Protein Kinase C βII Plays an Essential Role in Dendritic Cell Differentiation and Autoregulates Its Own Expression*

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Dendritic cells (DC) arise from a diverse group of hematopoietic progenitors and have marked phenotypic and functional heterogeneity. The signal transduction pathways that regulate the ability of progenitors to undergo DC differentiation, as well as the specific characteristics of the resulting DC, are only beginning to be characterized. We have found previously that activation of protein kinase C (PKC) by cytokines or phorbol esters drives normal human CD34+ hematopoietic progenitors and myeloid leukemic blasts (KG1, K562 cell lines, and primary patient blasts) to differentiate into DC. We now report that PKC activation is also required for cytokine-driven DC differentiation from monocytes. Of theα,βII isoforms, only PKC-βII was consistently activated by DC differentiation-inducing stimuli in normal and leukemic progenitors. Transfection of PKC-βII into the differentiation-resistant KG1a subline restored the ability to undergo DC differentiation in a signal strength-dependent fashion as follows: 1) by development of characteristic morphology; 2) the up-regulation of DC surface markers; 3) the induction of expression of the NFκB member Rel B; and 4) the potent ability to stimulate allo-reactive T cells. Most unexpectedly, the restoration of PKC-βII signaling in KG1a was not directly due to overexpression of the transfected classical PKC (α, βII, or γ) but rather through induction of endogenous PKC-β gene expression by the transfected classical PKC. The mechanism of this positive autoregulation involves up-regulation of PKC-β promoter activity by constitutive PKC signaling. These findings indicate that the regulation of PKC-βII expression and signaling play critical roles in mediating progenitor to DC differentiation.

Dendritic cells are professional antigen-presenting cells of hematopoietic origin that play a central role in the initiation and regulation of the adaptive immune responses (1–6). Unlike most hematopoietic cells that arise by classical divergent hematopoiesis, human DC can arise from a variety of different immediate precursors. These include CD34+ and CD34+CD86+ hematopoietic progenitor cells (HPC) (7, 8), myeloid progenitors (CFU-M/DC and CFU-DC) (9, 10), peripheral blood monocytes (11–13), and immature neutrophils (14) in what could be considered “convergent” hematopoiesis. In addition to this range of normal progenitors, myeloid leukemia blasts arrested in all stages of differentiation can be driven to undergo DC differentiation (15). On top of this complexity of progenitors, the process of differentiation itself can give rise to DC that are quite different in both their phenotype and function (10), and manipulation of specific signal transduction pathways during differentiation can yield DC with markedly altered function (5, 16). Conversely, exogenous stimuli can inhibit progenitor to DC differentiation in both normal (i.e. wound healing) and pathologic (tumors) settings, resulting in suppression of adaptive immune responses (17).

The intracellular signaling pathways that regulate progenitor to DC differentiation are only beginning to be defined. It has been well established that specific membrane receptors (GM-CSF, IL-4, TNF-α, FLT3L, CD40, etc.) can be activated in vitro by their exogenous ligands to initiate DC differentiation (7, 11, 18). Downstream of these membrane proximal events, signal transduction involving STAT3 (19, 20), intracellular calcium flux (21–24), specific NFκB family members (25), and Notch family members (26) has been implicated. However, how the various pathways are arrayed in relationship to each other has not been well characterized. We have shown previously that protein kinase C (PKC) activation is necessary and sufficient to drive human CD34+ HPC→DC differentiation (8). It is a likely downstream component of cytokine-mediated differentiation, as PKC inhibitors preferentially block DC differentiation in human CD34+ HPC driven by GM-CSF + TNF-α (27). PKC has been shown to be a downstream component of signaling through the GM-CSF receptor (28) and CD40 (29–31) in other cell types, and these receptors are also centrally involved in DC differentiation. Consistent with a role for PKC in DC lineage commitment, we and others have found that direct activation of PKC signaling by the 2,3-diacylglycerol (DAG) analogs (including phorbol 12-myristate 13-acetate (PMA) and bryostatin-1) in normal and myeloid leukemia blasts (27, 34, 35) specifically generates DC in the absence of cell proliferation. Of particular interest is the CD34+CD86+ myeloid leukemia cell line KG1, which we and others have found can be induced to undergo differentiation to fully functional dendritic cells that express DC-specific receptor/chemokines.

