Activated Apoptotic Cells Induce Dendritic Cell Maturation via Engagement of Toll-like Receptor 4 (TLR4), Dendritic Cell-specific Intercellular Adhesion Molecule 3 (ICAM-3)-grabbing Nonintegrin (DC-SIGN), and β2 Integrins*[^5]

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**Background:** Activated apoptotic lymphocytes provide activation/maturation signals to human monocyte-derived dendritic cells (DCs).

**Results:** Cell-cell contact-dependent signaling involved β2 integrins, DC-SIGN, and TLR4, which resulted in activation of multiple signaling pathways.

**Conclusion:** These studies provide mechanistic insight into DC responses during encounter with cells undergoing immunogenic cell death.

**Significance:** Learning how DCs respond to certain cell death has implications for vaccine design.

Dendritic cells (DCs) are professional antigen-presenting cells playing a central role in connecting innate and adaptive immunity. Maturation signals are, however, required for DCs to undergo phenotypic and functional changes to acquire a fully competent antigen-presenting capacity. We previously reported that activated apoptotic peripheral lymphocytes (ActApo) provide activation/maturation signals to human monocyte-derived DCs. In this paper, we have characterized the signaling pathways and molecules involved in ActApo-mediated DC maturation. We found that both cellular and supernatant fractions from ActApo are required for DC maturation signaling. ActApoSup-induced CD80 and CD86 expression was significantly blocked in the presence of neutralizing antibodies against tumor necrosis factor-α (TNF-α). Cell-cell contact-dependent signaling involved β2 integrins, dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), and TLR4 because ActApo-induced up-regulation of the maturation markers CD80 and CD86 was significantly inhibited in the presence of neutralizing antibodies against CD18, CD11a, CD11b, and DC-SIGN as well as TLR4. The role of TLR4 was further confirmed by silencing of TLR4 in DCs. In addition, the endogenous adjuvant effect exerted by activated apoptotic splenocytes (ActApoSp) was reduced after immunization with human serum albumin in TLR4^-/-^ mice. We detected activation of multiple signaling pathways and transcription factors in DCs upon coculture with ActApo, including p38, JNK, PI3K-Akt, Src family kinases, NFκB p65, and AP1 expression. These studies provide mechanistic insight into the responses of DCs during encounter with cells undergoing immunogenic cell death.

DCs[^3] reside in peripheral compartments, such as, for example, the epidermis, in an immature phenotype that is characterized by high CD1a expression but low expression of the co-stimulatory molecules CD80 and CD86. To elicit immunity, the immature, antigen-capturing DC has to transform into a mature antigen-presenting cell. This activation process includes morphological changes, such as loss of adhesion molecules, reorganization of the cytoskeleton and increased motility, transient increased followed by decreased antigen uptake, secretion of chemokines and cytokines, and up-regulation of MHC class II and co-stimulatory molecules (1, 2). All of these events occur to provide the DCs with the capacity to migrate to nearby lymph nodes and facilitate a successful priming of T cells.

DC maturation can be triggered by recognition of pathogen-associated molecular patterns that engage pattern recognition receptors (PRRs) (e.g., TLRs, NOD-like receptors, or C-type lectin receptors) expressed by DCs (3, 4). Alternatively, the DCs can also respond to endogenous danger-associated molecular patterns (DAMPs) from injured cells, and the list of endogenous DAMPs is long and growing and is nicely reviewed by Kono (5). Suggested receptors for the various endogenous dan-

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[^3]: The abbreviations used are: DC, dendritic cell; TLR, Toll-like receptor; ICAM, intercellular adhesion molecule; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; PBMC, peripheral blood mononuclear cell; ActApo, activated apoptotic PBMCs; RestApo, resting apoptotic PBMCs; ActApoSp, activated apoptotic splenocytes; HSP60, heat shock protein 60; HSA, human serum albumin; PRR, pattern recognition receptor; DAMP, danger-associated molecular pattern; CM, conditioned medium; CREB, cAMP-response element-binding protein; MFI, mean fluorescence intensity.
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EXPERIMENTAL PROCEDURES

Human Subjects and Blood Collection—Buffy coats from healthy human blood donors were obtained from the blood bank at Karolinska University Hospital Huddinge. Ethical approval was obtained from the medical ethics committee in Stockholm.

In Vitro Differentiation of DCs—CD14+ monocytes were enriched from buffy coats by negative selection using Rosette-Sep human monocyte enrichment (1 ml/10 ml blood; Stem Cell Technologies). Monocytes were separated using a Lymphoprep density gradient (Nycomed) and were cultured for 6 days in complete medium, containing RPMI 1640 (Invitrogen) supplemented with 1% HEPES (Invitrogen), 2 mM 1-glutamine (Invitrogen), 1% streptomycin and 1% penicillin (Invitrogen), and 10% endotoxin-free fetal bovine serum (FBS) (Invitrogen). The medium was further supplemented with recombinant human cytokines IL-4 (6.5 ng/ml; R&D Systems) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 250 ng/ml; PeproTech) to obtain immature DCs.

