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Distinct Maturation of, but Not Migration between, Human Monocyte-Derived Dendritic Cells upon Ingestion of Apoptotic Cells of Early or Late Phases

Wai-Kee Ip and Yu-Lung Lau

Cell death via apoptosis is a normal physiological process. Rapid, but silent, removal of apoptotic cells (ACs) plays an essential role in maintaining homeostasis in the immune system. Defective clearance of ACs allows ACs to accumulate and undergo late phase apoptosis, also known as secondary necrosis, which may generate danger signals, leading to inflammation or autoimmunity. In this study we investigate the outcome of dendritic cells (DCs), which are potent APCs, on the interaction with ACs of early or late phase. Immature DCs internalized ACs of both early and late phases with similar efficiency. However, DCs that had taken up ACs of early phase acquired a non-fully mature DC phenotype, expressing low MHC class II complex, costimulatory molecule CD40, and mature DC-restricted marker CD83, and had a low capacity to stimulate allogeneic CD4+ T cell proliferation, whereas DCs that had taken up ACs of late phase acquired a mature DC phenotype with enhanced T cell stimulatory capacity. Ingestion of either early or late ACs induced minimal production of IL-12 and modulated CC chemokine and CCR expression in DCs. In particular, there was down-regulation of CCR5 and up-regulation of CCR7, resulting in switches in responsiveness from inflammatory to lymphoid chemokines. We conclude from these data that after taking up ACs of either early or late phases, DCs acquire the capability of homing to draining lymph nodes, and the distinct maturation between DCs taking up early or late ACs may contribute to DC function in the induction of T cell tolerance or Ag-specific T cell response, respectively. The Journal of Immunology, 2004, 173: 189–196.

Dendritic cells (DCs) are potent professional APCs that display an extraordinary capacity to stimulate naive T cells and induce not only T cell immunity (1), but also T cell tolerance (2, 3). In peripheral tissues DCs are in a resting or immature state and can capture Ag. Upon receipt of maturation stimuli (such as bacterial product LPS), DCs down-regulate Ag-capturing machinery and up-regulate MHC-peptide complexes, T cell adhesion, and costimulatory molecules (e.g., ICAM-1, members of the B7 family, and CD40), DC-restricted marker CD83, and production of IL-12 as well as selected chemokine receptors that guide DC migration into draining lymph nodes for priming of Ag-specific T cells (4, 5). In Ag presentation, DCs provide three signals to naive CD4+ T cells to initiate T cell proliferation and differentiation. Mature DCs express a high density of MHC class II-peptide complexes on their cell surface for recognition by the TCR expressed on CD4+ T cells (signal 1) and costimulatory molecules (signal 2) to stimulate CD4+ T cell proliferation. Only the simultaneous provision of both signals ensures efficient T cell activation; signal 1 in the absence or low levels of signal 2 induces a state of Ag-specific T cell unresponsiveness, termed anergy or tolerance (2, 6). Secretion or lack of secretion of IL-12 by DCs (signal 3) is also of importance in the final differentiation of naive CD4+ T cells into Ag-specific Th1 or Th2 cells, respectively (7, 8).

Immature DCs acquire Ags by many pathways, including uptake of soluble Ags or protein complexes by endocytosis and macropinocytosis and ingestion of entire cells by phagocytosis (9). Ingestion of necrotic cells is capable of inducing DC maturation, whereas ingestion of apoptotic cells (ACs) fails to do so (10, 11). It was proposed that T cell recognition of self-peptide-MHC complexes on immature or non-fully mature DCs could induce tolerance (2, 12). ACs are the source of autoantigens, and the insufficient clearance of ACs results in the accumulation of ACs in the circulation, which leads to systemic autoimmunity (13–16). Accumulated ACs undergoing the late phase of apoptosis, also known as secondary necrosis, have been found to be associated with additional proteolytic degradation of specific autoantigens (17), which may send danger signals to the immune system and stimulate autoantibody responses, as seen in human systemic lupus erythematosus (SLE).