DC, dendritic cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; EMSA, electromobility shift assay; GM-CSF, granulocyte colony-stimulating factor; MHC, major histocompatibility complex; GFP, green fluorescent protein; TNF, tumor necrosis factor; IFN, interferon; cPKC, classical PKC; IL, interleukin; DAG, the 2,3-diacylglycerol; bis, bisindolylmaleimide I.
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Confocal Fluorescent Microscopy—CD34⁺ HPC, monocytes, K562, or KG1 were treated as indicated, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 1% bovine serum albumin, and stained with antibodies against CD11c, PKC-βII, PKC-α, PKC-βI, PKC-δ, PKC-ε, PKC-γ and CD34 (all from Immunotech, Westbrook, ME). Cells were then stained with fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) together with Texas Red-X phalloidin and TO Pro-3. Cells were imaged by confocal microscopy (Zeiss LSM-510). Images of typical mid-cell sections are shown.

Electromobility Shift Assay (EMSA)—EMSAs were done for NFκB family members as described previously (27). Briefly, equal amounts of protein from nuclear lysates were incubated with 32P-labeled primers containing consensus NFκB-binding sites (GAT CCA ACG GCA GGG GAA TAC CCC TCT CCT TA) or specific probes. For supershift assays, samples were first incubated with anti-Rel B antibody (Santa Cruz Biotechnology). Samples were visualized by autoradiography.

Northern Blot—Northern blot analysis was performed as described previously (47). Briefly, total RNA was isolated (RNeasy, Qiagen, Valencia, CA) and equalized by serial dilution and ethidium bromide visualization, separated on 1% formaldehyde/agarose gel, transferred to nylon membranes, and hybridized against specific PKC-βII-specific or glyceraldehyde-3-phosphate dehydrogenase probes.

PKC Kinase Assay—PKC kinase activity levels were determined using the PKC Kinase Assay Kit (Promega), according to manufacturer's instructions. Briefly, 7 × 10⁶ cells each of KG1, KG1a, KG1a 3.1 (vector alone), and KG1a E9 (PKC-βII transfected) were homogenized in 25 mM Tris buffer (pH 7.4) containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 50 µg/ml phenylmethylsulfonyl fluoride and purified through a DEAE column in a 5-ml final elute volume. A 5-µl aliquot of each sample was incubated in a buffer containing calcium, peptide target, and α-[γ-32P]ATP with and without the PKC inhibitors bisindolylmaleimide I (5 µM). Cell viability was assessed by trypan blue dye exclusion.

Flow Cytometry—Adherent and adherent-depleted cells were harvested using 3 mM EDTA and stained as reported previously (27) using the following monoclonal antibodies: HLA-ABC and HLA-DR (both from VMRD Inc., Pullman, WA), CD11c, CD14, CD34, CD40, CD80, CD83, and CD86 (all from Immunotech, Westbrook, ME). Appropriate isotype-matched antibodies were used as controls. 10,000 live cells were analyzed on a Coulter XL flow cytometer (Coulter, Hialeah, FL) using the software supplied by the manufacturer.

For intracellular cytokine staining, cells were cultured in media alone or with γ-interferon (250 units/ml, R & D Systems) for 72 h. Cells were then permeabilized using Cytofix/Cytoperm solution (BD Biosciences), as per the manufacturer's instructions, and stained for MHC class I and class II expression as above for flow cytometry.

T Cell Proliferation—Allogeneic T cell proliferation was performed as described previously (27). Progenitors were differentiated as indicated and γ-irradiated (5000 rad). Normal donor resting allogeneic T cells were purified (≥90% CD3⁺) as described previously (46) and co-cultured at various ratios with differentiated DC for 72 h. 1 µCi/well [methyl-3H]thymidine was added for the final 18 h of culture, and incorporation was measured using the Beta Plate scintillation counting system (Wallac Inc., Gaithersburg, MD). Where indicated, T cells alone stimulated with PMA (10 ng/ml) and ionomycin (100 ng/ml) were used as positive controls. The data are presented as the mean of triplicate wells ± S.D.
RESULTS

**Blockade of PKC Activation Inhibits Cytokine-mediated Monocyte→DC Differentiation**—We have shown previously that the PKC-specific inhibitor bisindolylmaleimide I (bis) could inhibit cytokine-driven DC differentiation in primary human monocytes (CD34/HPC) and primary human monocytes (27). To assess whether this was unique to CD34/HPC or represented a common role for PKC in downstream cytokine signal transduction, we first examined whether bisindolylmaleimide I could inhibit cytokine-mediated generation of dendritic cells from primary human monocytes in vitro. We evaluated the effect on three DC characteristics as follows: morphology, immunophenotype, and the ability to activate allogeneic T cells.