Purification and Activation of PBMCs—PBMCs were separated from blood from healthy blood donors using a Lymphoprep density gradient (Nycomed). Cells were frozen in FBS and 10% DMSO or were directly cultured in complete medium containing 1% sodium pyruvate. PBMCs (10^6/ml) were activated with anti-human CD3 (2 µg/ml, clone OKT 3; Ortho Biotech) and anti-human CD28 (2 µg/ml, clone L293; BD Biosciences). The monoclonal anti-human CD3 was adhered to plastic for 1 h in 4 °C before the addition of soluble monoclonal anti-human CD28 and PBMCs. After overnight incubation, cells were frozen in FBS/DMSO.

Phenotypic Characterization of DCs and PBMCs—DCs were washed and resuspended in PBS with 2% FBS. DCs were incubated for 30 min at 4 °C with the following anti-human mAbs: CD1a (clone NA1/34; DAKO), CD14 (clone TUK4; DAKO), CD3 (clone SK7; BD Biosciences), CD80 (clone L307.4), and CD86 (clone 2331/FUN-1; BD Biosciences). PBMCs were stained with anti-human mAbs CD19 (clone HD37; BD Biosciences), CD3 (clone SK7; BD Biosciences), CD4 (clone 2A3; BD Biosciences), and CD69 (FN50; BD Biosciences). Cell surface expression was measured by a FACS Calibur flow cytometer (BD Biosciences). 7-AAD (7-amino-actinomycin D) viability stain (eBiosciences) was used to exclude dead cells when analyzing.

DCs and Apoptotic PBMC Co-cultures—Differentiated, immature DCs were co-cultured with γ-irradiated (150 gray) PBMCs at a ratio of 1:2 (DCs/PBMCs) in complete medium supplemented with recombinant human IL-4 and GM-CSF. TLR4 ligands Escherichia coli LPS (100 ng/ml; Sigma-Aldrich) and monophosphoryl lipid A (100 ng/ml; Invivogen) were used as positive controls. For blocking experiments, DCs or PBMCs were preincubated with either isotype control IgGs or neutralizing antibodies for 60–120 min before co-culture. Neutralizing antibodies used in this study were anti-TNF-α (10 µg/ml, clone 1825; R&D Systems), anti-CD18 (10 µg/ml, clone 68–5A5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-CD11a (10 µg/ml, clone-38; GeneTex), anti-CD11b (20 µg/ml,
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cloned ICRF-44; Biolegend), anti-TLR4 (10 μg/ml, clone HTA-125; Santa Cruz Biotechnology, Inc.), anti-DC-SIGN (10 μg/ml, clone 1205077 (R&D Systems) and clone A2ND1 (Beckman Coulter), anti-HMG1B1 (high mobility group box 1) (10 μg/ml, clone 2G7 (kindly provided by Ulf Andersson, Karolinska Institutet) and clone DPH1.1 (Diapro), and anti-heat shock protein 60 (HSP60) (10 μg/ml, clone Mab11–13; Abcam). HMG1B1 antagonistic protein BoxA (10 μg/ml) was from HMG1Biotech.

For studies with inhibitors, DCs were pretreated with either vehicle alone or various inhibitors as indicated for 30–60 min, followed by the addition of apoptotic PBMCs. All inhibitors were purchased from Calbiochem, Merck4biosciences. DCs were analyzed by flow cytometry for expression of maturation markers after 48 h or cell lysates were prepared for immunoblotting or transcription factor ELISA at various time points.

Generation of Apoptotic Cells, Cellular Fraction, and Supernatant Fraction—Frozen PBMCs were thawed and washed three times in complete medium. Cells were induced to undergo apoptosis by γ-irradiation (150 grays). Apoptosis was confirmed by annexin V (Roche Applied Science) and propidium iodide (0.1 μg/sample; Sigma–Aldrich) staining according to the manufacturer’s protocol. To prepare cellular and supernatant fraction from apoptotic PBMCs, cells were centrifuged at 500 × g for 10 min. Pellet obtained (cellular fraction) was resuspended in fresh complete medium, and supernatants were further centrifuged at 16,000 × g (Eppendorf Centrifuge 5415D) for 30 min to remove any cellular part. Conditioned medium (CM) was prepared by co-culturing DCs with ActApoSp for 24 h.

ELISA for TNF-α—Conditioned medium was collected after 6, 14, and 24 h of co-culture and assayed for TNF-α by ELISA using the TNF-α assay kit (Biolegend) following the manufacturer’s instructions.

