Human CD34-Derived Myeloid Dendritic Cell Development Requires Intact Phosphatidylinositol 3-Kinase–Protein Kinase B–Mammalian Target of Rapamycin Signaling


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Human CD34-Derived Myeloid Dendritic Cell Development Requires Intact Phosphatidylinositol 3-Kinase–Protein Kinase B–Mammalian Target of Rapamycin Signaling

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Dendritic cells (DCs) are composed of different subsets that exhibit distinct functionality in the induction and regulation of immune responses. The myeloid DC subsets, including interstitial DCs and Langerhans cells (LCs), develop from CD34+ hematopoietic progenitors via direct DC precursors or monocytes. The molecular mechanisms regulating DC development are still largely unknown and mostly studied in mice. Phosphatidylinositol 3-kinase (PI3K) regulates multiple processes in myeloid cells. This study investigated the role of PI3K signaling in the development of human CD34-derived myeloid DCs. Pharmacologic inhibition of PI3K or one of its downstream targets mTOR reduced interstitial DC and LC numbers in vitro. Increased activity of this signaling module by introduction of constitutively active protein kinase B (PKB/c-Akt) increased the yields of human DC precursors in vitro as well as in transplanted β2-microglobulin−/− NOD/SCID mice in vivo. Signaling inhibition during differentiation did not affect the acquisition of a DC phenotype, whereas proliferation and survival strongly depended on intact PI3K–PKB–mTOR signaling. Interestingly, however, this pathway became redundant for survival regulation upon terminal differentiation, which was associated with an altered expression of apoptosis regulating genes. Although dispensable for costimulatory molecule expression, the PI3K–PKB–mTOR signaling module was required for other important processes associated with DC function, including Ag uptake, LPS-induced cytokine secretion, CCR7 expression, and T cell stimulation. Thus, PI3K–PKB–mTOR signaling plays a crucial role in the development of functional CD34-derived myeloid DCs. These findings could be used as a strategy to manipulate DC subset distribution and function to regulate immunity. The Journal of Immunology, 2010, 184: 000–000.
cell factor (SCF), the two functionally different myeloid subsets, intDC and LC, develop via independent pathways and can therefore be studied separately (21, 22). The differentiation in two phases, with pre-DCs developing prior to terminal differentiation, enables detailed analysis of CD34-derived myeloid DC development. In this by vitro system. In addition, CD34-derived DC development can also be studied in vivo, by xenotransplantation of human HPCs into immune-deficient mice (23–25).

In this study, we investigated the importance of PI3K signaling for the development of functional intDCs and LCs from human cord blood CD34+ HPC. PI3K-induced mTOR activation, mediated by PKB, was shown to be crucial for human myeloid CD34-derived DC development in vitro and in vivo. Precursor proliferation and survival strongly depended on this signaling module. Although inhibition of this pathway did not affect the acquisition of a DC phenotype, DCs generated under PI3K or mTOR inhibition were functionally impaired. In contrast to the requirement for PI3K signaling during development, the survival of terminally differentiated CD34-derived myeloid DCs was not dependent on PI3K or mTOR activity. However, this signaling module was still required for other important processes associated with DC function. These findings demonstrate the importance of the PI3K–PKB–mTOR signaling module during CD34-derived myeloid DC development, providing possibilities to manipulate DC subset distribution and function to regulate immunity.

Materials and Methods

Generation of CD34-derived myeloid DCs

Umbilical cord blood samples were obtained ex utero according to legal guidelines. CD34+ hematopoietic progenitor cells were isolated and cultured as described previously (22, 26). CD34+ cells were isolated from mononuclear fractions through positive selection using anti-CD34-coated microbeads and MS separation columns (Miltenyi Biotec, Bergish Gladbach, Germany) to a purity of 85–98%. After cryopreservation to standardize differentiation, these cells were cultured in complete medium containing RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 8% heat inactivated FCS (Hyclone; Thermo Fisher Scientific, Etten-Leur, The Netherlands), 10 mM Hepes (Invitrogen), 2 mM l-glutamine (Invitrogen), 50 mM β-mercaptoethanol (Merck, Darmstadt, Germany), and penicillin/streptomycin (Invitrogen). From day 0 to 6, the cells were cultured in complete medium supplemented with 100 ng/ml GM-CSF (Scherering-Plough, Houten, The Netherlands), 25 ng/ml SCF (PeproTech, London, U.K.), 2.5 ng/ml TNF-α (R&D Systems, Abingdon, U.K.) and 5% heat inactivated human serum, and 0.02% NaN₃. Labeling of cell surface markers was performed on ice, using fluorochrome-conjugated Abs against the following Ags: CD1a (HI149; BD Biosciences, Breda, The Netherlands), CD14 (M6P9; BD Biosciences), Langerin/CD207 (DCGM4; Beckman Coulter, Woerden, The Netherlands), CD1c (AD5-8E7; Miltenyi Biotec), CD20 (L27; BD Biosciences), CD86 (Fun-1; BD Biosciences), CD83 (HB15e; BD Biosciences), HLA-DR (L243, BD Biosciences) and CCR7 (150503, R&D Systems). Apoptosis was detected by determination of phosphatidyl serine exposure and membrane permeability. Cells were harvested, washed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4), incubated with Annexin V-FITC (BD Biosciences) for 30 min on ice and subsequently taken up in 1 μg/ml propidium iodide (PI; Sigma-Aldrich). Transduced cells were incubated with Annexin V-PE (BD Biosciences) and 7-aminoactinomycin D (7-AAD; BD Biosciences) in Annexin buffer for 30 min. Assessment was performed using a FACS Calibur or FACS CantoII (BD Biosciences), and data were analyzed using Cell Quest Pro and FACS Diva software (BD Biosciences).

