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Calcium Mobilization in Human Myeloid Cells Results in Acquisition of Individual Dendritic Cell-Like Characteristics Through Discrete Signaling Pathways

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We have shown previously that calcium ionophore (CI) treatment of various myeloid origin cells results in rapid acquisition of properties associated with mature, activated dendritic cells. These properties include increased CD83 and costimulatory molecule expression, tendencies to form dendritic processes, loss of CD14 expression by monocytes, and typically an enhanced capacity to sensitize T lymphocytes to Ag. We here analyze the intracellular signaling pathways by which CI induces acquisition of such properties. Thapsigargin, which raises intracellular Ca\(^{2+}\) levels by antagonizing its sequestration, induced immunophenotypic and morphologic changes that paralleled CI treatment. CI-induced activation was broadly attenuated by the Ca\(^{2+}\) chelator EGTA and by calmodulin antagonists trifluoperazine dimaleate and W-7. However, antagonists of signaling pathways downstream to calmodulin displayed more selective inhibitory effects. Calcineurin antagonists cyclosporin A and the FK-506 analogue, asemycin, diminished costimulatory molecule and CD83 expression, as well as formation of dendritic processes in CI-treated myeloid cells, and strongly attenuated the T cell allosensitizing capacity of CI-treated HL-60 cells. These calcineurin antagonists displayed minimal effect on CI-induced CD14 down-regulation in monocytes. In contrast, the calmodulin-dependent protein kinase antagonists, K252a and KT5926, while displaying only modest effects on CI-induced costimulatory molecule and CD83 expression, strongly blocked CD14 down-regulation. These results are consistent with a Ca\(^{2+}\)-dependent mechanism for CI-induced differentiation of myeloid cells, and indicate that multiple discrete signaling pathways downstream to calcium mobilization and calmodulin activation may be essential in regulating this process.

In previous studies, we demonstrated that the addition of calcium ionophore (CI)1 to human peripheral blood monocytes or immature dendritic cells (DC) resulted in the acquisition of many immunophenotypic, morphologic, and functional properties characteristic of activated myeloid DC (1). These included the rapidly (within 20 h) up-regulated expression of CD80, CD86, CD83, CD40, and CD54 (ICAM-1), the loss of CD14 expression by monocytes, and the later (48–96 h) acquisition of dendritic processes, as well as a capacity to sensitize T lymphocytes to Ag at efficiencies equivalent to that of purified, activated DC (1). We have also shown that CI induced many similar immunophenotypic and morphologic effects in 6-day cultured human CD34\(^{+}\) bone marrow cells, the promyelocytic leukemia line, HL-60, and chronic myelogenous leukemia cells freshly obtained from patients (2).

The fact that a single class of pharmacologic agents induced the coordinate expression of a number of surface proteins associated with an activated APC phenotype, additionally promoting remarkable functional and morphologic changes, suggested the existence of an important, previously uncharacterized, signaling pathway operative in many myeloid cells. The elucidation of this pathway is critical for determining how myeloid-lineage cells at many stages of ontogeny may rapidly respond to their environment by acquiring properties typically associated with activated DC and should provide clues to the regulation, at the molecular level, of a wide variety of genes associated with APC function.

In the studies presented here, we used well-characterized pharmacologic antagonists that block Ca\(^{2+}\)-mediated signaling pathways at defined points to elucidate the mechanisms by which CI promotes the acquisition of particular DC-associated properties in a wide variety of human myeloid cells, including both transformed (leukemia) cells and nontransformed cells at various stages of ontogeny (monocytes and cultured bone marrow progenitors). Strategies employing these agents have similarly been used by others to characterize Ca\(^{2+}\)-dependent signaling pathways in both T cell and myeloid systems (3–5). The human acute myeloid leukemia line HL-60, previously shown to mimic many of the responses to CI observed in normal monocytes, proved of particular value for its ability to withstand prolonged exposure to certain drug combinations. We also performed, for purposes of confirmation, limited studies on 6-day cultured CD34\(^{+}\) bone marrow progenitor cells, because a large subpopulation of these cells acquires a number of DC characteristics upon exposure to CI (2).