Cells undergoing physiologic apoptosis are removed efficiently during the early phase of apoptosis and do not result in maturation signal for DCs, which, in turn, prevent the generation of immune responses toward self-Ags contained in the ACs. However, little is known about whether DCs have different paths of maturation after interacting with cells undergoing different phases of apoptosis, such as ACs of late phase as compared with ACs of early phase. We hypothesize that the breakdown of tolerance in systemic autoimmunity, such as SLE, may be due to engulfment of accumulated ACs of late phase by DCs, which results in maturation that can induce a T cell response rather than tolerance. In this study we show the distinct maturation of, but not migration between, human monocyte-derived DCs upon the ingestion of ACs of early or late...
phase. Our data indicate that after taking up ACs of either early or late phase, DCs acquire the same capability of homing to lymphoid organs, but the distinct maturation between DCs upon the ingestion of early or late ACs contributes to different DC functions in terms of induction of T cell tolerance or Ag-specific T cell response, respectively.

Materials and Methods

Isolation and preparation of cells

PBMCs, DCs, and T cells were prepared as described previously (18). Briefly, peripheral blood was obtained from normal blood donors, and PBMCs were isolated by sedimentation over Ficoll-Plaque (Amersham Pharmacia Biotech, Piscataway, NJ). To generate immature DCs, monocyte-pancytes positively selected by anti-CD14 MACS magnetic beads in the MACS column purification system (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs were cultured in 24-well tissue culture plates (Corning-Costar, Corning, NY; 1 ml/well) at 1 × 10⁶ cells/ml in RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY) containing 10% FCS (Invitrogen Life Technologies), human rGM-CSF (R&D Systems, Minneapolis, MN), and human rIL-4 (R&D Systems) for 7 days at 37°C in 5% CO₂, GM-CSF (50 ng/ml) and IL-4 (10 ng/ml) were added to the cultures on days 0 and 3. On days 6–7, >95% of the cells were CD14+CD11c−CD1a+ DCs, as determined by immunophenotypic analysis. DCs were studied after 6–7 days in culture. CD4+ cells were purified from PBMCs to >98% purity by negative selection with the MACS column purification system (Miltenyi Biotec).

Induction and detection of apoptotic death

Jurkat T cells were used as a source of ACs. Cells were cultured in RPMI 1640 medium containing 10% FCS in 3% CO₂. When cell density reached 1 × 10⁶ cells/ml, cells were serum-starved for 36 h before induction of apoptosis by UV irradiation at a dose of 80 mJ/cm² in a UV Crosslinker (Spectronics, Westbury, NY). Irradiated cells were cultured in RPMI 1640 medium containing 0.4% BSA (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO₂ to undergo apoptosis for up to 50 h. Cell death was evaluated using an apoptosis detection kit (Immunotech, Marseilles, France). Briefly, cells were stained with Annexin V (Alexa 488) and propidium iodide (PI) according to the manufacturer’s protocol, and ACs were detected by flow cytometry performed on an EPICS Elite ESP (Coulter, Miami, FL). To ensure that we were studying the uptake of ACs of different phases, the kinetics of death were carefully worked out. Two hours after irradiation, 70–80% of cells became Annexin V+ PI− ACs. It was not until 36–50 h later that >98% of cells were Annexin V− PI+, and included trypan blue (Life Technologies) in the cytoplasm (data not shown). ACs 2 and 50 h after UV irradiation were used as the ACs of early and late phases (2-h ACs and 50-h ACs), respectively, in the present study. Apoptotic DNA fragmentation was also detected in cells of these two phases using TUNEL as performed according to the manufacturer’s instructions (In Situ Cell Death Detection kit; Roche, Indianapolis, IN).