Cell cycle progression analysis

Cells were harvested, washed in PBS and taken up in PBS/0.5% EDTA. Ethanol was added and cells were fixed in 50% ethanol for 30 min on ice. Next, cells were washed and subsequently incubated with PBS/0.5% EDTA with 40 μg/ml RNAse A (Promega, Madison, WI) for 30 min at room temperature. PI (50 μg/ml) was added and fluorescence intensity was immediately measured by flow cytometry (FACS Calibur).

Isolation of subset specific precursors or transduced cells by FACS sorting

Subset-specific precursors or retrovirally transduced cells were sorted using a FACS Aria (BD Biosciences). Control cultures were harvested at day 6, incubated with fluorochrome-conjugated Abs against CD1a and CD14, life gated, and sorted into CD14+CD1a+ and CD14+CD1a− fractions. eGFP+ cells were isolated 3–4 d after transduction.

Western blot

Cells were washed in PBS, lysed in Laemmli sample buffer (0.12 M Tris HCl [pH 6.8], 4% SDS, 20% glycerol, 0.05% w/v bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 min. Protein concentrations were determined by Lowry. Equal amounts of total lysate (10 μg/lane) were separated by 10% SDS-PAGE, transferred to a PVDF membrane (Millipore, Bedford, MA) and incubated with blocking buffer (Tris buffered saline/Tween20) containing 5% BSA or low-fat milk before probing with mouse anti-human phosphotyrosine (pTyr) 56 (rabbit polyclonal; Cell Signaling Technology, Danvers, MA) and tubulin (mouse monoclonal; Sigma-Aldrich). Subsequently, blots were incubated with HRP-conjugated secondary Abs (Dako, Glostrup, Denmark). Enhanced chemical luminescence was used as a detection method according to the manufacturer’s protocol (Amersham Pharmacia, Amersham, U.K.).

Reverse transcription-multiplex ligation-dependent probe amplification

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. OD260/262 ratios were measured to determine the quantity and purity of RNA preparations and RNA integrity was checked using the Agilent RNA 6000 nano assay kit according to manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). Reverse transcription-multiplex ligation-dependent probe amplification

24 h, viral supernatants were collected, filtered through a 0.2-μm filter, and subsequently frozen.

CD34+ progenitors were transduced in 24-well culture dishes precoated with 10 μg/cm² recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan) for 16 h. Transduction was performed by adding 0.5 ml viral supernatant to 0.5 ml medium containing 5 to 30 × 10⁶ cells. After 24 h, 0.7 ml medium was removed and 0.5 ml fresh viral supernatant was added together with 0.5 ml fresh medium. For in vitro experiments, cells were transduced in complete medium supplemented with GM-CSF, IFNα, SCF, and human serum in the concentrations described above. CD34+ progenitors used for in vivo experiments were transduced in IMDM medium (Invitrogen) supplemented with 8% heat inactivated FCS, penicillin/streptomycin, 50 μM β-mercaptoethanol, 2 mM l-glutamine, 50 mM NaFl3 ligand (Peprotech), and 50 ng/ml SCF.
Transplantation of β2-microglobulin−/− NOD/SCID mice with human CD34+ progenitors

Protocols for mouse experiments were approved by the local animal experimental committee. The β2-microglobulin−/− NOD/severe combined immune deficient (NOD/SCID) mice were bred and maintained under sterile conditions in microisolator cages and provided with autoclaved food and acidified water containing 111 mg/L ciprofloxacin (Ciproxin). Immune deficient (NOD/SCID) mice were virally transduced cord blood-derived CD34 + hematopoietic progenitors and transplants via tail vein injections with Eight- to 10-wk-old mice, sublethally irradiated with 250 cGy x-rays, re-

Cytokine production was measured in supernatants of stimulated cells by multiplex particle-based flow cytometry or ELISA. Concentrations of IL-6, IL-10, and TNF-α were measured with the Bio-Plex Manager software version 4.0 (Bio-Rad Laboratories, Hercules, CA), which uses Luminex xMAP technology as previously described (31, 32). The multiplex data (cytokine concentrations) were digitized to create a cytokine portrait enabling the complete spectrum of cytokines to be visualized. The detection limit of this procedure was 1.2 pg/ml. The commercially available ELISA kits for human IL-5 (eBioscience, San Diego, CA), IL-6 (eBioscience), IL-8 (Biosource, Invitrogen), IL-10 (eBioscience), IL-12p70 (eBioscience), IP-10 (Biosource, Invitrogen), and IFN-γ (eBioscience) were used according to manufacturer’s instructions. The detection limits of these assays were 2 pg/ml (IL-6, IL-10, and IP-10), 4 pg/ml (IL-5, IL-12p70, and IFN-γ), and 5 pg/ml (IL-8).