Among the acquired DC characteristics we examined in these studies are the enhanced surface expression of costimulatory molecules B7.1 (CD80) and B7.2 (CD86), which exist on the surface.
of matured DC and certain other APC as counterreceptors for CD28 and CTLA-4 on T lymphocytes. We also focused on the enhanced expression of CD83, a surface protein of undetermined function whose expression is strongly associated with DC activation/differentiation. Where technically practicable, we examined additional characteristics of DC including cellular morphology and APC function. The similar effects observed in each myeloid cell type is consistent with the presence of a Ca\(^{2+}\)-triggerable activation/differentiation pathway with common elements observed among the cell types tested. This pathway appears to use a pivotal calmodulin/calcineurin/calmodulin-dependent kinase axis for transmission of its activation signal through the cytoplasm, with some CI-induced characteristics proving more sensitive to calcineurin antagonists such as cyclosporin A (CsA), and others more sensitive to antagonists of calmodulin-dependent protein kinases (CaMK). This bifurcating pathway thus appears to share some features with the putative Ca\(^{2+}\)-dependent arm of the cellular activation pathway in T lymphocytes (3, 4) and also suggests the possibility that clinically important immunosuppressive calcineurin antagonists such as CsA may interfere significantly in certain circumstances with the acquisition of activated DC characteristics in vivo.

Materials and Methods

Reagents and Ab

CI A23187 was obtained from Sigma (St. Louis, MO), Thapsigargin, EGTA, trifluoperazine dimaleate (TpD), W-7, chelerythrine chloride (Chel CI), okadaic acid, ascomycin, KT5926, KT5720, KT5823, and K252a were obtained from Calbiochem (La Jolla, CA). CsA was obtained from Sandoz (East Hanover, NJ). These agents were chosen for their established specificity in cell culture systems and determined low toxicity in closed-cell systems. Exhaustive dose-response titrations were performed for each agent in our closed-cell myeloid cultures to determine the dose range in which toxicity was low and phenotypic modulations were reproducibly observed. Where available (see Results), drug analogues with identical toxicity profiles but different enzymatic specificities were examined to enhance the interpretation of data. (3–8). All biochemical reagent stocks were stored at -70°C before use, and individual aliquots were thawed only once immediately before culture addition. In some cultures, assays were supplemented with the following recombinant human (rh) cytokines: rh-\(\alpha\)-kit ligand (sp. act. 2.7 × 10\(^{5}\) U/mg), rhTNF-\(\alpha\) (sp. act. 1.1 × 10\(^{5}\) U/mg), and rhIL-4 (sp. act. 2.9 × 10\(^{5}\) U/mg) (all R&D Systems, Minneapolis, MN), and rhGM-CSF (sp. act. 5.6 × 10\(^{5}\) U/mg (Amgen, Thousand Oaks, CA).

Culture and evaluation of HL-60

The human promyelocytic leukemia-derived cell line, HL-60 (CCL 240) was obtained from American Type Culture Collection (Manassas, VA) and maintained as described previously (2) in RPMI 1640 with 10% FCS in supplemented with the following recombinant human (rh) cytokines: rh-\(\alpha\)-kit ligand, 10 ng/ml rhGM-CSF, and 10 ng/ml rhTNF-\(\alpha\)-fresh medium and factors but different enzymatic specificities were examined to enhance the interpretation of data. (3–8). All biochemical reagent stocks were stored at -70°C before use, and individual aliquots were thawed only once immediately before culture addition. In some cultures, assays were supplemented with the following recombinant human (rh) cytokines: rh-\(\alpha\)-kit ligand (sp. act. 2.7 × 10\(^{5}\) U/mg), rhTNF-\(\alpha\) (sp. act. 1.1 × 10\(^{5}\) U/mg), and rhIL-4 (sp. act. 2.9 × 10\(^{5}\) U/mg) (all R&D Systems, Minneapolis, MN), and rhGM-CSF (sp. act. 5.6 × 10\(^{5}\) U/mg (Amgen, Thousand Oaks, CA).

Culture and evaluation of human peripheral blood monocytes

Human CD14\(^{+}\)CD3\(^{+}\) peripheral blood monocytes from healthy volunteers (typically 95% purity) were prepared by leukapheresis and elutriation and cryopreserved as described previously (1). Thawed monocytes from these donors were studied under a variety of culture conditions. Preliminary experiments established that maximum viability was achieved during pharmacologic inhibitor studies by modifying our prior culture method (1) as follows: monocytes were plated in serum-free medium (Macrophage-SFM; Life Technologies, Grand Island, NY) with 50 ng/ml added rhGM-CSF (H. Nguy et al., manuscript in preparation), aliquoting cells at 3 × 10\(^{5}\) or 5 × 10\(^{5}\) per well in 24-well cluster plates (Costar). The monocytes were exposed to optimal doses of the inhibitory agents overnight before CI coexposure. The optimal activating dose of CI A23187 was 150 ng/ml for monocytes plated at 3 × 10\(^{5}\) cells/well and 225 ng/ml for monocytes plated at 5 × 10\(^{5}\) cells/well. Finally, the monocytes were harvested 20 h after addition of CI to provide maximum viability during analysis. Qualitatively similar results, but typically with lower viability, were observed in other media (e.g., RPMI 1640 with 5% human AB serum (1)), in the absence of rhGM-CSF, when CI treatment was initiated sooner after addition of the inhibitory agents, or when CI treatment was continued in the presence of the inhibitory agents beyond 20 h (not shown).