Phagocytosis assay

Two- or 50-h ACs were labeled with the red fluorescent dye PKH26 according to the manufacturer’s protocol (Sigma-Aldrich). Immature DCs were labeled with the green fluorescent dye PKH67 (Sigma-Aldrich) and then cocultured with the 2- or 50-h ACs in RPMI 1640 medium containing 10% heat-inactivated FCS at a DC:AC ratio of 1:3 for 3 or 12 h at 37 or 4°C. Phagocytosis of 2- or 50-h ACs by immature DCs was determined by flow cytometric analysis.

Transmission electron microscopy

Cell suspension was centrifuged to a cell pellet, which was fixed in 1.5% cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated in a series of ethanol, and embedded in PolyBed 812 (Polysciences, Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a CM10 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV.

Immunophenotype analysis

Cells were washed and resuspended in PBS and incubated with a series of mAbs to human Ags for 30 min at room temperature. Fluorochrome-conjugated Abs were acquired using a FACSCalibur (Becton Dickinson, San Jose, CA). The phenotype was analyzed by flow cytometry.

ELISA

The measurement of cytokines in the supernatants of cocultures of DCs and ACs was performed according to the manufacturer’s protocol. DCs (5 × 10⁵) were cocultured with 1.5 × 10⁴ 2- or 50-h ACs in RPMI 1640 medium containing 10% heat-inactivated FCS for 36 h at 37°C, after which the supernatants were assayed for the release of IL-12, IL-10, and TNF-α. Control supernatants were obtained by culturing immature DCs in medium alone or with addition of 10 μg/ml LPS (E. coli; Sigma-Aldrich) under the same conditions. GM-CSF-secreting DCs were cocultured with 2- or 50-h ACs for 36 h in the presence of 10 μg/ml LPS, and their supernatants were assayed for the release of IL-12. Color development was assessed using a microtiter plate reader (model 550; Bio-Rad, Hercules, CA).

RT-PCR

Total RNA from DCs of each sample (1 × 10⁶) was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized from 2 μg of RNA using SuperScript reverse transcriptase (Life Technologies). The primers used for PCR were: CCR1, 5′-5′-GGG AAT TCA CTC AGG-3′ and 5′-CAAG GAC GAC TTT GGA TT-3′, amplifying a 204-bp product; CCR5, 5′-ACT GCA AAA GGC TGA AGC-3′ and 5′-CGA TTG TCA GGA GGA TG-3′, amplifying a 204-bp product; CCR7, 5′-AGC GAC GAT TAC ATC ATC GA-3′ and 5′-GAG AGT CTT GGT CTT TC-3′, amplifying a 196-bp product; MIP-1α, 5′-CGA GCC CAC ATT CCT GCA TC-3′ and 5′-CGG ATG TTC CAA AGG AG-3′, amplifying a 390-bp product; MIP-3β, 5′-ATT CCT GGG TAC TTC CCA AG-3′ and 5′-CCT CGT CAC CCT GAG-3′, amplifying a 195-bp product; RANTES, 5′-CGG TGT CAT CTT CAG GCT-3′ and 5′-TTT GCA CAG TGG GTT CTG-3′, amplifying a 196-bp product; GM-CSF, 5′-TCC GGA GAA GAG GCA ATC-3′ and 5′-CCT CGT CAC CCT GAG-3′, amplifying a 390-bp product; and GAPDH, 5′-GACA GGG GGG GAG CAA AAG GG-3′ and 5′-GAG TCG CAC CAG CTT GCA-3′, amplifying a 196-bp product. cDNA were amplified by PCR with the number of cycles indicated using the following conditions: 30 s at 94°C, 20 s at 60°C, and 30 s at 72°C. PCR products were analyzed by electrophoresis through 2% agarose gels containing ethidium bromide and visualized under UV light.

