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Induced Dendritic Cell Differentiation of Chronic Myeloid Leukemia Blasts Is Associated with Down-Regulation of BCR-ABL

Inna Lindner,* Mohamed A. Kharfan-Dabaja,† Ernesto Ayala,† Despina Kolonias,* Louise M. Carlson,* Yasmin Beazer-Barclay,‡ Uwe Scherf,‡ James H. Hnatyszyn,§ and Kelvin P. Lee2*‡§

Although differentiation of leukemic blasts to dendritic cells (DC) has promise in vaccine strategies, the mechanisms underlying this differentiation and the differences between leukemia and normal progenitor-derived DC are largely undescribed. In the case of chronic myeloid leukemia (CML), understanding the relationship between the induction of DC differentiation and the expression of the BCR-ABL oncogene has direct relevance to CML biology as well as the development of new therapeutic approaches. We now report that direct activation of protein kinase C (PKC) by the phorbol ester PMA in the BCR-ABL+ CML cell line K562 and primary CML blasts induced nonterminal differentiation into cells with typical DC morphology (cytoplasmic dendrites), characteristic surface markers (MHC class I, MHC class II, CD86, CD40), chemokine and transcription factor expression, and ability to stimulate T cell proliferation (equivalent to normal monocyte-derived DC). PKC-induced differentiation was associated with down-regulation of BCR-ABL mRNA expression, protein levels, and kinase activity. This down-regulation appeared to be signaled through the mitogen-activated protein kinase pathway. Therefore, PKC-driven differentiation of CML blasts into DC-like cells suggests a potentially novel strategy to down-regulate BCR-ABL activity, yet raises the possibility that CML-derived DC vaccines will be less effective in presenting leukemia-specific Ags.


Chronic myelogenous leukemia (CML)3 is a malignancy of multipotential hemopoietic progenitor cells (HPC) caused by the BCR-ABL fusion oncogene, itself the result of a t(9;22)(q34;q11) chromosome translocation that juxtaposes the regulatory and 3' gene segments of BCR with the 3' catalytic domains of c-ABL (1, 2). From a clinical standpoint, the problem of residual disease, the efficacy of anti-CML immune responses (suggested by the graft-vs-leukemia effect following allogeneic bone marrow transplantation), and the presence of a tumor-specific Ag (BCR-ABL) whose expression is required even in advanced disease all underscore the rationale for developing anti-CML vaccine strategies (3–5). One such strategy involves directly differentiating leukemic blasts into dendritic cells (DC), potentially allowing the whole spectrum of known and unknown leukemia Ags to be presented (6). Of particular importance here is the effect of differentiation on BCR-ABL expression (as this oncogene is both the primary cause of the differentiation block and the predominant leukemia-specific vaccinating Ag) (5, 7, 8) as well as the comparison of leukemia-derived DC to DC derived from normal progenitors.

Previous studies showed that similar to their normal counterparts, myeloid leukemic blasts across a range of differentiation stages can be driven to differentiate to DC by exogenous cytokines and can elicit anti-leukemia CTL responses (9–16). DC generated directly from leukemic blasts potentially overcome the problems of other anti-tumor DC vaccine approaches, where normal progenitor-derived DC are exoenously loaded with a limited number of known tumor-specific Ags (17, 18). Ex vivo differentiation of DC from both normal and leukemic progenitors can be driven by both receptor-mediated exogenous stimuli (i.e., cytokine combinations GM-CSF, IL-4, TNF-α, and CD40 cross-linking) (9, 14, 19–22) and agents that directly activate intracellular signaling pathways (calcium ionophores [intracellular calcium] and phorbol esters [protein kinase C (PKC)] (23–29). Because leukemic cells may lack one or more components of membrane and proximal signal transduction pathways, direct activation of downstream pathways may drive DC differentiation more effectively (30). We have shown that PKC activation by the 2,3-diacrylglycerol analog PMA induces DC differentiation in acute myeloid leukemia (AML) blasts (26), while others have demonstrated the same following direct activation of intracellular calcium signaling by calcium ionophore (CI) (25, 27, 28, 31). Although these studies and model systems have also suggested which upstream signals initiate DC differentiation, the signal transduction pathways and genetic events involved remain largely undefined.