Allogeneic MLR

Responder T cells were isolated from a buffy coat. The mononuclear fraction was incubated with anti-CD15- and anti-CD234–coated microbeads (Miltenyi Biotec) and PE-labeled Abs against CD1c, CD14, CD19 (J4.119, Beckman Coulter, Woerden, The Netherlands), CD56 (MY31, BD Biosciences). A, Day 6 (n = 9) and day 13 (n = 4) cells were harvested and analyzed for the expression of CD14 and CD1a. Viable cells were gated on FSC/SSC. Representative FACS plots are shown. B, LPS-stimulated cells were harvested at day 15. Cells were analyzed for the expression of CD83, CD86, and HLA-DR. Viable cells were gated on FSC/SSC. Representative FACS plots (n = 2) are shown. C, At day 13, total cell numbers were determined by counting with trypan blue exclusion and absolute CD1a+ DC numbers (including both CD14+ and CD14− cells) were calculated and standardized to control cultures. Data shown represent mean ± SEM CD1a+ DC numbers of four independent experiments with different donors (* p < 0.05, Student paired t test on original data). D, LY, Rapa, or DMSO was added to control day 6 FACSorted pre-DCs. Cells were harvested after 2 d and whole cell lysates were prepared. Phosphorylation of S6 (p-S6) and tubulin expression were determined. FSC, forward scatter; SSC, side scatter.

FIGURE 1. PI3K and mTOR are required for CD34-derived myeloid DC development, but do not regulate differentiation. LY, Rapa or their solvent DMSO were added to CD34-derived myeloid DC differentiation cultures at day 0, 4, 6, and 10. At day 13, cells were thoroughly washed and subsequently stimulated with LPS. A, Day 6 (n = 9) and day 13 (n = 4) cells were harvested and analyzed for the expression of CD14 and CD1a. Viable cells were gated on FSC/SSC. Representative FACS plots are shown. B, LPS-stimulated cells were harvested at day 15. Cells were analyzed for the expression of CD83, CD86, and HLA-DR. Viable cells were gated on FSC/SSC. Representative FACS plots (n = 2) are shown. C, At day 13, total cell numbers were determined by counting with trypan blue exclusion and absolute CD1a+ DC numbers (including both CD14+ and CD14− cells) were calculated and standardized to control cultures. Data shown represent mean ± SEM CD1a+ DC numbers of four independent experiments with different donors (* p < 0.05, Student paired t test on original data). D, LY, Rapa, or DMSO was added to control day 6 FACSorted pre-DCs. Cells were harvested after 2 d and whole cell lysates were prepared. Phosphorylation of S6 (p-S6) and tubulin expression were determined. FSC, forward scatter; SSC, side scatter.

Ag uptake assays

Ag uptake was analyzed as described before (30). Fluid phase and lectin-mediated endocytosis were measured as the cellular uptake of 100 µg/ml Lucifer Yellow dipotassium salt ( Molecular Probes, Invitrogen) and 100 µg/ml dextran-FITC (dextranFITC, 40,000 MW; Molecular Probes, Invitrogen), respectively. Approximately 5 × 10⁶ DCs were incubated for 2 h at 37°C in the presence of culture medium containing Lucifer Yellow or dextranFITC. Negative controls were incubated with the respective Ag at 4°C. Staining was evaluated by flow cytometry, and Ag uptake was calculated by subtracting staining at 4°C from staining at 37°C.

Detection of cytokine production

Cytokine production was measured in supernatants of stimulated cells by multiplex particle-based flow cytometry or ELISA. Concentrations of IL-6, IL-10, and TNF-α were measured with the Bio-Plex Manager software version 4.0 (Bio-Rad Laboratories, Hercules, CA), which uses Luminex xMAP technology as previously described (31, 32). The multiplex data (cytokine concentrations) were digitized to create a cytokine portrait enabling the complete spectrum of cytokines to be visualized. The detection limit of this procedure was 1.2 pg/ml. The commercially available ELISA kits for human IL-5 (eBioscience, San Diego, CA), IL-6 (eBioscience), IL-8 (Biosource, Invitrogen), IL-10 (eBioscience), IL-12p70 (eBioscience), IP-10 (Biosource, Invitrogen), and IFN-γ (eBioscience) were used according to manufacturer’s instructions. The detection limits of these assays were 2 pg/ml (IL-6, IL-10, and IP-10), 4 pg/ml (IL-5, IL-12p70, and IFN-γ), and 5 pg/ml (IL-8).