**Morphology**—Undifferentiated monocytes were loose and round cells (Fig. 1A, left panel), whereas monocytes cultured with GM-CSF + IL-4 + TNF-α differentiated into clusters of DC (48) (center panel). Inhibition with bis resulted in the loss of these clusters (Fig. 1A, right panel), with a predominance of adherent spindle-shaped cells. Bis also blocked cytokine-mediated up-regulation of characteristic DC markers (MHC I and II, CD11c, CD40, CD80, CD86, and CD83) while preventing the complete down-regulation of the monocyte marker CD14 (Fig. 1B). The immunophenotype of these bis-treated monocytes appeared to be that of activated monocytes. Functionally, inhibition of PKC activation generated cells that were significantly less able to induce allogeneic T cell proliferation at all T cell:APC ratios tested (especially at the higher ratios) but were more immunostimulatory than undifferentiated monocytes (Fig. 1C). The latter observation would also be consistent with the bis-treated cells being activated monocytes. Together, these data suggest that PKC activation is also a downstream component of cytokine receptor-mediated signaling of DC differentiation from monocytes.

**PKC-βII in DC Differentiation**—To determine which PKC isoform(s) were being activated...
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Restoration of DC Differentiation in KG1a Transfected with PKC Isoforms—We and others have shown that the human CD34+/CD86+/CD14− myeloid leukemia cell line KG1, which has the same phenotype as primary CD34+/CD86+ human DC progenitors (50), similarly undergoes differentiation in response to cytokines or PMA to dendritic cells that have DC-specific molecular expression (e.g. DC-CK1 and DC-STAMP) and function (e.g. cross-presentation of antigen) (27, 35, 36, 51–55). KG1a is a less differentiated cell line spontaneously derived from KG1 and does not differentiate in response to phorbol esters (27, 56, 57). KG1a has less overall PKC activity and lower expression of β and δII compared with KG1 (27, 37). Given our demonstration that PKC-βII activation occurs during KG1 differentiation, we next asked if transfection of specific PKC isoforms into KG1a could restore the ability of these cells to differentiate to DC. As seen in Fig. 3A, KG1a was stably transfected with specific functional PKC isoform-GFP fusion constructs (58). Analysis of bulk cultures of the KG1a-PKC-βII transfectants revealed both high and low expressing clones. Single cell cloning resulted in isolation of each type of cell. Confocal imaging for GFP demonstrated cytoplasmic localization for PKC-α-GFP, PKC-βII-GFP, and PKC-γ-GFP in the unstimulated KG1a transfectants (Fig. 3B). PKC-α-GFP also appears as punctate cytosolic accumulations in unstimulated cells, which have been reported previously in NIH-3T3 cells (59). The cytosolic localization in unstimulated cells demonstrated that the majority of the transacted enzyme was not constitutively activated. Following PMA treatment, only PKC-βII-GFP underwent significant translocation to the plasma membrane. In addition to the plasma membrane, PKC-βII-GFP also translocated elongated subcellular structures that appeared to be contiguous with the plasma membrane on stacked views (Fig. 3B, arrow). These results are consistent with our findings with the endogenous PKC isoforms and indicate that the different PKC-GFP isoforms transacted into KG1a behave in a similar fashion to the endogenous PKC isoforms in KG1 (as well as in primary CD34+ HPC and monocytes).

We next assessed the ability of PKC-transfected KG1a to undergo DC lineage commitment in response to PMA. We have shown previously in KG1 and CD34+ HPC that this is characterized by development of typical DC morphology with extended branching dendrites, decreased proliferation and increased cell death, acquisition of DC surface markers (MHC I, II, CD40, CD80, CD86, and CD83), up-regulation of the NfκB family member Rel B, and the ability to induce allogeneic T cell proliferation.