Although myeloid leukemic blasts differentiated to dendritic-like cells (DLC) largely recapitulate DC differentiation from normal
progenitors, there is a significant variability among differentiated blasts (9, 32, 33), and certain aspects of differentiated blasts differ from normal progenitor-derived DC (25, 26, 34). One possible reason for these differences is normal DC differentiation from normal vs leukemic progenitors is the interaction between the differentiation program and the expression of leukemia-specific oncogenes. The ability to drive DC differentiation in vitro and evidence that leukemic blasts may differentiate to DC in vivo (35) raise a central biological question: how do stimuli that induce DC differentiation overcome the oncogene-mediated differentiation block in leukemia? Because BCR-ABL is both the primary cause of CML (36, 37) and a prototypic leukemic Ag, its regulation may represent a paradigm for the relationship between leukemic blast differentiation and oncogene expression. Previous studies have demonstrated that overexpression of BCR-ABL in multipotent progenitor cells does not prevent their differentiation (38, 39), yet BCR-ABL is down-regulated during induced differentiation of CML blasts along erythroid and granuloid lineages (40, 41), whereas direct inhibition of BCR-ABL activity in leukemic blasts can result in erythroid differentiation (42). The mechanisms that regulate BCR-ABL expression during differentiation and whether down-regulation of BCR-ABL is a necessary prerequisite to allow DC differentiation from CML blasts have not been well studied. Understanding the regulation of BCR-ABL during DC differentiation also has clinical implication, as its down-regulation may lead to new therapeutic approaches, but at the same time hamper the ability of leukemia-derived DLC to induce CML-specific CTL in a vaccine setting.

To further understand what intracellular signal transduction pathways drive progenitors to differentiate to DC and the relationship between differentiation and oncogene expression, we examined DC differentiation from CML progenitors. We now report that direct activation of PKC induces both the CML cell line K562 and primary CML blasts to differentiate into cells with phenotypic and functional characteristics of DC. However, this differentiation was not terminal, as the removal of the stimulus reversed differentiation-induced growth arrest as well as the ability to stimulate T cells. Furthermore, this differentiation process resulted in the down-regulation of BCR-ABL gene expression specifically involving downstream signaling through the mitogen-activated protein kinase (MAPK) pathway.

Materials and Methods

Cells and culture

K562 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in IMDM, 10% heat-inactivated FCS (both from HyClone, Logan, UT), 100 mM l-glutamine, and 100 U penicillin/streptomycin (Life Technologies, Grand Island, NY). Cultures were stimulated at 0.2 × 10⁶ cells/ml for 1–7 days with PMA (10 ng/ml; Sigma-Aldrich, St. Louis, MO), phorbol-12,13-dibutyrate (PDBu, 100 ng/ml; Calbiochem), TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN), the CI A23187 (385 ng/ml; Sigma-Aldrich), or GM-CSF (200 U/ml; Immunex, Seattle, WA), with a complete medium change every 2 days. Primary CML blasts were obtained from patient peripheral blood after informed consent was given, under protocols approved by the University of Miami institutional review board. Mononuclear cells (MNC) were separated by discontinuous density gradient centrifugation and cultured immediately or cryopreserved. To generate DLC, MNC were stimulated at 0.5 × 10⁶ cells/ml with PMA (3–10 ng/ml), CI (355 ng/ml), or PMA plus A23187 for 3 days or with GM-CSF (100 U/ml), TNF-α (10 ng/ml), or IL-4 (20 ng/ml; R&D Systems) for 14 days. Cell viability was assessed by trypan blue dye exclusion. Where indicated, cells were pretreated with bisindolylmaleimide I (1 μM), PD 98059 (5–60 μM), or Bay 11-7082 (5 μM; all from Calbiochem, San Diego, CA) for 3 h before the addition of PMA.

Monocytes were enriched from the MNC of normal donors by plastic adherence (43). To generate DC, monocytes were cultured in six-well plates in medium containing GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) for 12 days and TNF-α (10 ng/ml) for the last 4 days of culture (44).

Flow cytometry

Cells were stained as previously reported (26) using the following mAbs: HL-11 (anti-HLA-DR [both from VMRD, Pullman, WA] and CD80, CD11c, CD40, CD83, CD86 (all from Immunotech, Westbrook, ME). Appropriate isotype-matched Abs were used as controls. Cells were harvested using 3 mM EDTA, and 10,000 live cells were analyzed on a Coulter XL flow cytometer (Coulter, Hialeah, FL) using software supplied by the manufacturer.

