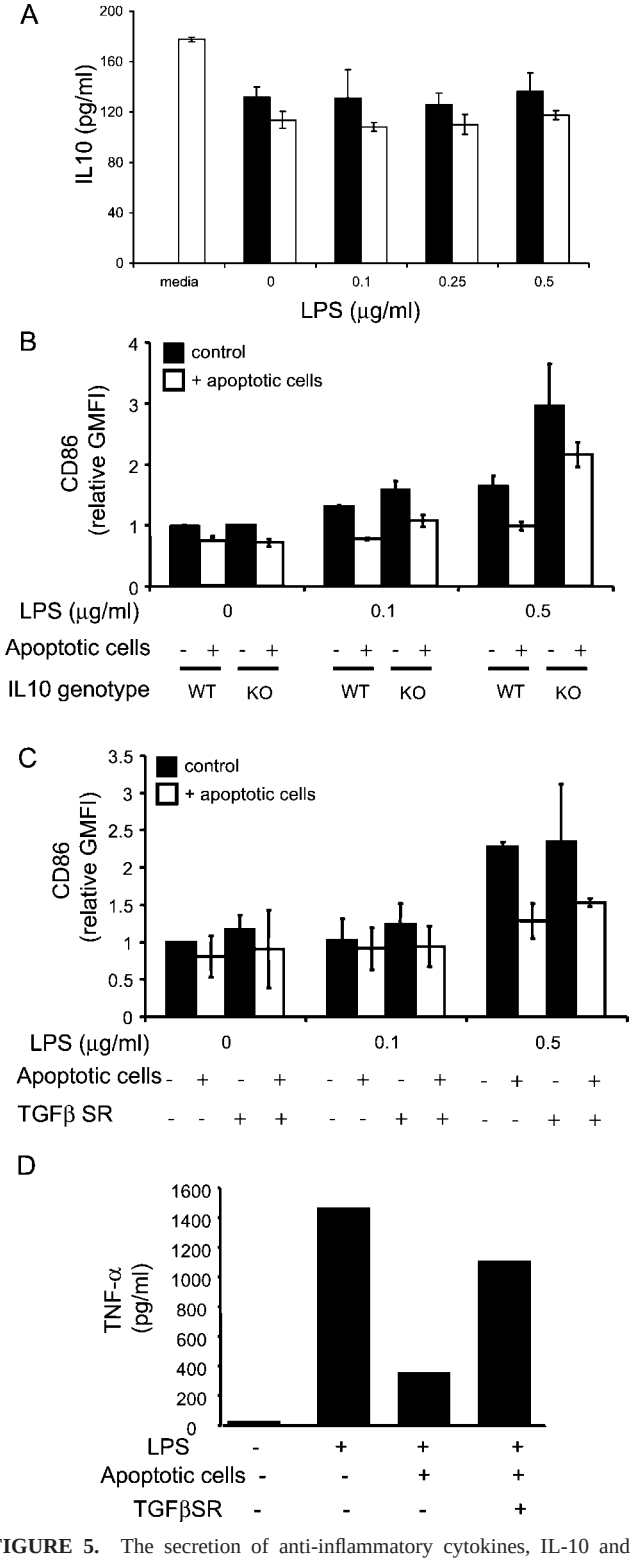


**FIGURE 5.** The secretion of anti-inflammatory cytokines, IL-10 and TGF-$\beta$, is not responsible for the observed changes in DC function. *A*, Apoptotic cells do not induce the release of IL-10 from DCs. Twenty-four-hour supernatants from control DCs (■) and DCs cocultured with apoptotic cells (□) stimulated with varying doses of LPS were assayed for IL-10 by ELISA. The mean ± SD of triplicate measurements from one culture supernatant is shown. Constitutive amounts of IL-10 detected in fresh medium containing hybridoma supernatant is shown (media) as baseline. Similar results were seen in three independent experiments. *B*, DCs derived from bone marrow of IL-10$^{-/-}$ mice were also inhibited by apoptotic cells. WT DCs with (□) and without (■) apoptotic cells demonstrate inhibition of CD86 expression. Similar results were seen when IL-10$^{-/-}$ DCs were incubated with or without apoptotic cells. Note the generally higher levels of expression of CD86 in IL-10-deficient DCs. Data are the mean ± SD from triplicate wells from one knockout or wild-type mouse representative of four similar mice. The presence of TGF-$\beta$ soluble receptor (*C*) does not affect the inhibitory capacity of apoptotic cells. DCs were cultured with apoptotic cells as described in *Materials and Methods* in the presence or the absence of TGF-$\beta$ soluble receptor. Data are the mean ± SD from triplicate wells from one culture. Similar results were seen in two independent experiments. *D*, TGF-$\beta$ soluble receptor does inhibit the effect of apoptotic cell ingestion on TNF-$\alpha$ production by LPS-stimulated mouse bone marrow-derived macrophages. Macrophages were incubated with LPS (1 $\mu$g/ml) and/or apoptotic cells, and TNF-$\alpha$ production was measured by ELISA after 24 h.

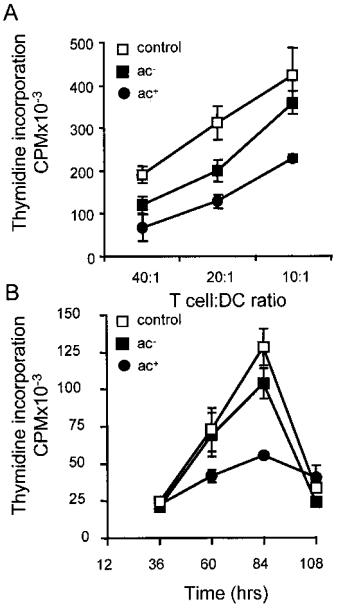


**FIGURE 6.** Ag-driven T cell stimulation was diminished in DCs that ingested apoptotic cells despite LPS stimulation. Mature DCs were pulsed with $OVA_{323–339}$ peptide, sorted into ac$^-$ (■) and ac$^+$ (●) cells, and incubated with T cells from DO11.10 mice. DCs that had not been incubated with apoptotic cells are included as a control (□). T cell proliferation was measured by [$^3$H]thymidine incorporation. *A*, Proliferation in response to different T cell:DC ratios, measured after 3 days of interaction. *B*, Time course of T cell/DC interaction demonstrated inhibition at all time points. Background proliferation in the absence of OVA peptide was <2000 cpm in all experiments. The mean ± SD from triplicate measurements in one experiment, representative of three independent experiments, are shown.

occur, our data imply that a direct interaction is necessary for these effects to be seen.