Morphology—Recent studies have demonstrated the importance of the extended dendrite morphology in the ability DC to activated T cells (60). We have found previously that in contrast to the clusters of cells with short dendrites induced by cytokine combinations, PMA-induced DC differentiation from primary CD34+ HPC or KG1 results in more tightly adherent individual cells with longer processes (either spindle shaped or branching) (27) as seen in Fig. 4A for KG1 (8). Both unstimulated (media) vector control KG1a and PKC-transfected cells were morphologically round and loosely adherent (Fig. 4A). Treatment with PMA had little effect on vector control KG1a morphology. However, similar to the parental KG1 line, PMA stimulation induced development of tightly adherent cells with extended cytoplasmic processes in the PKC-βII-GFP transfected cells. Although the KG1a PKC-α-GFP and PKC-γ-GFP cells also became adherent with some spindle-shaped cells, many of the cells were adherent round cells with very large vacuoles that is not a typical DC morphology.

during the initiation of DC differentiation, we took advantage of the fact that PKC activation results in translocation of the enzyme from the cytoplasm to the plasma membrane (49) and can be directly visualized by confocal microscopy. We focused on the DAG/Ca2+-dependent cPKC family (α, βI, βII, and γ), as we have found evidence for involvement of calcium in PMA-mediated DC differentiation (27, 34). In three different progenitor that we have shown previously differentiated to DC in response to PMA (primary CD34+ HPC, KG1, and K562), all four PKC isoforms are cytoplasmic when cells were unstimulated (media, Fig. 2A). Within 5–30 min of PMA stimulation, only the PKC-βII isoform translocated to the plasma membrane in all three cell types. Translocation of PKC-βII was also seen when primary monocytes were differentiated with GM-CSF + IL-4 for 24 h (Fig. 2B). This translocation took somewhat longer to detect compared with PMA treatment but is consistent with the slower kinetics of DC differentiation we see with cytokines versus PKC agonists. These findings indicate there is immediate/early activation of PKC during DC differ-
Differentiation-induced Cell Proliferation Arrest and Death—We have found previously that PMA-induced DC differentiation is associated with arrest of cell proliferation and induction of cell death (20–50%) in all the progenitors we have examined. Compared with KG1, the proliferation of KG1a was largely unaffected by PMA treatment (Fig. 4B). Although the KG1a-PKC transfecants have a higher basal rate of proliferation than either KG1 or KG1a, PKC-GFP transfection restored the ability of PMA to inhibit cell proliferation down to levels equivalent to PMA-treated KG1. Similarly, PMA induced

![Fig. 3. KG1a transfection with PKC-GFP. A, flow cytometry. Bulk populations of KG1a stably transfected with neomycin (vector control), PKC-α-GFP, PKC-γ-GFP, and single cell clones transfected with PKC-βII-GFP (E9 and E11) were analyzed for GFP expression by flow cytometry. B, confocal microscopy. The indicated KG1a-PKC-GFP transfecants were left unstimulated (media) or stimulated with PMA (phorbol ester) and imaged by confocal microscopy for the GFP tag (green). Arrow points to a PKC-βII-GFP-positive subcellular structure. Data are representative of three independent experiments.](http://www.jbc.org/)

![Fig. 4. PKC transfection restores morphologic changes, proliferation arrest, and cell death associated with PMA-induced DC differentiation. A, morphology. KG1a transfecants were left unstimulated (medium) or stimulated with PMA for 5 days (PMA). Photomicrographs were taken at ×400. B, proliferation. 5 × 10⁵ of the indicated cells were cultured in media or PMA for 5 days, and [³H] thymidine incorporation was measured for the last 18 h of culture. KG1a-PKC-α-GFP (KG1a-Alpha), KG1a-PKC-βII-GFP (KG1a-BetII), and KG1a-PKC-γ-GFP (KG1a-Gam) are bulk transfecnt cultures, whereas KG1a-PKC-βII-GFP clones E9 (BetII E9) and E11 (BetII E11) are single cell clones. Data are presented as mean proliferation ± S.D. of triplicate wells. Data are representative of three independent experiments. C, cell death. Cell viability was measured by trypan blue exclusion in the conditions indicated. Data are representative of two independent experiments.](http://www.jbc.org/)
progressively more cell death over time in KG1 but had no effect on KG1a (Fig. 4C). However, transfection of either PKC-α-GFP, PKC-βII-GFP, or PKC-γ-GFP restored the ability of PMA to induce cell death in the transected KG1a to levels equal to or greater than KG1.