Cell proliferation assays

For K562 proliferation, untreated, PDBu-treated (100 ng/ml), or PMA-treated cells were cultured for 5 days (PDBu) or for 1 or 2 days (PMA). In the PDBu washout experiments, PDBu was removed on day 5, and cells were cultured without PDBu for 0, 1, 3, or 5 days. Cells were plated in triplicate at 1 × 10⁶ cells/well. [Methyl-3H]Thd (0.5 μCi/well) was added for the final 18 h of culture, and incorporation was measured using the ϒ Plate scintillation counting system (Wallac, Gaithersburg, MD). For T cell proliferation assays, K562 cells, monocytes, or primary CML blasts were cultured with or without PMA (K562); GM-CSF, IL-4, and TNF-α (monocytes); or PMA, CI, or PMA plus CI (primary blasts). DC generated from CML blasts were gamma-irradiated (12,000 rad) for K562, 3,000 rad for primary CML blasts and monocytes and cocultured at various ratios with 1 × 10⁶ cells/well of purified resting allogeneic T cells (26). In GM-CSF washout experiments, K562 were cultured in PBUs for 5 days or in PDBu for 5 days, followed by 5 days in medium alone, irradiated at 12,000 rad, and then cocultured at a ratio of 1/1 with 1 × 10⁷ T cells. All conditions were performed in triplicate, and data are presented as the mean ± 1 SD.

In CFSE labeling, K562 were stimulated with PDBu for 5 days, resuspended in RPMI medium, and incubated with 8 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were then washed twice in RPMI and cultured for additional 3 or 5 days, untreated or treated with PDBu.

Western blot

Western blots for Rel B and C-ABL were performed as previously described (24). Briefly, cell lysates were made from untreated cells and K562 differentiated with PMA, with or without TNF-α, or K562 differentiated with PMA in the presence or the absence of PD 98059 (5, 15, 30, or 60 μM) or Bay 11-7082 (5 μM). Protein levels were quantitated by using the Micro BCA reagent kit (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE (4% stacking/6% (for BCR-ABL) or 10% resolving), electroblotted to nitrocellulose, and probed with Abs to β-actin (a gift from U. Siebenlist, National Institutes of Health). Samples were visualized by chemiluminescent detection (ECL; Amersham Pharma Biotech, Little Chalfont, U.K.).

EMSA

EMSA were performed for NF-κB family members as previously described (45). Briefly, equal amounts of protein were incubated with [32P]-labeled primer containing consensus NF-κB binding sites (GATCAGAGGCAGGGGTAATCCCTCTCCTTTA) and separated on 4% polyacrylamide gels. For supershift assays, samples were first incubated with anti-Rel B, anti-p52, anti-p65, anti-c-Rel (all from Santa Cruz Biotechnology) or anti-p50 Ab (a gift from U. Siebenlist, National Institutes of Health). Samples were visualized by autoradiography.

RT-PCR

Total RNA was isolated from cultured cells at the times indicated using RNAzol (Cinna Biotech, Friendswood, TX) according to the manufacturer’s instructions. First-strand RNA was synthesized from equal amounts of total RNA using Superscript II (Strategene, La Jolla, CA) and used as a template for PCR reactions with oligonucleotides specific for human β-actin (5′-TGACGGGCGTCACCCACACTGTGCACCTCTA, 3′-CTAGAA GCATTGGGTTGGACGAAGATGGAGGG) or BCR-ABL (5′-GCCACTG GTATTAGGACAG, 3′-GATAATGAGCGGTGATGATG). Cycle parameters were as follows: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles. Thirty microliters of each reaction was resolved on 1% agarose gels, transferred to nylon, and hybridized with either [32P]-labeled BCR-ABL (a 600-bp fragment spanning the junction region) or actin probes. Hybridization was quantified using the Scion Image program (Frederick, MD). For real-time fluorometric PCR, total RNA was isolated, and 500 ng was subjected to real-time PCR using the LightCycler (Roche, Idaho Falls, ID) with primers to b3 exon of BCR and a2 exon of ABL or...
with primers to GAPDH. PCR products were quantified with fluorescent SYBR Green I dye. Specific products were identified by melting curve analysis of the PCR products.

Northern blot

Northern blot analysis was performed as previously described (26). Briefly, total RNA was isolated from unstimulated or K562 stimulated by PMA with or without TNF-α, equalized by serial dilution and ethidium bromide visualization, separated on 1% formaldehyde/agarose gel, transferred to nylon membrane, and hybridized against human BCR-ABL, DC-CK1, or actin probes.

BCR-ABL autophosphorylation assay

BCR-ABL kinase activity was assayed as previously described (46). Briefly, 500 µg of protein lysate was immunoprecipitated with 5 µg of anti-c-ABL bound to agarose-G beads (Life Technologies), and the immunoprecipitated pellets were analyzed for kinase activity using [γ-32P]ATP. Samples were then electrophoresed on 8% SDS-PAGE gels, and radiolabeled proteins were visualized by autoradiography.