Apoptotic cells are poorly immunogenic and, unless they overload normal clearance mechanisms (probably becoming secondarily necrotic) or are associated with danger signals, rarely incite an immune response (31). Furthermore, UV irradiation, characterized by widespread apoptosis, is associated with generalized immunological hyporesponsiveness, demonstrating a potential immunosuppressive effect of apoptotic cells on the adaptive immune system. In an interesting recent report, injection of apoptotic cells was able to promote bone marrow engraftment even across MHC barriers in a species-independent manner (32). Although the exact mechanisms of immunosuppression in such systems are not fully understood, a bone marrow-derived cell, likely to be the DC, has been implicated. Exactly how this occurs is controversial, but an increasingly accepted view is that the immature DC, with low levels of costimulatory molecule expression, would fail to deliver

signal 2 and induce anergy or deletion of an interacting T cell. In support of this, repeated immunization with immature DCs does appear to induce Treg/Tr1 cells. In contrast, mature DCs, which are able to secrete a potent stimulatory cytokine, IL-12, and express high levels of costimulatory molecule expression, induce strong adaptive immunity. IL-12 has been shown to have a myriad of functions, including modulating Th1 vs Th2 switching, activation of NK cells, and production of IFN-$\gamma$. Furthermore, the autocrine effects of IL-12 have been shown to augment DC responses to exogenous stimuli, underscoring the importance of this cytokine in DC effector functions and the subsequent adaptive immune response. Although failure of $ac^+$ DCs to produce high levels of IL-12 might contribute to some of the subsequent phenotypic changes, including diminished T cell stimulation, we have no direct evidence of whether this is indeed the case and is the subject of ongoing investigation. Nevertheless, failure to produce IL-12 after apoptotic cell ingestion appears to correlate closely with these changes.