Preparation of Cell Lysates and Immunoblotting—After treatment, cells were collected by centrifugation at 200 × g for 5 min and washed with PBS. Whole cell lysates were prepared by resuspending the cell pellet in reducing sample buffer (Millipore), followed by heating at 95 °C for 10 min. In some experiments, cell lysates were prepared using the lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF) from Cell Signaling Technology following the manufacturer’s instructions. Briefly, control or treated cells were collected by centrifugation at 200 × g for 5 min and washed with ice-cold PBS. Pellets were resuspended in an appropriate volume of lysis buffer and incubated on ice for 15 min, followed by centrifugation at 14,000 × g for 10 min in a cold centrifuge. Supernatant were mixed with an equal volume of 2× reducing sample buffer (Millipore) and heated at 95 °C for 10 min. Proteins were separated on SDS-polyacrylamide gels and then transferred electrothermally to polyvinylidene difluoride membranes using the iBlot system (Invitrogen). The blots were blocked with Roti-buffer (Roth) for 1 h and subsequently incubated overnight at 4 °C with primary antibodies in blocking buffer. After washing with PBS containing 0.1% (v/v) Tween 20 (PBST), the blots were incubated with horseradish peroxidase-conjugated appropriate secondary antibody in blocking buffer for 1 h at room temperature, followed by washing with PBST and development with enhanced chemiluminescence reagent (Fisher).

Silencing of TLR4 and Real-time PCR Analysis—Day 4 immature DCs were transfected with 25 nM non-targeting siRNA (D-001206-13; Dharmaco) or TLR4 siRNA (M-008088-01; Dharmaco) with the transfection reagent DF4 (Dharmaco, Fisher) following the manufacturer’s instructions. This protocol resulted in a transfection efficiency of nearly 100%, as determined by flow cytometry of cells transfected with siGLO RISC-Free Control siRNA (D-001600–01; Dharmaco). RNA was extracted after 48 h of transfection using the RNeasy minikit (Qiagen), and cDNA was made using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Amplification of TLR4 and GAPDH cDNA was performed using the 7500 real-time PCR system (Applied Biosystems) and 6-carboxyfluorescein dye-labeled TaqMan MGB probes and primers (Applied Biosystems). Cycle threshold values for TLR4 were normalized to the value for GAPDH. Data are presented as -fold changes in mRNA copy number in the DC co-cultures as compared with mRNA in DCs cultured in medium only. 50–80% of silencing of expression of TLR4 was achieved as verified by real-time PCR analysis.

Transcription Factor Assays—Transcription factor assays were carried out using a transcription factor ELISA kit from Active Motif. Briefly, nuclear extracts from cells were prepared using a transcription factor extraction kit (Active Motif). After centrifugation for 5 min at 20,000 × g at 4 °C, supernatants were assayed for the presence of the respective transcription factors by the addition of equal amounts of lysates to wells pre-coated with DNA-binding consensus sequences. The presence of any particular transcription factor in the nucleus was then assessed by using a transcription factor kit according to the manufacturer’s instructions. Plates were read at 655 nm.

Mice and Immunizations—C57BL/6 wild type (WT), MyD88−/−, or TLR4−/− mice were bred and kept at the animal facility at MTC, Karolinska Institutet. The mouse experiments were performed under approval of the Stockholm North Ethical Committee on Animal Experiments. Mice (8–12 weeks of age) were immunized intraperitoneally with 50 μg of endotoxin-free HSA (Sigma) without any adjuvant or with the addition of the cellular adjuvant ActApoSp (1 × 106 cells/immunization). Additional control groups received only PBS. The ActApoSp were prepared by stimulating allogeneic (Balb/c) murine spleen cells in vitro with concanavalin A (2.5 μg/ml; Sigma). 2 × 106 cells/ml were cultured in RPMI 1640 medium containing 10% FCS for 24 h. The obtained cells were washed before being frozen in fetal calf serum (FCS) with 10% DMSO until the day of immunization. On the day of immunization, cells were thawed, washed two times in PBS, and exposed to γ-irradiation (150 grays) for apoptosis induction, as described previously (33, 34). In total, three washes were performed to remove cell culture content. The γ-irradiated cells were diluted in PBS (final immunization dose 0.2 ml/animal), and C57BL/6 mice were immunized intraperitoneally once. 10–12 days after immunization, the mice were sacrificed, and sera were analyzed for the presence of humoral immune responses by using ELISA. ELISA plates (Nunc) were coated with HSA and thereafter blocked with 2% fat-free milk and 1% FCS in PBS. Serum was diluted in...
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1% FCS in PBS, and 100 µl/well was added. HRP-labeled goat anti-mouse IgG (Bio-Rad) using o-phenylenediamine as a substrate was used to reveal the presence of antibodies by a color reaction. Plates were then developed for 5 min by adding o-phenylenediamine buffer (Sigma). The color reaction was stopped with 1.25 M H₂SO₄ and the optical density (OD) was read at 490 nm.

Statistical Analysis—Statistical significance was assessed using the Mann-Whitney test and the Wilcoxon rank sum test. Differences were considered significant at \( p \leq 0.05 \).

RESULTS

Both Cell-Cell Contact and Soluble Factors Are Required for Up-regulation of Co-stimulatory Molecules on DCs—Apoptotic cell-mediated DC maturation could theoretically be induced either by soluble factors released from dying cells or cell-cell contact-dependent mechanisms. To address this question, we co-cultured immature DCs with cellular or soluble fractions derived from ActApo. We detected partial up-regulation of the maturation markers CD80 and CD86 on DCs under both conditions with a more prominent effect by the cellular fraction (Fig. 1A). None of the fractions from RestApo induced up-regulation of maturation markers on DCs. In an approach to cover the whole apoptotic process, we co-cultured DCs and apoptotic cells using a transwell system, which did not allow any physical contact between two cells but allowed exchange of soluble factors. Under this condition, we observed partial up-regulation of maturation markers on DCs (Fig. 1A). Taken together, these results suggested that both cell-cell contact and soluble factors are required for ActApo-induced DC maturation.