Results

Direct PKC activation, induction of intracellular calcium, or exogenous cytokines induces K562 to acquire DC characteristics

We first sought to determine whether CML blasts, like their untransformed CD34+ HPC counterparts, could acquire DC characteristics in response to direct activation of intracellular signaling pathways, including PKC and mobilization of intracellular calcium. As a model of primary CML blasts we used the BCR-ABL+ CML cell line K562, isolated from a patient in blast crisis (47). Previous studies have characterized K562 as a multipotent cell line capable of differentiating along granulocytic/myelomonocytic or megakaryocytic lineages after PMA treatment, but differentiation to DC has not been described (48–54). As shown in Fig. 1A, untreated K562 cells were uniformly round, nonadherent cells. Similar to what we have reported for KG1, treatment with PMA (7 days) or PMA plus TNF-α (5 days) caused some cells to become adherent and develop cytoplasmic projections typical of DC seen in vivo (35), while inducing apoptosis in others (small, condensed cells in Fig. 1A, middle and right panels). Similar to other studies in myeloid leukemias (14, 27, 56), the DC morphologic change was not observed for all cells simultaneously, which could be due to a balance of cells in adherent and nonadherent states at any given time or to the transformed nature of the cells that alters their ability to develop DC morphology. In contrast to PMA-driven differentiation, treatment of K562 with the CI A23187, TNF-α alone, or GM-CSF plus TNF-α did not result in any adherence or gross morphologic changes (data not shown).

DC characteristically express MHC class I and II (MHC I and II); the costimulatory molecules CD80, CD86, and CD40; and the DC activation marker CD83 (57). Of these, untreated K562 expressed only CD80 (Fig. 1B). Both PKC activation and calcium mobilization induced acquisition of DC markers by K562. Although resulting in no morphologic change, treatment of K562 with A23187 rapidly induced up-regulation of CD83, CD40, CD80, and CD86, but not MHC I or II. Cells stimulated with PMA alone showed a more delayed up-regulation of DC markers, but by day 5 of culture the entire population of PMA-treated cells upregulated MHC I, MHC II (slight), CD40, and CD86. Similar to cells treated with PMA, PMA- plus TNF-α-treated cells uniformly up-regulated MHC II, with a more pronounced up-regulation of MHC I, CD40, and CD86. Compared with A23187-stimulated cells, K562 cultured with PMA and A23187 displayed a significantly greater up-regulation of CD40 and MHC I, a more modest and uniform up-regulation of CD83 and CD86, and a decrease in CD80. While the expression of these markers was maintained in differentiated K562 by day 5 of culture, the combination of PMA and A23187 induced more cell death than A23187 alone (data not shown). The fact that PMA induced a more modest up-regulation vs PMA plus TNF-α or PMA plus A23187 is consistent with our findings in KG1 that TNF-α (or CI) is not required for lineage commitment, but can drive more rapid and complete DC maturation (26). Treatment with the established cytokine combination GM-CSF and TNF-α up-regulated certain DC markers (CD11c, CD40, and MHC I, by day 14), but not MHC II, CD86, or CD83 (Fig. 1C). The differences observed between cytokine- and PMA- or CI-induced differentiation may be due to differences in the degree of intracellular signaling, the involvement of other signal transduction pathways in the cytokine-mediated differentiation, or defects in cytokine receptors or upstream signal transduction molecules that are bypassed by direct activation (58). Although K562-derived DLC expressed comparatively less MHC I and MHC II than DC derived from normal progenitors, other groups have found that leukemic blast-derived DLC may have very slight, if any, up-regulation or even down-regulation of these markers and yet acquire molecular and functional characteristics that are largely unique to DC (14, 25, 26, 33). To determine whether this is true for K562, we next examined the expression of DC-associated/speciﬁc genes. In the remaining studies we have focused on direct activation of PKC to more carefully characterize the signal transduction pathways involved in DC differentiation.

Direct PKC activation induces the expression of DC-associated/specific genes

The NF-κB transcription factor family member Rel B is up-regulated very early in DC differentiation (24, 26), but not in monocytes (59), and plays an important role in DC differentiation and function (60–63). Thus, up-regulation of Rel B may represent an early molecular marker of DC differentiation. As shown in Fig. 2A, untreated K562 did not express Rel B protein. Expression was rapidly up-regulated by 2 h after PMA treatment and was further up-regulated at 6 and 24 h, with sustained levels through day 5 (the last point measured; not shown). Up-regulation of Rel B by PMA plus TNF-α did not significantly differ from that by PMA, suggesting a primary role for PKC activation. Comparatively, GM-CSF plus TNF-α up-regulated Rel B by day 2, with further up-regulation by day 14. Consistent with our surface Ag phenotype findings, there was a more rapid Rel B up-regulation by PMA vs cytokines (hours vs days). To assess whether the overall increase in Rel B was associated with increased NF-κB signaling activity, nuclear extracts were assayed for DNA binding of free NF-κB heterodimers. As shown in Fig. 2B, EMSAs at 24 h demonstrated a concurrent increase in NF-κB binding activity, with supershift assays revealing increased nuclear Rel B heterodimers as well as p50, p52, and p65.