Culture and evaluation of human CD34\(^{+}\) cells

Normal donor bone marrow cells enriched for CD34\(^{+}\) cells (purity 91–98%) were obtained from Poietic Technologies (Gaithersburg, MD) or from cadaveric vertebral bodies by positive immunomagnetic selection using a CD34 isolation kit (Miltenyi Biotech, Auburn, CA). To promote enrichment and expansion of intermediate DC precursors (9, 10), CD34\(^{+}\) cells were resuspended to 4 × 10\(^{5}\) cells/ml and cultured for 6 days in an enriched IMDM (11) supplemented with 10% FCS (HyClone, Logan, UT), 20 ng/ml rh-\(\alpha\)-kit ligand, 10 ng/ml rhGM-CSF, and 10 ng/ml rhTNF-\(\alpha\)-fresh medium and factors but different enzymatic specificities were examined to enhance the interpretation of data. (3–8). All biochemical reagent stocks were stored at -70°C before use, and individual aliquots were thawed only once immediately before culture addition. In some cultures, assays were supplemented with the following recombinant human (rh) cytokines: rh-\(\alpha\)-kit ligand (sp. act. 2.7 × 10\(^{5}\) U/mg), rhTNF-\(\alpha\) (sp. act. 1.1 × 10\(^{5}\) U/mg), and rhIL-4 (sp. act. 2.9 × 10\(^{5}\) U/mg) (all R&D Systems, Minneapolis, MN), and rhGM-CSF (sp. act. 5.6 × 10\(^{5}\) U/mg (Amgen, Thousand Oaks, CA).

Abs and FACS analysis

The methods employed and the Ab reagents used against CD80, CD86, CD14, ICAM-1 (CD54), CD40, and CD1a were identical to previous published reports (1) except that PE-conjugated anti-CD38 Ab (Coulter) was substituted for indirect staining.

Photomicroscopy

Cells to be analyzed were resuspended in fresh medium and transferred to Lab-Tek 8 glass chamber slides (Nunc, Naperville, IL) previously coated with 1% polylysine (Sigma) and incubated for 30 min at 37°C, 5% CO\(_2\). Slides were then examined and photographed with an Olympus IX-70 inverted microscope using Nomarski differential interference contrast optics. Some preparations were fixed in absolute ethanol and Wright’s stained before photography.

Allosensitization studies

Human T lymphocytes obtained from lymphocyte-rich elutriation fractions were purified using T cell isolation columns (R&D Systems) (1). HL-60 cells preincubated with or without CsA (0.05 \(\mu\)g/ml) were subjected to CI (180 ng/ml) for 72 h and harvested. To ensure that any observed inhibitory effects of CsA resulted from direct inhibition of HL-60 cells, rather than from CsA “carryover” into T cell coculture, positive control HL-60 cells, which were CsA “carryover” into T cell coculture, control positive control HL-60 cells treated with CI were CsA-harvested at the time of harvest to an equivalent dose of CsA (0.05 \(\mu\)g/ml) and then immediately washed twice in CsA-free CM in parallel with all other treatment groups. This procedure normalized the amount of trace CsA carried over from CsA-harvested CI-treated CsA-harvested CI-treated HL-60 cells with or without CsA (0.05 \(\mu\)g/ml) in each well. The optimal activating dose of CI (375 ng/ml) was variably added 45 min later. Wells were harvested 20 h later for analysis.

Results

Treatments that modulate biologic availability of intracellular Ca\(^{2+}\) or inhibit the regulatory protein calmodulin broadly affect HL-60 expression of costimulatory, activation, and adhesion molecules

We had previously demonstrated (2) that treatment of the acute promyelocytic leukemia line HL-60 with optimized doses of CI...
A23187 (188–375 ng/ml) led to the rapid up-regulation of surface CD80, CD86, CD54, and CD83 within 20 h (see Figs. 1 and 2). During this culture period, CI-induced increased expression of nuclear-localized RelB was also observed (L. Lyakh et al., manuscript in preparation). At later time points (40–96 h), additional CI-induced modulations occurred, including the enhanced expression of CD40, CD1a, the formation of dendritic processes, and an enhanced capacity to allosensitize T cells (2) (see Figs. 3, 4, and 5).