TNF-α-dependent Up-regulation of Co-stimulatory Molecules on DCs—Apart from certain DC-stimulating soluble factors released from apoptotic cells, co-culture of ActApo and DCs could induce release of additional factors from DCs, which in turn could enhance maturation signaling. To assess involvement of additional soluble factors, we collected conditioned media from ActApo-DC co-culture at different time points and incubated them with fresh immature DCs. We measured up-regulation of maturation markers CD80 and CD86 on DCs cultured in CM, which was significantly higher than the effect of soluble fraction from ActApo alone, suggesting that there was release of additional stimulatory factors after co-culture (Fig. 1B). Our previous results have shown that there is rapid induction of TNF-α, a well known proinflammatory cytokine having DC-stimulatory capacity, in CM after co-culture (25). Preincubation of either soluble fraction or CM from ActApo with TNF-α-neutralizing antibody significantly inhibited up-regulation of maturation markers CD80 and CD86 on DCs (Fig. 1B). The HMGB1 protein is released from necrotic and late apoptotic cells (35–38), and DCs undergoing maturation have been shown to release their own HMGB1 (39). This protein has been shown to induce DC maturation in vitro and to have an adjuvant effect when added together with soluble antigen (8). We therefore assessed the possible involvement of HMGB1 in the present experimental system. ActApo-induced CD80 and CD86 expression on DCs was not affected in the presence of HMGB1 antagonistic protein BoxA (supplemental Fig. S1A) or two different neutralizing antibodies against HMGB1 (data not shown). These results suggested that TNF-α is the major soluble factor required for DC maturation signaling after co-culture with ActApo.

Cell-Cell Contact-dependent Signaling Involves β2 Integrins, DC-SIGN, and TLR4—Because cell-cell contact was crucial for the ActApo-induced maturation of DCs, we investigated the involvement of members of integrin family, which are well known for their role in cell-cell adhesion and uptake of apoptotic cells. ActApo-induced up-regulation of maturation markers CD80 and CD86 was significantly inhibited in the presence of neutralizing antibodies against the integrins CD18, CD11a, and CD11b (Fig. 2, A and B), suggesting the involvement of these molecules in cell-cell contact-dependent signaling. However, blocking of αvβ5, an integrin required for uptake of apoptotic cells (40), had no effect on ActApo-induced up-regulation of maturation markers, suggesting that uptake of apoptotic cells is not required for DC maturation signaling (supplemental Fig. S1B). In line with this, pretreating DCs with cytochalasin D, an inhibitor of actin polymerization and thereby phagocytosis, had no effect on ActApo-induced up-regulation of maturation.
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markers on DCs (supplemental Fig. S1C). Blocking phagocytosis of apoptotic cells with cytochalasin D treatment of DCs was previously confirmed by a flow cytometry-based uptake assay in co-cultures with Far Red-labeled DCs and PKH67-labeled apoptotic PBMCs, as described by Johansson et al. (25). This is in line with our finding that cell-cell contact is sufficient to trigger DC maturation signaling, and phagocytosis of apoptotic cells is not required.

DC-SIGN is a C-type lectin receptor shown to be involved in DC maturation induced by apoptotic neutrophils and Schistosoma mansoni worm glycolipids (41, 42). To examine the involvement of this lectin in DC maturation, we used two different neutralizing antibodies to block the receptor engagement. We observed a significant down-regulation of maturation markers CD80 and CD86 on DCs when co-cultured with ActApo in the presence of two different anti-DC-SIGN-neutralizing antibodies (Fig. 2, C and D), indicating that DC-SIGN is involved in DC maturation triggered by these stimuli. Several endogenous ligands have been shown to activate members of this family and has been shown to cooperate with DC-SIGN to induce activation of human DCs (42). Therefore, we investigated the possibility of involvement of this receptor in DC maturation signaling. Pretreatment of DCs with neutralizing antibody against TLR4 significantly inhibited up-regulation of maturation markers CD80 and CD86 on DCs (Fig. 3A) as well as release of TNF-α (supplemental Fig. S2A), suggesting the involvement of TLR4. The role of TLR4 was further confirmed by silencing of TLR4 in DCs using siRNA from Dharmacon. The transfection procedure per se neither altered the expression levels of TLR4 nor induced secretion of TNF-α (supplemental Fig. S2C), although some DC donors showed 2–10% increased up-regulation of CD80 and CD86 as compared with medium control. The efficiency of silencing as revealed by real-time PCR after 42 h was in the range of 45–80% in different DC donors (supplemental Fig. S2B). ActApo-induced TNF-α release (supplemental Fig. S2C) and CD80 and CD86 up-regulation (Fig. 3B) were significantly inhibited after silencing of TLR4 on DCs, further confirming the role of TLR4 signaling in ActApo-induced DC maturation.