The expression of the DC-CK1 chemokine is thought to be unique to DC and induces the migration of naïve T cells (64). Similar to Rel B expression, DC-CK1 expression was not detected in unstimulated K562, but was significantly up-regulated early during differentiation by both PMA and PMA plus TNF-α (Fig. 2C). Together these data demonstrate that PKC-mediated differentiation of K562 results in the expression of DC-specific genes.

K562 differentiated by PMA with or without TNF-α have characteristic DC function

A hallmark of DC function is the induction of T cell activation and proliferation. As shown in Fig. 3A, while untreated K562 cells induced minimal proliferation in purified allogeneic T cells, K562 differentiated for 5 days induced significant T cell proliferation (13.5-, 22.3-, and 27.9-fold higher at DC/T cell ratios of 1/10, 1/2, and 1/1, respectively, vs untreated cells). We have previously
shown that there is no PMA carryover from the differentiation cultures that causes T cell proliferation (24). Interestingly, although the addition of TNF-α/H9251 enhanced PMA-induced up-regulation of MHC I, MHC II, CD40, and CD86, this did not translate into greater allostimulatory ability (data not shown). In addition, down-regulation of CD80 and low expression of MHC II did not seem to impair the ability of PMA-differentiated K562 to activate T cells, as this proliferation was equivalent or superior to that induced by normal monocyte-derived DC (MODC) or T cells pharmacologically activated with PMA and ionomycin alone. Although MODC were capable of stimulating T cells at a lower DC/T cell ratio (1/100), their potency at higher ratios was equivalent to or less than that of differentiated K562. These results demonstrate that PMA-driven K562 differentiate into cells with comparable allostimulatory capacity compared with normal MODC. In addition to T cell proliferation, functional studies of Ag uptake by K562 derived DC (as assessed by FITC-dextran incorporation) demonstrated a loss in the ability to take up extracellular Ag on day 5 of differentiation, consistent with a decrease in macropinocytosis and micropinocytosis during DC maturation (data not shown).

To test whether the allostimulatory capacity of PMA-treated K562 is lost with the removal of the stimulating agent, we differentiated K562 in water-soluble phorbol ester, PDBu, for 5 days, followed by its washout and culture of cells in medium alone for an additional 5 days. Unlike PDBu-differentiated K562, K562 from which PDBu was removed were incapable of T cell stimulation, suggesting that PKC signal is required to keep leukemic

FIGURE 1. Morphology and surface phenotype of K562-derived DLC. A, Morphology. Photomicrograph of K562 cells cultured in medium, PMA alone, or PMA plus TNF-α (Wright stain; ×300 magnification). B, Surface Ag phenotype induced by PMA and PMA plus TNF-α. K562 cells were cultured in medium alone, PMA, or PMA plus TNF-α for 5 days or in CI (A23187) or CI plus PMA for 1 day and analyzed by flow cytometry. C, Surface Ag phenotype induced by GM-CSF plus TNF-α. K562 cells were cultured in medium or in GM-CSF plus TNF-α for 14 days and analyzed by flow cytometry. Staining with specific Ab; □, isotype-matched control. The y-axis indicates cell number.
blasts in a functionally differentiated state (Fig. 3B). Together, these phenotypic, molecular, and functional analyses demonstrate that the CML cell line K562 can be driven to differentiate to DLC.

**PMA-induced K562 differentiation does not involve proliferation**

Terminal differentiation is typically associated with loss of proliferation. We have previously shown that PMA (but not cytokine)-driven DC differentiation inhibits proliferation in CD34+ HPC and KG1 cells and induces apoptosis in 30–50% of cells (24, 26). Similarly, PMA-induced DC differentiation of K562 suppressed proliferation by 24 h (Fig. 4A), with cell viability reduced to ~45% by day 7 (Fig. 4B). In comparison, GM-CSF plus TNF-α treatment of K562 did not result in the loss of either cell viability (Fig. 4B) or proliferation (there was a 4-fold increase in cell number by day 7 compared with day 0, whereas neither PMA nor PMA plus TNF-α stimulation resulted in an increase in cell number). These findings suggest that pathways that induce DC differentiation can be separated from those triggering progenitor cell proliferation.