Chemotaxis assay

The in vitro migration of AC-engulfed DCs in response to CC chemokines was assessed in a Transwell cell culture chamber with 5.0-μm pore size polycarbonate filters (Corning-Costar). One hundred microliters of AC-engulfed DCs (1 × 10⁶) in serum-free RPMI 1640 medium was added to the upper compartment of the chamber. RANTES, MIP-1α, or MIP-3β (1–100 ng/ml; PeproTech, London, U.K.) diluted in serum-free RPMI 1640 medium (600 μl) was loaded in the lower compartment. After a 2-h incubation, the cells that had migrated through the polycarbonate filters into the lower compartment were collected and counted using a Coulter fluorochrome-conjugated mAbs included IgG1, IgG2a, IgG2b, and IgM (BD Pharmingen). Unconjugated mAb to human IgG1 was detected using an isotype-specific protocol (Roche). The data are expressed as absorbance at a wavelength of 450 nm (A₄₅₀) measured using the microtiter plate reader.

T cell proliferation assay

DCs (5 × 10⁵) were cocultured with 1.5 × 10⁴ 2- or 50-h ACs in RPMI 1640 medium containing 10% heat-inactivated FCS at 37°C. After 36 h, DCs were treated with mitomycin C (Sigma-Aldrich). Allogeneic CD4− T cells (1 × 10⁵/wells) were cultured in triplicate in 96-well, flat-bottom tissue culture plates (Corning-Costar) with DCs at different DC:T cell ratios, ranging from 1:10 to 1:1000. Sixteen hours before the end of the 5-day culture at 37°C in 5% CO₂, 20 ml of BrdU (100 μM; Roche, Mannheim, Germany) was added to each well, and its incorporation was measured using the BrdU Proliferation Kit according to the manufacturer’s protocol (Roche). The data are expressed as absorbance at a wavelength of 450 nm (A₄₅₀), assessed using the microtiter plate reader.
Results

Two- and 50-h ACs

ACs induced by UV irradiation were detected by AxV and PI staining. The kinetics of apoptotic death were defined by the positivity in AxV and PI staining (data not shown). As shown in Fig. 1A, 2- and 50-h ACs (2 and 50 h after UV irradiation) were different in their AxV and PI stainings. The 2-h ACs (70–80% routinely generated) were positive for AxV only (AxV+/PI−), whereas the 50-h ACs (>95% routinely) were double positive for AxV and PI (AxV+/PI−). Nucleosomal DNA fragmentation, a hallmark of programmed cell death, was found in both 2- and 50-h ACs (Fig. 1B). Differences in cell morphology and structure between 2- and 50-h ACs were also found by transmission electron microscopy (Fig. 2). The 2-h ACs were fragmented into multiple round bodies with nuclear budding, whereas the 50-h ACs were associated with cell swelling, vacuolization in cytoplasm, and loss of plasma membrane integrity. Chromatin condensation was found in both 2- and 50-h ACs.

Immature monocyte-derived DCs ingest both 2- and 50-h ACs

To generate immature DCs, we cultured freshly isolated blood CD14+ monocytes in GM-CSF and IL-4 for 7 days. By days 6–7, >95% of the cells became immature DCs, determined by cell surface phenotype (CD14+CD11c+CD1a+). Red fluorescent PKH26-labeled 2- or 50-h ACs were cocultured with green fluorescent PKH67-labeled immature DCs at a ratio of 3:1, and the interactions with DCs were quantified by flow cytometry. In a typical experiment, 3 h after coincubation >65% of DCs had interacted with 2- or 50-h ACs, whereas 12 h after coincubation, >92% of immature DCs had interacted with 2- or 50-h ACs (Fig. 3A). Such interaction was exhibited by <5% of DCs at 4°C (data not shown), demonstrating that the interaction assay used predominantly detected phagocytosis rather than binding. Ingestion was further confirmed by transmission electron microscopy (Fig. 3B).