To clarify the role of calcium mobilization in CI-induced differentiation, we began the present studies by examining the effects of various calcium-dependent signaling pathway agonists and antagonists. When, instead of CI treatment, HL-60 cells were exposed to an optimized dose of thapsigargin (250 nM), a compound which raises intracellular Ca\(^{2+}\) concentrations by impeding its sequestration (5, 12), the cells also up-regulated their expression of CD80, CD86, CD54, and CD83 (Fig. 1) and furthermore acquired...
costimulatory molecules and CD83 on HL-60 cells using the participated in CI-induced up-regulation of surface expression of (CaMK I, II, and IV/Gr). We looked for evidence that CaMK(s) family of calmodulin-dependent serine-threonine protein kinases Calmodulin is known to regulate the activity of members of a costimulatory molecules and CD83

Antagonists of CaMK modestly inhibit CI-induced expression of costimulatory molecules and CD83

Calmodulin is known to regulate the activity of members of a family of calmodulin-dependent serine-threonine protein kinases (CaMK I, II, and IV/Gr). We looked for evidence that CaMK(s) participated in CI-induced up-regulation of surface expression of costimulatory molecules and CD83 on HL-60 cells using the CaMK inhibitor K252a (IC_{50} = 2.8 nM) (8). K252a had a very modest yet consistent capacity to inhibit CI-induced up-regulation of CD80, CD86, and CD83 (Fig. 2). A second CaMK inhibitor, KT5926 (IC_{50} = 5.9 nM) (4, 8), had similar modest effects on the expression of these molecules (not shown). In contrast to EGTA and calmodulin inhibitors, optimized doses of CaMK inhibitors were not notably toxic to HL-60 during longer culture periods; nonetheless, they did not block the CI-induced acquisition of dendritic processes apparent at the end of such cultures (not shown). These results suggested that calmodulin-dependent kinase(s) may play only a subsidiary role in the particular modulations observed when HL-60 is treated with calcium-mobilizing agents.

Antagonists of protein phosphatase 2B (calcineurin) strongly inhibit immunophenotypic, morphologic, and functional activation of HL-60 induced by calcium-mobilizing agents

An enzyme target of the regulatory protein calmodulin is the serine-threonine protein phosphatase, calcineurin (PP2B) (15). Two compounds which inhibit the activity of calcineurin are the well-characterized immunosuppressive agents, cyclosporin A and the FK-506 analogue, ascomycin (3, 5, 16); inhibition is achieved via complexes these agents form with cellular proteins (cyclophilin and FK506-binding protein, respectively). Therefore, we tested the ability of these drugs to interfere with the CI-induced expression of DC-associated surface protein in HL-60 cells and compared these agents to okadaic acid, a compound which has little activity against calcineurin, but instead inhibits protein phosphatases 1 (IC_{50} = 10–20 nM) and 2A (IC_{50} = 0.1 nM). Preincubation with 10 nM okadaic acid had virtually no effect on the CI-induced expression of CD80, CD86, or CD83 in HL-60 cells after 20 h CI treatment (Fig. 2). On the other hand, ascomycin caused an almost total ablation of CI-induced expression of CD80, as well as strong inhibition of CD86 and CD83 expression at 20 h (Fig. 2). The second calcineurin antagonist, CsA, had virtually identical effects at 20 h (not shown). Because, in contrast to EGTA and calmodulin inhibitors, optimized doses of calcineurin antagonists were not notably toxic to HL-60 during longer culture periods, we next tested the capacity of CsA to block CI-induced alterations that are not observed until several days posttreatment, as well as CsA’s ability to sustain inhibition of otherwise early appearing immunophenotypic characteristics. HL-60 cells pretreated for 45 min with CsA before a 96-h exposure to A23187 not only remained inhibited in their expression of CD80, CD86, and CD83, but also failed to express de novo CD40 and CD1a, which normally appear after 72–96 h exposure to CI (Fig. 3) (2).

We also investigated the effect of CsA on acquisition of morphologic features characteristic of DC induced by calcium-mobilizing agents. As reported previously, untreated HL-60 cells cultured for 72 h and transferred onto polylysine-coated glass slides displayed no dendritic processes (Fig. 4A); the addition of CsA alone (0.5 μg/ml) throughout the culture period had no apparent effect (Fig. 4B). In contrast, as reported previously, cells treated with an optimal dose of A23187 (180 ng/ml) displayed pronounced morphologic alterations, with numerous cellular processes typical of activated DC (Fig. 4C). However, when HL-60 cells were preincubated with CsA for 45 min before the addition of
CI, such striking morphologic changes were virtually absent (Fig. 4D). Similar CsA-blockable effects were seen when dendritic morphology was induced in HL-60 with thapsigargin (Fig. 4, E–G).