We next asked whether leukemic cells undergo permanent growth arrest consistent with terminal differentiation or whether the growth arrest is reversible, similar to what was seen with the ability to stimulate T cells. Proliferation was measured in K562 cells cultured in the presence of PDBu and subsequently after PDBu washout. While cells treated for 5 days with PDBu stopped proliferating, they regained their ability to divide as soon as 1 day after PDBu removal (Fig. 4C). By day 5 post-PDBu removal, K562 proliferation was similar to that of untreated cells. Tracking of cell divisions by the membrane dye CFSE demonstrated that all cells were proliferating, rather than a subpopulation going back into cycle (Fig. 4D). Consistent with this is the loss of allostimulatory capacity following PDBu washout. The shift of CFSE fluorescence seen in PDBu-treated cells without washout (from days 0–3) is due to some leakage of the dye into the medium. These results suggest that unlike normal progenitors, leukemic cells do not undergo irreversible DC differentiation, possibly due to persistent oncogene activity.

**Direct PKC activation drives differentiation along DC lineage in primary CML blasts**

We next investigated whether our findings in a CML cell line were relevant in primary CML blasts. Dose-response experiments demonstrated that CML blasts from four patients (two in accelerated phase and two in blast crisis) could be induced to differentiate into DLC at PMA concentrations 3–10 times lower than those required for K562→DC differentiation. Unstimulated CML blasts were nonadherent, with uniformly round morphology (Fig. 5A). Similar to K562, PMA treatment induced CML blasts to become adherent and develop elongated cytoplasmic projections. The addition of CI to PMA (previously shown to act similarly to TNF-α by driving full maturation/activation of DC in AML blasts (26)) further enhanced DC morphology of CML blasts. Similar to K562, CML blasts stopped proliferating in PMA-containing combinations (data not shown).

PMA treatment also induced characteristic expression of DC-associated surface markers (Table I). While all four patients’ blasts expressed MHC I, MHC II was expressed in three of four patients and was up-regulated by PMA alone in the fourth patient. CD40 was up-regulated by PMA alone in three of four patients, and CD86 was up-regulated by PMA in one of four patients. The addition of A23187 enhanced the expression of CD86 in another patient, CD83 in two patients, and CD80 in three patients. GM-CSF-, TNF-α-, and IL-4-driven DC differentiation resulted in up-regulation of CD40 in all patients and more variable up-regulation of MHC I and II, CD86, and CD11c in individual patients. The down-regulation of CD34 expression was seen in three of the four patients in response to PMA plus A23187, suggesting differentiation to a more mature cell. FACs analysis for patient 9390 is shown in Fig. 5B. In this patient, untreated CML blasts expressed...
CD34 (which was nearly 100% of the cells analyzed), MHC I, and MHC II, but no detectable CD80, CD86, CD40, or CD83. PMA alone induced up-regulation of MHC II, CD40, and CD11c (slight) and down-regulated CD34. PMA plus A23187 induced a greater up-regulation of MHC I, MHC II, and CD40 vs PMA alone, and PMA plus A23187 induced the expression of CD80, CD83, and CD86. The two peaks seen for some surface markers (MHC I, CD80, and CD40) in the PMA- plus A23187-treated samples suggest the development of two distinct subpopulations. At the molecular level we also saw up-regulation of Rel B in primary CML blasts during differentiation (data not shown).

Functionally, PMA plus A23187 induced CML blasts from three of four patients to stimulate allogeneic T cell proliferation (patients 2025, 9390, and 0274; 26-, 75-, and 85-fold higher, respectively, than that induced by untreated blasts). The variability in allostimulatory capacity between patients may be due in part to T cells from different normal donors (with differing degrees of MHC mismatch) as well as differences in the extent of DC differentiation. The allopromotion induced by differentiated CML blasts from patient 9390 (the same blasts as those used for FACS analysis in Fig. 5B) is shown in Fig. 5C. Similar to the surface marker expression, CI synergized with PMA-induced differentiation to induce a more potent allostimulatory T cell response than did cells treated with either PMA or CI alone (75-, 7.5-, and 6.5-fold increase, respectively, in T cell proliferation over that induced by untreated blasts; data not shown). Interestingly, the expression of MHC I and MHC II on the undifferentiated blasts was not sufficient to induce T cell proliferation, suggesting that it is the up-regulation of costimulatory ligands (and

FIGURE 3. Induction of allogeneic T cell proliferation. A, T cell stimulation. K562 cells were left untreated or were differentiated in the presence of PMA for 5 days. Monocytes were left untreated or were differentiated in the presence of GM-CSF, IL-4, and TNF-α for 12 days. Cells were harvested, gamma-irradiated, and plated in triplicate. Purified resting allogeneic T cells (1 × 10^5) were incubated with increasing numbers of stimulator cells as indicated. Proliferation was measured in triplicate by [³H]TdR incorporation and is expressed as the mean ± SD. T cells alone stimulated with PMA and ionomycin were used as a positive control. B, Reversibility of allostimulatory capacity. K562 were left untreated or differentiated in the presence of PDBu (5 days) or PDBu (5 days) followed by PDBu washout and were cultured in medium for an additional 5 days. Purified resting allogeneic T cells (1 × 10^5) were incubated in triplicate with 1 × 10^5 stimulators, and T cell proliferation was measured as described in A.
possibly other molecules, such as adhesion receptors and cytokines) that is the basis of CML-derived DLC allostimulatory capability.