Surface Antigen Immunophenotype—KG1 expressed high levels of MHC I and II and low levels of CD86 in media alone and up-regulated CD80 and CD83 upon PMA stimulation (37). In comparison, unstimulated vector control KG1a in media expressed only MHC I, and PMA treatment down-regulated this expression without inducing other DC markers (Fig. 5A). Most unexpectedly, all three PKC isoform-transfected KG1a had down-regulated MHC I and up-regulated CD40 and CD86 in media alone. Conversely, unlike KG1a none of the unstimulated transfectants expressed surface MHC II, although it could be detected intracellularly (below). Constitutive expression was also seen for CD80 except for the low expressing βII clone E11 and CD83 (except for PKC-γ-GFP). These findings suggest that some PKC signaling is occurring in the transfected KG1a in the absence of exogenously added PMA. Stimulation with PMA results in variable up-regulation of MHC I in all the transfectants, MHC II (PKC-γ-GFP, PKC-βII-GFP (slight)), CD86 (PKC-α-GFP, PKC-γ-GFP), and CD83 (PKC-α-GFP). Conversely, PMA treatment down-regulated CD80, CD86, and CD83 in transfecteds that had high level constitutive expression of these markers. We have found previously that PMA treatment can down-regulate constitutive expression of MHC I, MHC II, CD80, and CD86 in DC progenitors (27, 34). This may represent a complex relationship between PKC signal strength and expression of these markers, which is also suggested by the immunophenotypic differences between the PKC-βII high (E9) and low (E11) expressing clones.

To determine why there was little MHC I and II surface expression in the KG1a-PKC transfectants, we examined whether they were present intracellulary and could be induced by γ-interferon stimulation. As seen in Fig. 5B (upper panels), KG1, KG1a-neo, and KG1a-PKC-βII (E9) cells all had low constitutive expression of intracellular MHC I and II. γ-IFN treatment induced expression of both in KG1, whereas there was less (MHC I) to no effect (MHC II) in KG1a-neo cells. In contrast to the control neo cells, KG1a-PKC-βII (E9) regained the ability to up-regulate both intracellular MHC I and II in response to γ-IFN. Although this up-regulation resulted in increased MHC I surface expression, there was virtually no increase in MHC II surface expression (Fig. 5B, lower panels). We have also found that the addition of TNF-α (as a maturation factor) to γ-IFN treatment does not alter MHC I or II expression compared with γ-IFN alone, nor does the addition of γ-IFN during PMA-induced differentiation alter MHC II expression compared with γ-IFN alone (not shown). These findings suggest that although γ-IFN-inducible MHC II gene expression is reconstituted by PKC-βII transfection, MHC II transport to the cell surface remains impaired. We have also found that compared with KG1, there is low expression of the chaperone protein Erp57 (which is involved in peptide loading onto MHC I complexes) in KG1a that is not increased in KG1a-PKC-βII cells (data not shown), which may explain the low basal levels of MHC I surface expression in these cells.

Rel B Expression—Immediate/early up-regulation of expression of the NFκB family member Rel B is an essential event for both DC differentiation (61) and function (62). It is the inducible nature of Rel B that is important, as it has been shown that constitutive expression of Rel family members will actually inhibit DC differentiation (63). Although KG1 have low levels of Rel B that was rapidly induced following PKC activation, KG1a had higher levels of constitutive expression that was not inducible (Fig. 6A). Transfection of PKC-α, -βII, or -γ down-regulated Rel B expression in unstimulated cells, which was then inducible by PMA stimulation. The restoration of a KG1-like pattern of inducible Rel B protein expression was also reflected in the regulation of nuclear Rel B capable of binding NFκB DNA motifs as detected by EMSA (Fig. 6B). Most surprisingly, the total NFκB DNA binding activity was down-regulated in the unstimulated KG1a-PKC-βII and was inducible by PMA, suggesting a common mechanism of NFκB regulation that is not specific for Rel B.

T Cell Proliferation—Functionally, restoration of the ability of KG1a to undergo DC differentiation by transfected PKC isoforms should be reflected in the re-acquisition of T cell stimulatory capacity. As seen in Fig. 7A (left panel), undifferentiated KG1, KG1a, or KG1a transfectants did not induce alloimmune T cell proliferation. The fact that the undifferentiated PKC-βII E9 clone expresses MHC I, MHC II (slight), CD40, CD80, and CD86 and yet still did not elicit T cell prolif-
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Fig. 6. Rel B expression. A, protein. KG1 and the KG1a transfectants were stimulated with PMA as indicated; protein lysates were isolated, and equal amounts were analyzed for Rel B by Western blots. Arrow points to the specific band for Rel B. Results are representative of three independent experiments. B, EMSA. KG1 and the KG1a transfectants were stimulated with PMA as indicated; protein lysates were isolated, and equal amounts were analyzed for binding to labeled DNA probes containing the consensus NF-κB-binding site. Arrow points to the complexes supershifted by an anti-Rel B antibody. Data are representative of two independent experiments.