Allogeneic MLR

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Biosciences), and CD123 (SSDCLY107D2, Beckman Coulter), followed by incubation with anti-PE-coated microbeads (Miltenyi Biotec). T cells were isolated through negative selection according to manufacturer’s instructions (Miltenyi Biotec). Irradiated DCs (30 Gy) were added in graded doses to 2 × 10^6 allogeneic T cells in 96-well round bottom plates in RPMI 1640 containing 8% heat inactivated FCS. Proliferation was quantified by incubation with 1 μCi (37 kBq) [methyl-3H]thymidine (NEN Life Science Products; Boston, MA) during the last 18 h of 6-d cultures.

**Results**

**Inhibition of PI3K or mTOR strongly reduces CD34-derived myeloid DC yield**

The importance of PI3K signaling for the development of CD34-derived myeloid DCs was investigated by addition of a specific pharmacologic PI3K inhibitor, LY, during DC development in vitro. Continuous exposure to LY from day 0 did not affect differentiation, as shown by the presence of CD14^+CD1a^- precursor intDC (pre-intDC) and CD14^-CD1a^- precursor LC (pre-LC) at day 6 and 90% CD14^-CD1a^- myeloid DCs present at day 13, regardless of the presence of PI3K inhibition (Fig. 1A). Also, the specific myeloid DC marker CD1c was expressed by the majority of the CD1a^- cells in the control as well as inhibitor cultures (data not shown). In addition, stimulation of these cells with LPS in the absence of LY induced comparable expression of CD83, CD86, and HLA-DR for all cells (Fig. 1B). Thus, blockade of PI3K during DC development did not significantly affect the phenotype of the DCs. However, a strong reduction (64.4 ± 7.9%) in the yield of CD1a^- DCs was observed at day 13 (Fig. 1C).

To determine the mechanism by which PI3K regulates DC development, the role of mTOR, one of the proteins whose activity is regulated by PI3K signaling, was examined. Both LY and Rapa, a specific pharmacologic inhibitor of mTOR, reduced the phosphorylation of S6 (Fig. 1D), a target downstream of mTOR (33). Similar to LY, addition of Rapa allowed development of pre-DCs and terminally differentiated DCs from CD34^+ progenitors, but significantly reduced the DC yield (Fig. 1A,1C). Also as observed for LY, LPS-induced costimulatory molecule expression was unaffected for these cells (Fig. 1B). Because LY and Rapa strongly reduced DC numbers without affecting their phenotype, we conclude that both PI3K and mTOR activity are required for CD34-derived myeloid DC development, but these proteins seem to regulate processes other than differentiation.

**PI3K-mediated regulation of pre-DC numbers by a combined effect on survival and proliferation**

To investigate how PI3K and mTOR regulate DC development, we first focused on pre-DC development (day 0 to 6) and examined the consequences of PI3K and mTOR inhibition in more detail. Depending on the donor, control cultures resulted in a 3- to 50-fold increased cell yield after 6 d, compared with day 0. Addition of either LY or Rapa significantly reduced cell yields in a dose-
dependent manner (Fig. 2A, 2B). At a concentration of 2 μM LY or 20 nM Rapa, which are both within the range of concentrations used in literature, cell growth was inhibited with 67 ± 9.7% and 83 ± 12.5% (Fig. 2A), respectively. Increasing the concentration of Rapa hardly induced additional effects (Fig. 2B). Increased concentrations of LY, however, appeared to be extremely toxic (Fig. 2B), which might be explained by mechanisms other than mTOR inhibition (34). These data show that the expansion of DC precursors requires both PI3K and mTOR activation.

To determine whether the reduced expansion in PI3K- or mTOR-inhibited cultures was due to decreased cell survival and/or proliferation, cell viability and cell cycle profiles were analyzed at different time points during the 6-d culture period. As demonstrated by increased V binding in both the PI− and PI+ cell population upon culture in the presence of LY or Rapa, PI3K and mTOR inhibition induced apoptosis at all time points (Fig. 2C, 2D). In addition, a decreased cell cycle progression was observed, as shown by the increased proportion of cells in G0/1 (Fig. 2E, 2F). These data show that a combination of reduced survival and decreased proliferation was responsible for the reduction in pre-DC yields found upon inhibition of PI3K or mTOR activity.

PI3K or mTOR inhibition during terminal differentiation induces specific loss of less differentiated cells

Then, the importance of PI3K and mTOR activity during development of pre-DCs into intDCs and LCs was investigated. Day 6 pre-intDCs and pre-LCs were isolated by FACS sorting and subsequently cultured in the presence or in the absence of LY or Rapa. In both pre-intDC and pre-LC cultures, inhibition of PI3K or mTOR resulted in reduced cell survival by the induction of apoptosis within 1 d (Fig. 3A). Under control conditions, CD1a−CD14− pre-intDCs differentiate to CD1a+CD14− intDC via an intermediate CD1a−CD14+ state, while CD1a+CD14+ pre-LCs remain CD1a+ and acquire upon terminal differentiation (22). Interestingly, in intDC cultures both PI3K and mTOR inhibition induced a time-dependent relative increase in differentiated cells (Fig. 3B, 3C). After 1 d, the ratio of differentiated CD1a+ cells and CD1a−CD14+ preintDC was similar for all intDC cultures irrespective of PI3K or mTOR inhibition. Two and 3 d of incubation with inhibitors resulted in a relative increase in CD1a+ cells compared with control cultures. Also, the percentage of langerin+ cells in end-stage LC cultures was increased upon addition of LY or Rapa from day 6 (Fig. 3D, 3E). However, this relative increase was not due to an increased differentiation, as demonstrated by the unchanged CD1a+ and langerin+ absolute cell numbers (Fig. 3F, 3G). Instead, it resulted from a specific loss of the less differentiated CD1a− and langerin− cells. Thus, PI3K and mTOR activity is required for the survival of pre-intDC and pre-LC, whereas further differentiated cells seem to be unaffected by inhibition of this pathway.