The development of typical DC morphology, enhanced expression of MHC, costimulatory and DC activation markers, and enhanced T cell allostimulatory capacity are all consistent with differentiation of primary CML blasts to DLC by PMA (with or without A23187). These results suggest that similar to the K562 cell line, activation of PKC in primary CML blasts drives DC differentiation.

**DC differentiation is associated with down-regulation of BCR-ABL expression**

The ability of PKC activation or exogenous cytokines to drive DC differentiation in CML blasts indicates that these signals can overcome the differentiation block in CML. Given the central role of BCR-ABL in CML oncogenesis, we next assessed the effect of CML→DC differentiation on the oncogene’s expression. As shown in Fig. 6A, PMA-induced K562 differentiation resulted in down-regulation of BCR-ABL protein by day 5, which was not significantly changed by the addition of TNF-α. Blocking PKC activation by the PKC inhibitor bisindolylmaleimide I also blocked BCR-ABL down-regulation, consistent with PKC activation being the primary pathway involved in PMA-induced differentiation of K562. To determine whether this down-regulation was unique to PMA-driven activation or more generally related to the process of DC differentiation, we examined the effect of cytokine-mediated differentiation on BCR-ABL expression. Similar to PMA, GM-CSF plus TNF-α-driven K562 differentiation down-regulated BCR-ABL protein expression, but with the same slower kinetics (day 14 vs day 5) as those seen for surface marker up-regulation. Consistent with down-regulation at the protein level, we found a parallel decrease in total BCR-ABL kinase activity as assessed by in vitro autophosphorylation (Fig. 6B), suggesting that there was not a separate effect on the oncogene’s kinase activity. Whether the loss of BCR-ABL is reversible after the removal of the stimulus is currently being studied.

We next sought to determine at what level BCR-ABL down-regulation was occurring. As shown in Fig. 6C, both PMA and PMA plus TNF-α induced early down-regulation of BCR-ABL
pH3 expression. Interestingly, in the PMA- plus TNF-α-treated cultures by 24 h of differentiation, mRNA expression began to rebound (and by 72 h in PMA-treated cultures), but BCR-ABL protein expression remained reduced even on day 5. Whether this represents additional mechanisms regulating BCR-ABL expression is under investigation.

Signal transduction pathways in BCR-ABL regulation
To begin to characterize the signal transduction pathways that led to down-regulation of BCR-ABL expression, we examined the

Table 1. Surface marker expression in unstimulated and PMA-, PMA plus A23187-, or GM-CSF, TNF-α, plus IL-4-stimulated primary CML blasts

<table>
<thead>
<tr>
<th>Patient ID</th>
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<th>P</th>
<th>PI</th>
<th>GT + IL-4</th>
<th>UT</th>
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*Primary CML blasts were left untreated (UT) or were treated with PMA (P), PMA plus A23187 (PI), or GM-CSF, TNF-α, and IL-4 (GT + IL-4) for 3 days. Cells were analyzed by flow cytometry. Data are represented as the log increase in mean fluorescence intensity over the isotype control (-, <0.5 log; +1, ≥0.5 log <1 log; +2, ≥1 log <1.5 log; +3, ≥1.5 log). Patient 6227, peripheral blood from blast crisis; 9390, peripheral blood from blast crisis; 2025, bone marrow from accelerated phase; 0274, peripheral blood from accelerated phase.*
Discussion