The importance of ingestion of apoptotic cells by DCs is underscored by circumstantial evidence implicating such DCs in maintaining tolerance. For example, a specific population of rat lymph DCs, characterized by being $OX41^-CD4^-$, has been described. These cells represent a major population found in the lymph draining the intestinal epithelium and have blunt pseudopodia and coarse granular inclusions, identified as being derived from apoptotic intestinal epithelial cells. Functionally, these $OX41^-CD4^-$ rat lymph DCs also demonstrate an impaired ability to stimulate T cells in vitro and have been implicated in the ability of the gut to handle large amounts of foreign Ags in a tolerogenic fashion (33–35). Interestingly, our in vitro cultured $ac^+$ DCs also share some of these physical characteristics with the $OX41^-CD4^-$ rat lymph DCs (our personal observations). While comparisons between in vivo/ex vivo studies of rat DCs and our in vitro work on murine DCs need to be made with caution, taken together these data lend strong support to the concept that ingestion of apoptotic cells by DCs modulates their function.

In conclusion, it is essential for DCs to mature before they can activate naive T cells, and our data and two recent studies (4, 5) confirm that ingestion of apoptotic cells alone did not provide sufficient maturation stimulus. However, some necrotic cells or virally infected apoptotic cells are effective stimulators of DC maturation. Therefore, DCs ingesting apoptotic cells must be exposed to additional agents, such as necrotic cells, monocyte-conditioned medium, or viral products, before they become capable of stimulating T cells. Many of these agents will be present in inflamed sites alongside apoptotic cells in vivo, and the potential for DCs both to acquire apoptotic cell-derived self-Ags and receive maturation signals is high. However autoimmunity is uncommon, and the response of the DC is likely to be tightly regulated. We suggest that ingestion of apoptotic cells is not immunologically null, but is capable of regulating DC maturation, providing a counterbalance for inflammatory stimuli. A failure to see these inhibitory effects of apoptotic cells in other studies may reflect the percentage of DCs ingesting apoptotic cells and the strength of the maturation stimulus used. In the future, defining whether apoptotic cells themselves are sufficient to alter DCs effector functions in vivo will be of great interest. Further investigating this process will increase our understanding of the mechanisms controlling peripheral self-tolerance while giving us new insights into strategies for Ag delivery that might generate tolerance rather than immunity. In contrast, understanding how apoptotic tumor cells or pathogens might also use this phenomenon for immune evasion will increase our understanding of tumor immunology and infectious disease.

## Acknowledgments

We thank Prof. N. A. Mitchinson and Dr. E. Lightstone for their helpful discussion, and S. MacCall and M. Clay for their technical support.