Our previous studies demonstrated that CI treatment of HL-60 increased its allosensitizing capacity by up to 25-fold, although on a per cell added basis CI-treated HL-60 remained less allostimulatory than CI-treated normal monocytes, consistent with the aberrant MHC regulation often observed in this type of leukemia (2, 17–19). Such dysregulation could be corrected partially by combining CI treatment with adjunct rhGM-CSF and rhIFN-γ, further tripling HL-60’s allosensitizing capacity (2). In the present studies, we examined the functional impact of CsA on HL-60 cells treated with CI alone. HL-60 cells were either untreated or treated with CI for 72 h in the presence or absence of 0.05 μg/ml CsA, the lowest dose of this drug in dose-titration studies found to give maximal sustained inhibition of HL-60 cells (not shown). After rigorous washing and irradiation, treated HL-60 cells were tested for their capacity to induce normal donor T lymphocyte alloproliferation. As demonstrated previously (2), untreated HL-60 displayed a negligible capacity to allosensitize T lymphocytes (Fig. 5). In contrast, HL-60 cells treated with CI demonstrated markedly enhanced allosensitizing capacity. However, coexposure to CsA during CI treatment virtually abolished the enhanced allosensitizing capacity conferred by CI treatment. The observed inhibition was not attributable to carryover of CsA from the antecedent HL-60 culture into the T cell coculture (see Materials and Methods).
treatment of monocytes was conducted in Ca²⁺-free medium (not shown). As similarly observed in HL-60 (Fig. 1), thapsigargin treatment, which raises intracellular Ca²⁺ levels by antagonizing its sequestration, resulted in increased monocyte expression of CD80, CD86, CD54, and CD83, as well as down-regulated CD40 expression, similar to modulations induced by CI treatment (not shown). Such studies confirmed that calcium mobilization can serve as a profound differentiating stimulus for monocytes. However, concomitant exposure of monocytes both to CI and to calcium signaling pathway antagonists proved to be more toxic to

Calcium pathway antagonists downstream to calmodulin have discrete effects on costimulatory molecule and CD83 vs CD14 regulation in peripheral blood monocytes

As previously reported, treatment of normal peripheral blood monocytes with CI induces many phenotypic modulations typical of activated myeloid DC (1). Therefore, we tested the effects of individual pharmacologic agents on CI-treated normal human peripheral blood monocytes to determine whether their response would parallel those observed in HL-60 cells. Because, in contrast to HL-60 and bone marrow cells, monocytes constitutively and uniformly express high levels of CD14, down-regulation of CD40 expression induced by CI treatment could also be analyzed in this population (1).

As for HL-60, CI activation of monocytes proved to be calcium-dependent. Up-regulation of costimulatory molecule expression and CD14 down-regulation were markedly impaired when CI treatment of monocytes was conducted in Ca²⁺-free medium (not shown). As similarly observed in HL-60 (Fig. 1), thapsigargin treatment, which raises intracellular Ca²⁺ levels by antagonizing its sequestration, resulted in increased monocyte expression of CD80, CD86, CD54, and CD83, as well as down-regulated CD40 expression, similar to modulations induced by CI treatment (not shown). Such studies confirmed that calcium mobilization can serve as a profound differentiating stimulus for monocytes. However, concomitant exposure of monocytes both to CI and to calcium signaling pathway antagonists proved to be more toxic to
monocytes than to the HL-60 cell line. Therefore, it was necessary to modify our existing monocyte culture system to improve viability during multiagent exposure.

This proved possible by substituting serum-free medium for human serum-containing medium (1). In either culture condition, CI treatment induced monocytes to up-regulate CD80, CD86, and CD83, as well as markedly down-regulate CD14, within 20 h of CI exposure. However, several differences were observed: 1) a lower concentration of CI proved optimal for activation in serum-free medium (90–180 ng/ml vs 375–750 ng/ml with 10% human serum present); 2) whereas early (0–8 h) exposure to CI was necessary in serum-containing medium to achieve maximal activation, CI exposure could be delayed for 24 h or longer in serum-free medium without impeding subsequent acquisition of DC characteristics (H. Nguyen et al., manuscript in preparation); 3) serum-free medium enabled preservation of high viability when monocytes were exposed to combinations of CI and inhibitors, particularly when the monocytes were pretreated overnight with the inhibitors in the presence of rhGM-CSF, followed by a limited (20–30 h) exposure to CI. This enabled meaningful study of those immunophenotypic modulations that occurred within 20 h of CI exposure. Similar results were also obtained when pharmacologic inhibitors were added synchronously with CI treatment, when rhGM-CSF was not included, and when CI treatment was extended to 40 h, but with less acceptable viability.