Ingestion of 50-h, but not 2-h, ACs significantly enhances the ability of DCs to up-regulate CD40, CD83, and MHC class II complex

To evaluate whether ingestion of ACs at different phases would result in different alteration in DC phenotype, cell surface expression of selected markers was studied by flow cytometry on DCs after 36-h coincubation with 2- or 50-h ACs at a DC:AC ratio of 1:3. Mannose receptor expression was significantly down-regulated on DCs that had ingested 2-h ACs (2-h AC+ DCs) and those that had ingested 50-h ACs (50-h AC− DCs), whereas ICAM-1 expression was significantly up-regulated on 2-h AC+ DCs and 50-h AC+ DCs compared with immature DCs cultured in medium alone (AC− DCs; Fig. 4). No significant difference in the cell surface expression of MHC class I complex was seen among 2-h AC− DCs, 50-h AC− DCs, and AC− DCs (Fig. 4). A significant up-regulation of MHC class II was seen on 50-h AC− DCs, but not on 2-h AC+ DCs, compared with that on AC− DCs (Fig. 4). The 50-h AC− DCs were also found to have significant up-regulation of CD40 and CD83 compared with that of 2-h AC+ DCs or AC− DCs (Fig. 4). Both 2- and 50-h AC+ DCs significantly up-regulated CD80 and CD86 compared with AC− DCs, although there was a trend for higher expression of CD80 and CD86 on 50-h AC+ DCs than on 2-h AC+ DCs (Fig. 4).

Modulation of IL-12p70, IL-10, and TNF-α production by DCs occurs upon the ingestion of 2- or 50-h ACs

Cytokines produced by DCs are also of importance in regulating DC function and especially in determining subsequent naive T cell differentiation. We therefore evaluated the effect on the ingestion of 2- and 50-h ACs on cytokine production by DCs. DCs were cocultured with 2- or 50-h ACs at a ratio of 1:3 or with medium alone, and culture supernatant collected after 36 h was assayed for cytokine production. Both 2- and 50-h AC+ DCs produced minimal amounts of IL-12p70 (Fig. 5). The effect of 2- and 50-h ACs on IL-12p70 production was inhibitory when coculturing DCs with either 2- or 50-h ACs in the presence of LPS (Fig. 6). Furthermore, 50-h AC+ DCs produced significantly higher levels of IL-10 and TNF-α compared with 2-h AC+ DCs (Fig. 5).

Ingestion of 2- or 50-h ACs modulates the expression of CC chemokines and CCRs by DCs

It has been reported that DC maturation is linked with their migration from the peripheral tissue to the draining lymphoid organs, with modulated expression of chemokines and chemokine receptors (20, 21). The production of inflammatory and lymphoid CC
chemokines, RANTES, MIP-1α, and MIP-3β, and of CCR1, CCR5, and CCR7 by DCs upon the ingestion of 2- or 50-h ACs was analyzed by semiquantitative RT-PCR. Similar patterns and kinetics of these CC chemokine and CCR mRNA production were observed in 2- and 50-h AC+ DCs as well as DCs stimulated with LPS (LPS+ DCs; Fig. 7). mRNA for the inflammatory chemokines RANTES and MIP-1α were already present in immature DCs. RANTES mRNA was up-regulated very rapidly at 4 h after ingestion of either 2- or 50-h ACs, whereas MIP-1α mRNA was produced in a more sustained fashion. For lymphoid chemokine MIP-3β, mRNA was also up-regulated upon the ingestion of either 2- or 50-h ACs, but only at late time points. mRNA production for CCR1 and CCR5, receptors for inflammatory chemokines, was high in immature DCs and down-regulated in DCs upon the ingestion of either 2- or 50-h ACs. In contrast, for the receptor for lymphoid chemokine CCR7, mRNA was low in immature DCs and up-regulated at late time points after the ingestion of either 2- or 50-h ACs, as in LPS+ DCs.

As expected, the down-regulation of CCR5 and the up-regulation of CCR7 were also observed when their cell surface expression levels were evaluated on DCs 36 h after the ingestion of either 2- or 50-h ACs using flow cytometric analysis (Fig. 8). Surface expression of CCR5 was significantly down-regulated, whereas that of CCR7 was up-regulated, in 2- and 50-h AC+ DCs as well as LPS+ DCs compared with immature DCs cultured in medium alone (Fig. 8).