Terminally differentiated DCs are less dependent on PI3K and mTOR activity than pre-DCs

To further investigate this apparent reduction in dependency, the role of PI3K and mTOR in the survival of pre-DCs and terminally differentiated DCs was compared. LY or Rapa were added to pre-intDCs and pre-LCs, or to intDCs and LCs generated from these pre-DCs. In contrast to pre-intDCs (Fig. 3A), terminally differentiated intDCs survived in the presence of LY or Rapa as shown by an V/PI staining comparable to control cultures (Fig. 4A, 4B). Similarly, LY- and Rapa-induced apoptosis was reduced in LC cultures compared with pre-LC cultures (Fig. 4C), leading to a similar survival in inhibitor and control cultures (Fig. 4A). Thus, whereas pre-intDCs and pre-LCs highly depend on PI3K and mTOR activity, these proteins seem redundant for the survival of terminally differentiated DC.
To investigate whether these differences in survival regulation could be explained by changes in the expression of known regulators of apoptosis, RT-MLPA was used to compare the mRNA composition of pre-intDCs and intDCs as well as pre-LCs and LCs. Large differences in relative expression profiles were induced during development of both subsets (Table I). Some of these changes, including the increased relative expression of antiapoptotic IAP family members and the reduced expression of proapoptotic proteins such as Apaf, could reflect an overall reduced susceptibility to apoptosis. However, other differences, such as the reduced relative expression of the mTOR-regulated McI-1, suggest a specific reduction in PI3K/mTOR dependency for survival.

**PI3K–PKB–mTOR signaling regulates CD34-derived myeloid DC development**

Although treatment with LY or Rapa induced overlapping results (Figs. 1–4), it remains possible that the observed effects of LY and Rapa treatment are due to unrelated consequences of PI3K and mTOR inhibition. Because PI3K-induced mTOR activation is mediated by the activation of PKB (11), the relation between LY- and Rapa-induced effects on DC development was investigated by transducing CD34+ HPCs with a bicistron viral DNA construct coexpressing eGFP and a constitutively active form of PKBα (myrPKB). HPCs transduced with myrPKB showed increased expansion in relation to progenitors transduced with the control construct (Fig. 5A), and the viability of pre-DCs expressing myrPKB was increased compared with control cells (Fig. 5B). Furthermore, LY-induced apoptosis was completely abolished in myrPKB-transduced cells, whereas apoptosis induced by mTOR inhibition was unaffected (Fig. 5C), placing PKB downstream of PI3K and upstream of mTOR. Moreover, myrPKB was able to rescue cells from apoptosis induced by deprivation of GM-CSF (Fig. 5D), a cytokine known to trigger PI3K activation and involved in myeloid cell survival (34, 35). These data indicate that the roles of PI3K and mTOR in CD34-derived myeloid DC development can be ascribed to their function in the PI3K–PKB–mTOR signaling module and suggest that GM-CSF stimulation results in activation of this pathway in vitro.

**Constitutive PKB activation increases CD34-derived myeloid DC development in vivo**

To investigate human CD34-derived DC development in vivo, myrPKB- or control-transduced human CD34+ HPCs were transplanted into NOD/SCID β2-microglobulin−/− mice. Six weeks after injection, human myeloid DCs originating from transduced HPCs could be recognized in the bone marrow by their expression of eGFP and CD1c+. The proportions of eGFP+CD1c+CD20− human DCs in mice transplanted with progenitors expressing constitutively active PKB were enhanced 20-fold compared with mice that had received a transplant of control-transduced HPCs (Fig. 5E, 5F). Thus, in vivo CD34-derived human myeloid DC development is augmented by the ectopic expression of constitutively active PKB, which confirms the role of PI3K–PKB–mTOR signaling in the development of these cells.

**A role for PI3K and mTOR in functional, but not phenotypic maturation**

While we could show that PI3K and mTOR activity was not critical for the phenotype or survival of terminally differentiated DC, inhibition of PI3K or mTOR activity during either DC differentiation or DC activation has been suggested to negatively affect DC function (13, 36–38). We therefore investigated the functionality of CD34-derived myeloid DC differentiated or activated in the presence of LY or Rapa. Both lectin-mediated and fluid phase endocytosis, as investigated by incubation with dextran FITC and Lucifer Yellow, respectively, were reduced in cells generated in the presence of Rapa, while cells generated with LY showed only a slightly reduced Ag uptake capacity (Fig. 6). Ag uptake capacity
of control CD34-derived myeloid DC, however, was not affected by the addition of LY or Rapa during incubation with dextran\textsuperscript{Hoechst 33342} or Lucifer Yellow (data not shown).