When cultured in serum-free medium alone for 20 h, normal human monocytes typically maintained high CD14 expression, failed to express detectable levels of CD80, displayed low/absent CD83, and down-regulated expression of CD86 (Fig. 8). When rhGM-CSF alone was also added during the culture, a modest but variable degree of CD14 down-regulation typically occurred whereas significant CD80 or CD83 expression did not occur, and little alteration was observed in CD86 expression (Figs. 8 and 9). In contrast, monocytes treated with CI for 20 h down-regulated CD14 expression nearly to control levels, expressed de novo CD80, and up-regulated CD86 and CD83 expression, similar to effects previously demonstrated in serum-containing medium (1). Paralleling prior observations in HL-60 cells (Fig. 2), exposure of monocyte cultures to the calmodulin inhibitor W7 or the calcineurin inhibitor CsA markedly inhibited CI-induced up-regulation of CD80, CD86, and to a lesser and more variable extent CD83 expression (Figs. 8 and 9). Strikingly, however, CI-induced CD14 down-regulation was inhibited by the calmodulin antagonist, W-7, but minimally affected by the calcineurin antagonist CsA. In contrast, the CaMK antagonist KT5926, like W-7, markedly inhibited the CD14 down-regulation induced by CI (Fig. 8). Unlike W-7, KT5926 had little or no effect on CI-induced expression of CD80, CD86, or CD83 (Fig. 8). Structurally related compounds with reduced activity against CaMK (the protein kinase G antagonist KT5823 and the protein kinase A antagonist KT5720) as well as the protein kinase C antagonist Chel CI displayed little suppression of A23187-induced CD14 down-regulation (Fig. 8). We also examined the inhibitory effects of a second CaMK antagonist, K252a (Fig. 9). This compound, in comparison to CsA, behaved identically to KT5926 in its selective block of CD14 down-regulation. Not only were costimulatory molecule and CD83 expression not appreciably inhibited by K252a, but in roughly half the donors tested, CD80 and CD86 expression was modestly enhanced, even in the face of profoundly blocked CD14 down-regulation (Fig. 9).

Discussion

The present studies employed a variety of pharmacologic agents, predominantly enzymatic inhibitors, to gain insight into the mechanism(s) by which CI induce a variety of myeloid-origin cells to acquire many DC characteristics. Although the data derived by studying such inhibitors in whole-cell systems are by nature indirect, the judicious comparison of structurally unrelated agents with similar specificity, as well as structural analogues with alternative specificities, markedly enhances the meaningful interpretation of results (3, 4, 20–22). Furthermore, the use of very conservative concentrations of each inhibitor increases the likelihood that observations in whole-cell studies will reflect the specific activity of these agents in open-cell studies.

We originally hypothesized that the immunophenotypic, morphologic, and functional changes in human myeloid cells induced by A23187 were linked to this compound’s well-characterized properties as an intracellular calcium-mobilizing agent. This was by no means a foregone conclusion, because secondary pharmacologic effects could be responsible for these observed changes. Therefore, we conducted experiments to determine the ability of...
Ca^{2+} per se to trigger differentiation in myeloid cells. Thapsigargin is a compound that raises cytoplasmic Ca^{2+} levels not through the permeabilization of cell membranes to Ca^{2+} (as do CI agents such as A23187), but rather through the pharmacologic inhibition of the smooth endoplasmic reticulum-associated ATP-dependent Ca^{2+} pump (12), thereby preventing endoplasmic reticulum sequestration of Ca^{2+} and causing higher cytoplasmic levels of Ca^{2+}. Consistent with the hypothesis that CI produces the observed effects in myeloid cells through Ca^{2+} mobilization, thapsigargin induced qualitatively similar changes in human peripheral blood monocytes and HL-60 cells (Figs. 1 and 4). Furthermore, CI-induced acquisition of DC-associated characteristics was sensitive to the chelation of free Ca^{2+} by EGTA (Fig. 2) or its removal from CM by dialysis (data not shown).

Such results render Ca^{2+} mobilization the probable explanation for the observed effects of CI on myeloid cells. Furthermore, the Ca^{2+} signaling pathway antagonists employed in the present studies often resulted in impressive, if sometimes incomplete, inhibition of many DC-associated characteristics induced by calcium-mobilizing agents. Because, in closed-cell studies, complete

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Effect of pharmacologic inhibitors on A23187-induced surface molecule expression in human peripheral blood monocytes. Previously cryopreserved human peripheral blood monocytes from a healthy volunteer were thawed and resuspended in Life Technologies Macrophage-SFM at 3 × 10^6 per well in 24-well cluster plates as described in Materials and Methods. Then, 45 min after culture initiation, the following inhibitors were each added at the designated final concentration, which previously had been found to maximize inhibition while minimizing toxicity: W-7 (10 μM), CsA (0.5 μg/ml), KT5926, KT5823, and KT5720 (each at 1 μM), and Chel Cl (10 μM) 45 min after inhibitor addition, rhGM-CSF (final concentration 50 ng/ml) was added to selected groups, and 20 h later A23187 (final concentration 150 ng/ml) was added to selected groups. After 40 h total culture period, cells were harvested and analyzed by FACS as described above. Before culture, monocytes had an identical profile to the “No Treatment” group after 40 h culture, except for higher initial expression of CD86 (not shown). Data is representative of three to eight experiments for each tested reagent.
enzymatic inhibition cannot always be achieved with maintained high cell viability, we cannot rule out the formal possibility of some Ca$^{2+}$-independent differentiating effects resulting from CI treatment. Nonetheless, the bulk of evidence points to a leading role for Ca$^{2+}$ mobilization in this process.