**Discussion**

An important difference between programmed (apoptotic) and accidental/toxic (necrotic) death is that programmed cell death results in the ordered fragmentation of the cell, leading to rapid phagocytosis by neighboring cells and/or professional phagocytes without cell activation and inflammation (22, 23). ACs abnormally accumulated, such as in patients with SLE or other autoimmune diseases, may, however, develop into the late phase of apoptosis, also known as secondary necrosis, that is believed to send danger signals to activate the immune system (24, 25). DCs that have captured ACs have been suggested to be able to maintain homeostasis by inducing peripheral tolerance to self (10, 11, 26). However, the role of DCs in the clearance of ACs of different phases remains largely undefined. In this report we investigated whether DCs, after taking up ACs of early or late phase (i.e., 2- or 50-h ACs), could lead to distinct development of DCs in terms of maturation, migration, and stimulation of T cell proliferation.

The major observations reported in this study are that 1) DCs that have taken up ACs of early phase acquire the non-fully or semimature DC phenotype, whereas DCs that have taken up ACs of late phase acquire the mature DC phenotype; 2) DCs that have taken up either early or late ACs are able to produce TNF-α and IL-10, but have a diminished ability to produce IL-12p70 even in response to stimulus LPS; 3) ingestion of either early or late ACs modulates the expression of chemokines and their receptors on DCs and enhances migratory capacity of DCs in response to lymphoid chemokine MIP-3β; 4) DCs that have taken up late ACs have a higher capacity to stimulate T cell proliferation than those that have taken up early ACs. These data suggest that after taking up ACs of either early or late phase, DCs acquire the ability to...
home to lymphoid organs, and the distinct maturation between DCs that have taken up early or late ACs may contribute to DC function in the induction of T cell tolerance or Ag-specific T cell response, respectively. Normally, the removal of dying cells is so efficient that during the steady state ACs are present at extremely low level even in tissues with very high rates of apoptosis, such as thymus or germinal centers of lymph nodes (27). The clearance of most ACs probably occurs in the very early phase of apoptosis in vivo. To mimic such a normal physiological situation, Jurkat T cells 2 h after the induction of apoptosis (i.e., 2-h ACs) were used as the ACs of early phase in the present in vitro study. We demonstrated that after engulfing early ACs, immature DCs developed into a non-fully mature state, as DCs did not up-regulate MHC class II complex, T cell costimulatory molecule CD40, or mature DC-restricted marker CD83 and produced only a minimal amount of IL-12p70. These non-fully mature DCs, like immature DCs, had a poor capacity to stimulate autologous CD4\(^+\) T cell proliferation. Not surprisingly, a recent report has demonstrated that the protein synthesis of CD83 is required for enhancing the T cell stimulatory capacity of DCs (28). Our data suggest that the Ag presentation by such non-fully mature DCs with delivery of both low signal 1 and signal 2 to naive T cells may lead to T cell unresponsiveness or anergy. Although not yet clear, there is growing evidence that anergic T cells can mediate T cell suppression and may be precursors of T regulatory (Tr) cells (29). Tr cells have been recognized as a subset of T cell repertoire playing an important role in mediating tolerance (30). Indeed, the immature developmental stages of DCs are thought to induce T cell anergy or Tr cells (31–35). Further clarification about whether the non-fully mature DCs that have taken up ACs in the early phase of apoptosis mediate peripheral tolerance by inducing anergic T cells or Tr cells is required.

DCs that had ingested ACs 50 h after the induction of apoptosis (ACs of late phase) were found to develop into a mature state with the cell phenotype of up-regulatedphenotypic markers The expression levels are expressed as MFI, determined by flow cytometry, and the data shown are the mean of five experiments ± SEM. Paired t tests were used for comparisons. *, p < 0.05; **, p < 0.01.

**FIGURE 4.** Surface expression of phenotypic markers on DCs that had ingested 2- or 50-h ACs. DCs after 36-h coincubation with 2-h ACs, 50-h ACs, or culture in medium alone were analyzed for surface expression of phenotypic markers. The expression levels are expressed as MFI, determined by flow cytometry, and the data shown are the mean of five experiments ± SEM. Paired t tests were used for comparisons. *, p < 0.05; **, p < 0.01.