In order to identify the molecule involved in triggering TLR4 signaling, we tested the possible involvement of HSP60, which was previously shown to activate TLR4 (5). We found that ActApo-induced CD80 and CD86 expression was significantly inhibited in the presence of neutralizing antibodies against HSP60 (Fig. 3C). In addition, we detected a significantly higher expression of HSP60 on PBMCs after activation (Fig. 3D). These results indicated a role for HSP60 in TLR4 activation.

We previously reported on the endogenous adjuvant effect exerted by ActApoSp but not resting apoptotic splenocytes after immunization in mice using a DNA vaccine (26). Here we assessed whether ActApoSp can provide an adjuvant effect in combination with HSA administered intraperitoneally and if TLR4 is involved in the endogenous adjuvant response. WT and TLR4−/− animals were immunized with 50 μg of HSA with and without ActApoSp, followed by measurements of anti-HSA antibodies in sera using ELISA. We detected a significantly improved titer of anti-HSA antibodies when ActApoSp was used as adjuvant in WT mice (Fig. 3E). The amounts of anti-HSA IgGs were significantly decreased in the TLR4−/− mice compared with WT, showing involvement of TLR4 in pro-
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FIGURE 3. TLR4 is required for up-regulation of co-stimulatory molecules on DCs. A, day 6 immature human monocyte-derived DCs were preincubated with either control IgG (Ctrl IgG) or neutralizing anti-TLR4 antibody (10 μg/ml) for 1 h, followed by co-culture with ActApo for 48 h. Data are represented as MFI of CD80 and CD86 expression on DCs in multiple donors (n = 13). Data shown in control IgG sets are results obtained from both control IgG-treated and untreated cells as described in the legend to Fig. 2. Significant differences were assessed by the non-parametric Wilcoxon test and are indicated as *** (p < 0.001). B, day 4 immature DCs were transfected with control siRNA (siNon Target) or TLR4 siRNA (siTLR4) as described under “Experimental Procedures.” 72 h after transfection, cells were left untreated (med) or exposed to ActApo or RestApo or TLR4 ligand monophosphoryl lipid A (100 ng/ml). Data are represented as MFI of CD80 and CD86 expression on DCs from five donors, and analyses were performed 48 h after co-culture. Data represent mean ± S.D. C, ActApo were preincubated with neutralizing anti-HSP60 antibody (10 μg/ml) for 1 h, followed by co-culture with day 6 immature DCs for 48 h. The frequency of CD80- and CD86-positive DCs was analyzed by flow cytometry in multiple donors (n = 5). Significant differences compared with medium control were assessed by the non-parametric Mann-Whitney U test, and significance is indicated by * (p < 0.05). D, lysates from live unactivated PBMCs (Rest), RestApo, live activated PBMCs (Act), and ActApo were immunoblotted with anti-HSP60 antibody (top) and reprobed with actin antibody (bottom) to confirm equal loading. Blots shown are representative of results obtained in three PBMC donors. E, WT or TLR4−/− mice were treated intraperitoneally with 50 μg of HSA alone or in combination with murine ActApoSp. PBS was injected as a control. The mice were sacrificed 10–12 days postimmunization, and serum levels of anti-HSA antibodies were measured with ELISA. Data are from three independent experiments. Significant differences were assessed by a non-parametric Mann-Whitney test and are indicated as ** (p < 0.01) and * (p < 0.05). Error bars, S.D.

viding the adjuvant effect. We obtained similar results using MyD88−/− mice (data not shown), which further supports a role for TLR signaling after exposure to the endogenous cellular adjuvant ActApo. Taken together, these findings provide evidence for a role of TLR4 upon contact with ActApo in vitro and in vivo.

Activation of Multiple Signaling Pathways in DCs upon Co-culture with ActApo—In order to characterize some of the differences in intracellular signaling that leads to different responses to ActApo and RestApo, we assessed activation of several key intracellular molecules known to be involved in DC maturation signaling. p38, ERK, and JNK MAPKs were rapidly activated in DCs after co-culture with ActApo in a time-dependent manner (Fig. 4). We also observed a strong activation of Akt, detected by phosphorylation-specific antibodies (Ser-473 and Thr-308) against activated Akt, in DCs co-cultured with ActApo but not with RestApo (Fig. 4B). The transcription factor NFκB p65 regulates expression of several key cytokines, including TNF-α. In addition, CD86 expression is also regulated by NFκB p65. We therefore assessed the activation of NFκB p65 by monitoring the level of inhibitory protein IκB.