There are several recognized mechanisms whereby increased levels of cytoplasmic Ca$^{2+}$ may play a role in regulating gene expression. One of the best-characterized systems is the transcriptional activation of the IL-2 gene in T lymphocytes induced through TCR stimulation (23, 24). Increased intracellular Ca$^{2+}$ levels activate the regulatory protein calmodulin, which can bind to and positively modulate the activity of multiple enzymatic targets. Among such targets are calmodulin-dependent serine/threonine protein kinases (CaMKs). In particular, CaMK IV has been shown in T lymphocytes to induce or contribute to induction of both c-jun (25) as well as p21ras-independent c-fos transcriptional activation (26). Both c-fos and c-jun are critical components of the transcriptional activator protein, AP-1, which participates in the regulation of a variety of genes. Calmodulin also positively modulates the activity of the serine-threonine protein phosphatase 2B, calcineurin (15). Unlike many protein phosphatases, calcineurin appears to have a very narrow substrate specificity (27). One such substrate, NFAT (28), is fundamental for subsequent IL-2 gene transcriptional activation in T lymphocytes. Dephosphorylation of NFAT by activated calcineurin appears to unmask a nuclear localization signal, leading to the translocation of NFAT across the nuclear membrane and into the nucleus. There, NFAT may form critical associations with AP-1 and interact with appropriate binding sites on the IL-2 promoter region. Therefore, we used a number of well-characterized Ca$^{2+}$ signaling pathway antagonists to look for evidence of a calmodulin-calcineurin-CaMK signaling axis in CI-induced myeloid differentiation. An analogous approach, using pharmacologic antagonists of protein kinase C-dependent signaling pathways, was recently employed successfully by others (20) to infer protein kinase C’s role in phorbol ester-induced acquisition of DC characteristics by in vitro cultivated human bone marrow progenitors.

In the present studies, our ability to monitor effects of the calcium chelator EGTA and the calmodulin antagonists W-7 and TpD were limited to 24 h treatments, due to associated toxicities during longer cultures. Furthermore, only for HL-60 did it prove possible to maintain viability during longer cultures that incorporated exposure to calcineurin antagonists or CaMK antagonists. Within these timing constraints, we were nonetheless able to perform key studies of costimulatory molecule and CD83 regulation not only in the relatively robust HL-60 cell line, but also in normal bone marrow-derived myeloid progenitors and peripheral blood monocytes. Our combined observations among these myeloid cells, coupled with our studies of CD14 regulation in monocytes, are consistent with the presence of a calcium-dependent signaling pathway, which can lead to the acquisition of DC-like characteristics in human myeloid cells. However, our studies employing antagonists of downstream regulatory targets of calmodulin suggest that this pathway is at least bifurcary in nature, with the enhanced expression of costimulatory molecules and CD83 proving more sensitive to calcineurin antagonists, and the down-regulation of CD14 expression in monocytes conversely more sensitive to antagonists of CaMKs.

We were also able to investigate the effects of prolonged exposure to CI plus calcineurin or CaMK inhibitors in the HL-60 cell line, due to the latter’s ability to maintain high viability in the face of prolonged treatments.
of such treatment. Notably, calcineurin antagonists inhibited CI-induced acquisition of dendritic processes in HL-60 and also prevented CI enhancement of HL-60’s T cell-sensitizing capacity. Such studies suggest that activation of the calcineurin pathway can, at least in HL-60, lead not only to the expression of individual molecules associated with enhanced Ag presentation, but also to the initiation of a multifaceted differentiation program. Consistent with this hypothesis, we have recently determined that the induced expression of nuclear-localized RelB in both HL-60 and monocytes, demonstrable within 20 h of CI treatment, is also significantly blocked by cotreatment with calcineurin antagonists (L. Lyakh et al., manuscript in preparation). Such data reinforce the possibility that calcineurin activation is critical to the full acquisition of DC characteristics in myeloid cells following calcium mobilization stimuli.