DC generated ex vivo from CML blasts may be potent APC (endogenously expressing a gamut of leukemia Ags) with the ability to effectively stimulate antileukemia immune responses and thus have clinical utility in antileukemia vaccine strategies. However, the biology of CML differentiation (including the signal transduction pathways involved, genetic changes initiated, etc.) and how this differentiation compares to normal progenitor-derived DC are largely uncharacterized. As BCR-ABL is both a critical oncogene and a tumor-specific Ag in CML, characterization of its expression during differentiation may yield important observations for both leukemia biology as well as the potential for CML-derived DLC to present tumor-specific Ag in a vaccination context. We have found that the CML cell line K562 retained the ability to acquire DC characteristics in response to direct activation of PKC by PMA. Similar to PMA-induced differentiation in the CD34+ AML cell line KG1, TNF-α also potentiated PMA-induced DC differentiation in K562. PMA induced K562 to become adherent cells with typical DC morphology, stop proliferating, and up-regulate characteristic DC surface markers, consistent with what we have previously reported for primary CD34+ HPC and KG1 (24, 26). CI mobilization, also used as an intracellular signal for DC differentiation, resulted in greater up-regulation of CD80, CD86, and CD83, but failed to up-regulate MHC I or MHC II, consistent with what has been previously reported for other leukemic cell lines (25). At a molecular level, we found that Rel B expression and activity were rapidly up-regulated, in addition to other NF-κB family members. Additionally, the DC-specific chemokine DC-CK1 was up-regulated shortly after PKC activation. Functionally, PMA-differentiated K562 up-regulated their ability to activate T cells to levels comparable to those of monocyte-derived DC, consistent with differentiation to mature DC. Similar to studies in primary CML blasts (6, 9, 11), GM-CSF plus TNF-α resulted in K562 acquisition of certain DC characteristics, but with slower kinetics than PMA. In contrast to PMA, CI and GM-CSF did not synergize or fortify these DC characteristics in K562, probably reflecting the full maturation of these blasts for ultimate DC differentiation (12, 56, 68), and they underscore how agents that bypass these proximal elements to directly activate downstream signaling pathways may be more effective in driving DC differentiation in leukemic blasts. Our results for primary CML blasts (accelerated and blast phase) corroborated our K562 findings, in that
activation of PKC induced the differentiation of cells with morphological, phenotypic, molecular, and functional characteristics of DC.

Compared with DC derived from normal progenitors, we have found that CML-derived DLC are considerably more variable in terms of DC.

The data are plotted as the negative change in $x$-axis (s-axis). A greater change in fluorescence at a given temperature represents a higher product concentration. The line marking the peak of the curve represents the predicted melting temperature of the BCR-ABL product. The BCR-ABL product of the same reaction was visualized by agarose gel electrophoresis (inset). BCR-ABL-specific primers spanning the translocation point were used in the reaction.

FIGURE 8. BCR-ABL expression during DC differentiation in primary CML blasts. A, Semiquantitative RT-PCR. CML blasts from bone marrow or peripheral blood of patients with accelerated phase or blast crisis were cultured in PMA for 7 days. Total RNA was isolated from untreated and PMA-treated blasts and subjected to semiquantitative RT-PCR with BCR-ABL- or $\beta$-actin-specific primers. The products were hybridized with $^{32}$P-labeled BCR-ABL-specific probe or $\beta$-actin probe as a control. Bands were visualized by autoradiography. BCR-ABL levels were measured by densitometry, normalized to $\beta$-actin, and are shown as the percent expression relative to untreated blasts. B, Real-time fluorogenic PCR. CML blasts from the peripheral blood of a patient with accelerated phase disease were cultured in PMA for 7 days. Total RNA was isolated from untreated blasts at time zero or from PMA-treated blasts and subjected to real-time PCR. The data are plotted as the negative change in fluorescence (y-axis) over melt temperature (x-axis). A greater change in fluorescence at a given temperature represents a higher product concentration. The line marking the peak of the curve represents the predicted melting temperature of the BCR-ABL product. The BCR-ABL product of the same reaction was visualized by agarose gel electrophoresis (inset). BCR-ABL-specific primers spanning the translocation point were used in the reaction.
76) appeared to be important for BCR-ABL down-regulation, consistent with our findings that these two signal transduction pathways control different aspects of DC differentiation (our manuscript in preparation). How down-regulation of BCR-ABL affects the ability of CML-derived DC to induce an antileukemic T cell response (and the nature of this response) is currently being examined.

Although our data do not prove a direct link between BCR-ABL down-regulation and the onset of DC differentiation, other studies have shown that direct inhibition of BCR-ABL kinase activity restores growth factor dependence in BCR-ABL-transformed hematopoietic cells, regulates erythroid differentiation in human CML lines, and induces apoptosis (42, 77, 78). On the other hand, PMA-treated K562 are capable of initiating differentiation (e.g., morphologic changes, surface Ag up-regulation, Rel B up-regulation, decreased proliferation, etc.) despite continued BCR-ABL kinase activity, albeit at a reduced level compared with that in untreated cells. Cells differentiated in PMA in the presence of MAPK inactivity, albeit at a reduced level compared with that in untreated cells. Cells differentiated in PMA in the presence of MAPK inactivity, albeit at a reduced level compared with that in untreated cells. 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