Also the expression of costimulatory molecules was unaffected by PI3K or mTOR inhibition, and neither LY nor Rapa affected LPS-induced upregulation of CD83, CD86, or HLA-DR (Fig. 7A). However, LY and Rapa inhibited CCR7 expression (Fig. 7A), indicating that this signaling module may be involved in the regulation of lymph node migration. Although only low cytokine concentrations could be measured due to low cell densities, LPS stimulation induced the production of detectable levels of IL-6, IL-8, and IP-10, which were reduced by the addition of LY and Rapa during either differentiation or activation of the cells (Fig. 7C). The secretion of IL-10 and TNF-α depended on intact PI3K and mTOR activity (Fig. 7C). Secretion of IL-12p70, which could be detected only after stimulation with CD40L, was also reduced by the presence of LY or Rapa during either differentiation or activation (Fig. 7C and data not shown). In accordance with the stronger effects of Rapa compared with LY (Fig. 7), T cell proliferation induced by myeloid DCs pretreated with Rapa, but not LY, was reduced compared with control DCs (Fig. 8). Only when using DCs treated with Rapa during activation was this reduced proliferation clearly associated with reduced IFN-γ concentrations (Fig. 8). A reduction in IL-5 production was also observed in cocultures of T cells with Rapa-treated DCs, whereas IL-10 concentrations were similar in all cultures (Fig. 8). Like for the nonstimulated DCs, neither LY nor Rapa affected the survival of LPS-activated DCs (data not shown), indicating that the effects on DC function were not caused by reduced DC viability. These data show that whereas PI3K and mTOR activity are dispensable for the phenotype and survival of LPS-activated DCs, whereas activation of this pathway increased DC development (19, 20), the requirement of DC development and survival for both immunogenic and tolerogenic responses, the molecular mechanisms regulating these processes are still largely undefined. In this study, we have shown that PI3K–PKB–mTOR signaling is required for proliferation and survival, but not differentiation, of human CD34-derived myeloid DCs. Inhibition of PI3K–PKB–mTOR activity is dispensable for the phenotype and survival of terminally differentiated CD34-derived myeloid DCs, these proteins play an important role in the functionality of these cells.

**Discussion**

DCs play a crucial role in the induction and regulation of immunity. Despite the requirement of DC development and survival for both immunogenic and tolerogenic responses, the molecular mechanisms regulating these processes are still largely undefined. In this study, we have shown that PI3K–PKB–mTOR signaling is required for proliferation and survival, but not differentiation, of human CD34-derived myeloid DCs. Inhibition of PI3K–PKB–mTOR activity is dispensable for the phenotype and survival of terminally differentiated CD34-derived myeloid DCs, these proteins play an important role in the functionality of these cells.

### Table I. Differentiation-induced changes in genes regulating apoptosis

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Pre-intDCs and pre-LCs were isolated by FACSort at day 6. Cells were further cultured and harvested at day 7 or 14 after reseeding at day 13. Total RNA was isolated and analyzed by RT-MLPA. The expression of 34 apoptosis genes was determined, and relative expressions were calculated as percentages of total mRNA. Per gene, the difference of expression (Δ) was calculated as Δ = log<sub>2</sub>(relative expression in DC/relative expression in pre-DC). Δ ≥ 1 and Δ ≤ −1, with the difference between pre-DC and DC being statistically significant (p < 0.05, paired Student t test), are seen as biologically significant and shown in bold (n = 3–4).

<sup>a</sup>Values <0.5 are subject to fluctuation. Therefore, when values for both pre-DC and DC were ≤0.5, no further analysis has been performed.

<sup>b</sup>When only one value was <0.5, analysis was performed as for all other genes, but care must be taken when interpreting these data.
HUMAN MYELOID DC DEVELOPMENT REQUIRES PI3K–PKB–mTOR