**FIGURE 5.** IL-12p70, IL-10, and TNF-\(\alpha\) production by DCs upon the ingestion of 2- or 50-h ACs. DCs after 36-h coincubation with 2- or 50-h ACs or culture in medium alone were assayed for the release of IL-12, IL-10, and TNF-\(\alpha\) by ELISA. The data shown are the mean of 11 experiments ± SEM. Paired t tests were used for comparisons. *, p < 0.05; **, p < 0.01.

**FIGURE 6.** Inhibitory effect of ingestion of either 2- or 50-h ACs on LPS-induced IL-12p70 production by DCs. DCs were coincubated with 2- or 50-h ACs or were cultured in medium for 36 h in the presence or the absence of 10 \(\mu\)g/ml LPS, then assayed for the release of IL-12p70 by ELISA. The data shown are the mean of three experiments ± SEM. Paired t tests were used for comparisons. *, p < 0.05.
and members of the B7 family (CD80 and CD86), but minimal production of IL-12p70, which was also seen in the DCs that had ingested ACs of early phase. Such alterations of the DC phenotype (e.g., up-regulated MHC class II, CD40, CD83, and members of the B7 family on DCs that had ingested ACs of late phase) were also observed when using U937 monocytes, Raji B lymphocytes, and EBV-transformed B lymphocytes as the sources of ACs (data not shown) and are therefore unlikely to be restricted to only after ingestion of apoptotic T cells. In addition, these mature DCs stimulated CD4+ T cell proliferation as strongly as that by DCs fully matured with LPS, suggesting that ingestion of ACs of late, but not early, phase generates DCs that can provide high signal 1 and signal 2 to naive T cells in Ag presentation. The absence or low levels of IL-12 production (signal 3) by mature DCs has been shown to differentiate naive CD4+ T cells into Th2 cells (36–38).

Although IL-10 is suggested to be required to induce tolerance (32, 39), accumulated evidence has shown that Th2 differentiation involves the effects of IL-10 on DCs (40–42). Whether the mature DCs with minimal IL-12p70, but high IL-10, production after taking up ACs of late phase, e.g., 50-h ACs in the present study, induce an Ag-specific T cell response such as the Th2 type remains to be addressed.
Ingestion of ACs of either early or late phase induced minimal IL-12p70 production by DCs and even had an inhibitory effect on IL-12p70 induction by LPS. Such suppression of IL-12 production may be mediated by ligation of receptors expressed on DCs, as also suggested by a recent report showing a similar observation for the inhibitory effect of AC ingestion in murine DCs (43). A previous study has indeed demonstrated that the IL-12 induction by LPS is suppressed after the ligation of macrophage receptors (44). CD36 and integrins, αvβ3 and αvβ6, expressed on DCs have been shown to mediate the engulfment of ACs (45, 46). The uptake of ACs via these receptors or other surface molecules may lead to the suppression of IL-12 production by DCs. It is also possible that some surface receptors can bind self-Ags and foreign ligands, but the consequences of bindings may not be the same. For example, CD14, the LPS pattern recognition receptor on macrophages, may mediate clearance of microorganisms that evoke a proinflammatory response, whereas, in contrast, the clearance of ACs results in an anti-inflammatory response (47).

The different outcomes of DC maturation after ingesting early and late ACs may also be due to the involvement of different downstream signals that instruct DC activation. Our data showing the DC engulfment of ACs associated with the formation of tight and nonspacious endosome (Fig. 3B) indeed suggests that ingested ACs engage multiple receptors that trigger different downstream signals. It has been shown that many nuclear or cytoplasmic autoantigens are located on the surface of ACs (48, 49). It is possible that further change in membrane composition or post-translational modification of nuclear constituents by proteolytic cleavage during the late phase of apoptosis (17) may lead to the expression of cryptic epitopes, to which the immune system has not achieved tolerance, and hence alter immunogenicity. The engagements of Ags from early ACs, such as phosphatidylserine, or the cryptic Ags from late ACs with multiple receptors of DCs may trigger different, or affect the balance between, downstream signals responsible for DC activation directing toward an anti- or proinflammatory response, i.e., tolerogenic or immunogenic DCs. It also remains to be addressed whether the interaction of DCs with ACs, but not the actual ingestion of ACs, is responsible for the different outcome of DC maturation on the uptake of ACs of different phases.