Both ActApo and RestApo induced degradation of IκB, albeit with different kinetics (Fig. 4C). However, we could detect Ser-536 phosphorylation of p65 only in DC co-cultures with ActApo but not with RestApo (Fig. 4D). IκB kinase (IKK)-dependent p65 ser536 phosphorylation has been suggested to be required for interaction with co-activators CREB-binding protein/p300 to obtain subsequent acetylation-dependent complete activation of p65 (44). In the present experimental system, it appears that both ActApo and RestApo induce degradation of inhibitory IκB subunit, but full activation of NFκB p65 is achieved only in DCs co-cultured with ActApo. In addition, ActApo, but not RestApo, induced activation of AP-1 transcription factor family members c-Jun and c-Fos in a time-dependent manner (Fig. 4, E and F), as assessed in nuclear extracts by ELISA-based binding assays to consensus oligonucleotides. There was no activation of other AP1 family members JunB, FosB, and Fra-1 in DCs co-cultured with ActApo (data not shown).

p38, JNK, PI3K-Akt, and Src Family Tyrosine Kinases Are Required for ActApo-induced DC Maturation—To confirm whether activation of the different intracellular signaling mol-
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ecules studied is actually linked to DC maturation signaling, we blocked activity or activation of target molecules using specific inhibitors and assessed their effect on expression of maturation markers on DCs. Blocking of p38 and JNK MAPKs, using SB2035800 and SP600125, respectively, significantly reduced up-regulation of maturation markers CD80 and CD86 on DCs. However, inhibition of ERK MAPK had no effect on ActApo-induced up-regulation of maturation markers (Fig. 5A), suggesting that only p38 and JNK MAPKs are required for DC maturation signaling. The role of Akt activation in DC maturation signaling was confirmed by blocking activation of PI3K, which is an upstream kinase required for activation of Akt. PI3K inhibitor wortmannin significantly reduced up-regulation of maturation markers CD80 and CD86 on DCs. Although signaling through DC-SIGN has been suggested to be involved in DC maturation, intracellular signaling molecules involved in this process are not known. DC-SIGN-dependent activation of DC and NFκB p65 has been suggested to be dependent on Raf-1 activation. We could not detect activation of Raf-1 in DCs co-cultured with ActApo (data not shown). Also, pretreatment of DCs with Raf-1 inhibitor GW5074 had no effect on ActApo-induced up-regulation of maturation markers on DCs (data not shown), suggesting involvement of other molecules in the present experimental system, which needs to be explored further. Taken together, these results suggested that multiple signaling pathways involving p38 MAPK, PI3K-Akt, and the Src family of tyrosine kinases are required for ActApo-induced maturation signaling.

Akt Activation Is Dependent on TLR4 and β2 Integrins—PI3K-Akt is known to signal downstream of TLR4 (47). Therefore, we examined the role of TLR4 signaling in activation of Akt using...
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neutralizing antibodies against TLR4. The activation of Akt following pretreatment with neutralizing antibody against TLR4 was assessed by immunoblotting against Ser-473 of Akt and shown to be abrogated (Fig. 6A). In addition, pretreatment of cells with neutralizing antibodies against β2 integrin CD18 also blocked Ser-473 phosphorylation of Akt, suggesting the critical role of cell-cell contact-dependent signaling on activation of Akt (Fig. 6A). Pretreatment of cells with neutralizing antibodies against DC-SIGN had no effect on activation of Akt (Fig. 6A).

**PI3K-Akt and Src Family Kinase-dependent Activation of p38 MAPK Regulates NFκB Activation**—Pretreatment of cells with neutralizing antibodies against β2 integrin CD18 and TLR4 significantly inhibited activation of p38 MAPK, whereas blocking of DC-SIGN had no effect on p38 MAPK activation (Fig. 6B). Because both Akt kinase and p38 MAPK were required for ActApo-induced up-regulation of maturation markers CD80 and CD86 on DCs, we sought to examine whether activation of these kinases was dependent on the other. The activation of p38 MAPK, as assessed by Western blot analysis of phospho-p38 MAPK, was partially abrogated in cells pretreated with PI3K kinase inhibitor wortmannin (Fig. 6C). In addition, activation of p38 MAPK was also dependent on signaling via the Src family of tyrosine kinases because ActApo-induced p38 MAPK phosphorylation was significantly inhibited in cells pretreated with Src family tyrosine kinase inhibitor PP1 (Fig. 6C).

ActApo-induced NFκB p65 Ser-536 phosphorylation was abrogated in the presence of neutralizing antibodies against β2 integrin CD18 and TLR4, whereas neutralizing antibody against DC-SIGN had no effect on Ser-536 phosphorylation of p65 (Fig. 6D), which further supports previous reports showing that NFκB p65 Ser-536 phosphorylation is not regulated by DC-SIGN (48). In addition, pretreatment of DCs with wortmannin, PP1, or SB203580 blocked NFκB activation (Fig. 6E). Taken together, these results indicate that signaling involving β2 integrin dependent cell-cell contact, TLR4-PI3K/Akt, and the Src family of tyrosine kinases is required for activation of p38 MAPK-dependent activation of NFκB.