In contrast to moDC development, the development of myeloid DCs from human CD34+ progenitors involves not only differentiation and survival, but also proliferation. The reduced intDC and LC yields found upon inhibition of PI3K or mTOR activity were partly caused by a block in cell cycle progression during the development of pre-DCs. An increase of cells in G0/1 has also been reported for other cell types in which inhibition of PI3K–PKB–mTOR signaling caused a G1 cell cycle arrest (8, 40–42). The LY- or Rapa-induced apoptosis could be either a direct consequence of cell cycle arrest or an independent process. A combined reduction in cell cycling and survival has also been reported in developing erythrocytes (8), but is not a prerequisite in all cell types (19, 41). Because only minimal proliferation was present from the stage of pre-DC while these cells underwent apoptosis upon PI3K or mTOR inhibition, it appears that the LY- and Rapa-induced effects on proliferation and survival are independent consequences of reduced signaling along this pathway. PI3K signaling plays a critical role in the regulation of survival in various hematopoietic cell types, including murine bone marrow-derived DCs and human moDCs (8, 18, 43). As demonstrated here, also during CD34-derived myeloid DC development, survival was decreased upon inhibition of PI3K or mTOR activity and increased in pre-DCs with increased activity of this signaling module by transduction with myrPKB. Whereas CD34-derived myeloid pre-DC survival required PI3K–PKB–mTOR signaling, terminally differentiated CD34-derived DCs survived without active PI3K or mTOR. This lack of apoptosis induction by PI3K or mTOR inhibition in CD34-derived intDCs is in sharp contrast to moDCs, which share phenotypic and functional characteristics with CD34-derived intDCs, but die upon inhibition of this pathway (19, 44). Moreover, whereas CD34-derived pre-DCs require mTOR for their survival, monocytes, the direct precursors of moDC, are insensitive to Rapa (19, 44) (Fig. 9). These disparities in survival signaling challenge the hypothesis that DCs developing from monocytes and from direct DC precursors are completely identical (45).

Although the effects of PI3K and mTOR inhibition on survival and proliferation could theoretically be unrelated, this seems unlikely. The lack of effects induced by only mTOR or only PI3K inhibition as well as the complementary consequences of PI3K/mTOR inhibition and increased PKB activity suggest that the observed effects reflect a critical role of the PI3K–PKB–mTOR signaling module. In addition, the myrPKB-induced rescue from GM-CSF withdrawal- or PI3K inhibition-induced apoptosis, but not mTOR inhibition-induced apoptosis, supports the suggestion that PI3K regulates CD34-derived myeloid DC development via the activation of PKB and mTOR.

The apparently absent PI3K–PKB–mTOR dependency of terminally differentiated DCs could reflect a generally reduced apoptosis sensitivity of these cells compared with their precursors, for example owing to the decreased Apaf expression in these cells (46). Alternatively, signaling pathways other than those including mTOR might be involved in the survival regulation of terminally differentiated myeloid DC. This hypothesis is supported by the observation that the expression of the antiapoptotic Bcl2 like proteins Mcl-1 and Bcl-XL is reduced in differentiated CD34-derived DC compared with their precursors (Table I). Although the regulation of these proteins by mTOR is at the translational level (47, 48), the reduced mRNA expression suggests a diminished role in survival regulation and thus a reduced requirement of mTOR mediated translational control of these proteins. The increase in Bfl-1/A1 expression, a Bcl2 family member with similar functions as Mcl-1 but regulated by NF-κB (49, 50), may point toward a role for other signaling pathways in the survival regulation of differentiated CD34-derived myeloid DC.
Because the surviving cells demonstrated normal pre-DC and DC phenotype, PI3K–PKB–mTOR signaling appears to be redundant for differentiation of CD34-derived myeloid DC. However, tolerogenic effects of Rapa addition during DC differentiation have been reported (38, 51), and in particular activation-induced cytokine secretion has been shown to be regulated by PI3K and mTOR (13–16, 36, 52). In accordance, the function of CD34-derived myeloid DCs differentiated or activated in the presence of PI3K or mTOR inhibition was inhibited. As previously reported for mouse bone-marrow derived DCs and human moDCs (39, 53), human CD34-derived myeloid DCs differentiated in the presence of Rapa showed reduced macropinocytosis and lectin-mediated endocytosis. In contrast, LY or Rapa did not affect Ag uptake by control DCs when inhibitor and Ag were added simultaneously (data not shown), indicating that neither PI3K nor mTOR was directly involved in the regulation of endocytosis. Rather, mTOR inhibition during differentiation seems to induce development of altered DCs with relatively poor Ag uptake capacity. The reduced cytokine secretion by DCs generated in the presence of LY or Rapa also indicates the development of alternatively functioning DCs. However, because LY and Rapa also inhibited DC function when added during activation, we cannot fully exclude the possibility that

![FIGURE 6. Impaired Ag uptake by cells differentiated in the presence of mTOR inhibition. LY, Rapa, or DMSO was added to CD34-derived myeloid DC differentiation cultures at days 0, 4, and 6. Cells were harvested at day 10, thoroughly washed, and incubated with dextranFITC or Lucifer Yellow. Lectin-mediated (dextranFITC) and fluid phase (Lucifer Yellow) endocytosis were determined after 2 h of incubation at 37 °C. Surface binding was determined by incubation at 4 °C. Ag uptake was calculated as MFI 37 °C – MFI 4 °C. Data shown are mean ± SEM Ag uptake standardized to control (n = 2). MFI, mean fluorescence intensity.](image)