eration suggests factors other than antigen presentation receptors and co-stimulatory ligands are necessary to elicit T cell activation. KG1 differentiated with PMA, and more so with addition of the maturation signal TNF-α, can stimulate T cell proliferation. By comparison, PMA + TNF-α-treated KG1a, KG1a vector control, and KG1a-GFP do not acquire allostimulatory capacity. These controls also demonstrate that PMA carryover from the differentiation cultures does not account for the T cell proliferation seen in the co-cultures. In contrast, PMA-induced differentiation of all three PKC-transfected KG1a resulted in the ability to drive significant T cell proliferation. This was significantly greater than undifferentiated PMA-induced differentiation of all three PKC-transfected KG1a expressed very little PKC-βII, the ability to undergo DC differentiation as seen for KG1.

PKC Induction of Endogenous PKC Isoform Expression—Because we have consistently observed activation of PKC-βII and not other cPKC isoforms during DC differentiation, we were surprised by the ability of the α and γ isoforms to also restore aspects of DC differentiation in the KG1a cells. We sought to understand the underlying mechanism by first comparing the relative levels of endogenous PKC versus PKC-GFP expression in the transfected KG1a. Because of the GFP tag, we can distinguish transfected PKC from endogenous PKC by molecular weight on Western blots. Most unexpectedly, we found relatively low levels of transfected PKC-GFP protein expression (Fig. 8A). Except for the high expressing PKC-βII-GFP clone E9, the transfected PKC-GFP proteins were only detectable upon long exposures. Remarkable, however, was the up-regulation of all the endogenous cPKCs in each of the KG1a transfectants. This is most marked for PKC-βII and demonstrates that although untransfected KG1a did not express much PKC-βII, the ability to up-regulate this expression was still intact. These findings suggested that the transfected PKC-GFPs were not up-regulating PKC activity by their own overexpression but rather by inducing endogenous PKC expression. This would also explain why PKC-α or -γ transfection can restore the ability of KG1a to differentiate to DC, namely via up-regulation of endogenous PKC-βII.

How do the transfected PKC-GFPs induce endogenous PKC-βII expression? Because PKC-β and -βII are alternatively spliced variants from the PKC-β gene that can be generated in a regulated fashion (64), we first examined if increased PKC-βII expression was because of a switch of splicing from β to βII. KG1a expressed very little PKC-βII to begin with (Fig. 8B), making it unlikely that changes in splicing account for the increased βII expression. The fact that both PKC-βI and -βIII protein were induced in the PKC-transfected KG1a suggests that expression of the PKC-β gene is up-regulated. Consistent with this, PKC-β mRNA expression was significantly up-regulated in the KG1a transfectants (Fig. 8C). Because PKC enzymes are degraded following activation (65), we determine whether the induced endogenous PKC-βII could be activated by following protein levels during PMA treatment. As seen in Fig. 8D, PKC-βII was degraded during PMA stimulation in KG1, KG1α, and all the PKC-transfected KG1a. This indicates that the induced endogenous PKC-βII can be activated and is likely to be signaling. Together, our data indicate that the transfected PKC-GFP are up-regulating endogenous PKC isoform gene expression (in particular PKC-βII), and this confers on KG1α the ability to undergo DC lineage commitment. These findings also suggest that regulation of PKC-βII expression may in turn regulate the ability of progenitors to undergo DC differentiation.