**TLR4, DC-SIGN, and Src Family of Tyrosine Kinases Regulate JNK MAPK Activation**—In order to identify the signaling pathways regulating JNK MAPK, DCs were pretreated with different neutralization antibodies or inhibitors, followed by co-culture with ActApo for 40 min. Pretreatment of cells with neutralizing antibodies against β2 integrin CD18 completely blocked JNK MAPK activation, whereas neutralizing antibodies against TLR4 and DC-SIGN had a partial effect (Fig. 7A). In addition, pretreatment of DCs with PP1 blocked JNK activation, whereas wortmannin had no effect (Fig. 7B). Because pretreatment of DCs with PP1 inhibited ActApo-induced NFκB activation (Fig. 6E) and also JNK MAPK activation (Fig. 7B), we tested the possibility of JNK-dependent NFκB activation. Pretreatment of DCs with SP600125 had no effect on NFκB activation (data not shown), suggesting that the NFκB activation is independent of JNK MAPK in DCs co-cultured with ActApo. Taken together, these results indicated that signaling involving β2 integrin-dependent cell-cell contact, TLR4, DC-SIGN, and the Src family of tyrosine kinases are required for activation of JNK MAPK.

**Regulation of c-Jun and c-Fos Activation**—In order to identify the signaling pathways leading to activation of c-Jun and c-Fos, DCs were pretreated with various inhibitors, followed by co-culture with ActApo for 60 min. Nuclear extracts were prepared, and activation of c-Jun and c-Fos was assessed by ELISA-based binding assays to consensus oligonucleotides. Pretreatment of DCs with either JNK inhibitor SP600125 or Src family tyrosine kinase inhibitor PP1 partially blocked c-Jun activation (Fig. 7C), whereas c-Fos activation was blocked in DCs pretreated with either p38 MAPK inhibitor SB203580 or Src family tyrosine kinase inhibitor PP1 (Fig. 7D). Neutralizing antibodies against β2 integrin CD18 significantly blocked ActApo-induced c-Jun and c-Fos activation in DCs (Fig. 7, E
and F). Taken together, these results indicated that cell-cell contact-dependent signaling involving p38 MAPK, JNK MAPK, and the Src family of tyrosine kinases regulate activation of c-Jun and c-Fos.

DISCUSSION

In this study, we have characterized some of the molecules and signaling pathways regulating DC activation in response to dying cells that were activated prior to apoptosis induction (ActApo). Our results indicated that both soluble factors released from ActApo and cell-cell contact between immature DCs and ActApo are required for generation of maturation signals, with TNF-α being the main soluble factor. The role of TNF-α in DC maturation signaling is well documented (49–51), and here it is important to note that ActApo secrete very low amount of TNF-α, and there is strong induction of TNF-α in DCs after co-culture with ActApo, which in turn acts in an autocrine manner to induce maturation signaling in DCs.

Over the past several decades, much has been revealed about the nature of the host innate responses to microorganisms with the identification of PRRs, including TLRs, NOD-like receptors, and C-type lectin receptors. It is now apparent that the same PRRs can also be activated by endogenous, non-microbial signals, many of which are considered as DAMPs. Several endogenous ligands have been reported for PRRs (43). Besides foreign antigens, the C-type lectin receptor DC-SIGN binds to a number of endogenous ligands, particularly ICAM-2 on endothelial cells and ICAM-3 on T cells. The physiological function of DC-SIGN has been suggested to be induction of tolerance by immature DCs, after recognition of glycosylated self-antigens, for homeostatic control (6). However, we found that interaction of ActApo with DC-SIGN is necessary for ActApo-induced DC activation because expression of
maturation markers were significantly inhibited in the presence of neutralizing antibodies against DC-SIGN. This suggests that DC-SIGN is involved in recognition of endogenous danger molecules expressed in activated dying cells. In addition, we also found that TLR4 plays a crucial role in ActApo-mediated DC maturation signaling. Our findings that ActApo expresses more HSP60 than RestApo and that pretreatment of ActApo with neutralizing antibody against HSP60 results in significantly reduced ability to mature DCs indicate that this endogenous DAMP is likely to be involved in ActApo-induced TLR4 activation although this needs to be explored further. TLRs have been a target for several vaccine adjuvants, and our immunization studies in TLR4−/− knock-out mice confirmed a role for TLR4 signaling in the endogenous adjuvant effect of dying cells that were activated before apoptosis induction. It is possible that other members of the TLR family, requiring Myd88 as an adaptor protein, are also involved because we observed a greatly reduced adjuvant effect of ActApoSp in Myd88−/− mice (data not shown). It has been reported that DC-SIGN and TLR4 cooperate to induce an inflammatory phenotype in human DCs in response to S. manosii worm glycolipids (42). Therefore, it is possible that similar cooperation is occurring on DCs in presence of ActApo. In addition, an interaction between Mac-1 (CD11b/CD18) and DC-SIGN was shown to be required for apoptotic PMN-induced DC maturation (41). We also observed that upregulation of maturation markers was significantly reduced on DCs in the presence of neutralizing antibodies to CD18, CD11b, and DC-SIGN, suggesting that this interaction is probably involved in DC maturation signaling in the present condition, although this needs to be explored further.