## References

1. Steinman, R. M., S. Turley, I. Mellman, and K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med. 191: 411.*
2. 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.*
3. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
4. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med. 5:1249.*
5. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med. 191:423.*
6. Bellone, M., G. Iezzi, P. Rovere, G. Galati, A. Ronchetti, M. P. Protti, J. Davoust, C. Rugarli, and A. A. Manfredi. 1997. Processing of engulfed apoptotic bodies yields T cell epitopes. *J. Immunol. 159:5391.*
7. Albert, M. L., S. F. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med. 188:1359.*
8. Albert, M. L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature 392:86.*
9. Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, et al. 1998. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med. 188:2163.*
10. Rosen, A., and L. Casciola-Rosen. 2001. Clearing the way to mechanisms of autoimmunity. *Nat. Med. 7:664.*
11. Scott, R. S., E. J. McMahon, S. M. Pop, E. A. Reap, R. Caricchio, P. L. Cohen, H. S. Earp, and G. K. Matsushima. 2001. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature 411:207.*
12. Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J. Exp. Med. 192:359.*
13. Lechler, R., W. F. Ng, and R. M. Steinman. 2001. Dendritic cells in transplantation: friend or foe? *Immunity 14:357.*
14. Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-$\beta$, $PGE_2$, and PAF. *J. Clin. Invest. 101:890.*
15. Fadok, V. A., M. L. Warner, D. L. Bratton, and P. M. Henson. 1998. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor ($\alpha_v\beta_3$). *J. Immunol. 161:6250.*
16. Fadok, V. A., D. L. Bratton, D. M. Rose, A. Pearson, R. A. Ezekewitz, and P. M. Henson. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature 405:85.*
17. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods 223: 77.*
18. Savill, J., N. Hogg, Y. Ren, and C. Haslett. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest. 90:1513.*
19. Langenkamp, A., M. Messi, A. Lanzavecchia, and F. Sallusto. 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol. 1:311.*
20. Faulkner, L., G. Buchan, and M. Baird. 2000. Interleukin-10 does not affect phagocytosis of particulate antigen by bone marrow-derived dendritic cells but does impair antigen presentation. *Immunology 99:523.*
21. Corinti, S., C. Albanesi, A. la Sala, S. Pastore, and G. Girolomoni. 2001. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol. 166: 4312.*
22. Voll, R. E., M. Herrmann, E. A. Roth, C. Stach, J. R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature 390:350.*
23. Chen, W., M. E. Frank, W. Jin, and S. M. Wahl. 2001. TGF-$\beta$ released by apoptotic T cells contributes to an immunosuppressive milieu. *Immunity 14:715.*
24. Savill, J., and V. Fadok. 2000. Corpse clearance defines the meaning of cell death. *Nature 407:784.*

25. Meagher, L. C., J. S. Savill, A. Baker, R. W. Fuller, and C. Haslett. 1992. Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2. *J. Leukocyte Biol. 52:269.*

26. Albert, M. L., J. I. Kim, and R. B. Birge. 2000. $\alpha_v\beta_5$ integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nat. Cell Biol. 2:899.*

27. Sutterwala, F. S., G. J. Noel, R. Clynes, and D. M. Mosser. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J. Exp. Med. 185:1977.*

28. Sutterwala, F. S., and D. M. Mosser. 1999. The taming of IL-12: suppressing the production of proinflammatory cytokines. *J. Leukocyte Biol. 65:543.*

29. Urban, B. C., D. J. Ferguson, A. Pain, N. Willcox, M. Plebanski, J. M. Austyn, and D. J. Roberts. 1999. Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. *Nature 400:73.*

30. Urban, B. C., N. Willcox, and D. J. Roberts. 2001. A role for CD36 in the regulation of dendritic cell function. *Proc. Natl. Acad. Sci. USA 98:8750.*

31. Ronchetti, A., P. Rovere, G. Iezzi, G. Galati, S. Heltai, M. P. Protti, M. P. Garancini, A. A. Manfredi, C. Rugarli, and M. Bellone. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol. 163:130.*

32. Bittencourt, M. C., S. Perruche, E. Contassot, S. Fresnay, M. H. Baron, R. Angonin, F. Aubin, P. Herve, P. Tiberghien, and P. Saas. 2001. Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers. *Blood 98:224.*

33. Liu, L., M. Zhang, C. Jenkins, and G. G. MacPherson. 1998. Dendritic cell heterogeneity in vivo: two functionally different dendritic cell populations in rat intestinal lymph can be distinguished by CD4 expression. *J. Immunol. 161:1146.*

34. Liu, Y. J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell 106:259.*

35. Huang, F. P., N. Platt, M. Wykes, J. R. Major, T. J. Powell, C. D. Jenkins, and G. G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med. 191:435.*