Autoregulation of PKC Promoter Activity—The ability of the transfected PKC-GFP to up-regulate endogenous PKC-βII mRNA expression suggested that this is happening at the transcriptional or post-transcriptional (e.g. mRNA stabilization) level. Previous studies have demonstrated a phorbol ester-inducible site in the PKC-β promoter (66, 67), suggesting that PKC activity may positively autoregulate its own gene expression. The relationship of the enzyme activation constant, $K_{act} = \text{(active enzyme)}/\text{(inactive enzyme)}$, predicts that under steady state conditions, an increase in the total concentration of PKC enzyme (by transfection or induction of endogenous expression) will result in an increase in the number of activated
PKC molecules. We first measured total PKC kinase activity under steady state conditions. As seen in Fig. 9A, unstimulated KG1 had more kinase activity than KG1a or KG1a-neo, whereas KG1a/neomycin (E9) had significantly more than both. To determine whether this increased PKC kinase activity resulted in increased PKC-β promoter activity, we cloned a 1.2-kb fragment of the human PKC promoter containing the previously reported PMA-inducible site (−11 to +43 (66)) into a luciferase reporter construct (pPKC-Forward). There was significantly higher luciferase activity when pPKC-Forward (but not the reverse orientation and empty vector negative controls) was transfected into E9 compared with the KG1a-neo control transfectants, demonstrating a 5-fold increase in promoter activity under unstimulated conditions (Fig. 9B). Finally, if an increase in constitutive PKC activity is inducing PKC-β expression, inhibition of this kinase activity would result in decreased expression. Consistent with this, unstimulated PKC-βII (E9) transfectants cultured with the PKC inhibitor bis I undergo a loss of both PKC-βII protein and mRNA expression (Fig. 9C). Together, these data indicate that constitutive PKC kinase activity can positively autoregulate PKC-β gene expression by PKC-responsive elements in the promoter.

DISCUSSION

Our findings support a model where quantitative and qualitative PKC-βII activation is a key component of the signal transduction pathways that induce DC differentiation and that regulation of this activation is in part through regulation of PKC-β.
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Fig. 8. Up-regulation of endogenous PKC-βII expression. A, induction of endogenous PKC-βII protein expression. Equal amounts of protein lysate from the unstimulated cells indicated were analyzed for PKC isoform expression by Western blot. Arrows point to the (larger) transfected PKC-GFP and (smaller) endogenous PKC bands. Blots were reprobed with anti-actin antibodies as a loading control. B, PKC-βI versus -βII expression. Protein lysates were analyzed as above using antibodies specific for PKC-βI or -βII. C, mRNA expression. Total mRNA was isolated from the indicated (unstimulated) cells, equalized by serial dilution (top panel, Total RNA), and analyzed by Northern blot for PKC-βII expression (using a PKC-βII-specific probe) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as a loading control). D, degradation of PKC-βII following PMA activation. The indicated cells were cultured in PMA for the times indicated; protein lysates were made and equal amounts were analyzed for PKC-βII expression by Western blot. All results are representative of two independent experiments.

gene expression. Specifically, these data indicate the following. 1) PKC is a critical downstream signaling component of cytokine-induced monocyte→DC differentiation, consistent with our previous findings in CD34+ HPC→DC differentiation (27). 2) Of the PKC isoforms, PKC-βII is the most centrally involved in DC differentiation. 3) Different aspects of DC differentiation are induced by differing degrees of PKC signal strength. 4) PKC signal strength is regulated (at least in part) by PKC-β gene expression,
which involves a positive autoregulatory feedback loop.

The ability of the PKC inhibitor bisindolylmaleimide I to inhibit cytokine-driven monocyte (or CD34+/HPC) progenitor to DC differentiation indicates that PKC is a downstream component of these signaling pathways. It is not yet clear which cytokine receptor is triggering PKC activation, as these receptors typically signal via Janus tyrosine kinase/signal transducers and activators of transcription and TRAFs, whereas PKC has been best characterized to be activated by G-coupled proteins and phospholipase C. However, the PKC adapter protein RACK-1 associates constitutively with the common β chain of the GM-CSF/IL-3/IL-5 receptor, and PKC-βII rapidly associates with this complex upon receptor activation (28).

Involvement of PKC-βII in GM-CSF receptor signaling is consistent with our observation that this isoform is activated in CD34+/HPC by GM-CSF + TNF-α stimulation, and appears to be the cPKC isoform that is predominantly activated by PMA in three different DC progenitors. PKC-βII has also been implicated previously in myeloid differentiation (40), and our findings demonstrate a correlation between PKC-βII expression and the ability to undergo DC differentiation in KG1 and KG1a. The rapid activation of PKC-βII indicates that it is involved in the primary signaling rather than a later secondary response. Together, these data suggest that DC differentiation is specifically signaled through PKC-βII. However, we could not rule out the involvement of low level activation of the other cPKCs, activation of novel nPKC, and/or atypical PKC isoforms, and the formal possibility that PMA is acting through a non-kinase phorbol ester receptor (68) and that PKC-βII activation is an epiphenomenon.