Despite abundant expression of β2 integrins on DCs, their functional relevance is poorly understood. It has been reported that expression of maturation markers is not altered on murine bone marrow-derived DCs from CD18−/− mice (52). However, in another study, it was shown that β2 integrins expressed on human DCs interacted with Thy1 and ICAM-1 expressed on fibroblasts to induce phenotypic maturation of DCs (53). It was recently reported that signaling via β2 integrins on human DCs is required for expression of maturation marker CD86 in response to biomaterials (54). Thus, additional studies are required to further characterize the role of β2 integrin signaling leading to DC maturation. We observed a significantly reduced expression of maturation markers on DCs in the presence of neutralizing antibodies against the β2 integrin CD18. In order to assess the role of β2 integrins in ActApo-induced DC maturation signaling, we tried to knock down CD18 on DCs. Unfortunately, we were unable to achieve any significant silencing using two different siRNAs. Interestingly, the expression of maturation markers on DCs did not differ significantly in experiments where either DCs or ActApo were preincubated with neutralizing antibodies against CD18 before the co-culture. The inhibitory effect of the neutralizing antibodies was visible even in experiments with a very short time period (20–60 min) of co-culture. Therefore, it is possible that integrins expressed on both DC and ActApo are involved in the interaction. CD11b/CD18 has been reported to be an essential component of a TLR4-containing complex involved in uptake of LPS in hepatocytes, and engagement of this complex leads to p38 MAPK activation, not via TLR4 but in a CD11b/CD18-dependent manner (55). Integrin αM (CD11b) has also been shown to negatively regulate TLR-triggered inflammatory responses in monocytes and macrophages (56). However, the roles of the β2 subunit (CD18) and other α subunits in TLR signaling were not studied. Another explanation is that integrins involved in adhesion are not sufficient to activate DCs but rather allow for other co-receptors to be in close proximity with the ligands expressed on the apoptotic cell. In summary, it appears that ActApo engages multiple receptors simultaneously to induce DC maturation.

Several intracellular signaling molecules and pathways have been suggested to be involved in DC maturation signaling. Available reports clearly indicate that the role of each pathway is stimulus-specific, having both positive and negative regulatory roles. Therefore, in order to obtain a correct picture, it is important to characterize the role of these pathways in specific contexts. Activation of Akt/protein kinase B, a major effector of PI3K, has been reported as a critical factor in both activation and survival of DCs (57, 58). In a recent study, Kim et al. (59) reported an improvement in the potency of a DC vaccine with an siRNA targeting phosphatase and tensin homologue, which is known to be a central negative regulator of the PI3K/Akt signal transduction cascade. Several studies have suggested that Akt kinase could also negatively regulate DC maturation signaling (60, 61). Our results, however, indicated that ActApo-induced DC maturation signaling is positively regulated by Akt kinase. Mascanfroni et al. (62) have suggested that PI3K-independent activation of Akt could also regulate DC maturation, but our results clearly indicated that ActApo-induced Akt activation occurs via PI3K because ActApo-induced Ser-473 phosphorylation of Akt and expression of co-stimulatory molecules on DCs were significantly inhibited after pretreatment with the PI3K inhibitor wortmannin. In addition, we observed a role for the Src family of tyrosine kinases in ActApo-induced DC maturation signaling. It has been reported that PP1 and PP2, inhibitors of the Src family of tyrosine kinases, inhibit release of proinflammatory cytokines without affecting the expression of co-stimulatory molecules upon stimulation of TLRs in human DCs (29, 45). We observed that both release of proinflammatory cytokines (data not shown) and expression of CD80 and CD86 were significantly inhibited upon PP1 or PP2 pretreatment of DCs co-cultured with ActApo. Therefore, our results indicate that apart from regulating the release of proinflammatory cytokines, the Src family of tyrosine kinases could also regulate the expression of maturation markers on DCs. In addition, we found that both Akt and the Src family of tyrosine kinases regulate activation of MAPKs in DCs that were co-cultured with ActApo. Several studies have previously shown that members of MAPKs play a critical role in DC maturation signaling. Although we observed a strong activation of MAPK members ERK, p38, and JNK, the ActApo-induced DC maturation involved signaling only via p38 and JNK MAPK, whereas activation of ERK MAPK is linked to other responses in DCs. Activation of p38 MAPK occurred in a DC-SIGN-independent but TLR4- and CD18-dependent manner, whereas activation of JNK MAPK was dependent on TLR4, DC-SIGN, and CD18.
again suggesting a cooperative signaling between these molecules.

NF-κB and the AP1 family of transcription factors play an important role in DC activation and release of proinflammatory cytokines. Signaling through NF-κB determines the increased expression of MHC II and co-stimulatory molecules, release of proinflammatory cytokines and chemokines, and DC migration and recruitment (46). Src family kinase-dependent activation of AP1 family member c-Jun regulates the release of proinflammatory cytokines from DCs (29, 45). Our results indicate that ActApo-induced DC activation involves both NF-κB and the AP1 family of transcription factors. The proposed pathway for ActApo-induced DC activation is shown in supplemental Fig. S2D. Collectively, these results provide an insight into the complex series of events occurring inside DCs after an encounter with immunogenic dying cells. We have identified some of the mechanisms involved in DC activation, which could be beneficial in the field of vaccine research.

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