Clinically important antagonists of calcineurin-dependent signaling pathways, such as CsA and FK-506 are already used routinely to attenuate organ transplant rejection and to treat autoimmune disease. Although it is believed that these agents act in vivo principally by interfering with the previously described calcium/calcineurin-dependent T cell activation pathway, other investigators have provided evidence that calcineurin antagonists can also affect the maturation/activation of APC. For example, topically applied FK-506 was observed to inhibit the expression of costimulatory molecules on epidermal APC in a murine contact-sensitivity model (29), and Panhans et al. (30) have described FK-506 suppression of CD80 and CD86 expression by in vitro cultivated human Langerhans cells. The data presented in such studies as well as in our own are consistent with the hypothesis that a wide variety of activated myeloid cell characteristics, including the expression of costimulatory molecules, the development of dendritic processes, and perhaps the enhanced capacity to sensitize T cells can be regulated through a pathway that is sensitive to calcineurin antagonists. In addition, the apparent delayed toxicity observed in vitro when monocytes are calcium-mobilized in the presence of CsA raises the possibility that CsA may act therapeutically not only by blocking calcium-activated DC differentiation, but also by killing calcium-activated APC. Thus, some of the clinically observed immunosuppressive effects of CsA and FK-506 may be exerted through suppression of myeloid APCs as well as T lymphocytes.

Previous studies by others have suggested that the expression of CD80 (31) and CD54 (32, 33) in a variety of cell types may be regulated, at least in part, at the transcriptional level. Because the data presented here suggest that elements of the Ca\(^{2+}\)-dependent signaling pathways found in myeloid cells bear similarities to those found in T lymphocytes (3, 4), it seems possible or even likely that some of the same or similar transcriptional factors used by T lymphocytes to regulate genes such as IL-2 might also transcriptionally regulate CD80, CD54, and additional surface proteins in myeloid cells. The striking inhibition by CsA and an FK506 analogue of CI-induced alterations in surface molecule expression by myeloid cells raises the possibility that a likely candidate transcriptional factor may be NFAT or an NFAT-like protein. It should be noted that the tissue distribution of NFAT is by no means restricted to T lymphocytes. In fact, NFAT has been detected in some murine myeloid cell types including monocytes (34), and we have observed both constitutively produced and CI-inducible forms of NFAT in HL-60 cells (L. Lyakh et al., manuscript in preparation). However, an alternative explanation is that NFAT plays a subsidiary or even no role in the regulation of these processes in myeloid cells. Recent studies regarding the regulation of human CD80 in B lymphocytes by cAMP strongly indicate a central role for NF-kB in the inducible expression of this gene (31). Other studies have suggested that phorbol esters, LPS, and TNF-α regulate ICAM-1 expression through NF-κB as well (35, 36). Whereas there is presently no evidence that cAMP or treatment with LPS or phorbol esters achieved CD80 or ICAM-1 up-regulation through elevations in intracellular Ca\(^{2+}\) levels, Ca\(^{2+}\)-mobilizing agents have been observed to contribute to the activation of NF-κB (37). Additionally, some studies have shown that the activation of NF-κB can also be inhibited by CsA, at least in part through modulating the degradation of the NF-κB inhibitory subunit IκB (38). Other studies have implicated RelB, a member of the NF-κB family, as playing a crucial role in DC development and activation (39–42). Indeed, we have observed enhanced levels of RelB induced by CI in both HL-60 and cultured peripheral blood monocytes (L. Lyakh et al., manuscript in preparation). We are currently evaluating the role of NFAT, NF-κB, and other nuclear factors in CI-induced myeloid differentiation.

Finally, it is of obvious interest to determine whether the calcium-dependent signaling pathway we have described here is distinct from, or overlaps with, activation pathways induced by the several cytokine/ligand treatments previously shown to induce DC characteristics in myeloid cells (43–56). Because cytokine/ligand treatments typically require multiday culture to induce DC characteristics, it is challenging to incorporate studies with inhibitory agents whose toxicity becomes more pronounced with prolonged treatment. Logical candidates for cytokine/ligand treatments employing calcium mobilization pathways are those whose action displays similar rapidity to CI or thapsigargin treatment. Interestingly, a recent study focusing on the transendothelial migration of human monocytes demonstrated that monocytes can acquire pronounced DC characteristics within 2 days of exposure to collagen matrix and endothelial cells (57). This rapid time frame of conversion kinetically resembles CI-induced differentiation more than the lengthy differentiation process associated with typical cytokine/ligand treatments. We are continuing our efforts to define culture conditions to determine those myeloid differentiating treatments that are sensitive to calcium-pathway antagonists. Furthermore, refinements in our understanding of calcium-dependent activation pathways, especially if additional signaling branchpoints are found to exist, may allow specific pharmacologic agents to be used as modulatory adjuncts to prepare APC with highly defined sensitizing, tolerizing, or apoptosing characteristics.

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References