To address these possibilities, we asked the seemingly straightforward question whether reconstitution of specific
PKC isoform expression would reconstitute the ability of KG1a to undergo DC differentiation, providing strong evidence for a central role for that isoenzyme. The inability of KG1a to undergo differentiation (57) has been attributed to lower expression of PKC isoforms/activity (37). Confocal studies of the KG1a-PKC-GFP transfectants demonstrated that only PKC-βII-GFP was clearly activated by PMA, consistent with what we saw for the endogenous cPKC. Most surprisingly, however, each of the PKC-GFP isoforms could reconstitute the ability of KG1a to undergo DC differentiation as assessed by inhibition of cell proliferation and induction of cell death, costimulatory ligand expression, re-establishment of inducible Rel B expression, and acquisition of allostimulatory capability. Although PKC-GFP transfection did not restore the high level MHC II surface expression seen on KG1, it did restore γ-interferon inducibility as seen by intracellular staining. These findings suggest that although some aspects of MHC II expression are regulated by PKC signaling, transport/processing for surface expression is not. The transfected KG1a also down-regulated constitutive MHC I expression, which is likely due to the increased constitutive PKC activity in these cells (and is consistent with the observation that PMA-induced PKC activation reduces cell proliferation and induction of cell death, costimulatory ligand expression, re-establishment of inducible Rel B expression, and acquisition of allostimulatory capability. Although PKC-GFP transfection did not restore the high level MHC II surface expression seen on KG1, it did restore γ-interferon inducibility as seen by intracellular staining. These findings suggest that although some aspects of MHC II expression are regulated by PKC signaling, transport/processing for surface expression is not. The transfected KG1a also down-regulated constitutive MHC I expression, which is likely due to the increased constitutive PKC activity in these cells (and is consistent with the observation that PMA-induced PKC activation down-regulates MHC I (e.g. Fig. 4C)).

The unexpected expression of CD40, CD80, CD86, and CD83 on the unstimulated KG1a-PKC-GFP transfectants is also likely due to increased constitutive PKC activity that is seen in the kinase assays. Most interestingly, this is the same “semi-activated” phenotype of the pathogenic Langerhans cells in Langerhans cell histiocytosis (69). It is unclear whether the variation in surface marker expression between the PKC-α, βII, and γ transfectants is due to qualitative and/or quantitative differences between the clones. However, the difference in MHC I, MHC II, CD40, and CD80 expression between the low (E11) and high (E9) expressers of PKC-βII suggests that the degree of PKC signal strength plays a defining role in determining which DC characteristics are manifested. This is supported by the observation that even though unstimulated KG1a-PKC-GFP expresses MHC and costimulatory ligands, they are still unable to activated T cells unless PKC is fully activated by an exogenous PKC agonist. Why there is no direct correlation between MHC/costimulatory ligand expression and the ability to activate T cells is unclear, although we and others have seen the same phenomenon in other myeloid DC progenitors (15, 70). Recently, Benvenuti et al. (60) have reported that even though DC from Rac 1/2−/− mice have the same degree of MHC/costimulatory ligand expression as WT DC, they are far less capable of priming naïve T cells. This appears to be in part due to the inability of these knockout DC to form the DC membrane extensions that physically interact with the T cell. Similarly, we believe that factors responsive to PKC signaling are involved in these initial physical/adhesive interactions and may be playing an important role in the immunogenecity of the PKC transfectants.

Varying degrees of PKC signaling is also supported by the confocal microscopy studies, where significant translocation of activated PKC-βII-GFP in the KG1a transfectants is only seen after PMA treatment. Further evidence for a differential effect of PKC signal strength is seen in the regulation of Rel B expression. These data and additional transcriptional run-off studies suggest that the absence of PKC-βII activity (KG1a) results in constitutive/non-inducible Rel B expression; intermediate activity (unstimulated KG1 and KG1a-PKC-GFP) suppresses expression, and high PKC activity (PMA-stimulated KG1, KG1a-PKC-GFP) induces Rel B expression. Most inter-

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elements have been described between −3000 and −690 of the 5′-untranslated region (which may not be included in our promoter construct) (67). If exogenous stimuli can inhibit PKC-β expression through these elements, this may provide a mechanism by which progenitor to DC differentiation can be inhibited in inflammatory settings where an adaptive immune response is unwanted (e.g., sterile trauma and wound healing). Although such a PKC-specific checkpoint has not been described, it has been well established that a number of cytokines (in particular those secreted by tumors) can inhibit the differentiation of myeloid progenitors to DC through signaling to yet undefined downstream targets (17).

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