The higher TNF-α production by DCs we observed after ingesting ACs of late phase than ACs of early phase may be of importance in response to the danger situation due to ACs not being cleared fast enough in peripheral tissues. TNF-α, which has been widely used to generate in vitro bone marrow-derived DCs (50), may boost recruitment of DC precursors to sustain the removal of ACs and presentation in the lymph nodes. Also, we could not exclude the possibility that the autocrine secretion of TNF-α can mediate the maturation of DCs we observed after ingesting ACs of late phase, although TNF-α has been shown to be less effective than LPS in activating the expression of genes involved in Ag presentation and T cell stimulation (51).

It has been proposed that there is a balance in vivo between macrophage- and DC-mediated clearance of ACs, in which macrophages may maintain homeostasis of immune system by promoting silent clearance of ACs, whereas clearance by DCs favors an immune response (24, 52). Phagocytosis of ACs by macrophages is believed to be so rapid and so efficient that the presentation of ACs of early phase is markedly inhibited. If macrophages fail to clear ACs normally or ACs are present in large number, overwhelming the phagocytic capacity, ACs will accumulate and undergo the late phase of apoptosis. DCs in this case may function as a second route for the clearance of ACs. Our observation in DCs that have taken up ACs of late, but not early, phase developing into the mature DC phenotype may represent the possible outcome of this second route. The implication of increased apoptosis or impaired clearance of apoptotic cells in the pathogenesis of human SLE (13–16) suggests that late apoptosis may occur in vivo. ACs of late phase have indeed been shown to serve as a source of modified forms of specific autoantigens that may stimulate an autoimmune response (17). Furthermore, pathogenic Th clones specific for autoantigens, such as nucleosome, were found in lupus patients (53). DCs may therefore be critical in presenting specific autoantigens derived from ACs of late phase and differentiating naive T cells into Ag-specific Th clones. More interestingly, our observation of up-regulation of CD40 in DCs that have ingested ACs of late, but not early, phase may also be implicated in mediating the autoimmune response. Recent reports demonstrate that the autoimmunity in a mouse model of SLE is dependent on CD40-CD154 (CD40 ligand) interactions, and an elevated level of CD154-mediated costimulation may be linked to an imbalance of DC homeostasis, forming an autostimulatory loop that maintains autoimmunity (54, 55).

The migration of DCs to the lymphoid organs after capturing Ags is one of the critical events for induction of immune responses. Maturation of DCs is associated with striking switches in chemokine and chemokine receptor usage to facilitate the homing of DCs to lymphoid organs (20, 21). In this report we found that the ingestion of ACs of either early or late phase led to the switches in DCs with patterns and kinetics of expression similar to those observed in DCs matured with LPS. The observed up-regulations of the inflammatory CC chemokines RANTES and MIP-1α at the most early time points and of the lymphoid CC chemokine MIP-3β at the late time points upon taking up ACs of either early or late phase was associated with an enhanced responsiveness to MIP-3β, a CCR7 ligand constitutively expressed in T cell areas of lymph nodes, demonstrating the capability of DC homing. This suggests that the induction of either tolerance or specific T cell response by DCs that have captured self-Ags, such as those derived from ACs of different phases, takes place in the lymphoid organs. The checkpoints for DCs in the lymphoid organs to support either the induction of effective T cell response or T cell tolerance may then depend on the delivery of signals through the MHC class II complex, CD40, and CD83 during Ag presentation.

References