![FIGURE 7. PI3K signaling is required for functional maturation. CD34-derived myeloid DCs were differentiated under control conditions and subsequently stimulated with LPS or CD40L-transfected L-cells in the presence or absence of LY or Rapa (2 h preincubation; A,C) or differentiated in the presence or absence of LY or Rapa, thoroughly washed, and stimulated with LPS without inhibitors (B). A, LPS-stimulated cells were analyzed for the expression of CD83, CD86, and HLA-DR (cells harvested at day 14; n = 4), and CCR7 (n = 2). Viable cells were gated on FSC/SSC. Representative FACS plots are shown. B, LPS-induced IL-6, IL-8, and IP-10 production was determined by ELISA (n = 2). Mean ± SD of duplicate cultures of a representative experiment are shown. Dotted line shows detection limit of the assay. C, IL-6, IL-8, IL-10, IP-10, and TNF-α concentrations were determined in supernatants from LPS cultures. IL-12p70 concentration was determined in supernatants from CD40L-stimulated cultures. Cytokine concentrations were determined by luminex (IL-6, IL-10, TNF-α; supernatants harvested at day 14; n = 3) or ELISA (IL-8, IP-10, IL-12p70; n = 2). Mean ± SD of duplicate cultures of a representative experiment are shown. Dotted line shows detection limit of the assay. FSC, forward scatter; SSC, side scatter.](image)
these effects result from ongoing PI3K and/or mTOR inhibition during activation. Although both LY and Rapa inhibited LPS-induced DC function, LY did so to a lesser extent, which could explain the reduced T cell stimulatory capacity observed for Rapa- but not LY-treated DCs. These findings seem contradictory to some studies describing repression of cytokine production by PI3K and mTOR in moDC (16, 36), but are in accordance with others (38, 54–56). These data indicate that although PI3K–PKB–mTOR signaling is not required for the acquisition of a DC phenotype or the survival of terminally differentiated CD34-derived myeloid DCs, this pathway may still be involved in the regulation of other important processes in these cells.

The molecular mechanism underlying the impaired response to activation signals following mTOR inhibition is relatively unknown. Minor changes in TLR4 mRNA expression were observed (data not shown), which might provide an explanation for the reduced response to LPS. However, as LPS induced normal upregulation of costimulatory molecule expression, loss of TLR4 stimulation cannot fully account for the diminished function observed after mTOR inhibition. Whereas the role of mTOR in the regulation of proliferation and survival is mainly ascribed to translational control (11, 57), cytokine production has been suggested to be regulated on the transcriptional level (15, 58, 59). However, the exact mechanisms of the processes involved remain to be elucidated.

Whereas the decreased T cell proliferation induction by DCs treated with Rapa could result from the induction of regulatory T cell differentiation as suggested before (38, 51), Foxp3 expression in these T cell cultures was unchanged compared with control (data not shown). Whether the altered secretion of Th1 and Th2 cytokines after coculture with Rapa-treated DCs only reflects reduced T cell numbers or results from changed Th1 and Th2 differentiation as shown for murine Rapa-differentiated DCs (37) remains to be determined. Also, although the reduced IL-5 secretion but unaffected IFN-γ concentrations found in T cell cultures stimulated with Rapa-differentiated DCs might suggest skewing of T helper differentiation, this should be investigated further. In addition to reduced DC–T cell interaction as a consequence of mTOR inhibition in DCs, the reduced CCR7 expression following LY or Rapa treatment may also have consequences for the induction of immune responses. These data suggest that lymph node migration requires intact activity of the PI3K–mTOR signaling module, as has been suggested before (60).

**FIGURE 8.** PI3K signaling is required for myeloid DC function. CD34-derived myeloid DCs were differentiated in the presence or absence of LY or Rapa, thoroughly washed, and stimulated with LPS without inhibitors (A) or differentiated under control conditions and subsequently stimulated with LPS in the presence or absence of LY or Rapa (2 h preincubation; B). After 18 h LPS stimulation, cells were thoroughly washed and used in an allogeneic MLR (n = 2). T cell proliferation was quantified by incubating the cells with [methyl-3H]thymidine during the last 18 h of 6-d cultures. ELISA determined IFN-γ, IL-5, and IL-10 concentrations in supernatants harvested at day 5 (DC:T cell ratio of 1:2). Data represent mean ± SD of triplicate cultures of a representative experiment.

**FIGURE 9.** Role of PI3K–PKB–mTOR signaling in human myeloid DC subset development. CD34+ HPCs differentiate into intDCs and LCs via subset-specific pre-DCs. Early in CD34-derived myeloid DC development, PI3K–PKB–mTOR signaling is required for proliferation and survival. The dependency on this signaling module for survival is decreasing upon differentiation, with terminally differentiated intDC and LC survival being unaffected by inhibition of PI3K or mTOR. In contrast, mTOR activity has been reported to be crucial for the survival of moDCs, whereas their direct precursors, monocytes, are insensitive to mTOR inhibition (19, 44).
In conclusion, PI3K–PKB–mTOR signaling is required for human CD34-derived myeloid DC development by regulating the proliferation and survival of DC precursors. Whereas this signaling module is redundant for differentiation and phenotypic maturation as well as survival of terminally differentiated CD34-derived iDCs and LCs, its activity is required for the generation of fully functional DCs. These findings could be used as a strategy to manipulate DC subset distribution and function to regulate immunity.

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Disclosures

The authors have no financial conflicts of interest.

References


